Molecular and Translational Medicine
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Dwaine F. Emerich
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Cell Therapy

Current Status and Future Directions

Humana Press

Molecular and Translational Medicine

Series editors

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As we enter into this new era of molecular medicine with an expanding body of knowledge related to the molecular pathogenesis of human disease and an increasing recognition of the practical implications for improved diagnostics and treatment, there is a need for new resources to inform basic scientists and clinical practitioners of the emerging concepts, useful applications, and continuing challenges related to molecular medicine and personalized treatment of complex human diseases. This series of resource/reference books entitled Molecular and Translational Medicine is primarily concerned with the molecular pathogenesis of major human diseases and disease processes, presented in the context of molecular pathology, with implications for translational molecular medicine and personalized patient care.

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Editors

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I would like to dedicate this book to a collection of people. First, and foremost, to my wife (Renee), son (Julian), and daughter (Simone) who have tolerated this crazy biotechnology life for 30 years and have always made me want to be a better person. Secondly, to Dr. Gorka Orive, co-editor of this book. We have had a nearly decade old, very fruitful, collaboration and the funny thing is-we have never met. Here's looking forward to that beer when we finally get together. Lastly, I would like to thank Mastery Martial Arts for adding years to my life following a life-threatening illness. Special thanks to Mr. West, a gifted instructor, for physical and mental guidance together with effective arm (and sometimes leg) twisting.

Dwaine F. Emerich

I would like to dedicate this book to my wife (Raquel), my two sons (Ander and Mikel), my parents (Ramón and Araceli) and my brother (Ibon). I am very fortunate to have them always by my side, receiving all their support and love. I also would like to thank my co-editor in this book, Dwaine Emerich. We have been working together "virtually" for more than 10 years and he has always been and remains being a benchmark for me. Dwaine inspires me and helps me to improve everyday. Last but not least, I would like to thank my instructors and my closest friends for their friendship.

Gorka Orive, Ph.D.

Preface: A Brief Introduction to the Span of Cell Therapy

Cell-based therapy is a very old concept dating back thousands of years. Today's modern concepts of cell therapy can be traced to studies by researchers such as Claude Bernard and Alexis Carrel in the early 1800s to 1900s to Dr. Paul Niehans in the 1930s who successfully treated a patient with a damaged parathyroid by macerating an ox parathyroid gland injecting it into a patient's pectoral muscle. Contemporary cell therapies are more targeted than the "general revitalizing whole body therapies" envisioned by these early investigators and, as illustrated in this, volume span a virtually unlimited range of therapeutic applications with cells derived from an equally diverse range of sources. Most cell therapies are experimental or are in early stage clinical trials with some notable exceptions including hematopoietic stem cells, dendritic cells (Provenge®), cartilage-derived chondrocytes (ChondroCelect® and MACI®) and corneal stem cells (Holoclar®). Together with rapid advances in many fields these therapies illustrate how recently intractable translational challenges have been overcome and how we can anticipate cycles of research and clinical development to lead to an acceleration in product approval.

It is impossible to classify all of the cell types under investigation for cell therapy or to list all of the possible indications that those cells could be applied to in an easily digestable format. But to give a flavor of the breadth of the field we list a few general approaches here.

1. Non-modified, somatic cells have been used as general medical practice for many years. Blood transfusions and bone marrow transplants are routine and other cells types including mesenchymal stem cells are under intense pre-clinical and clinical investigation. The reasons behind their routine use are based on the relatively easy ability to isolate and manipulate the cells into a reliable product without any associated significant co-technologies. Other cell types, especially immune cells for oncology, can also be considered under this classification but the complexity of manipulation and difficulties in cost-effective manufacturing and clinical translation make their use more challenging. It is also likely that in many cases, adjunct technologies for modification of immune cells will be needed to optimize their benefit. It is also likely, however, that these cells will not benefit in the near term from currently transformative immortalization technologies. Despite decades of basic research these technologies still remain unproven.

2. Viral manipulation of cells can be, in principle, carried out in vivo or ex vivo. This is really a subset of gene therapy and involves directly administering genes into the desired portion of the body. Commonly, this involves the use of viral vectors and can be applied to a vast range of indications including cancer, brain diseases, and cardiovascular diseases. Still the translation from animal model to human has been hampered somewhat by uncertainty over the ability to regulate or discontinue gene expression once the virus is injected. Ex vivo gene therapy involves transferring genes in culture prior to reintroducing the modified cells into the patient. This is also an area where the technology might be applicable for a variety of cell types, although the most common cell type are T cells where the cells are isolated and modified to activate the cells for selective destruction of cancers. This is one of the few areas where large pharmaceutical companies have invested considerable resources into developing large scale capacity.
3. Stem cell technologies hold the promise of a holy grail of an unlimited supply of an infinite repertoire of cell types. Beginning with the development of mouse and human embryonic stem cell lines the field has now been set on fire with the discoveries of transdifferentiation (or lineage reprogramming) and human induced pluripotent stem cells (iPS). It is difficult to understate the impact of the iPS revolution. While still in its infancy and still controversial, the field is developing rapidly in areas including using iPS cells to recapitulate neurodegeneration in vitro to understand disease pathogenesis and is hurtling towards clinical evaluation. Reprogramming approaches allow investigators to generate stem cells from poorly defined or accessed progenitor pools. As exciting as this field is it is still unknown whether iPS-based cell treatments will provide significant therapeutic benefits. As of now, nearly 2000 clinical trials are open and registered at www.clinicaltrials.gov. so many answers will be forthcoming. As a note of caution, such a large number of clinical trials can easily form unrealistic expectations. After all, clinical trials with these cells will still need to elucidate the optimal means of utilization including suitable trial designs, manufacturing processes that control cell composition, genetic stability/drift, optimal dosing and route of administration, and potency: all considerations that other cell therapies must traverse.
4. Biomaterials are increasingly being combined with cells to provide three–dimensional constructs that are otherwise unachievable with conventional approaches. Biomaterials initially were used as simple scaffolds for promoting cell growth, providing controlled drug delivery, or protecting cells from immunological destruction but have evolved considerably to provide support for tissue regeneration, control of cell fate, three-dimensional scaffolds for developing complex tissue and organ constructs. Even more contemporary materials are so–called "smart" and are capable of combining all of the above advantages with complex receptor-ligand profiles, thermo-responsive properties, and self-assembly.

5. In the future gene editing may become a viable means of targeted and efficient genetically engineering live cells by inserted or deleting DNA using engineered nucleases. The field has rapidly run through meganucleases, zinc finger nucleases, transcription activator-like effector-based nucleases and most recently the CRISPR-Cas system. CRISPR-Cas9, in particular, has moved into a mainstream technological method with enormous potential. Initial target indications will likely be blood cell and monogenetic diseases.

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Molecular Considerations in Cell Transplant Imaging

1

Aline M. Thomas and Jeff W.M. Bulte

Introduction

The potential of cell transplantation as a therapy is unparalleled to conventional approaches. Cell-based therapies are flexible in their delivery and their utility. Cells can be administered to the patient as a single injection, in clusters as grafts, or with biomaterials [1, 2]. Transplanted cells have the ability to interact with and sniff out their microenvironment—interrogating signals from proteins, sugars, and host cells—and, therefore, unlike today's drugs, can adapt to and change their microenvironment [3]. The result is a dynamic, fine-tuned therapy that can adjust to interindividual variations and day-to-day changes in patients. Transplanted cells have been used to replace diseased organs, to regenerate injured tissues, and to change immune responses. Yet, few candidate cell therapies have reached, let alone, passed clinical trials [4–6].

The chief obstacle of cell transplantation therapy stems from its greatest strength: harnessing the body's own power to heal itself. On one hand, their ability to adapt and change based on the environment are exposed to permit dynamic therapies that can respond to change in stimuli in real time. On the other hand, this ability can result in transplanted cells with phenotypes that are unintended [7], ineffective [8–10], and sometimes harmful [11] to the patient. Furthermore, the specific use of transplanted cells (e.g., regeneration, immune modulation) poses unique obstacles for clinical implementation. To maximize efficacy and safety for the patient, extensive characterization of the cell's behavior in vitro and in vivo is required to develop a better understanding of how the choice in patient, cell, and transplantation method determines clinical outcomes.

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The Need for Molecular Imaging: Limitations of Cell-Based Therapies

Strategies for real-time monitoring are needed to assess both the transplanted cells and possible reasons for therapeutic failure, which can vary greatly depending on the patient-specific use. Cell-based therapies can "fail" at all stages of transplantation due to misinjection, occlusion of vessels, lack of proper cell differentiation, or lack of cell survival. The delivery method can initiate cellular apoptosis in the transplanted cells [12]. To add insult to the injury, the surgical procedures associated with the delivery can initiate blood-mediated inflammatory reactions (BMIR) whose resulting cytokine secretions reduce successful engraftment [13, 14]. If the cell source is allogeneic (donor-derived) or xenogeneic (animal-derived) instead of autologous (patient-derived), the transplanted cells are also exposed to an adaptive, chronic immune response [15, 16]. As a result, an insufficient number of cells with the appropriate phenotype may reach the site of action and survive these events to be effective therapeutically.

Should the cells survive the inflammatory and immune responses after transplantation, variations in transplantation procedures can influence the phenotypes and function of transplanted cells, thus creating variations in clinical outcomes. Mechanical stress from the act of delivering the cells to the patient can influence transplanted cells toward unhealthy states and prime them toward undesired fates and phenotypes [17]. Additionally, if the delivery site is not the desired final location, changes in cellular environments due to the inherent differences in cellular and molecular compositions of the tissues traversed by the cells will further prime their phenotypes to the point of inefficiency [18] or prevent their migration and homing to the desired site [19, 20].

Furthermore, the cellular environment within patients can vary and affect the efficacy of transplanted cells [21]. Diseases within the same tissue are initiated by different key players, requiring unique cell-based therapies for effect [22]. Patients diagnosed with the same disease can present with distinct subtypes, stages, severities, and prognoses that may require different cell types to attenuate the disease. Environments in these tissues may alter after transplantation due to changes in disease state within the patient and result in changes in chemical, cellular, and molecular composition as the disease progresses [7, 23–25].

In regenerative medicine, cell transplantation is used to replace or repair host cells that are damaged by injury or disease. The most common source for cells in regenerative medicine are allogeneic (donor-derived), which typically involves extraction of the organs of interest from cadavers, isolation of the desired cell type, and cell culture while awaiting test results for purity, contamination, and donor-recipient matching [26, 27]. However, long-term culture can reduce cell viability and can prime the cells toward non-ideal phenotypes, resulting in a less functional transplantation [28]. For long-term survival, transplanted cells need access to a vascular system to ensure sufficient nourishment and waste excretion [29]. The environment at the transplantation site or along of the path of migration may be hypoxic, resulting in death or reduced functionality of the transplanted cells [30]. Throughout

this process, an immune response further reduces their numbers, unless shielded by isolating biomaterials or immunosuppressive drugs [31, 32]. The additional stressors experienced by allogeneic cells result in more cells being required for successful transplantation than if autologous cells were used [33].

Cellular transplantation has more recently been utilized for immune modification (e.g., vaccination, tolerance induction), also described in more detail in other chapters. More often than not, the cell source for tolerance induction is allogeneic, often derived from an organ donor due to enhanced efficacy [34]. However, tolerogenic immune cells are notoriously sensitive to their environment [35]. Many stimuli are known to alter their phenotype and switch their response to an activated one. As a result, despite defined culture conditions, immune cells tend to lose their tolerogenic properties [36] and potentially exaggerate the immune responses they were tailored and (co)transplanted to subdue. Surface markers have been established to distinguish immune cells, namely, B cells, T cells, dendritic cells, and macrophages. However, distinguishing the phenotypes of these cell types (e.g., activated versus tolerogenic) has been difficult historically. Thus, current research has focused on distinguishing these phenotypes [37, 38]. The resulting expansion of available surface markers has permitted the use of molecular imaging to simultaneously detect the presence of distinct immune cell phenotypes and to deduce the type of immune response generated.

Stem cells are frequently transplanted in regenerative medicine because of their ability to secrete growth factors that promote regeneration, reduce inflammation, and suppress transplant immunity [39]. For tissue replacement, stem cells have an advantage over their adult progeny to survive the transplantation process [40], proliferate [41], migrate, and home to the site of interest. In culture their "stemness," i.e., the ability to proliferate into cells that can differentiate into the various progenies, is maintained by preexposing the cells to a combination of growth factors. However, after transplantation these cells are no longer in that controlled environment. Without proper precautions, transplantation of stem cells may result in an increase in problematic cell types that exacerbate the disease instead of beneficial ones that mitigate the disease [7, 11]. The lack of suitable growth factors provided in culture can induce differentiation without any additional stimuli. The cellular and molecular composition of the transplantation site can then direct the differentiation of the stem cells toward undesired fates. The inflammatory and immune responses initiated by the transplantation may further direct their differentiation toward such fates. The timing [23, 42] and location [43, 44] of transplantation may determine the success of stem cell-based therapies. Thus molecular imaging strategies need to assess the extent and direction of differentiation of stem cells in addition to their survival and location.

The ability to monitor the disease in patients and cells upon transplantation is paramount to maximizing the likelihood of clinical effect. Conventional, assay-based approaches are well-established tools used both in research and in the clinic. These assays can monitor diseases and transplantation at the genetic, molecular, cellular, and systemic levels. "Omics" databases created from their results are extensive and constantly expanding; however, two factors limit their utility in the

clinical setting. First, most of these assays require invasive extraction of tissue for analysis. Second, development of these assays requires the creation and optimization of kits and devices that interact with and report target biomolecules, which is costly and time-consuming [45, 46]. On the other hand, today's imaging methods can monitor several molecular processes simultaneously using the same instruments and machines, greatly reducing the development time and costs for novel targets.

Recent Advances in Molecular Imaging

Imaging methods—optical, nuclear, (e.g., positron electron tomography (PET) combined with or without computer tomography (CT)), and magnetic resonance imaging (MRI)—can all monitor biological processes on a molecular level noninvasively and in real time compared to conventional assays. These imaging methods vary in their clinical strengths as discussed further below. However, technological advances in these imaging techniques, especially in combination (i.e., multimodal imaging), have permitted the rapid assessment of diseases and the cell transplantation-based therapies used to treat them with a comparable level of discernment as conventional assays.

Bright-Field and Fluorescent Optical Imaging

Optical imaging, which can be performed both in bright-field and fluorescent mode, was initially developed as a microscope on a laboratory bench used to look at eukaryotic and prokaryotic cells in a petri dish. At first, optical imaging was limited by the penetration depth that could be monitored due to low optical clarity of skin and overlying tissue, both in bright-field and fluorescent mode, which necessitated chambers for serial or long-term imaging (Fig. 1.1) [47]. However, technological advancements in optical devices have removed this necessity. For imaging in bright-field mode, developments in optical fibers and probes have expanded its use in clinical and surgical settings with products such as the PillCam™. For imaging in fluorescent mode, recent advancements in multiphoton and confocal microscopy have improved the penetration and resolution [48, 49].

Optical imaging in bright-field mode permits the visualization of cells and tissues in "real" color. Changes in color have long been used as a metric to assess the health of tissues on a gross scale—including vascularization, inflammation, and necrosis. The administration of colorimetric dyes expands the capabilities of bright-field imaging to detect more difficult to find organs and tissues, e.g., lymph nodes [50], and the leaks of vascular systems and other fluid-filled tissues [51]. However, toxicity and other medical complications upon administering these dyes [50] have limited their use to extracorporeal assays.

Optical imaging in fluorescent mode provides more options due to the abundance of fluorescent moieties including simple dyes, quantum dots, and transgenic proteins [50, 52]. The sensitivity of fluorescence readers permits monitoring the presence of

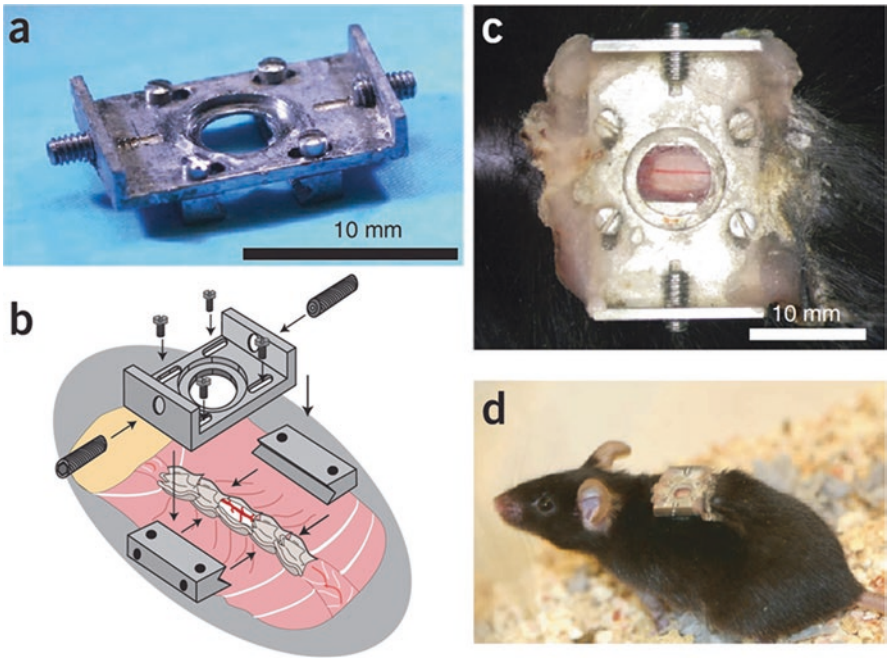

Fig. 1.1 Spinal cord injury progression can be monitored in vivo with an optical imaging chamber (**a**) that removes the need for repeated surgeries. (**b**) Schematic illustration of the procedure required for implanting the imaging chamber in mice. (**c**) Spinal cord of a mouse as seen through the implanted chamber. (**d**) Implanted chamber shown on a live mouse. Reproduced, with permission, from [47]

biomolecules to be monitored in patients and calculating their binding rates [53]. Unlike bright-field images, fluorescence images are often false colored for the purpose of easy visualization. The unique excitation and emission frequencies of fluorescent probes permit monitoring several biomolecules simultaneously using the same device—from infrared to visible and ultraviolet frequencies. Co-localization and interactions of cells and biomolecules can thus be quantified. Additionally, selectively activated fluorescent probes have also been developed to expand the use of fluorescence imaging for interrogating biomolecular processes in vivo [54].

Fluorescent probes have been paired in Förster resonance energy transfer (FRET) imaging to provide three distinct excitation/emission sets that can reveal biological interactions. In the most basic scheme, the close proximity of the two probes permits the exchange of energy from one to the other which changes the fluorescence characteristics of the pair to the excitation of the first and the emission of the second [55]. This phenomenon has allowed researchers to determine whether two proteins (or other tagged biomolecules) interact with or reside proximal to each other as part of a signaling pathway [55]. When tethered with a linker, FRET has revealed whether a single protein changed conformation and/or activation state in response to external stimuli [56]. More recently, the development of bioactive linkages has

permitted the use of FRET technology as a biosensor to detect pH [57] and thiolation [58], as well as energy transfer paths on macromolecules [59]. Since its discovery the number of available FRET probes has expanded due to improved linkage design [56], novel fluorescent tags [60], and the addition of quenchers [61]. With more probes available, multiple biological processes can be assessed simultaneously using FRET [62, 63].

Nearly a decade ago, the first photo-convertible fluorescent probe, EosFP, was developed. EosFP is normally green but upon exposure to ultraviolet light fluoresces red [64, 65]. In the preclinical setting, this feature permits the tracking of the same starting cell population in vivo after transplantation to differing sites or via differing methods for side-by-side comparisons with minimal confounding factors. At first, this technology was limited by its tendency to blink, which made quantitative assessment problematic; however, longer-lasting versions have been developed since then [64]. Furthermore, both the light source used for conversion of photo-convertible fluorescent molecules and the colors that are changed upon conversion have been expanded to include those with the ability to convert fluorescence from green to red using white light [66], switch fluorescence from blue to yellow under ultraviolet light [67], and change colors under near-infrared light [68]. Additionally, photoactivatable fluorescent probes are available now [69].

PET Imaging

PET by itself primarily visualizes molecular processes and thus is often combined with CT and recently also MRI (PET/CT and PET/MR imaging, respectively) for anatomical localization. The biggest advantage of PET compared to optical imaging is the use of radioisotopes as the labeling agent because the absence of radioactivity in the body permits "hot spot" images with limited cases of reported false-positive images [70]. Fluorine-18 (F-18) is considered a premier labeling agent due to its relatively long half-life (110 min), which permits widespread applications due to the ability to transport regionally to imaging centers [71, 72]. Gallium-68 (Ga-68) has emerged as another popular tracer due to the long half-life of its precursor Germanium 68 (271 days), which permits longer transport and storage, though side-by-side comparisons have revealed F-18-based compounds detect better than Ga-68 compounds [73]. Most advances in types of biological processes that PET can image using these radiolabels parallel that of fluorescent optical imaging as similar constructs (e.g., antibodies, ligands, quantum dots) used for detection [74–76]. As an example, the well-known PET tracer Pittsburgh compound B (PiB) is widely used for imaging beta-amyloid plaques in Alzheimer's patients [77]; it is based on a fluorescent dye, thioflavin T, that was developed in the late 1950s for histopathological detection of these plaques [78].

The focus of PET imaging research of late has been improved spatiotemporal modeling of the radioactive signal. The development of time of flight (ToF) PET improved the temporal resolution of PET imaging to enhance the precision in detection compared to traditionally used interrogation methods [79]. Thus, ToF permitted

the expansion of radioactive isotopes used for PET from those with longer half-lives (e.g., F-18 and Ga-68) to those with shorter ones. ToF increased the signal-to-noise ratio to enhance the contrast with fewer iterations which reduced the presence of artifacts and false positives of scans with shorter imaging times [80, 81]. The development of point spread function (PSF) improved the spatial resolution of PET imaging, which when combined with ToF resulted in resolutions as low as 1 mm in the clinic [82]. At the other end of the spectrum, combining ToF with depth-of-interaction (DoI) has permitted total body imaging in humans [83].

Bioluminescence Imaging

Bioluminescence imaging (BLI) emerged as an alternative to fluorescent optical imaging for preclinical in vivo applications [84, 85]. Typically, in BLI, the gene for the enzyme luciferase is delivered to the cell and/or animal of interest, which in the presence of a light-generating substrate and ATP emits photons that can be measured using a CCD camera [86]. Two main applications have been explored for this technology: tracking of transplanted cells (typically for cancer research applications) [87–90] and the delivery of transgenes [91–93]. A promising technique is the use of spectral deconvolution to permit the measurement of multiple bioluminescent moieties simultaneously (Fig. 1.2) [94], permitting the tracking of a cellular response in regenerative medicine.

The identification and generation of cell state-specific promoters has expanded the use of bioluminescent imaging to monitor molecular processes, specifically transcription factor presence and activity [95]. The primary advantage of monitoring transcription factors is obtaining a systemic view of biological responses using orders of magnitude fewer biomolecules. Monitoring transcription factor activation has been used both in vitro as a cellular array [95] and in vivo to assess the pathology of diseases and the efficacy of regenerative medicine therapy (Fig. 1.3) [92]. This molecular biology approach has also been developed for fluorescence imaging [96] but, due to the reduced sensitivity when compared to bioluminescence, has been restricted to ex vivo or in vitro use.

MR Imaging and Spectroscopy

The original use of ^{1}H (proton) MRI was to visualize anatomical structures. Tissues were distinguished by their water content, chemical structure, and relaxation times which determine their contrast. To visualize anatomical structures that are difficult to distinguish, contrast agents were developed to change the longitudinal (T1) or transverse (T2) relaxation time of the region of interest. Contrast-enhanced MRI has been used to locate cancers [97], detect lesions [98], and track transplanted cells [99]. Gadolinium is the most widely used contrast agent due to its greatest efficiency for shortening T1 relaxation times; however, due to concerns of renal toxicity [100] and cumulative deposition into neural tissue upon repeated exposure [101, 102],

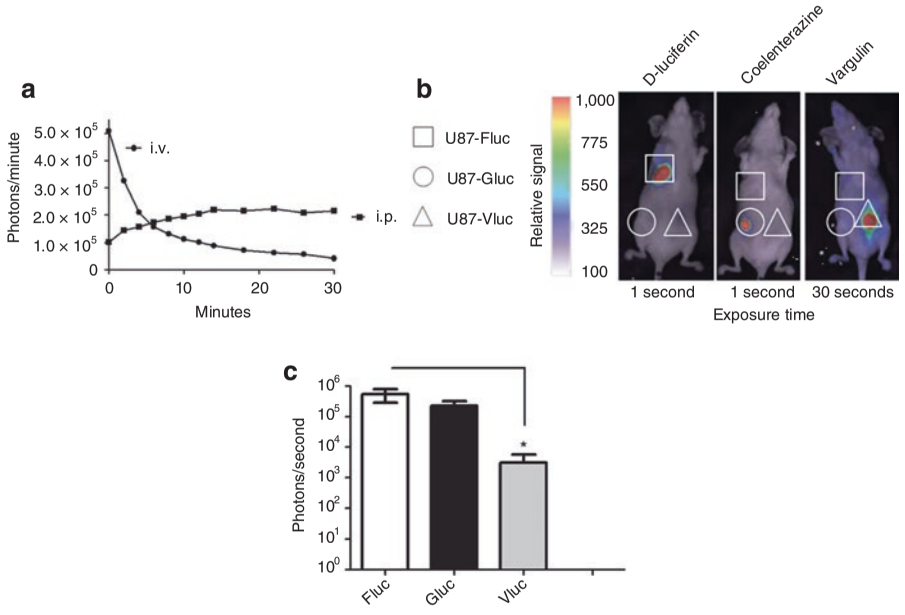

Fig. 1.2 Multiple implanted tumor cells expressing luciferase are tracked with BLI. (**a**) Tumor-associated luciferase signal after intravenous (i.v.) or intraperitoneal (i.p.) injection of vargulin (Cypridina luciferin). (**b**) BLI of U87 glioma cells stably expressing Firefly (Fluc), Gaussia (Gluc), or Vargula (Vluc) luciferase that were injected subcutaneously, implanted subcutaneously in nude mice at different sites. (**c**) Sequential (1 day interval) imaging of luciferase-expressing cells (10 days after implantation) after injection of coelenterazine, vargulin, and D-luciferin, respectively. Reproduced, with permission, from [94]

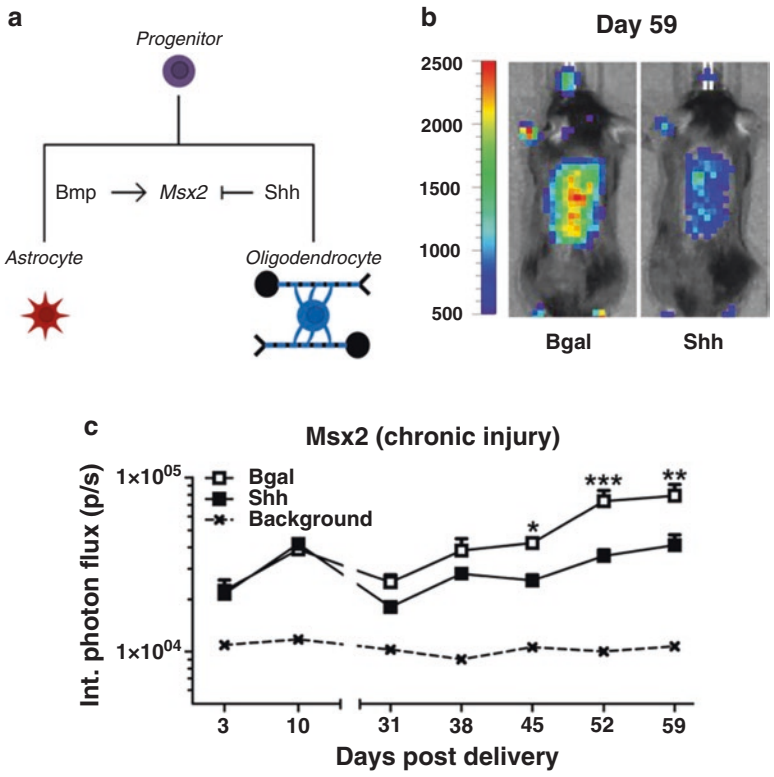

Fig. 1.3 Differentiation of stem cells after spinal cord injury is assessed using a bioluminescent probe sensitive to the activity of transcription factor muscle segment homeobox 2 (Msx2). (**a**) Schematic illustrating that Msx2 activity decreases in the presence of oligodendrocyte-promoting sonic hedgehog (Shh) and increases in the presence of astrocyte-promoting bone morphogenic protein (Bmp). (**b** and **c**) Msx2 activity in the injured spinal cord decreases when lentivirus encoding Shh is delivered compared to delivery of B-galactosidase as control. Reproduced, with permission, from [92]

other paramagnetic agents are being explored. Manganese has been investigated as an alternative to gadolinium as a T1 contrast enhancer [103, 104]. Superparamagnetic iron oxide (SPIO) particles have been used as a T2 contrast enhancer to monitor cell populations following its intracellular incorporation (Fig. 1.4) [105, 106].

While the most commonly used form of MRI is proton based, the elements that can be directly detected using MRI have expanded to include fluorine (^{19}F) MRI (Fig. 1.5). The advantage of using fluorinated tracers in ^{19}F MRI compared to metallic contrast agents in ^{1}H MRI is the tracer "hot spot" interpretation [107] that can be co-registered with the anatomical ^{1}H MRI images using the same hardware [108–110]. Intracellular incorporation of ^{19}F has been shown to be efficient with minimal changes in cell behavior, permitting its use for tracking cell transplants [111]. ^{19}F MRI has also been used to simultaneously track multiple cations as its chemical shift is ion specific [112]. Chemical exchange saturation transfer (CEST) MRI

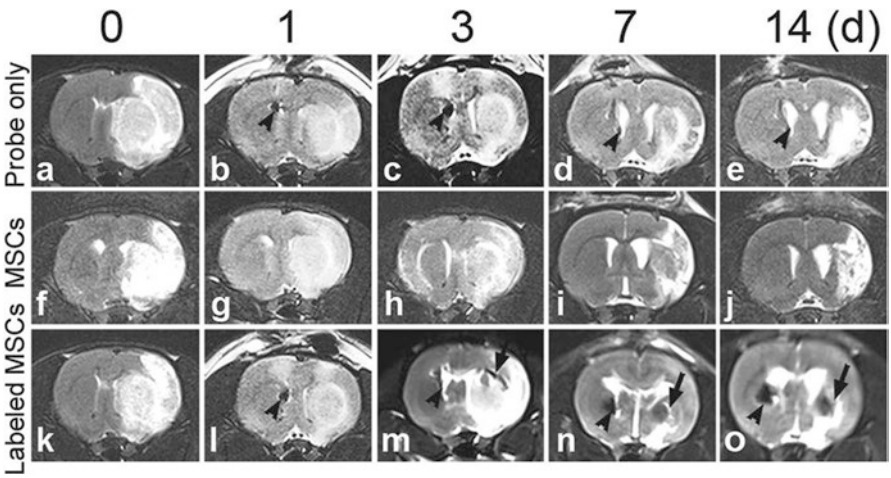

Fig. 1.4 In vivo ^{1}H MRI of transplanted ^{125}I-fSiO4@SPIO-labeled mesenchymal stem cells pre-labeled with fluorescent silica-coated ^{125}I containing SPIO in stroked rats after intracerebral injection. T2-weighted MR images of ischemic rat brain before (**a**, **f**, and **k**) and after transplantation of the probe (**b**–**e**), unlabeled MSCs (**g**–**j**), or labeled MSCs (**l**–**o**). Shown are images at day 1 (**b**, **g**, and **l**), day 3 (**c**, **h**, and **m**), day 7 (**d**, **i**, and **n**), or day 14 (**e**, **j**, and **o**) after injection. *Arrows* indicate hypointensity from the probe and labeled MSCs after injection. Reproduced, with permission, from [106]

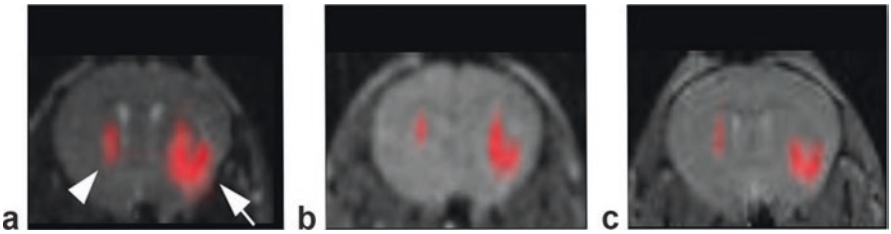

Fig. 1.5 In vivo ^{19}F MRI of transplanted NSCs pre-labeled with cationic perfluoro-15-crown-5-ether, with the ^{19}F signal superimposed on the ^{1}H MR images. MR images were acquired at 1 h (**a**), 3 days (**b**), and 7 days (**c**) after injection of 4×10^4 (left hemisphere, *arrowhead* in **a**) or 3×10^5 (right hemisphere, *arrow* in **a**) C17.2 neural stem cells. Reproduced, with permission, from [108]

permits the tracking of certain diamagnetic biomolecules, whose protons can be selectively magnetized [113]. After saturation, their saturation transfers to water protons, upon which the ^{1}H MRI signal is decreased. Monitoring neurotransmitters [114] and metabolites [115] that exchange magnetization energy with water has permitted the characterization of disease pathology in real time. With the development of other CEST contrast agents, pH [116, 117] and temperature [118] can now also been monitored. Due to the selectivity of magnetization, multiple CEST-detectable compounds can be tracked simultaneously to interrogate biological responses as part of a multicolored MRI approach [119].

A big push has emerged for the development of theranostics, especially therapies that are MR visible to monitor their location and efficacy. Two strategies have been employed: developing MR techniques on the therapeutic molecule itself or developing nanoparticles that can co-localize a MR-visible agent with the candidate. The former is limited to use with therapeutic molecules that have CEST-able bonds [120, 121] or can be conjugated to MR-visible contrast agents [122]. The latter has expanded the number of MR-visible therapies to those that can be incorporated into CEST- and contrast agent-containing vehicles [123–125].

Concluding Remarks and Outlook

Multimodal strategies have recently been developed to combine the sensitivity and easy interpretation of hot spot-based PET and SPECT imaging with the anatomical location of the signal and its relation to tissue structures as seen on MRI or CT. Incorporating transcranial magnetic stimulation (TMS) and electric encephalography (EEG) has permitted the simultaneous evaluation and tracking of conductivity and function for neurological disorders, e.g., stroke [126]. On the preclinical side, incorporating bioluminescent and fluorescent moieties has sped the validation of novel clinically translatable imaging approaches [127–129]. Given the promise of multimodal imaging, the development and clinical evaluation of multimodal equipment is now in full swing [130].

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Considerations for Successful Encapsulated β-Cell Therapy

2

Christopher G. Thanos, Jason L. Gaglia, and Felicia W. Pagliuca

Introduction

Type 1 diabetes (T1D) is a disease of insulin insufficiency that can be clinically managed with delivery of various exogenous insulin formulations, typically via subcutaneous injection or infusion. Otherwise, insufficient insulin can lead to acute complications including ketoacidosis or chronic complications including nephropathy, retinopathy, macular edema, neuropathy, and macrovascular disease. With intensive insulin therapy, risks of developing these complications can be reduced [1]. However, the burden on the patient makes long-term compliance a difficult proposition, often resulting in increasingly poor control and secondary health issues [2]. One of the greatest concerns of tight glycemic control is the possibility of life-threatening iatrogenic hypoglycemia. In clinical practice, approximately 90% of all patients self-administering insulin have experienced some degree of hypoglycemia [3]. Once plasma glucose falls below about 70 mg/dL, a cascade of released hormones leads to reduced glucose uptake in peripheral tissues [4] and initiation of counter regulatory mechanisms that are observed over hours. Hypoglycemia can lead to seizures, coma, and death [5].

The pharmacological challenge of treating T1D involves utilization of insulin, a drug with a rather narrow and variable therapeutic window, to achieve

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normoglycemia, which under normal physiologic conditions involves the interplay of multiple hormone mediators, activity, and carbohydrate intake as major factors. Intravenous insulin as is delivered by the pancreas has much more rapid kinetics than insulin that is subcutaneously delivered. The variable requirements for insulin delivery in T1D may be better suited for delivery systems that can respond dynamically and that incorporate glucose sensing and control of insulin release in a way that behaves more similarly to the innate pancreas. Since the timing and magnitude of insulin dosing is dependent on plasma glucose, the system must be able to couple the two activities (sensing and delivery) without significant delays due to secretion, diffusion, uptake, or other transport-limiting phenomena. Many strategies for such systems have been evaluated, including pulsatile-release polymeric microspheres [6], multiphasic insulin conjugates [7, 8], insulin pumps [9], naked islet therapy [10], and the bioartificial pancreas [11, 12].

In September of 2016, Medtronic announced FDA approval of its automated insulin delivery device for T1D [12], the first approval of its kind. The MiniMed 670G hybrid closed-loop system measures glucose every 5 min through a body-attached sensor and delivers the appropriate amount of insulin to the subcutaneous space with an external insulin pump. This closed-loop system operates within limits defined by an algorithm with the assistance of patient inputs signaling timing of meals. The technology is particularly compelling because it can control fasting glucose with minimal patient input, protecting against unaware hypoglycemia throughout the night. The device was evaluated in clinical trials in 123 patients for 3 months, with no episodes of extreme hypoglycemia or ketoacidosis, although 24 severe hyperglycemia events were reported [13].

With this significant success using a completely artificial closed-loop system, the technology has advanced through various failure modes that have plagued its development, including blockage, sensor fouling, and insulin stability issues [14], as well as poorly predictive compensation for onboard insulin [15]. Even with pump-based insulin delivery at this advanced state, a monohormonal approach may not be sufficient to completely eradicate unaware hypoglycemia. The device's algorithm that controls insulin delivery is the only means by which glycemia is regulated, while under normal physiologic conditions during hypoglycemia [16], the alpha cells of the endocrine pancreas secrete glucagon to mobilize glycogen stored in the liver into the bloodstream as glucose. Dual hormonal therapies have been evaluated [17], and such closed-loop systems are in development [9].

The complex activities of the endocrine pancreas may be best recapitulated in a biological system wherein islet or islet-like tissue is placed within an environment in the patient that promotes viability and function through engraftment, diffusion, or integration with the host.

Primary islets from human or animal origin have been used in this role [18], but recently, islet-like tissue derived from stem cells has been developed to a level of functionality and safety that enables its use in commercial development. This chapter will detail the development of cell-based therapies for T1D, with a specific focus on the bioartificial pancreas (cells encapsulated in biomaterials) and the emerging

prospect of stem cell-derived islet-like tissue as a functional and renewable cell source.

Islet Transplantation

Clinical islet transplantation has been in practice for decades now as a therapy for T1D [18]. Originally envisioned as a panacea limited only by the availability of human cadaveric islets, a commercially viable cell therapy for T1D has remained an elusive target due in large part to the mass and metabolic demands of the β-cells required for insulin therapy. The diffusive constraints of the encapsulation systems employed to deliver islets, the variability in the quality and performance of cadaveric islets, and the host response to the encapsulation materials and secreted contents are all additional compounding factors that impact the success of the graft by activating elements of the immune system.

Portal delivery of naked islets with immunosuppression but without encapsulation, as performed in the Clinical Islet Transplantation (CIT) Consortium and the earlier Edmonton Protocol [19, 20], is perhaps the most direct way to introduce beta cells into organ parenchyma. In these studies, human cadaveric islets are infused directly into the portal vein, where they travel into the small sinusoids of the liver vasculature. This transplant site provides direct access to oxygen and nutrients in the blood while minimizing travel to distant sites through physical entrapment within the tissue. However, it is estimated that of the infused islets, only 10–20% survive the immediate posttransplant period [21]. Still, in select cases, these marginal beta cell masses have been sufficient to maintain euglycemia.

In the CIT-07 clinical trial [19], 48 subjects were implanted with an average of about 800,000 IEQ with about half of the group receiving a second transplant. Patients were immunosuppressed acutely with sirolimus (rapamycin), tacrolimus, etanercept (a TNF inhibitor), and rabbit antithymocyte globulin (rATG) and then with sirolimus and tacrolimus maintenance. After 1 year, about 40% of patients were insulin independent with 100% demonstrating graft function as measured by circulating C-Peptide values greater than 0.3 ng/mL. After 2 years, the median exogenous insulin requirement was zero with the highest doses still remaining less than 10 IU/day. Compared to the Edmonton Protocol, which did not demonstrate this level of success, CIT-07 differed by several peritransplant procedures [22]. This included a short period of islet culture, early initiation of rATG, and modulation of anti-inflammatory therapy to mitigate instant blood-mediated inflammatory reaction (IBMIR) with a combined insulin and heparin infusion followed by a week of additional low molecular weight heparin and intensive insulin therapy for 2 months. A cohort of 11 patients in the trial treated at the University of Pennsylvania demonstrated improvement of beta cell secretory capacity, and all showed complete insulin independence after a year [22].

Human islet allotransplants, when properly administered, cultured, and immunosuppressed, can clearly provide useful therapy for years. The caveat to this approach is the potential side effects of chronic immunosuppression including direct

toxicities and increased malignancy risks. Tacrolimus and other calcineurin inhibitors are associated with nephrotoxicity and beta cell toxicity [23]. Other side effects include peripheral edema, hypercholesterolemia, abdominal pain, headache, nausea, diarrhea, pain, constipation, hypertriglyceridemia, hypertension, fever, urinary tract infection, anemia, arthralgia, and thrombocytopenia [24]. While many of these side effects are not life-threatening, they present a significant burden of potential morbidity to the patient.

Islet Transplantation: Site Selection Considerations

In their native location, β-cells are contained within islets which are dispersed within the exocrine pancreas. The human pancreas contains approximately one million islets, each consisting of about 1500 cells but ranging from only a few cells to over 12,000 cells [25]. The majority of the venous drainage of the pancreas is via the pancreaticoduodenal veins into the portal vein. This supports the liver receiving higher levels of insulin than the rest of the body. Such portal insulin delivery supports liver glycogen synthesis and suppression of endogenous glucose production [26]. Ideally, an islet replacement strategy would recapitulate this physiology, but portal insulin delivery is not required to achieve normoglycemia. Evidence for this comes from whole organ pancreas transplants, where venous drainage is most often into the systemic circulation via the recipient's vena cava or an external iliac vessel and less frequently into the portal circulation via the superior mesenteric vein. While fasting insulin is significantly lower with portal drainage, no difference has been found in comparing portal verses systemic drainage with regard to fasting and stimulated glucose or hemoglobin A1c [27].

Within the pancreas, islets are richly vascularized by direct arteriolar blood flow. Microsphere flow studies in animals demonstrate that islets, which make up only about 1–2% of the mass of the pancreas, receive greater than 10% of the pancreatic blood flow [28]. This high degree of arteriolar blood flow is likely important for rapid nutrient sensing from the blood. Indeed, mice with decreased vascularization of the islets due to β-cell-reduced VEGF-A expression have impaired stimulated insulin secretion that has been shown to be related to these vascular alterations and not β-cell dysfunction [29].

The portal vein is currently the most common transplant site for islets. Pancreatic islets infused into the portal vein lodge in distal tributaries. These islets are revascularized by branches of the hepatic artery [30]. In an animal model, glucose and arginine administered through the hepatic artery, but not through the portal vein, induced insulin release from intraportally implanted islets [31]. Unfortunately, this intraportal site has generally had low revascularization of islets [32]. Other transplant sites that are being explored include muscle, pre-vascularized subcutis, and omentum. Of these, the omental pouch appears to have the best glucose kinetics with naked islets [33, 34]. However, with appropriate vascularization, as seen in whole organ pancreas transplant, there are likely many suitable sites.

Encapsulating Islets: Biomass Considerations

Encapsulated β-cell therapy has not yet demonstrated the level of success of naked islets, but remains a very active area of R&D in the pursuit of a therapy that obviates the need for chronic immunosuppression. In theory, encapsulating materials can be designed to support the flux of therapeutic molecules, oxygen, and nutrients while creating a permselective barrier against harmful elements of the immune system. But these ideal transport properties are difficult to achieve in the dynamic environment of the host in a way that is predictable in vitro, owing to interaction with host proteins, and involvement of the immune system against either materials or cell debris. Rather, several encapsulation systems have been fabricated out of membranes with much larger pore structures that allow some elements of the immune system to pass, favoring transport of the necessary nutritive elements at the expense of potentially injurious immune molecules or inflammatory cytokines. The sections below highlight some of the challenges of encapsulation system design, both in the context of primary islets and stem cell-derived islet-like tissue.

In the endocrine pancreas, islets are organized around a rich network of capillaries with about ten times more fenestrae than the exocrine tissue that surrounds them [35]. In this arrangement, β-cells are typically distanced by a single cell from the bloodstream [36], allowing efficient sensing of glucose and secretion of insulin with minimal diffusive resistance, as well as enhanced oxygenation and nutrient transport. There is also data suggesting that the presence of endothelial cells and their secreted growth factors is necessary for β-cell function. In several animal models, deletion of VEGF-A is associated with glucose intolerance [37, 38].

Fully contained cell encapsulation systems necessarily separate islets or β-cells from the bloodstream, relying instead on diffusive or assisted transport of key solutes through the biomaterial and encapsulated (and avascular) biomass. For a human dose of islets, the distance that insulin must travel is quite far. At a dose of 800,000 IEQ as evaluated in the Edmonton Protocol and CIT-07, and a diameter of 150 μm for each IEQ, the dose would occupy a packed volume of about 1.4 cc of solid tissue. Considering that the abdominal cavity of an adult could probably comfortably house a 10-cm disc-sized encapsulation system, depending on location, the disc would need to be at least 176 μm thick to accommodate an internal volume of 1.4 cc, not including any of the materials used to make the device. Estimating membrane thickness at about 25 μm, this hypothetical encapsulation device would be 226 μm in total thickness with 100% packing density inside the device, e.g., the spherical islet clusters would be compressed into a solid tissue mass. The β-cells in the device core would be separated from the outside by 88 μm of biomass and 25 μm of biomaterial, for a total distance of 113 μm. This is 5–10 times the distance in the innate pancreas where β-cells are only separated by a one to two 12 μm cells. In addition to greater distance from an oxygen source, the diffusion of oxygen through solid tissue encased within a permselective membrane is significantly slower than diffusion through a liquid [39]. Further complicating matters is the limited vascularization that can be accommodated by the surface of indwelling abdominal implants,

which in simple formats cannot approach the relative density of vessels to β-cells in the pancreas [40].

Microencapsulated islets have the advantage of potentially uniform distribution throughout a cavity and, depending on size and configuration, a greater surface area to volume ratio (SA:V). Conformally coated islets have a barrier thickness of about 25 μm and SA:V of about 300 cm^{-1} and are administered as a suspension that can distribute throughout the peritoneal cavity or can be localized within an enclosed pocket in the renal subcapsular space, omentum, or epididymal fat pad [41]. In this configuration, the 800,000 cell clusters that make up a human dose can be delivered separately in that suspension, allowing the full surface area of the membrane to have contact with the host, with the diffusion distance dictated by the thickness of the barrier layer and surrounding tissue. In terms of surface area to volume, this encapsulation modality is about three times higher than the 10-cm disc described earlier. Conformally coated C57BL/6 islets were evaluated in vitro for insulin transport kinetics and were implanted into the renal subcapsular space to assess control of diabetes [41]. In dynamic perifusion experiments, no delay in insulin secretion was observed in coatings comprised of crosslinked poly(ethylene glycol) (PEG) and alginate in comparison to naked islets. Further, the duration of glucoresponsiveness was enhanced in culture, demonstrating a high stimulation index for multiple days. In vivo, the subcapsular coated syngeneic islets controlled glucose levels for 112 days, with little evidence of fibrosis or macrophage infiltration. These results highlight the significant advantage of maintaining a high SA:V ratio, which translates into more desirable insulin kinetics and the ability to transplant into environments such as the renal subcapsular space that would not be permitted with larger format microcapsules or macrodevices.

Traditional microcapsules of about 1 mm in diameter, with a thinner membrane wall and about three islets per capsule, have a twofold lower SA:V than the bilayer example device described earlier but with an even greater diffusion distance. But these capsules have other advantages, including rapid solute flux through a very hydrophilic membrane, high levels of biocompatibility, anti-fibrotic properties, and durability. Neither microcapsule system is completely retrievable, a significant pitfall in the case of an adverse event or complication. A comparison of conformally coated islets and microcapsules is shown in Fig. 2.1.

With these physical limitations relative to the innate pancreas, micro- and macroencapsulation platforms have evolved to address these and other failure modes through years of research. This sequence of potentially adverse events begins with issues during fabrication and filling, to acute implant responses that cause damage to encapsulation devices or the cells inside, to chronic failure involving the autocatalytic process evoked when cells within the device die, releasing antigens that are shed into the surround tissue provoking an immune attack, to ramifications of mechanical failure at the implant site. Some of the challenges facing cell encapsulation are shown in Fig. 2.2. This diagram attempts to depict the relative magnitude of some encapsulation failure modes, either process-related or that occur in vivo, as a series of peaks and valleys.

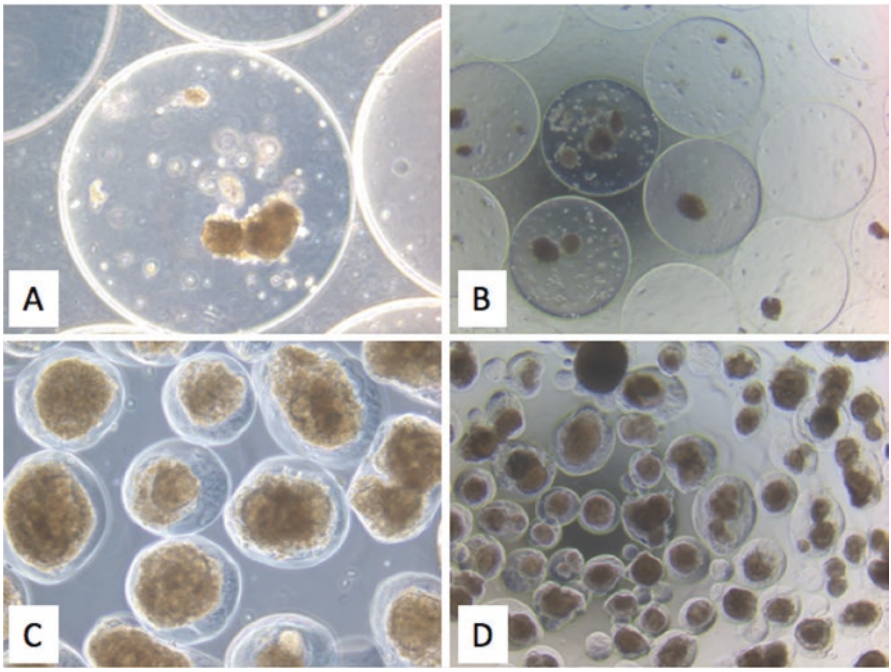

Fig. 2.1 Microencapsulated and conformally coated islets. (**a**) 10× and (**b**) 4× magnification of alginate-encapsulated islets. (**c**) 10× and (**d**) 4× magnification of PEG conformally coated islets (Image courtesy of Alice Tomei, Diabetes Research Institute)

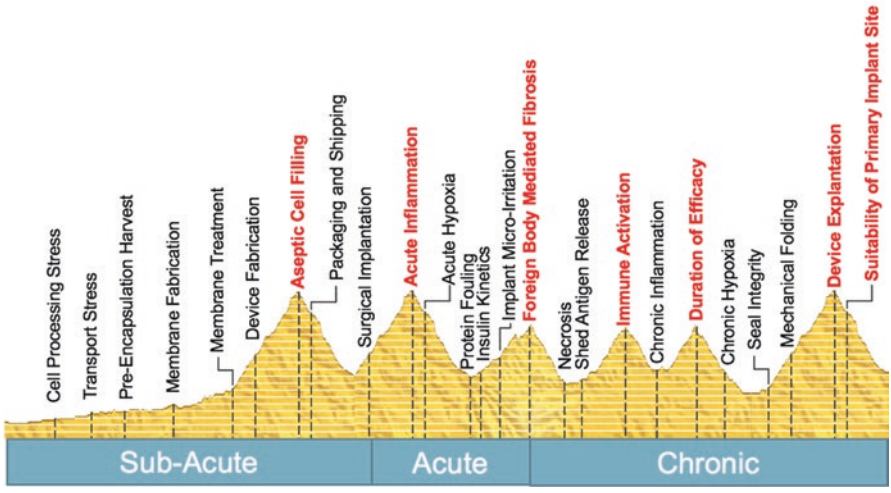

Fig. 2.2 Potential failure modes along the path to long-term device success. The *x*-axis represents time, and the height of the peaks represents a conceptual magnitude of the challenge

Islet Encapsulation Failure Modes

Subacute Processing Stress

Islets isolated from donor pancreata are subjected to an enormous amount of processing stress, due in large part to the variability in the enzyme blends [42], age and health status of the donor [43], and other islet-processing variables [44]. Once healthy and viable cells are achieved in culture, they must be harvested for encapsulation. This process involves concentration of the cell suspension used in culture such that it can be efficiently encapsulated, usually involving sedimentation, centrifugation, and a series of washes. During the encapsulation process, islets or cell suspensions can be adversely impacted by temperature, shear stress due to fluid path limitations, hypoxia, osmotic gradients, and stress associated with exposure to the encapsulation materials [45]. Post-encapsulation storage and transport conditions mark the final hurdles prior to implantation.

Acute Implant Failure

After implantation, encapsulation devices are exposed to a site of injury created by the incision, through blunt dissection and trauma to the surrounding tissues, potentially by anchoring sutures, and a transition from media with defined content to the physiologic environment. Some level of acute inflammation will undoubtedly accompany implantation, as proteins are adsorbed onto the biomaterial surface leading to varying activation of the coagulation cascade and complement system [46]. Fibrin deposition and platelet activation prime polymorphonuclear cells to attach to the surface via integrin receptors [47], secreting proteolytic enzymes and reactive oxygen species (ROS), followed by IL-8. As IL-8 secretion is decreased, migration of monocytes and macrophages leads to activation and potentially foreign body giant cell formation [48].

Macrophage polarization is an important component of the inflammatory response that can be impacted by the size, shape, and chemistry of the biomaterial [49]. M1 or classically activated pro-inflammatory macrophages secrete toxic reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α. These cells are the inducer and effector cells in the Th1 type response. In contrast, M2 macrophages activated through the alternative pathway are involved in polarized Th2 reactions and are associated with pro-healing responses. In cutaneous wound healing, the early inflammatory stage of wound healing is dominated by expression of genes associated with M1 macrophages, while the later stages involving tissue remodeling and angiogenesis are associated with mostly M2 genes [50]. The plasticity of macrophages enables a transition between the two phenotypes that is required at a particular time and place in the wound healing response. Fibrosis associated with a protracted inflammatory response can result from dysregulation of macrophage phenotype transitioning.

The inflammatory response associated with implantation of biomaterials and regulated by macrophage phenotype can be modulated by manipulation of chemical and physical material properties. Macroporous biomaterials with pores on the order of 30–40 mm have been associated with minimal fibrosis and high levels of vascularization [51], while non-porous versions of equivalent materials elicit a foreign body response and become encapsulated in fibrosis. In the myocardium of nude rats, acellular scaffolds composed of collagen-modified pHEMA-co-MAA hydrogels were evaluated for fibrous encapsulation and vascularization. In this study, the ratio of M2/M1 macrophages was significantly higher in the group with the highest porosity, with reduced fibrotic response and increased density of neovascularization [52]. In nanofibrous electrospun fiber scaffolds, filament diameter and orientation have also been shown to modulate acute inflammation, with a reduction in foreign body giant cells compared to a film of the same material composition [53]. Similarly, an M2 macrophage phenotype was associated with large or widely separated features on PVDF surface, as the expression of CD163 was significantly enhanced in the presence of a microtextured surface in comparison to a smooth control [54].

The mechanism and extent of inflammation can impact encapsulated cell systems by secreting the so-called cytokine storm, a set of inflammatory cytokines, as well as reactive oxygen species (ROS) such as superoxide, nitric oxide, hydrogen peroxide, and hydroxyl radical that induce oxidative damage [55]. One strategy to prevent oxidative damage is to incorporate a superoxide dismutase (SOD) mimetic into the polymer structure of the immunoisolatory barrier, which can provide protection against ROS injury as an antioxidant [56]. In this work, β-cells incubated in soluble Mn(III) tetrakis[1-(3-acryloxy-propyl)-4-pyridyl] porphyrin, an SOD mimetic, and superoxide was generated by adding xanthine and xanthine oxidase. Based on alamar blue staining, metabolic activity was shown to be increased about fourfold in the presence of the SOD mimetic. Poly(ethylene glycol) diacrylate (PEGDA) was also used to encapsulate MIN-6 cells together with the SOD mimetic, crosslinking with a photoinitiator and 365 nm light. Incorporated at a concentration of 100 μM, the mimetic provided protection against 25, 50, and 100 μM xanthine in a dose-dependent fashion.

A unique approach to counteracting the cytokine cascade is to co-encapsulate an immunomodulatory chemokine together with islets [57]. CXCL12, a CXCR4-binding chemokine, has demonstrated anti-inflammatory suppression of effector T cells in sites of injury. Murine islets incubated in media containing CXCL12 were implanted under the kidney capsule of C57BL/6 mice. The PBS control and 100 ng/mL CXCL12 group showed control of diabetes for only a couple of weeks, while mice implanted with 1 μg/mL CXCL12 were controlled for over a month. CD3+ cells within the graft were reduced by about fourfold in the high-dose CXCL12 group compared to the PBS control. Allo-sensitized NOD/LtJ mice were implanted with murine islets co-encapsulated with 1 μg/mL CXCL12 in ultrapure, low-viscosity alginate high in mannuronic acid content. In comparison to the control group, which demonstrated normoglycemia for about 10 weeks, the group loaded with CXCL12 maintained glucose control for the duration of the experiment, 15

weeks. This was extended to a xenogeneic model wherein porcine islets were implanted into diabetic C57BL/6 mice, demonstrating glucose control for 300 days.

Chronic Failure of Cell Encapsulation Systems

If encapsulated cells are able to withstand the early stresses after implantation, and engraft or exist in a particular anatomical site without encountering destructive fibrosis, the timeline for therapeutic benefit becomes limited by the ability of the delivery system to sustain adequate diffusion and mechanical integrity for the lifespan of the cells. In the case of macrodevice membranes, this requires resistance to protein fouling such that diffusivity is maintained sufficient to support oxygen transport to the cells within. Mechanical integrity of macrodevices, including individual components as well as the seals that hold them together, is another important site of potential failure as the implant encounters physiologic conditions for months or years. Membrane coatings, sealants, and frames are all susceptible to material changes due to potential degradation, leaching of critical excipients, swelling, hydrolysis, or protein and cell deposition. Grossly, a change in the shape or topography of the implant can result in stimulation of an inflammatory event leading to a second wave of acute inflammation. Of particular concern to large-footprint macrodevices, especially those comprised of thin bilayer membrane sections with relatively little mechanical support, is the propensity for such devices to fold, crease, or contract. In surgical sites such as the subcutaneous space, this can be exacerbated by the micromotion-triggered inflammation that is inherent to regions of the body or tissues that incur the most movement [58].

The failure modes highlighted in this section make up a significant amount of the research focus in the field of cell encapsulation. The following sections will describe some of those efforts in the context of both micro- and macroencapsulation systems and whether there is any inherent advantage to using primary tissues compared to stem cell-derived or genetically modified cell lines.

Immune Rejection

Immunoprotective encapsulation systems are based on the premise that permselective membranes can limit or deny the passage of harmful elements of the host into the encapsulated microenvironment, while nutrients and oxygen flow freely. Depending on the size and shape of the solute of interest, their flux can be controlled by altering membrane parameters such as porosity and thickness, as well as the hydrophobicity of the surface, the radius and tortuosity of the pores, and by creating gradients within the membrane [59]. Membranes originally developed for other biological applications such as protein purification have been used in this capacity for decades and has led to the development of a number of materials with so-called immunoisolatory capacity.

Much has been written on the theory of immunoisolation as it applies to cell therapy devices, but many questions remain regarding the relative impact of cellular vs. humoral immune protection. A cellular immune response to live, encapsulated

cells is minimized due to the physical separation of encapsulated cells from the host immune system [60]. But the release of small components of dead or dying cells, or shed antigens, can stimulate such a response. A humoral immune response mediated by immunoglobulin and complement is another likely pathway for immune rejection, although there is some evidence that exclusion of the elements of the complement system is not an absolute requirement as they are inactivated during outward diffusion [61]. For an immunoprotective membrane that excludes antibodies and complement to provide utility in the treatment of T1D, it must allow the outward flux of insulin and cellular waste products and inward flux of glucose and oxygen. Insulin is a 5.8 kDa molecule with a Stokes radius of about 1.35 nm, while IgG is about 5.4 nm in diameter, and elements of the complement system are only slightly larger at about 13 nm [62, 63]. The ability to deliver insulin to the outside freely without diffusion of IgG to the inside requires a very narrow range in which pore size can be optimized.

Diffusion through a membrane by passive processes requires movement of the solute throughout the matrix as dictated by the physical constraints of the membrane (thickness, porosity, tortuosity), the concentration gradient, and the available surface area. Fick's law describes this relationship as shown below, where J = flux, ε m = porosity, C = concentration, D = diffusion coefficient, and τ = tortuosity. Solute flux can be viewed as the absolute performance of a membrane with a given set of characteristics and physical properties and is inherently tied to porosity and tortuosity.

$$J_{eff} = -\varepsilon D_{eff} \nabla C = -\varepsilon \frac{D_o}{\tau} \nabla C$$

Conceptually, a porous structure with pores smaller than 5.4 nm in diameter could provide an immunoisolatory interface. But rather than a monodisperse assembly of transmembrane pores, most membranes contain a range of pore sizes with varying interconnectivity and tortuosity that together govern solute transport. These membranes include materials produced by phase inversion, controlled stretching, and sintering [59]. Phase inversion produces primarily anisotropic membranes from materials like polysulfone, polyethersulfone, cellulose, and others. As a diffusive barrier, such membranes typically achieve permselectivity within a thin region or limiting skin, surrounded by a less dense region that serves as a protective layer (Fig. 2.3). Together, these membrane layers achieve a very high level of porosity, in some cases up to 80%, but usually with very high tortuosity. Controlled stretching, sintering, and electrospinning [64] also produce potentially immunoprotective membranes that are more uniform in character with minimal pore gradients. These structures can also have extremely high porosities but are inherently very tortuous.

The relationship between porous tortuosity and absolute porosity may be the most impactful on defining immunologically relevant molecular weight cutoffs. While the spinning chemistries described above produce a range of pore sizes and pore gradients, other techniques can create a discrete pore size with very minimal tortuosity. Track etching is a process that produces almost perfect cylindrical pores

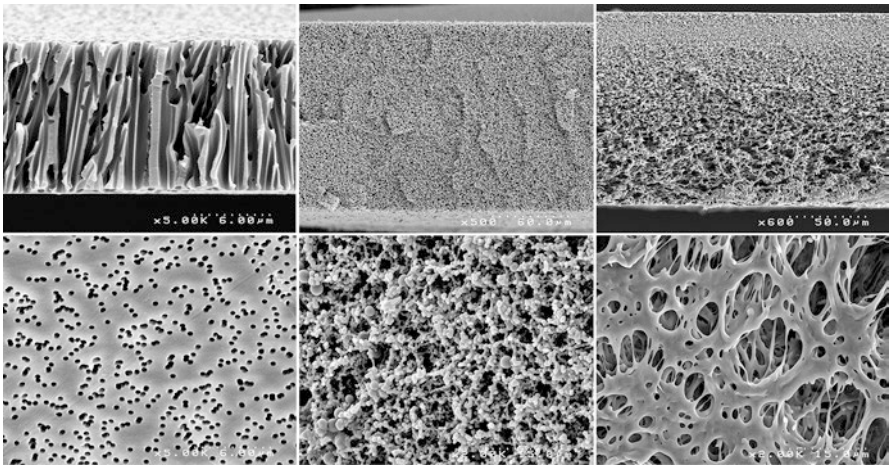

Fig. 2.3 Scanning electron micrographs of membrane materials. (*Left*) *Top*, membrane wall of a track-etched polycarbonate membrane; *bottom*, surface of the same membrane. (*Middle*) *Top*, membrane wall of a commercially available polyvinylidene difluoride membrane; *bottom*, surface view of same membrane. (*Right*) *top*, wall of a poly(ether sulfone) membrane; *bottom*, surface of the same membrane

using a technique developed by GE in the 1960s [65]. The process bombards a polymer thin film, typically polycarbonate or PET, with a collimated beam of high-energy nuclear particles resulting in tracks that are later opened by a heated peroxide or NaOH solution. This type of membrane is theoretically well suited for immunoprotection, as a truly uniform pore distribution [66] can be achieved within a relevant pore size range. The caveat to this approach is that porosity is limited due to the inability to create tracks at very high density without loss of mechanical integrity, with a maximum of about 20–40% depending on pore size, thereby decreasing the resulting flux of the membrane. Similarly, inorganic membranes have been produced with low-tortuosity nanopores from silicon [67], aluminum/aluminum oxide, and titanium/titanium oxide [67]. While these membranes offer an exceptionally narrow pore distribution, less than 5% in some cases [68], at a very minimal thickness, they have varying degrees of biocompatibility that confound their utility. Nanoporous polycaprolactone, a very slowly biodegradable membrane, has been engineered using zinc oxide nanorod assembly to produce pores in the range of 30–100 nm [69]. These membranes would not completely restrict passage of a 5.4 nm IgG molecule, but they are extremely biocompatible, flexible, and could theoretically be produced with much smaller pore sizes. However, the porosity of these membranes is not described and would play an important role in determining diffusive behavior. Membrane coatings have also been used to restrict the diffusion of membranes with large pore size. Expanded polytetrafluoroethylene (ePTFE) membranes with nominal pore size of 400 nm were evaluated for the ability to pass IgG or C1q [70]. When impregnated with ultrapure alginate high in mannuronic acid content and then crosslinked with barium chloride, the diffusion of IgG and

C1q was reduced to 0.5% after 20 h of incubation, while the diffusion coefficient of insulin only decreased from 2.38×10^{-7} cm^2/s in the untreated membrane to 1.11×10^{-7} cm^2/s for the alginate-impregnated membrane.

Alginate as a microencapsulation system has also demonstrated permselective properties depending on the type of alginate employed, the crosslinking agent, and whether or not there is an intermediate polycation [71]. Alginate capsules made by the traditional A-P-A process (alginate-polycation-alginate) were compared to alginate capsules that were simply crosslinked with barium chloride and left as solid hydrogel spheres [72]. In this study, encapsulated rat islets were transplanted into the peritoneal cavity of diabetic rats and evaluated for glycemic control over the course of months in comparison to renal subcapsular implants. In both cases, the encapsulated allogeneic transplants corrected diabetes more quickly than the unencapsulated control. However, both capsule formulations were associated with kinetic delays in vitro and in vivo suggesting that significant diffusion limitations prevented adequate transport. Alginate high in mannuronic residues is also associated with greater diffusivity than capsules made from alginate containing primarily guluronic acid due to the reduction in available crosslinking sites present in G-blocks [73]. In an experiment comparing APA capsules fabricated using the same polyornithine intermediate layer and the same crosslinking agents, only the base alginate material was varied by selecting a high-M Keltone alginate or a high-G Novamatrix alginate. The release of a 20 kDa FITC-dextran marker was about only 8% higher in the high-M group than the high-G group, and the molecular weight cutoff values were very similar at 165 kDa for the high-M group and 156 kDa for the high-G group. This illustrates that in the presence of a polycation, the diffusive control of the capsule formulation is dominated by the ability of the polycation to intercalate within the inner and outer layers of alginate. In comparison to alginate slabs crosslinked with only calcium, the diffusion coefficient of bovine serum albumen was several log orders of magnitude faster (2×10^{-6} cm^2/s vs. 1×10^{-10} cm^2/s).

Hypoxia

Pancreatic islets are organized in a trilaminate structure with vessels that circulate along both of its sides, providing access to oxygen within a distance of 2–3 cells for any given β-cell within the islet [74]. This translates into higher oxygen exposure for β-cells in comparison to other cell types in the body during development and in the adult pancreas. As a result, β-cells are extremely reliant on oxygen for function. The transition from the intact pancreas to isolated islets involves significant stress and disruption of the organization that is present when residing in the pancreas. All functional vascular supply is lost, and the impact on islet structure is primarily one of diffusion constraints, as the diffusion distance to the cells in the core increases from about 2–3 cells to about 15 cells. Hypoxic centers can be visualized in isolated islets in culture within a matter of days [39], and in the context of encapsulation systems, diffusion distances and consumptive demand are increased significantly. As described previously, about a cubic centimeter of solid tissue is required for human therapy. To organize such a tissue mass in a way that circumvents both diffusion limitations and the consumptive load of the β-cell mass, e.g., the cumulative

amount of oxygen that is consumed in relation to the amount of oxygen that is present requires a reduction in the packing density, thickness, and the materials in the encapsulation system must not inhibit transport of oxygen.

The threshold for islet hypoxia may be much lower than for glucose-stimulated insulin secretion, indicating that islets could potentially be transplanted and remain viable without providing function. This oxygen concentration threshold may be as low as 10 mmHg for function and 0.1–0.44 mmHg for viability [75, 76]. In vitro, second-phase insulin secretion was reduced at only 60 mmHg, and the total secretion rate was reduced by 50% at 27 mmHg [77]. In a human islet with about 1560 cells and a diameter of about 150 μm [78], the outer surface would need a pO_2 of about 35–40 mmHg to keep even the innermost cells alive and about 45–50 mmHg to keep those same cells functional [75].

The partial pressure of oxygen in the transplant site can be variable depending on the disease state, the anatomical location, and the extent of injury incurred during the implantation process. In mice maintained under normal ambient conditions with 20% oxygen, tissue oxygen partial pressure varied from about 50 mmHg in fat, 26 mmHg in muscle, 27 mmHg in liver, 25 mmHg in kidney, and 42 mmHg in the subcutaneous space [79]. The oxygen partial pressure of the peritoneal cavity is about 40 mmHg. Given the published threshold for an islet to function of about 45–50 mmHg, and the increased diffusion distance and decreased diffusivity when on the other side of a permselective membrane, none of these transplant sites would seemingly be able to support a biomass of any significant size without a supplemental oxygenation strategy.

The oxygen that diffuses into encapsulation devices is quickly consumed by the viable biomass that remains. As β-cells consume more oxygen than most other cell types including tumor cells [80], the consumptive load of encapsulated islets is compounded by the absolute number of cells required, presenting a serious obstacle to longevity. The oxygen consumption rate (OCR) of human islets has been characterized extensively and has recently been retrospectively correlated with clinical trial outcomes [81]. In patients with severe pancreatitis that received total pancreatectomy and intraportal autotransplantation, dosing by total islet equivalents (IE dose) was compared to dosing by viable islet equivalents as inferred by measuring oxygen consumption rate (OCR dose). In this analysis, both metrics correlated well with insulin independence and OCR doses greater than 150 nmol O_2/min*mg DNA supported previous studies in mice suggestive of a functional dose threshold [82, 83].

Resting islets consume less oxygen than islets stimulated with glucose, which triggers ATP-dependent insulin secretion and significantly higher mitochondrial respiration [84]. Rat islets exposed to 3 mM glucose followed by 20 mM glucose showed a roughly sixfold increase in OCR upon glucose stimulation, increasing to about 0.25 nmol/min/100 islets. Similarly, nonhuman primate islets were evaluated in microplate-based assay (BD Biosciences) using an oxygen-sensitive fluorescent dye. Compared to a basal OCR of about 2.7×10^4 fmol/min, glucose-stimulated

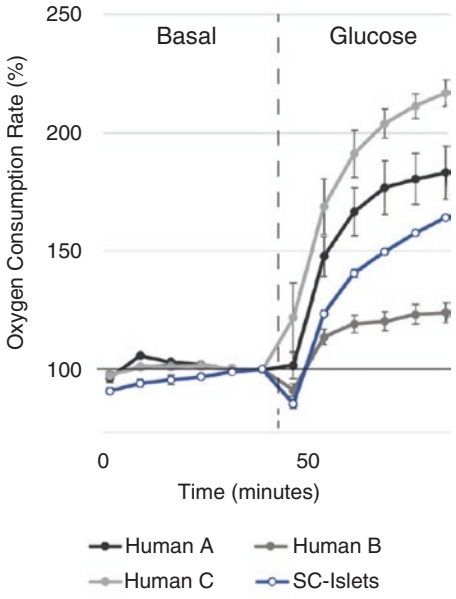

Fig. 2.4 Oxygen consumption rate (OCR) of human or stem cell-derived islets as a function of glucose stimulus. *Y*-axis represents OCR values normalized by basal levels (Image courtesy of Semma Therapeutics)

NHP islets increased to about 1.1×10^5 fmol/min in 16.7 mM glucose. In the same study, the group established that human islets secreted insulin at a similar magnitude as they consumed oxygen. In 5.6, 16.7, and 33.3 mM glucose solutions, OCR increased from about 2.5×10^4 to 5×10^4 to 5.8×10^4 fmol/min/100 IEQ. Accordingly, insulin secretion increased from about 600 to 900 to 1000 ng/mL/100 IEQ. A linear correlation was calculated with $P < 0.01$, highlighting the critical dependence of insulin secretion on oxygen consumption.

Human islets as well as stem cell-derived islets show similar behavior as demonstrated by the Seahorse extracellular flux technique (Fig. 2.4). Here, cells are exposed to 2.8 mM glucose for roughly 40 min and then the solutions are spiked to 20 mM glucose, leading to a 50–220% increase in OCR values. Three separate human preparations are shown to highlight the inherent variability of primary cell preparations. The interdependence of insulin secretion on oxygen consumption illustrates the importance of oxygen supply in encapsulation systems. While a viable cell may consume 150 nmol O_2/min*mg DNA, a functioning cell consumes 300 nmol O_2/min*mg DNA under peak load. As an encapsulated biomass of about 800,000 islets as in the CIT-07 trial, or about a billion cells (roughly 6.6 mg DNA based on 6.6 pg per diploid cell [85]), the graft would need about 2 µmol/min to support insulin secretion. In comparison, 1 mL of peritoneal fluid at an oxygen partial pressure of 40 mmHg only contains about 0.056 µmol oxygen or roughly 3% of the amount needed to support this function for a single minute.

Strategies for Addressing Hypoxia

Refillable Gas Supply

Because of this serious oxygen limitation , several strategies are in development to support encapsulated cells with oxygen from an external source. Beta-O_2 has developed a macrodevice built around a central oxygen chamber wherein patients can replenish the device's oxygen supply percutaneously by injecting oxygen daily into a small port [80]. The islets are contained within an alginate matrix 600 μm in depth at the surface of the device, separated from the internal oxygen chamber by a silicone rubber membrane (Fig. 2.5) that provides a gas permeable barrier between the central cavity and the cell chambers that flank it. This creates a gradient of oxygen concentration to develop from the supply (95% oxygen) to the cell chamber (~77%). The device offers immunoprotection by incorporating an ePTFE membrane with 400 nm pores that are impregnated with a highly crosslinked high-M alginate, resulting in significant reduction of IgG and complement diffusion [70].

The device is designed for hyperoxic conditions (<550 Torr) to support packing densities as high as 3600 IEQ/cm^2, or 11% V/V, and has progressed through rodent prototypes to pig and human sizes [80]. The device design process made extensive use of mathematical modeling, both for evaluating the geometric requirements of the cell cavity and the pressure and concentration of the supplied oxygen gas. The result was that a 95% oxygen mixture could be administered into the chamber at a pressure of 1000 Torr, but the multi-chamber design of the device, separated by silicone membranes, maintained the critical pO_2 of less than 550 Torr and greater than 100 Torr. In studies using rat islets implanted into diabetic Sinclair pigs, glucose levels were controlled for 75 days with an initial dose of about 6700 IEQ/kg. But as these pigs grew quickly, glycemic control was lost when weight gain was sufficient to bring the dose below 4000 IEQ/kg [70]. A similar sized device was also evaluated

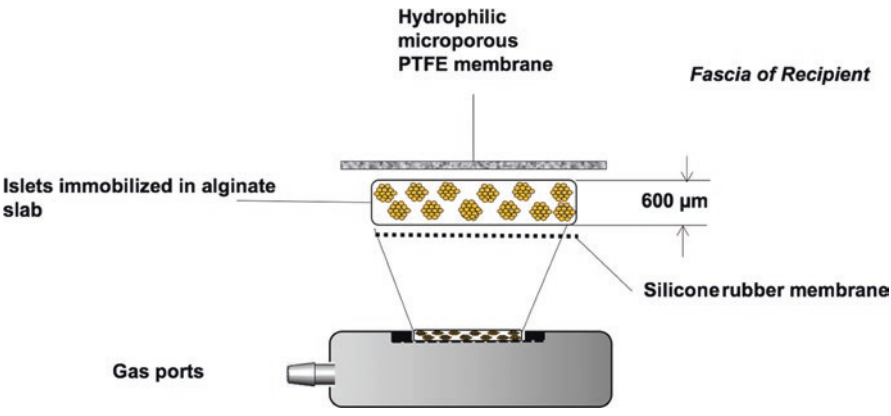

Fig. 2.5 β-Air oxygenated macroencapsulation device developed by Beta-O_2 (Image courtesy of Avi Rotem, Beta-O_2)

in human clinical trials using a sub-therapeutic dose of human islets (2100 IEQ/kg). After 10 months, this marginal mass resulted in a reduction of exogenous insulin requirements of 15% and a reduction in HbA1c from 7.4% to 6.4%. [86]. The device is currently in clinical evaluation in a pilot trial (NCT02064309), and a next-generation device is in development that requires less frequent oxygen refilling and that has a larger cell capacity [87].

Artificial Oxygen Carriers

The use of artificial oxygen carriers (AOCs) in microencapsulation systems has been explored as a means of improving the diffusion of oxygen in alginate systems. AOCs bind, transport, and release oxygen into the target site. Perfluorcarbons have been studied in this role since the 1960s but their toxicity profile includes prolonged presence in the reticuloendothelial system and is associated with chemical pneumonitis [88]. Perfluorocarbon products have been approved by the FDA but their use was discontinued in 1994 [89]. Still, AOCs represent a chemical means by which an oxygenation solution could be incorporated into encapsulation devices. Perfluorocarbon (PFD) AOCs have been evaluated as a 400 nm emulsion making up 36% V/V of an alginate capsule formulation crosslinked with barium [90]. In these studies, capsules containing 2.4×10^7 cells/mL were prepared in alginate and compared to naked islets and alginate PFC microcapsules. The diffusion coefficient of oxygen in individual and combined systems was calculated, highlighting that PFC increased diffusion from 2.77×10^{-6} cm^2/s for 2% V/V alginate to 9.36×10^{-6} cm^2/s in a 70% emulsion with PFC. After 2 days in culture in low oxygen (3%), tissue recovery in the PFC formulation was significantly lower than in the alginate capsule control. OCR recovery showed no difference with or without the incorporation of PFC. Indeed in this study, histology revealed that there was minor toxicity of the PFC component to the islets.

Rather than using an emulsion, PFC has also been grafted directly onto alginate using a PEG-amide linker [91]. Alginate beads were made at 1.6% W/V and crosslinked with barium, but the gelation process was inhibited at higher PFC levels. Oxygen diffusion was increased in the PFC-grafted alginate by about 10% with a PFC loading of 0.05% W/V, demonstrating its potential in this application. Encapsulation of MIN-6 cells showed that the material had a high level of biocompatibility, with increases in cell number by MTT assay compared to the alginate control after a week.

Oxygen-generating materials have recently been evaluated as a means of introducing oxygen into the internal volume of encapsulation systems. These materials can be particularly useful in device strategies that rely on vascularization to support the oxygen demands of the encapsulated cells. As the vascularization process can take days or weeks to reach steady state, the encapsulated biomass must survive hypoxic conditions for at least some period of time, almost certainly in conditions that are not ideal for function. Oxygen-generating materials provide a potential short-term oxygen supply to overcome the immediate posttransplant period wherein

hypoxia is most extreme. Calcium peroxide (CP) has been used in this role as it can readily dissociate into oxygen and calcium hydroxide with the introduction of water [92]. One unique approach is to incorporate CP particulate into a polymer solution prior to casting of a membrane or cell scaffold. Scaffolds comprised of CP impregnated in polycaprolactone were fabricated by an electrospinning process [93]. In this work, scaffolds were generated with up to 10% CP which demonstrated antimicrobial activity in cultures of gram-negative and gram-positive bacteria. Osteoclasts cultured on these fibers, however, showed some evidence of cytotoxicity. CP was also incorporated into poly(D,L-lactide-co-glycolide) (PLGA) scaffolds designed to release oxygen for up to 10 days [94], a critical timepoint in the immediate post-implant period prior to engraftment. The PLGA-CP scaffolds were produced to be highly porous by incorporating paraffin particles, followed by removal in hexane. Scaffolds measuring 10 mm × 4 mm were maintained in hypoxic conditions at 1% oxygen, and generated oxygen was measured over 10 days in media supernatant, which maintained slightly higher oxygen levels than the scaffold controls (roughly 6 mmHg vs. 7 mmHg). NIH 3T3 fibroblasts seeded onto the scaffolds showed markedly improved survival after 2 weeks in culture in comparison to unmodified PLGA scaffolds. CP has recently been evaluated in an islet scaffold fabricated from porous PDMS [95]. In this work, the use of a very hydrophobic PDMS matrix was hypothesized to reduce the rate at which CP was converted to oxygen. The study showed that over 3–4 weeks of oxygen generation above 0.05 mol/m^3 was possible, and in culture with MIN6 cells, PDMS-CP scaffolds rescued metabolic function in hypoxic conditions of 0.01 mM oxygen for 24 h. To demonstrate long-term utility, MIN6 cells were cultured for 3 weeks with or without PDMS-CP scaffolds. Both metabolic activity and recovered DNA were significantly higher in the group containing CP, indicating that oxygen generation by this method was supportive and nontoxic. Finally, MIN6 viability in both normoxic and hypoxic conditions was shown to be enhanced in the presence of CP at a cell loading of 250,000 cells per disc, whereas loading of 50,000 and 150,000 cells were associated with an equal or detrimental effect when compared to the PDMS controls, indicating a dose-dependency between the number of β-cells and the amount of CP.

Alginate microcapsules containing CP or sodium percarbonate (SP) has been produced by doping alginate solutions with oxygen-generating particulates [96]. SP particulates with average diameter of 330 μm and CP particles with average diameter of about 3 μm were incorporated into alginate solutions at a loading of 10 mg/mL with an islet loading of 5000 IEQ/mL. The droplet formation and coating processes were carried out at 4 °C to avoid premature degradation of SP particulates. Since alginate is readily crosslinked with divalent cations, calcium liberated during the dissolution of CP can accelerate gelation kinetics. The authors state that both of these formulations protect encapsulated islets from hypoxic conditions and reduce damage from oxidative stress.

Another strategy for oxygenated cell delivery is based on a core-shell microparticle containing peroxide in the core and catalase, an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen, within the outer shell [97]. In vitro, oxygen was generated by the 3.5 μm microparticles at a concentration

of 1 mg/mL for about 12 days before reaching the level of the PBS control at day 14 (0.83–0.06 mg/L O_2). In hypoxic conditions, MIN6 cells cultured with the peroxide/catalase microparticles showed a statistically significant decrease in the presence of lactate dehydrogenase in the culture medium, indicating that the cells maintained higher levels of viability than the microparticle-free control. Additionally, cultures with microparticles showed no evidence of HIF-1α translocation to the nucleus in comparison to controls. In VEGF-containing collagen scaffolds, rat islets co-loaded with oxygen-generating particles showed improved function for 30 days in comparison to naked islets and other combination matrices in alloxan-induced mice.

These temporary oxygenation strategies have merit in device designs that rely on vascularization for chronic oxygen supply to encapsulated islets. The device design and vascularization strategy can have a tremendous impact on the rate at which functional vascularization becomes available to support the cells, but incorporating a bridging strategy using the techniques described may provide a higher level of viability once a vascular network is established. This critical post-operative period may be the decisive factor not only in determining the functional capacity of the device but also by avoiding acute hypoxia-related cell death; there is additional benefit in mitigating shed antigen release and a potentially catastrophic immune response against the device. The next section will highlight several vascularization strategies that have been used to promote angiogenic support both for oxygen supply and as an interface for glucose sensing and insulin diffusion.

Vascularization

Bringing vessels from the host to the surface or around a device is an oxygenation strategy that does not necessarily require additional biologic molecules and that provides the added benefit of more direct access to the blood supply for glucose sensing and insulin secretion. The same kinetics that govern the diffusion of oxygen from hemoglobin within capillary red blood cells to tissue parenchyma would be applicable to capillary or arteriolar networks that form within or around devices. This exchange occurs between any two regions in which a P_{O2} difference exists [98]. If the device is vascularized with functional arterioles, oxygen transport proceeds in a convective manner defined by the concentration of oxygen in the arteriole as shown in the equation below, where R = radius, v = velocity, and S = saturation.

$$Q_{O_2} = \pi R^2 v S_{O_2} \left[Hb\right] C_{Hb}$$

Capillary exchange of oxygen follows diffusive kinetics as governed by Fick's first law:

$$\frac{\Delta N}{\Delta t} = DA\Delta\left(\alpha P_{O_2}\right) / \Delta x$$

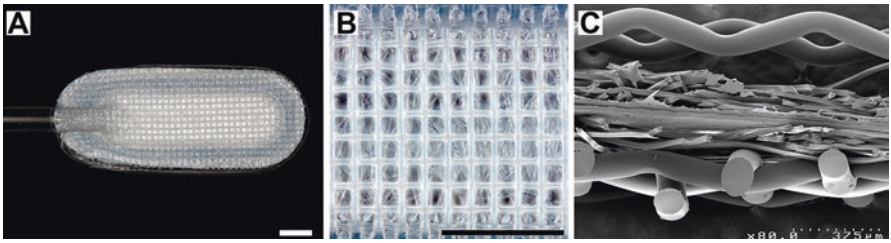

Fig. 2.6 (**a**) Low magnification and (**b**) high magnification of a rodent Theracyte device. Both **a** and **b** have scale bars that are equivalent to 2 mm in length. (**c**) Cross-section of a Theracyte device showing the outer polyester mesh, separated by small filaments from the inner ePTFE membranes

In diffusive transport, the extent of vascularization of the device and the density of capillaries within the vascularized tissue can impact kinetics by increasing the area (A) and distance (x) for diffusion [99].

One of the most thoroughly studied macrodevices, the Theracyte device, employs this strategy by incorporating multiple layers of engrafting material that stimulate ingrowth and vascularization [100]. The layered device is shown in Fig. 2.6 and comprises an outer polyester mesh, a smaller filament polyester mesh, an ePTFE membrane with internodal distance of 5 μm, and an interior 0.4 μm ePTFE membrane. The assembly is sealed by ultrasonic welding and connected to a rigid polyethylene port for loading cells. Early in development of the Theracyte, a number of membrane candidates were evaluated in the rat subcutaneous space for the presence of a foreign body reaction and vascularization. The materials that were evaluated included a number of cellulosic membranes with pore size ranging from 0.8 to 8.0 μm, as well as polyester and ePTFE materials. Notably, 10 μm nylon membranes and 1–12 μm polycarbonate membranes were not associated with the formation of new vessels in proximity to the test materials. The group hypothesized that the large features of the nonvascularizing membranes enables cells from the host to attach and flatten out, leading to a classic foreign body response. In comparison, vascularizing membranes were thought to allow the cells to exist in a rounded configuration resulted in the release of pro-angiogenic factors.

Since this early work, the Theracyte device has been evaluated in a variety of cell encapsulation studies and has advanced through clinical design in several efforts to treat type 1 diabetes. To evaluate the transport properties of the device over the course of an implantation period, Theracyte devices were loaded with insulin and implanted in the subcutaneous space of rats. Using microdialysis sampling, the device was confirmed to deliver an equivalent dose to a subcutaneous injection, indicating that the membranes used in the device did not negatively impact insulin transport. The device was evaluated after 1, 2, 4, and 12 weeks following transplantation, demonstrating that as vascularization of the device progressed, the concentration of insulin in the serum was significantly higher in devices showing high vascular density within 15 μm of the device [101]. Vascular density in this study was highest after 12 weeks. In the same model, the permeability of glucose was

measured in the device using the microdialysis technique in subcutaneously implanted rats [102]. These results also demonstrated that transport kinetics were retarded at all timepoints except for 12 weeks, which showed glucose concentration equivalent to the control animals. Finally, the group evaluated microcirculation around the device by introducing a laser Doppler probe through the loading port [103]. Flow was analyzed over the course of 3 months and was found to decrease significantly between day 1 and 4 weeks, from 158 to 72 perfusion units. This increased by 2 months to 138 PU and reached steady state after 3 months at 165 PU, again highlighting that the Theracyte device implanted subcutaneously may have relatively poor transport kinetics within the first months after implantation.

Based on these findings, several groups have explored the possibility of prevascularizing a device or a transplant site prior to introduction of the cells. This would allow for a transplant environment that is higher in oxygen supply and that would permit diffusion more freely at the time the cells are implanted into the host. Preimplantation of the Theractye device was evaluated in Sprague-Dawley rats for 3 months prior to introduction of allogeneic islets [104]. Subcutaneous devices were accessed and filled with 1500 rat islets via the device port after 3 months, and filled devices were implanted contralaterally to serve as a prevascularization control. Devices explanted after 2 weeks revealed that preimplantation was associated with a 40% reduction in the amount of fibrotic tissue contained in the device with almost twice as many insulin-positive β-cells. Similarly, cure rates of preimplanted Theracyte devices in diabetic athymic mice were significantly higher than devices that were freshly implanted, with 100% of preimplanted animals reaching euglycemia compared to only 17% in the group implanted with preloaded devices [105].

Syngeneic pancreatic islets were implanted into a non-immunoprotective, prevascularized chamber device that was allowed to reside in the subcutaneous space for 40 days [106]. The device is comprised of a stainless steel mesh that is filled with a PTFE insert that can be removed after sufficient ingrowth occurs. The plunger was removed, and 3000 syngeneic rat islets were administered in saline solution, followed by closure of the device with a threaded PTFE seal. Seven out of the eight recipients achieved diabetes reversal within 6 days, in comparison to a cohort infused via the portal vein which showed reversal after 1 day. Both treatment groups demonstrated efficacy for 160 days. A comparison of insulin kinetics was conducted after 160 days by performing an intravenous glucose tolerance test. In this evaluation, islets contained in the device were about 30 min slower than islets in the liver to reach blood glucose levels less than 200 mg/dL.

Another approach that does not involve an indwelling device is to create a potential space, or "device-less" site that can act as a vascularized bed for cell or device implantation [34, 107, 108]. One iteration of this strategy involves implantation of a vascular catheter 4 weeks prior to transplantation, creating a vascularizing lumen into which islets are injected. In C57BL/6 mice induced with streptozotocin, a marginal mass of 150 syngeneic islets with 90% purity was implanted under the kidney capsule, in an unmodified subcutaneous space, or in the device-less site. Islets transplanted under the kidney capsule yielded euglycemia after about 18 days (94%), while the group implanted into the device-less site corrected glucose by day 34

(72%). Despite the delay, the grafts showed similar efficacy in glucose correction until they were removed after 100 days. Transplants into the unmodified subcutaneous site were not successful at correcting hyperglycemia. This approach certainly shows a potential benefit for naked cell injection and may be amenable to implantation of micro- or macroencapsulation modalities within a pre-vascularized site to improve acute oxygenation and transport properties.

Strategies for Mitigating Chronic Fibrosis

Coatings and Covalent Modifications

The foreign body response to biomaterials is an encapsulation failure mode that can, on its own, lead to a nearly impermeable, avascular granulomatous sheath surrounding the diffusive surface of devices effectively sealing it off from the host [109]. In a more moderate context, the biomaterial selected for an encapsulation system can act as an adjuvant to sway a potentially steady-state engraftment into irrecoverable inflammation. While the previous section on vascularizing strategies seeks to bring host tissue into or around the device, the ramification of doing that could very well lead to terminal fibrosis. Theracyte devices loaded with cadaveric parathyroid tissue were evaluated in nonimmunosuppressed humans for up to 14 months [110]. In this study, a cohort of patients undergoing parathyroidectomy were re-implanted with their own autologous tissue in Theracyte devices, while other patients with chronic hypoparathyroidism were implanted with allogeneic parathyroid loaded into Theracyte devices. Autologous transplants contained 22% endocrine tissue after up to 14 months and 63% fibrosis and about 15% was completely necrotic. In comparison, the explanted volume of devices loaded with allogeneic cells was dominated by fibrosis after only 4 weeks, with endocrine tissue accounting for only 1%, 5%, and 23% by volume. In all cases, there was no detectable increase in parathyroid hormone (PTH) level, potentially due to marked fibroblast overgrowth resulting from inflammation or an immune response.

Both micro- and macroencapsulation systems are susceptible to fibrotic encapsulation. A series of different sized spherical materials composed of hydrogels, metals, glass, and polymers was evaluated in C57BL/6 mice to evaluate biomaterial-specific foreign body responses. All of the materials were very smooth with no surface features greater than 1 μm. In surface area-matched doses implanted in the peritoneal cavity for 14 days, it was observed that spheres of 500 μm in diameter produced a significantly higher fibrotic response, while 1-mm diameter materials were associated with only very thin, mild responses [111]. The phenomenon was not altered at very long timepoints, as alginate spheres composed of SLG20 displayed this size-dependent fibrosis after 6 months when comparing 0.3-mm diameter spheres, which were significantly fibrosed, to 1.5-mm spheres, which were relatively free of cellular attachment. This observation was extended to the ability of alginate-encapsulated rat islets to cure streptozotocin-induced diabetic mice, where the cure rate of 1.5-mm capsules was maintained at 100% for about 120 days,

compared to 0% at the same timepoint for 500 μm capsules. The size dependency of foreign body response observed in these studies has important ramifications for micro- and macrodevice designs, as the features integral to both (capsule diameter, mesh spacing) may be optimized by geometry alone.

A combinatorial library of molecules was evaluated in a similar manner attached to a low molecular weight, ultrapure high-G alginate [112]. The library was generated based on the presence of amines, alcohols, azides, and alkynes in candidate materials that were evaluated for feasibility based on gelation kinetics and acute inflammation. Of these candidates, 16 were formulated into barium-alginate microspheres with diameters of 300–350 μm, within the size range discussed previously for propensity toward a foreign body response. Compared to unmodified alginate microcapsules, which displayed significant deposition of macrophages and myofibroblasts, the alginates modified with triazole functionality showed little to no presence of an inflammatory response. After 14 days in the peritoneal cavity, three candidate formulations were identified by FACS that were sufficiently biocompatible in mice that they warranted investigation in a nonhuman primate. In primates, the larger 1.5 mm alginate spheres made from unmodified material did lead to fibrosis, displaying fibrotic overgrowth and dense myofibroblast coverage. Three modified candidate materials that were also evaluated in primates showed little to no fibrosis. A lead candidate was identified that was evaluated as a potential encapsulation system for stem cell-derived β-cells (SC-Islets) in immune-competent diabetic mice [113]. In these studies, three densities of SC-Islets were encapsulated in an alginate modified with thiomorpholine dioxide (TMTD). As demonstrated previously, SC-Islets encapsulated in 1.5-mm TMTD-alginate spheres showed enhanced performance compared to 0.5-mm spheres, which controlled glucose for only 15 days at the highest density. The 1.5-mm TMTD-alginate spheres controlled glucose for over 70 days at each of the densities tested, with much higher levels of human C-peptide.

Nanolayer shells of polyphenolic antioxidant materials are also being investigated as dual antioxidant and immunosuppressive ancillary encapsulation systems capable of reducing fibrosis [114]. The core-shell particles are produced by depositing hydrogen-bonded multilayers of polyphenol, poly(methacrylic acid) along with amino-containing poly(*N*-vinylpyrrolidone), or poly(*N*-vinylcaprolactam) onto silica particles. After deposition of four layers, the core can be removed, resulting in a hollow shell that can be coadministered with any number of encapsulation systems. The shells were shown to scavenge ROS generated by stimulation of OT-II splenic T cells with phorbol 12-myristate 12-acetate and ionomycin. TA shells were also effective in reducing the production of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-2. Other polyphenolic compounds are in development for anti-fibrotic strategies, including the flavonoids taxifolin and quercitrin [115]. In an in vitro model comprised of human mesenchymal stem cells and human gingival fibroblasts, flavonoid-modified titanium surfaces demonstrated anti-fibrotic potential and increased differentiation of MSCs into osteoblasts.

Zwitterionic coatings have recently been developed as an alternative to traditional brush polymers, which are susceptible to coating loss in high shear

applications [116]. In general, hydrophilic coatings that are required on hydrophobic biomaterial substrates and permselective membranes can be eroded over time, gradually increasing the exposure of the hydrophobic material and the likelihood of membrane de-wetting. A zwitterionic macrocrosslinker has been developed as a polyurethane-grafted coating to provide better stability and long-term anti-fouling properties. In comparison to brush coatings, the addition of the macrocrosslinker retained the same low contact angle for up to 2 weeks in a flow cell, compared to only several days for the individual components. Protein fouling was also reduced by about sixfold. This type of coating strategy could be employed in many types of macroencapsulation devices, not only potentially improving anti-fibrotic properties but potentially improving the rate and stability of insulin and glucose transport kinetics.

Stem Cell-Derived B-Cells as an Alternative to Isolated Cadaveric Islets

In addition to the challenge of immunoprotection, cadaveric islet transplantation is limited by the scarcity of high-quality and consistent islet material. Both cause of death and islet isolation procedures and culture can negatively impact the performance of the tissue. Donor-to-donor variability is very high and has confounded development of clinical therapies. Thus, an alternative source has long been sought. Pluripotent embryonic stem cells (ESC), which were first isolated from human tissue in 1998 [117], are capable of expanding for dozens of generations and generating virtually all tissues of the body. This latter feature has meant that pluripotent stem cells could theoretically become the starting material for nearly infinite supplies of stem cell-derived β-cells to replace cadaveric islets as a transplantation source.

Although the idea was an obvious one, the path to achieve this goal has not been straightforward or rapid. In fact, since the derivation of the first ESC lines in 1998, it took 7 years to develop a robust method for inducing these cells to efficiently differentiate in the first step into endoderm tissue, based on molecular signals observed in the early development of model organisms like frogs, fish, and mice [118, 119]. This is the very first fate decision for ESCs, choosing between the three germ layers—ectoderm, mesoderm, or endoderm. The endodermal lineage forms the organs of the gut tube including the pancreas.

Generation of Glucoresponsive Stem Cell-Derived B-Cells

Following induction of the endodermal lineage, cells must be sequentially directed to differentiate into pancreatic specific progenitor cells [120, 121]. These cells could not be further differentiated to β-cells in vitro at the time, but they could be transplanted into rodents and over the course of several months undergo further differentiation in vivo to generate islet cell types, including a small population of β-cells, as

well as pancreatic ductal tissue. Though this in vivo differentiation process remains largely a black box even today, the fact that even some β-cells can be generated via this approach prompted Viacyte to proceed to clinical trials using stem cell-derived pancreatic progenitors in a macroencapsulation device [122]. One key challenge in the pancreatic progenitor step is to avoid the generation of progenitor cells for adjacent organ types along the gut tube, like the intestine or liver. Organ development and differentiation make repeated use of the same sets of signaling pathways, like retinoic acid or bone morphogenetic protein (BMP) pathway signaling [123]. The key to developing directed differentiation protocols is identifying precisely the right factors in the right combination for the right duration in the right sequence. This combinatorial challenge becomes even more difficult in the later stages of directed differentiation where the pathways and expression patterns in mice and humans diverge. Thus the last steps of directed differentiation from pancreatic progenitor cells to glucose-responsive insulin-secreting cells were particularly challenging and required empirical screening approaches. As a result, it took another 8 years following the first report of efficient stem cell-derived pancreatic progenitors in 2006 to the first reports of stem cell-derived β-cells (or β-like cells, SC-β-cells) in 2014 [124–126]. Today, these SC-β-cells (Fig. 2.7) are being developed by Semma Therapeutics.

These studies directed the differentiation of ESCs into pancreatic progenitors and then, using a combination of signaling pathways, induced those cells to differentiate into glucose-responsive insulin-secreting cells. A common theme of signaling pathways used for the final induction of β-cells was the combination of thyroid hormone signaling and TGF-β inhibition. The identification of this specific combination of signaling pathways was the result of empirical screening of many different pathways. Additional molecules have been added in certain protocols and cell lines to enhance maturation further including the calcium channel agonist BayK [126] and *N*-cysteine and AXL inhibition [125].

The most important feature of the cells produced by these techniques is their ability, for the first time, to secrete insulin in response to a glucose challenge. The generation of glucose-responsive insulin-secreting β-cells from stem cells has been a holy grail for this field for at least two decades. Importantly these cells

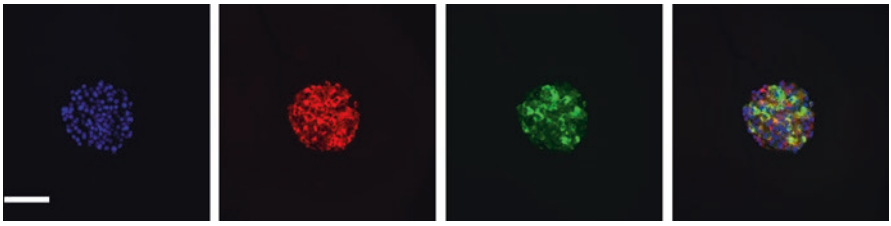

Fig. 2.7 SC-Islets produced by Semma Therapeutics. The *blue* signal is DAPI, staining nuclei. The *red* signal is chromogranin A, staining endocrine cells of the islet. The *green* signal is c-peptide, staining insulin-producing SC-beta cells. Scale bar = 100 μm (Image courtesy of Yeh-Chuin Poh, Semma Therapeutics)

demonstrated this functional capacity both in vitro and in vivo, including the ability to provide glycemic control in diabetic animals. These achievements represent a turning point for the field in terms of its ability to generate islet cell types useful for drug screening, disease modeling, and ultimately cell therapy. Although this achievement was indisputably a major breakthrough, there remains a significant capacity for improvement in the efficiency of the protocols, the transferability across cell lines, and the ultimate functionality of the cells both in magnitude of insulin secreted and in reproducibility of the stimulation index (ratio of insulin secreted at high vs low glucose). In particular, in all cases published to date, these metrics for functionality were measurably less than for cadaveric human islets.

A second common theme of the published SC-β-cells is the expression of a number of β-cell-specific transcription factors, like Pdx1 and Nkx6.1, but not identical gene expression patterns to human islet β-cells. Some of these gene expression differences may be related to issues of genetic background or cause of death and methods of processing for the human islets. Alternatively, some of these gene and protein expression differences likely represent gaps in the complete differentiation or maturation of β-cells derived through an accelerated developmental process performed in the dish. Finally, most comparisons are made between SC-β-cells and adult human β-cells in cadaveric islets. A more appropriate comparison may be of neonatal or juvenile human islets, which may be closer in type and function to newly derived stem cell-derived islets. Nonetheless, ongoing and future improvements to protocols will likely move the expression patterns of these cells closer to that of endogenous tissue, whether adult or juvenile.

Clinical Translation of Stem Cell-Derived β-Cells

An obvious application of these newly described SC-β-cells is in cell transplantation for type 1 (or insulin-dependent type 2) diabetes. Given the ability of starting with a single pluripotent stem cell line of virtually unlimited expansion capacity, it should be possible to generate nearly unlimited supplies of standardized and qualified SC-β-cells for cell therapy. However, several key challenges exist in translating these initial academic discoveries into therapeutic candidates. These challenges include identifying clinically suitable starting cells lines and enhancing the differentiation protocols to produce cell product with appropriate composition, purity, and activity to move into clinical trials.

The choice of clinically suitable cell lines starts first with the identification of pluripotent cell lines that have been consented, documented, and derived in a manner consistent with FDA guidelines and preferably under xeno-free conditions. In addition, SC-β-cells have been generated from both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC). ESC lines are derived from discarded blastocysts from in vitro fertilization clinics [117], and iPSC lines are derived from somatic tissue, like skin fibroblasts or peripheral blood, using the transient induction of exogenous pluripotency genes [127]. Although a number of trials are ongoing in the United States using ESC lines as starting material [128], none have begun

yet with iPSC lines in part due to the relative newness of the technology and in part due to technical questions related to the potential for acquisition of genetic mutations during reprogramming or the retention of exogenous reprogramming material. However, the first trial of an iPSC-derived product has started in Japan [129], and others are likely to follow. In addition to regulatory considerations and the choice of ESC vs iPSC, the most important features of a suitable line are its ability to expand through sufficient passages during manufacturing while maintaining phenotype and genotype and its ability to differentiate efficiently to the cell type of choice.

In addition to selecting suitable starting material, the other key challenge for clinical translation of SC-β-cells into the clinic is improving the efficiency of the differentiation protocols. This means improving the composition (increasing the number of β-like cells in the final mixture and potentially also increasing other islet cell types like the α- or γ-cells), improving the purity (removing any residual undifferentiated cells or cells that have differentiated into undesirable off-target lineages), and improving the potency (insulin content and stimulated release.) Each of these features is critical to a safe and effective cell product, and each individually is a challenging biological problem, raising the bar considerably from the first-generation protocols published in 2014. However, the ability to modulate these features through careful control of the developmental biology involved also present unprecedented opportunities for improving the cell product transplanted back into patients. For example, if high potency cell preparations can be developed, the ultimate dose for transplantation may be lessened, having a major impact on device volume requirements. Similarly, this in vitro process also provides multiple points in which to intervene to alter the ratios of cell types within the composition, which could impact long-term graft durability. Methods of genetically engineering the starting material in ways to achieve these or other design goals are certainly within reach, at least for first demonstration in preclinical proof of concept studies.

Finally, the key hurdle for the clinical translation of SC-β-cells is developing methods to safely deliver these cells to patients. These potential paths include immunosuppression-based approaches (substituting SC-β-cells for cadaveric islets) or autologous transplantation (making patient-specific SC-β-cells from patient iPSC lines). Both of these approaches are limited—the first by the limited patient population for whom the benefits outweigh the risks and the second by the logistical, manufacturing, and preclinical challenges to developing patient-specific therapies. Cells genetically engineered to be "invisible" to the immune system present another possible path, albeit with a lengthy and complicated path to clinical trials and unique risks.

The Future of β-Cell Therapy

Since the first demonstration that encapsulated islets could control hyperglycemia in animal models of diabetes, the field of cell encapsulation has evolved with the availability of new materials, new sources of islet tissue, and the scale of federal and private funding for encapsulation research and development. Achieving long-term

glycemic control with an encapsulated cell product remains a holy grail of cell therapy, impacted significantly by the reproducibility, reliability, and scale of available of cell sources. The viability and functionality of isolated primary islets can have a dramatic impact on the performance of encapsulation devices and can exacerbate or change the way that the host responds to these materials. One of the primary concerns of macrodevices is the nonspecific inflammatory response associated with the biomaterials that they are composed of. But limited access to a relevant number of islet cells required to recapitulate the requirements of a therapeutic dose for a human type 1 diabetic has contributed to poor translation from rodent models to the clinic, resulting from the inability to test encapsulation hypotheses empirically. While modeling is useful in predicting diffusive and oxygenation gradients within devices, the encapsulation process becomes quite different when encapsulating several million cells for a rodent compared to nearly a billion cells for a human. Stem cell-derived β-cells change that dynamic and for the first time may allow for the proper number and type of experiments to generate the empirical support required for human product development. With large-scale production of SC-β-cells on the horizon, the development of successful encapsulation approaches will be significantly accelerated, and the advances described in this chapter may serve as a road map toward several potential pathways to success.

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Stem Cell Therapy for Neurovascular and Traumatic Brain Diseases

3

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Introduction

Stroke and traumatic brain injury (TBI) are classified as neurological disorders but more precisely belong to the heterogeneous subclass of neurovascular disease. This broad field of diseases is characterized by pathological dysfunction of the cerebral vasculature which invariably results in some degree of ischemia and metabolic restriction [1]. Generally, these diseases are associated with poor clinical outcomes and an under-availability of effective therapeutic options. While stroke abides more intuitively to the definition of neurovascular disease, our improved understanding of the pathology which accompanies TBI makes it an appropriate member of this disease group as well.

In the United States, stroke affects nearly 800,000 people annually, posing a significant medical and economic burden [2]. Stroke disproportionately occurs in the aging population and is a leading cause of disability among this population [2, 3]. Stroke is defined as a temporary or permanent reduction in blood flow to a brain region which can occur in one of two ways, either ischemic or hemorrhagic, depending on the origin of the circulatory reduction [4]. Ischemic strokes are more common, resulting from embolic vessel blockage, while hemorrhagic stroke occurs less frequently and results from the leaking or rupturing of blood vessels, reducing the appropriate circulation to downstream brain regions [4]. In both cases, the lack of blood causes a depletion of metabolic resources and triggers a cascade of events which are detrimental to neural cell health. This primary cell death can then lead to a host of progressive secondary complications.

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TBI presents many pathological commonalities to stroke and is also highly prevalent in the United States. TBI is a leading cause of death and disability in both civilian and military populations [5, 6]. More recently, the implications of mild TBI—also known as concussion—have received much attention due to mild TBI's ubiquity in sports as well as an appreciation for the long-term complications which can result from repeated impacts [7–9]. TBI affects 1.7 million people in the United States each year, with undiagnosed mild TBIs likely making that number a gross underestimate [10]. With the support of accumulating clinical and laboratory evidence, TBI is no longer considered only an acute injury but is now defined as a disease state which ensues a physical insult to the brain—be it blunt, penetrating, or explosive impact—and can manifest symptoms which progress for decades [11, 12]. The causative insult may result in a focal impact core (often the case with penetrating wounds) or a more diffuse area of initial damage. Regardless, this primary injury often triggers a set of secondary pathways similar to stroke, which proceed to worsen patient outcomes [13].

Developing effective clinical therapies for these neurovascular disorders has posed a steep challenge. One aspect of these diseases' pathology in particular, neuroinflammation, has become the target of choice for most potential therapies. Particularly, attenuating the chronic neuroinflammation following stroke and TBI shows the greatest promise in providing significant functional improvements. Increasing evidence has directed researchers to approach the development of therapies in neurovascular diseases from the perspective of the entire neurovascular unit, instead of targeting a single entity. The concept of the neurovascular unit highlights the phenomenon in which a single disruption within the neurovascular unit can promote a cascade which affects all other parts of the system [14]. In a simplified view, the neurovascular unit is broken into three main parts—neurons, glial cells, and endothelial vasculature [14]. These components are connected via cell-to-cell "cross talk," and the disturbance of one cell type can impact the whole system. New data also suggests that the neurovascular unit is further supported by non-CNS components, including important interactions observed within the spleen and the gut. In this light, a systems biology approach may be advantageous in making clinical progression for stroke and TBI research. This chapter will discuss stroke and TBI pathology, with a focus on how stem cells can be used to attack this inflammatory response on multiple fronts, with emphasis on the recent advancements in our understanding of how stem cells carry out their therapeutic mechanisms.

Acute Pathology of Stroke and TBI

Despite differing etiologies, ischemic stroke and TBI display remarkably similar pathologies, particularly in chronic stages. Because of these commonalities, much of the research on the disease progression or treatment of one disease is

relevant to the other. Directly following stroke or TBI onset, a number of cells are subjected to immediate cell death, and thus the necrotic core forms. This necrotic brain tissue is quickly fixed and unable to be saved [15]. Much of the acute phase is marked by irreversible damage, primarily mitochondrial dysfunction and cell membrane disturbance [16]. Damage to both cellular systems may result from mechanical trauma or ischemic conditions. Mitochondrial dysfunction, in particular, leads to an accumulation of harmful reactive oxygen species (ROS) and a release of many cytotoxic elements [16]. Upon the necrosis caused by these factors, toxic compounds are subsequently released into the surrounding tissue. Cells within the perimeter of the necrotic core, termed the peri-infarct or penumbral region, are faced with the challenges posed by this harsh microenvironment [17]. Changes in plasma membrane permeability often occur during energy-depleted states and, without restoration, cause a solute imbalance and loss of ionic homeostasis [18]. Specifically, sodium and calcium ion infiltration follows this increased permeability and may contribute to cell death [18]. Finally, the necrotic neurons flood the extracellular space with the previously intracellular glutamate. Glutamate excitotoxicity rapidly occurs after the initial cerebral insult, and this can further accelerate the elevation of cytoplasmic calcium concentration by glutamate-mediated release of calcium stored within the endoplasmic reticulum [19]. The damage directly corresponding to glutamate toxicity is short-lived, however, as glutamate concentrations peak at only about 10–30 min after insult [20].

Chronic Pathology of Stroke and TBI

Both the stroke and TBI brains are accompanied by the phenomenon of secondary cell death and its gradual progression. This notion of secondary cell death embodies the chronic phases of these diseases. Two major facets of the chronic disease progression observed in stroke and TBI are alterations in growth factor/apoptotic factor levels as well as an extensive neuroinflammatory response. Neuroinflammation has been shown to persist for years in both diseases—up to 17 years after TBI—and is a complex process involving microglia, peripheral immune cells, inflammatory cytokines, and chemokines [21–23]. The gradual neurodegeneration caused by the inflammatory response means the potential for worsening symptoms long after the original insult [24]. As a result of this extended time-point, a large therapeutic window exists when targeting the inflammatory states of stroke and TBI brains. Especially in TBI, the infiltration of systemic immune cells into the brain parenchyma is permitted by a loss of blood-brain barrier fidelity and can hyperactivate the brain's immune response [25–27]. Therefore, the chronic inflammatory response observed in both stroke and TBI brains has been revealed as the most advantageous secondary cell death factor to attack when proposing new treatment options for the diseases. Neuroinflammation poses both a promising and challenging target though.

The inflammation seen post-insult is a double-edged sword; inflammation seen in the acute phase has been shown to serve a protective role, while the chronic inflammation can become self-perpetuating and lead to significant neurodegeneration [28–31]. Precisely defining this transition from neuroprotective to neurodegenerative inflammation is nearly impossible and can pose practical challenges in developing ideal treatment plans.

Secondary cell death is caused by a host of metabolic changes, reactive species, and persistent inflammation within the regions surrounding the primary insult [32, 33]. These changes are detrimental to neural cells and have a propensity to spread to adjacent tissue, creating the outwardly expanding region of unhealthy brain tissue called the penumbra [33–35]. This tissue is at risk of succumbing to irreparable damage, and the expansion of the penumbra correlates to an increase in functional deficits experienced by patients. Thus, the region of dying, yet not dead, tissue in the penumbra represents a more practical therapeutic target for stroke and TBI.

Importantly, the chronic inflammatory response which accompanies neurovascular insults is now understood to be a global event. In particular, recent research has implicated the spleen as a key player in the global inflammatory response, with alterations in the brain-spleen inflammatory coupling system having been shown to affect experimental outcomes [36]. As a result, both neuroinflammation and the spleen have become highly valuable targets in developing effective stroke and TBI therapies. Current research is attempting to find traditional pharmaceuticals, as well as cellular therapy options, which can aid in abrogating the persistent and complex inflammatory response which occurs after stroke and TBI.

Stem Cell Therapy for Neurovascular Disorders

Currently, there exists an unmet clinical need for effective treatments in neurological disorders, including stroke and TBI. Only one FDA-approved drug is available for stroke patients, and it is only beneficial to a small percentage of patients [37]. This drug—tissue plasminogen activator (tPA)—is severely limited due to its required delivery within a 4.5 h window [37]. There are several other restrictions to tPA use which primarily affect patients with an increased risk for hemorrhaging, such as those taking oral anticoagulant medication [38]. In addition, patients who have undergone recent surgeries or CNS trauma may also be excluded [38]. There is a desperate need for treatment options which are available to a larger scope of patients, as well as treatments without such high risk for serious complications. Similarly for TBI victims, there is not a singular effective drug at this time; therefore, treatment for such injuries is limited to rehabilitation and symptom management. Ideal treatment options are those which possess a large therapeutic window and work to impede the progression of secondary cell death. Due to the complexities of this cell death cascade, the development of a multipronged therapeutic option will be required to observe robust clinical recovery after stroke or TBI. Accumulating research suggests that transplantation of stem cells may check off all the boxes of an

optimal therapy: by rescuing the reduction of growth factors, limiting apoptotic factor levels and neuroinflammation, being chronically applicable, and potentially benefiting a larger percentage of patients.

Stem Cell Transplantation in Stroke

Researchers have established a number of mechanisms by which transplanted stem cells may be utilized to offer neuroprotection in the stroke brain (which will be discussed in the following sections). Stem cell transplantation is approaching feasibility as a stroke treatment, with numerous clinical trials completed and more in progress. With practically all stroke therapeutics having failed in the clinic, stem cell therapy offers a unique and more holistic approach by targeting multiple facets of the complex physiopathology of stroke. In both clinical cases and laboratory models of stroke, stem cell therapy has been shown to reduce infarct size, increase neuron survival, decrease chronic inflammation, and aid in blood-brain barrier repair [39–43]. The means by which these neuroprotective phenomena are achieved remains poorly understood; over the last 25 years of stem cell research in stroke patients, therapeutic benefits by way of cell replacement and growth factor release have been established as part of the regenerative process after transplantation yet cannot account for the entirety of the neuroregeneration displayed. Thus, less intuitive and poorly understood mechanisms must be contributing to the effects displayed. Regardless, the use of stem cells within the stroke brain has been proven to offer the potential for significant improvement of functional outcomes.

Stem Cell Transplantation in TBI

Originally, TBI was categorized as an acute injury, but is now recognized to possess chronic pathological symptoms—particular secondary cell death driven by aberrant neuroinflammation—and is closely associated with lifetime behavioral deficits [44, 45]. Currently, the treatment options for TBI are limited [46] and typically consist solely of rehabilitation therapy [47–51]. Bearing in mind the extensive secondary cell death facilitating the progression of symptoms with TBI, new potential treatments have gravitated toward targeting the wide therapeutic window of TBI pathology, aiming to promote "neuroregeneration" instead of the relatively narrow window for "neuroprotection" associated with the acute TBI phase [52, 53]. Stem cell-based therapeutics have become a central theme in regenerative medicine, displaying promising results in animal models of TBI [54–57] but have reached scarce success in reaching the clinic [58]. Additional translational research is needed to gain a better understanding regarding the mechanisms of stem cell action and their capacity to confer neuroregeneration in the brain, as well as establish optimal treatment regimens—all in an effort to drive successful trials into the clinic. In addition, identifying a well-defined stem cell source is necessary for assuring quality of graft origins,

as well as being a measure for insuring validity and reproducibility of experimental results. The establishment of optimal cell populations that are both safe and effective is also an area that requires further investigative efforts. There is, however, accumulating evidence to suggest that stem cells produce neuroprotective effects via multipronged neuroregenerative pathways including anti-inflammation and enhanced neurogenesis [59–61], in addition to improving angiogenesis and vasculogenesis [62–64]. Also, poor graft survival has been reported in the TBI brain, likely attributed to the harsh conditions manifested from the secondary neuroinflammatory response [15, 65, 66]. These data suggest significant survival may not be a prerequisite for behavioral recovery; however, abrogating the hostile microenvironment in which stem cells are transplanted may achieve higher graft survival and boost the degree to which bystander effects occur. In view of that, it is thought that by taming the incompatible microenvironment (i.e., reducing neuroinflammation), stem cell therapy can be optimized, therefore appealing to the advancement of regenerative medicine for treating the injured brain.

Mechanisms of Stem Cell Therapy

Stem cell therapy has received an increasing amount of attention within the realm of regenerative medicine. Multiple neurological diseases, including stroke and TBI, have been a special area of focus for stem cell therapy research. It was initially proposed that stem cell transplantation into the CNS would result in the replacement of dead or dying neuronal cells, as this is the most intuitive mechanism. However, it was observed in many studies that stem cell transplantation into damaged tissue resulted in poor engraftment rates. Interestingly, a robust functional recovery and reduction of infarct core were observed in animals that received stem cell transplantation despite this poor retention [37]. Thus, the therapeutic effects of stem cells appear to not be dependent on their long-term survival and differentiation as initially anticipated.

A far more prominent mechanism of stem cell action has since been brought to light. This mechanism involves the secretion of neurotrophic factors from the transplanted cells. Growth factors play a role in pro-survival pathways; therefore, increasing their concentration has the capacity to thwart impending apoptosis in vulnerable tissues such as the regions of the penumbra in stroke or TBI [67, 68]. Stand-alone administration of BDNF [69], VEGF [70], GDNF [71], SDF-1α [72], and SCF [73] has been shown to have a positive effect on neurological disease outcomes. However, it has been suggested that individual treatment with any one of these growth factors would not result in significantly improved clinical outcomes. Stem cells provide an impressive cocktail of growth factors which contribute to an overall anti-inflammatory and anti-apoptotic effect. In addition, stem cell therapy avoids the complication of establishing the correct dosage of growth factors by having self-regulating secretion. Importantly, it has been observed that drug-induced

overproduction of growth factors may lead to detrimental neurological effects, such as the development of epilepsy as a consequence of BDNF overexpression [74, 75].

A third mechanism of action for stem cell transplantation was first observed in a rat model of TBI. This newly discovered mechanism functions to enhance the host's natural neuroprotective processes which are initiated upon injury through the activation of endogenous stem cells. Until recently, a long-standing belief was held that mammalian adults lacked the ability to generate new neurons. This paradigm was quickly reversed after the discovery of endogenous stem cells, found predominantly within the SVZ of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus [76, 77]; these regions also may be referred to as the neurogenic niches. The capacity for neurogenesis therefore extends into adulthood and is amplified following neurological insults such as stroke [78]. The host's attempt to reverse or stop the gradual progression of damaged tissue by stem cell mobilization is highly inefficient. Endogenous stem cells are limited in their ability to differentiate, commit to neuronal lineage, and migrate from these neurogenic niches [79]. Proper migration of endogenous stem cells is imperative if they are to elicit therapeutic benefit to regions distant from their origin in the SVC or SGZ. Recent research revealed assisted transportation of host stem cells via transplantation of exogenous stem cells. The first study to observe this phenomenon used a TBI model in which a controlled cortical impact (CCI) was delivered to the frontal cortex—a region too distal for significant endogenous stem cell migration—followed by intracerebral injection of mesenchymal stem cells (MSCs) [80]. Interestingly, observations utilizing immunohistochemistry and laser capture microdissection exposed the presence of transplanted MSC creating a cellular pathway connecting the neurogenic niche to the impacted cortex [80]. This discovery led to the biobridge theory as a third and novel mechanism in which transplanted stem cells provide a neuroprotective and/or neuroregenerative effect. The biobridge is thought to facilitate the migration of endogenous stem cells from their residential origin to the injured region, therefore rendering the host's natural regenerative mechanism more effective [80]. This novel mechanism is entirely unique to stem cells and is yet to be achieved by any other therapy.

Finally, recent data have revealed a fourth mechanism of action executed by stem cells. The vast secretome produced by stem cells has displayed the ability to confer therapeutic effects on neurovascular diseases. Microvesicles and exosomes secreted from stem cells contain growth factors, cytokines, chemokines, microRNAs, and long noncoding (lncRNA), all of which may contribute to the therapeutic effect observed following transplantation [81]. Treatment with isolated exosomes and microvesicles derived from multipotent MSCs has been explored in liver, kidney, cardiovascular, and lung disease with promising results [81]. In the transition to neurovascular diseases, studies focusing on exosome transplantation following CNS insult include an in-depth analysis of neuroinflammation, as the magnitude and persistence of inflammation in neurological disorders are rather unique. As emphasized previously, neuroinflammation is an important target for the establishment of an effective therapy in neurovascular diseases.

The Promise of Combination Therapies with Stem Cells

While stem cells alone have been shown to be therapeutic in many applications, an increasing body of research is exposing the additive effects that certain combination therapies can afford. Combination therapies may take multiple forms, such as pairing multiple stem cell types, delivering stem cells in conjunction with a more traditional therapeutic agent, or delivering stem cells with biostructural material. In some cases, these adjunct agents serve as independent therapeutics which complement the stem cells, while other adjuncts play a supporting role by helping to bolster the effectiveness of stem cells. Both approaches possess merit and may serve to improve patient outcomes in the future.

Depending on their tissue of origin, stem cells can exhibit significant variation in morphology, protein markers, differentiation capabilities, and treatment efficacy. This has been established by the use and comparison of a number of different stem cell types in the stroke brain, including mesenchymal stem cells (MSCs), bone marrow-derived stem cells (BMSC), adipose-derived stem cells (ADSC), and neural stem cells (NSC). A trail of investigation which is now being explored aims to characterize the effectiveness of heterogeneous stem cell populations. In one study, the effects of MSC-NSC combination therapy was explored in a middle cerebral artery occlusion (MCAO) rat stroke model [82]. Twenty-four hours after occlusion, MSCs were injected into the right lateral ventricle, and 6 days later, NSCs were injected in the same manner [82]. Histological and behavioral analysis revealed that the MSC + NSC group showed reduced functional deficits and smaller lesion volume when compared to sham animals, MSC-only animals, and NSC-only animals [82].

In a study utilizing a percussion model of TBI, the use of olfactory ensheathing cells (OEC)—supporting glial cells derived from the olfactory system—was delivered in conjunction with neural stem cells (NSC) [83]. The olfactory system is unique in its ability to perform significant neurogenesis throughout the mammalian life cycle [84, 85]. This experiment explored the idea that these supporting cells may be instrumental in encouraging the neural proliferation unique to this region. Using a vehicle control group, OEC alone, NSC alone, and OEC + NSC group, a notable trend was observed whereby neuron survival in the OEC + NSC was significantly greater than in either individual cell treatment, nearing sham levels [83]. Similarly, apoptosis was reduced in the OEC + NSC group to a significant degree when compared to individual cell treatments [83]. While the mechanism of this additive improvement was not studied, the ability of different cell types to synergistically ameliorate the effects of TBI supports further investigating this type of therapy.

Stem cells have also been delivered with a number of therapeutic compounds such as mannitol, granulocyte colony-stimulating factor, minocycline, and progesterone [68, 86–88]—often with compounding benefits. It is plausible that a major leap in stem cell effectiveness could be found in their simultaneous delivery with neurotrophic factors, anti-inflammatories, or cytoprotective agents. Stem cell treatment in neurovascular diseases has also been paired with alternative therapies such as hypothermia and hyperbaric oxygen treatment [89–91]. The benefit of a

co-treatment with stem cells and one of these alternative therapies would be eliminating some of the complications that come along with pairing complex pharmaceuticals. Similarly, delivering stem cells in a formulated biomaterial may aid in improving the therapeutic effects of stem cells [92, 93]. Certain materials have been shown to reformat the extracellular matrix and make the brain microenvironment more conducive to stem cell survival, migration, and proliferation. In general, the search for compatible biomaterials and effective co-treatments represents a worthy endeavor and may help to expedite the transition of stem cells from the laboratory to the clinic.

The Spleen as a Novel Target for Stem Cells

Once thought to be isolated, CNS disorders, stroke, and TBI are now recognized to be affected by distal regions of the body. One organ that has been implicated in these diseases to a surprising degree is the spleen. A reciprocal relationship exists between the spleen and the brain following neurovascular insult, with the injury altering the physical size and function of the spleen and the spleen in turn, affecting brain health [94]. In fact, the spleen has been shown to release splenocytes in response to ischemic events, exacerbating neurodegeneration [94]. Additionally, studies have shown the spleen to be especially critical in the physiological processing and therapeutic mechanisms of stem cells [95]. The cornerstone of this relationship lies in the post-insult inflammatory response. We now understand the brain inflammatory response to include both central and peripheral components following stroke and TBI—local inflammation and edema persist in the brain parenchyma, while systemic inflammation helps to propagate the cerebral inflammation [96–98]. The peripheral aspect of neuroinflammation allows for invading immune cells—such as T cell, lymphocytes, monocytes, and macrophages— to extravasate through the compromised BBB which results from neurovascular damage [95, 98]. As mentioned previously, the chronic inflammation seen following stroke represents a prime target for therapeutics; thus, understanding both the local and the global nature of the inflammatory response is critical in attacking neuroinflammation from all possible angles.

Interestingly, studies in which the spleen was removed prior to MCAO demonstrated smaller infarct volumes, reduced infiltration of peripheral inflammatory cells, and reduced pro-inflammatory cytokines [99, 100]. This demonstrates that the spleen's innate processes contribute to worsening outcomes following an ischemic event [94, 100]. As routine splenectomies are not a viable clinical option, the use of other approaches to attenuate the spleen's pro-inflammatory role may serve as a practical approach.

Stem cells have been shown to preferentially migrate to the spleen when delivered intravenously, both in the acute and chronic stroke brain [95, 101]. These stem cells demonstrated an ability to downregulate pro-inflammatory molecules and immune cells which are released by the spleen, effectively altering the blood composition in a way that promotes stroke recovery [95]. This exhibits that stem cells

may potentially function therapeutically, in part, by modulating this brain-spleen axis inflammatory response. Our understanding of the spleen's implications in the stroke and TBI brain remains incomplete, but its role in the chronically inflamed state which exists after stroke gives insight into how stem cells may be an effective option in later stages of the disease. Additionally, this spleen-mediated mechanism furthers the notion that entering the brain tissue may not be a prerequisite for an effective stem cell therapy [95]. If the presence of stem cells in the blood stream can indirectly alter the brain parenchyma environment, the inability for stem cells to efficiently cross the BBB may not be a restriction to effective therapy. Importantly, this helps to circumvent the quandary whereby blood-brain barrier repair makes peripherally delivered stem cells less potent. To date, only one preclinical study has investigated the use of stem cell administration in the chronic phase of stroke [102]; understanding how stem cells confer neuroregeneration by interacting with the spleen to sequester the inflammatory response at these later time-points may pave the way for a continuous, long-term stem cell treatment plan.

Challenges in Stem Cell Therapy

A number of major hurdles stand in the way of progressing stem cell therapy to patient availability. These include difficulties concurring on the most effective dosages, establishing consistent time-points, and determining practical routes of delivery. Three collaborative meetings have produced a set of guidelines for stem cell research called STEPS—stem cells as an emerging paradigm for stroke—guided by field experts in both the laboratory and clinical setting [103]. Many of their suggestions have addressed the issues mentioned above, stressing the importance of basic science-inspired clinical trial design [103]. A common disconnect in clinical trial design exists in deciding the appropriate dosage; the number of cells administered in human patients is rarely proportional to the most effective cells-per-kilogram dosage established in animal models. This cripples clinical trials from the start and contributes to most clinical trials agreeing on the safety of stem cell therapy in stroke, but not definitively concluding on the efficacy [102, 104–108].

In addition to dosage concerns, defining an ideal time-point or set of time-points which produce the greatest possible patient recovery remains an elusive target. A limitless number of possible delivery time-point combinations exist, with different studies finding varied effectiveness depending on the chronology of treatment(s) [109]. Importantly, the route of administration also affects stroke and TBI outcomes. It is not surprising that injection of stem cells directly adjacent to the infarct or impact core consistently delivers the greatest functional improvements, but the practicality of intracerebral delivery for patients en masse is questionable.

The difficulty in developing treatment plans exists not only as a result of the chasm between laboratory and clinical research, but also arises from the heterogeneity of stroke and TBI. Stroke patients experience extensive variation in infarct region, stroke severity, and capacity for natural recovery [110]. TBI is equally varied, with location, severity, and affected area differing greatly depending on the

circumstances of the injury. Thus, a key to pushing stem cell treatment forward is to conduct more clinical trials, produce more substantial data, and use this to tailor future experimental designs in order to define the best treatment plans for general insults or develop patient-specific plans. Of course, this must be in addition to tighter collaboration between basic scientists and clinicians.

Potential Adverse Effects of Stem Cell Therapy

While stem cells undoubtedly offer hope as a stroke treatment, the administration of stem cells is not without its risks. The two predominate concerns with cell transplantation are the danger of teratoma formation and graft rejection—a host immune response to the exogenous cells. The danger of teratoma formation is especially prevalent in embryonic-derived stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [42]. When analyzing the safety of potential stem cell therapies, regulatory institutions prefer to see a loss of stemness over time, as this indicates a loss of unwanted replicative potential. All stem cells hold a certain degree of overproliferation risk, but this is largely dependent on the cell type's differentiation capabilities. On the other hand, the risk of immune response is primarily dependent on the host; thus, immunosuppression may be necessary in certain clinical applications [111].

A novel therapeutic concept may be able to deliver the benefits associated with stem cell transplantation while curtailing these associated risks; this approach uses cellular materials which offer functional effects comparable to those seen with traditional stem cell transplantation [42]. These cell-derived therapies take advantage of an emerging notion; significant evidence now points to the secretome, the sum total of a cell's secreted factors, as a leading contributor to stem cells' therapeutic actions and anti-inflammatory effects observed in stroke and other neurological disorders [42, 112–115].

Cell-Free Materials and Exosomes in Neurovascular Therapy

Different means of preparing cell-derived materials exist, but the harvesting of conditioned media is perhaps the simplest. Conditioned media refers to the chemically altered, secretome-infused media which results from the culturing of a specific stem cell type [116]. This is typically achieved by growing the cells within a 3D scaffolding to maximize the desired secretions [117]. A number of studies have exposed the potential which lies in the use of conditioned media [116–119]—highlighting a reduction in apoptosis, decrease in inflammation, improved neuron survival, and cell proliferation—yet, the practicality of employing conditioned media as an effective stroke treatment in humans remains to be verified.

The cultivation of stem cell-produced exosomes is another cell-free therapeutic option for stroke which has received significant attention in recent years. As mentioned previously, exosomes are small secreted vesicles which contain a variety of cellular products—including mRNA, lncRNA, lipids, and proteins—and maintain

the ability to act as both paracrine signals as well as extracellular environmental modulators. Experiments have demonstrated that these isolated exosomes retain the ability to confer neuroprotection and neuroregeneration comparable to that of stem cell transplantation [120, 121].

TBI and Stroke: Implications of Cell-Free Treatment

Currently, the use of isolated stem cell-derived exosomes to treat stroke and TBI is a relatively new area of research. This has both aided in furthering our understanding of exosomes' role in stem cell treatments and offered a new therapeutic option all together. This subsection will detail three studies and their unique findings on this subject.

A recent study explored the utilization of human adipose-derived stem cells (hADSCs) in TBI rats using the CCI injury model [122]. In addition to treatment with transplanted stem cells, another experimental group received cultured media (CM) derived from these cells [122]. This CM contained the entire secretome extracted from the cultured cells and included the exosomes carrying growth factors, protein, microRNA, and lncRNA [122]. Previous studies of experimental TBI have evaluated hADSCs for their ability to secrete large amounts of the anti-inflammatory cytokines IL-10 and IL-4 [119, 123, 124] and reduce the production of pro-inflammatory cytokines, such as TNF-α and IFN-γ [125]. For this reason, the anti-inflammatory effect observed with CM treatment was expected. In order to characterize the importance of exosomes and their lncRNA, knockdown groups were utilized which shed light onto the vital role these specific lncRNA exosomes play in the neuroprotective properties contributed by the CM. Many types of lncRNAs are secreted by proliferating stem cells that are not in a differentiating state.

Two specific lncRNAs were selected for knockdown, nuclear-enriched abundant transcript 1 (NEAT1) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). They were chosen because of their essential role in cell survival, inflammation, and gene expression. Specifically, these lncRNAs are modulators of cellular differentiation due to their ability to take part in the alternative splicing of numerous pre-mRNA [126–128]. Upon knockdown of the two lncRNAs, the functional recovery seen in the CM experimental group was significantly reduced. The following proteins—VEGF, stem cell factor (SCF), and tissue inhibitor of metalloproteinases-3 (TIMP3)—were analyzed for their concentrations in CM versus CM-knockdown groups. The CM-mediated improvement in VEGF and SCF levels was significantly reduced in the knockdown group, as well as an increase in TIMP3, a VEGF inhibitor [129, 130]. Based on these results, it was concluded that the stem cell-derived secretome yields a neuroprotective and anti-inflammatory effect that is largely dependent on the action of lncRNAs.

The next two studies explored the potential in direct exosome transplantation following isolation from cultured MSCs. One study used a TBI model [81], the other stroke [131], and both resulted in improved cognitive and motor function in

exosome-treated rats. The stroke study included a particular focus on exosomal transfer of microRNA, specifically microRNA-133b (miR-133b) [131]. The selection of miR-133b was based on prior in vitro studies that revealed an elevation in miR-133b within MSC-derived exosomes after exposure to ischemic cerebral extracts [112]. This increment in miR-133b attributed to heightened neurite growth due to transportation to astrocytes and neurons via exosomes [112]. For the following in vivo study, knock-in and knockdown experimental groups were utilized. The data showed that increased miR-133b concentration in exosomes provides a more robust neurological recovery, and a significant decrease of therapeutic capacity results when miR-133b is in reduced concentrations [131]. Exosomes with miR-133b + MSC lead to improved axonal plasticity and neurite remodeling that contributed to functional recovery [131].

The TBI study of MSC-derived exosome transplantation illustrated similar results in that the treatment was neuroprotective [114]. This study revealed the role of angiogenesis in the functional recovery seen with administration of cell-free exosomes generated by MSCs [114]. In addition, exosome treatment reduced neuroinflammation and raised the number of newly formed neuroblasts and mature neurons in the dentate gyrus [114]. Interestingly, treatment groups did not display any downsizing in cortical lesion volume but still showed improved cognitive and sensorimotor functional recovery [114]. This further exemplifies the importance of angiogenesis and sequestration of neuroinflammation and their contribution toward functional improvements following stroke or TBI.

Feasibility of Exosome Transplantation Therapies

It remains unclear whether exosomes present a practical clinical approach. For one, the difficulty of accumulating sufficient quantities of this cell-free product is both challenging and expensive. Individual stem cells secrete an unsubstantial amount of exosomal product; therapeutic dosages for humans could only be obtained with vast quantities of stem cells using presently available methods. It is also yet to be determined if the reduced risk offered by cell-free options—both conditioned media and exosomes—is marginalized by a reduction in therapeutic effectiveness. While the potential for therapeutic has already been unequivocally demonstrated, if its effects are significantly less than that offered by traditional stem cell therapies, it may be worth the associated risks to proceed with stem cell therapy as usual. Further studies comparing the neuroprotection conferred by stem cells to that offered by exosomes alone would allow for an appropriate risk-versus-reward analysis. Regardless, the study of exosomes and their constituent compounds may be instrumental in furthering our knowledge of how stem cells deliver their therapeutic benefits.

Conclusion: Connecting the Dots of Stem Cell Therapy

With such a large population of patients being affected by either stroke or TBI, the need for neurovascular therapeutics cannot be overstated. Current best medical practice for stroke involves the use of tPa if possible, yet utilizing this drug can be difficult as a result of its narrow therapeutic window and adverse effects. TBI patients are yet to have a viable option. The use of cell transplantation offers a promising solution to these clinically difficult disorders.

The therapeutic effects of stem cells have been described as paradoxically robust. As mentioned, the engraftment rate of transplanted stem cells is surprisingly low considering the significant benefits they exhibit. In reality, this paradox is just a reflection of our rudimentary understanding of how stem cells function within the body. Fortunately, great strides are being made in unveiling the complex mechanisms by which stem cells confer neuroprotection. These strides help to push stem cell therapy closer to efficient clinical applications. Specifically, the topics covered in this chapter—inflammation, the spleen's role in neurovascular disease, and stem cells' tendency to hone in on this organ—reveal much about how stem cell therapy for stroke and TBI operates. By targeting the pathology of neurovascular diseases in a holistic manner and by respecting the cellular interplay which is presented in the neurovascular unit concept, stem cell therapies may be able to be maximally utilized.

The role of the inflammatory response in stroke and TBI has been discussed extensively, but it is important to understand how this novel idea of splenic mediation of peripheral inflammation impacts stem cell therapy. Again, inflammation offers the most accessible target in stroke and TBI pathology. With intravenous stem cells honing in on the spleen—the newfound mediator of systemic inflammation—this provides a convenient link whereby stem cells attack the most accessible point of neurovascular pathology, the inflammatory response, at a convenient location.

Similarly, the rising notion that stem cells confer their benefits in part through the release of therapeutic exosomes is an important revelation in how stem cells function. In addition to the secretion of anti-inflammatory cytokines, growth factors, and anti-apoptotic molecules, we have recently revealed the role of exosomal secretions. Within these exosomes, important mediators of cell growth, division, and survival help to confer neuroprotection. This revelation that stem cell products—not necessarily the cells themselves—contribute to their therapeutic profile opens up new possibilities of cell-free treatments. These treatment options may take advantage of similar mechanisms as traditional stem cell therapy while circumventing the dangers that accompany it.

Stem cells are a non-conical therapy option. Because of this, transitioning stem cells from the laboratory to the clinic has proven challenging, but the difficulties associated with their use are matched by equal promise. Further research is needed to understand how stem cells operate in the human body and by which means they extend their neuroprotection. Improving our understanding of stem cell therapeutic function is a critical step in making their use widely available to patients suffering with stroke and TBI.

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Cell Replacement Strategies for Parkinson's Disease

4

Diptaman Chatterjee, Dustin R. Wakeman, and Jeffrey H. Kordower

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is a synucleinopathy that is characterized in large part by the loss of putamenal dopamine secondary to the degeneration of dopaminergic neurons in the substantia nigra (SN). Canonical symptomatology exhibited by PD patients includes a tremor at rest, bradykinesia and akinesia, cogwheel rigidity, and postural instability. In addition to the primary hallmarks of classical motor defects, PD patients often experience age-dependent dementia or progressive cognitive impairment as well as several nigrostriatal-independent behavioral phenotypes. As the clinical understanding of PD has expanded over the past 40 years, the scope of symptoms now frequently features sleep disturbance, depression, and autonomic dysfunction [1].

Although no cure exists, many current therapeutics target the pharmacological replacement of dopamine. Treatment with L-dopa, or catecholamine precursors, provides significant motor relief for PD patients. However, most patients on extended L-dopa courses develop involuntary dyskinesias and/or off-target behavioral effects [2]. Similarly, surgical alternatives using deep-brain stimulation in the globus pallidus and the subthalamic nucleus, though effective in relieving motor symptoms, have also elicited side effects affecting both movement-related and non-motor phenomena and do not generally affect disease progression or non-levodopa responsive features [3]. In addition, PD patients express compounded aberrance of

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dopamine-independent synaptic signaling pathways that have not been addressed through treatment strategies.

As current therapies can feature a vast range of debilitating side effects or fail to comprehensively challenge diffuse disease pathophysiology, the need for therapies promoting DA neuron cell replacement in place of compensation is still a forefront in PD research. Here we synopsize the historical evolution of cell graft treatments for Parkinsonian disorders and evaluate the future clinical outlook for therapeutic cell transplantation.

Ventral Mesencephalon Grafting

Transplanting neural tissue from healthy control subjects to diseased sites of action has been conceptualized since the late 1800s. As PD primarily presents in loss of a specific subtype of dopamine neuron, cell replacement in PD was an appealing option for early testing to counteract symptoms. However, progress was inevitably slow as researchers found difficulty integrating grafted cells due to the intractability of mature, postmitotic neural tissue.

In the context of PD, replacing degenerating DA neurons became a tangible venture after seminal works in the 1970s describing in vitro and in vivo effectiveness of grafted fetal tissue in DA depletion models [4–11]. These works validated the potential of cell populations derived from donor DA-rich ventral mesencephalon (VM) as enduring grafts capable of host tissue integration. Evidence from the Bjorklund group of fetal graft catecholamine restoration in concert with electrophysiological integrity further corroborated the prospective benefit of VM cell grafts [12, 13]. As more studies were carried out to assess the viability of grafts, transplant localization became a vital factor in the supply of DA in target sites relevant to disease. VM tissue from 6- to 9-week-old human fetuses showed favorable integration and consequent improvement in phenotype when implanted in the striatum of 6-hydroxydopamine (6-OHDA)-lesioned rats, markedly contrasting the results of cells transplanted homotopically into the SN [14–17]. The inability of cells grafted into the SN to adequately develop rostral projections to the caudate and putamen proved challenging for hopes of rescue through return of striatal-DA tone.

Following the success of counteracting the effects of DA denervation in small animal models, several groups showed fetal VM grafts to improve multiple aspects of disease in severe Parkinsonian monkey models [18–22]. Nonhuman primate models became important for demonstrating the effects of trophic factor supplementation and, recently, a valuable tool in addressing the PD non-motor symptoms escaping rodent models [23]. Additionally, data from nonhuman primate models validated the use of multidose micrografts in sustaining graft integrity [24].

Clinical Trial Outcomes

After mixed results in animal models, a number of clinical studies sought to verify potential efficacy of non-VM cell grafts, particularly adrenal medullary cells [25]. Unfortunately, patients showed minimal motor improvement and highly variable

graft survival upon postmortem analysis, ultimately leading to cessation of trials [25]. Attempted use of porcine mesencephalon xenografts also proved ineffective in comparison to VM allografting [26].

The first clinical trial of VM allografts was performed in the late 1980s by Lindvall et al. [27], followed by multiple other open-label studies that validated long-term survival of grafts and appropriate integration into host tissue [28–33]. During this time, the Core Assessment Program for Intracerebral Transplantation was developed as a method of standardization of practice [34, 35]. However, although fluorodopa scanning in positron-emission tomography (PET) highlighted graft survival, most early trials showed striking variability in benefit of motor symptoms as evaluated by the Unified Parkinson's Disease Rating Scale (UPDRS). Many attributed this variability to the number of donors per patient, target site of graft, and inconsistent methods of immunosuppression [36]. Additionally, initial trials lacked placebo controls, casting doubt on these data sets. As a result, the National Institutes of Health (NIH) sponsored two double-blind, placebo-controlled trials in PD patients to rectify the ills of previous efforts.

The first of these studies by Freed and colleagues examined a comparison of 40 patients, 20 of whom received sham surgery in which stereotaxic delivery needles stopped short of the dura mater [37]. Although no significant differences were observed in UPDRS scores among treatment and control groups, secondary endpoint revealed suspicion of treatment benefit seen between patients ±60 years old [37]. The isolated motor component of UPDRS showed an even greater improvement among younger patients. The second NIH trial featuring 6-month, postsurgical administration of cyclosporin, carried out by Olanow and colleagues, also did not reach the primary clinical endpoint, although adequately demonstrated graft survival and integration [38]. Importantly, both trials uncovered a critical roadblock in the form of graft-induced dyskinesia, independent of levodopa treatment. The mechanism behind these graft-induced dyskinesias among fetal VM-grafted patients remains unknown, although some theorize the improper neurotransmission of grafted serotonergic neurons as a potential cause [39–42]. Furthermore, there is now evidence that nigral Girk2-positive DA neurons that are A9-specific are vital for proper innervation and catecholamine delivery along the nigrostriatal pathway, potentially calling into question mechanisms of heterogeneous cell-type integration via donor graft material [43, 44].

Another important consideration is the viability of DA neurons immediately after graft transplantation. Barker et al. demonstrated apoptotic or necrotic elimination of a majority of VM-graft neurons within 1 week of transplantation [45]. These data, in concert with preclinical efficacies of GDNF graft supplementation, suggest innate hostility toward graft material in PD neural environments [46–48]. Recently, positive results from GDNF supplementation in ALS preclinical studies have indicated the therapeutic benefit of intramuscular GDNF delivery in enhancement of synaptic connectivity through trophic support of the neuromuscular junction [49]. Thus, increasing the percentage of surviving neurons by bolstering the graft environment may be essential in procuring sustainable phenotypic improvement and on-target growth.

Recently, our lab has shown superior graft survival and integration from the postmortem analysis of a patient that died 16 years after VM cell transplantation [50].

Additionally, upon reappraisal of their initial study, Lindvall and colleagues determined the patients that benefited most from striatal VM grafts were those that featured minimal extrastriatal denervation [51]. Thus, cell specificity and localization of cell-specific denervation are critical factors in the potential effect size of VM allografts. Thus far, the variable conditions and isolated patient factors that have confounded VM graft results have rendered it difficult to conclusively assess embryonic transplantation in the context of PD. Currently, a new open-label trial (TRANSNEURO) with standardized grafting procedures and immunotherapy measures, and more tightly controlled inclusion criteria, is underway with potential use as a standard method of comparison for future cell-based therapies [52].

Stem Cell Grafting

Although grafts derived from fetal VM tissue show promise, insufficient tissue abundance and ethical controversy have plagued practical application for the large PD patient cohort. Furthermore, lack of consistent efficaciousness and frequent induction of dyskinesia events sprouting from fetal graft heterogeneity have not warranted displacement of DBS and levodopa therapy as the gold standard, countermeasure practices in PD treatment. These findings warrant further investigations into cell sources with specific control of composition and specialized targeting of the A9-DA neurons that play such a vital role in nigrostriatal organization and dopaminergic tone.

Primary pathogenesis in PD cases, specifically in nigrostriatal DA neurons, makes tractable stem cells ideal candidates for graft components best equipped for microenvironmental adaptation. Indeed, graft cells sourced from bone marrow-derived mesenchymal stem cells, retinal pigmented epithelial cells, and stromal adipose tissue have all been tested on PD models [53–55]. In the early 1990s, adult neuroblast progenitor cells were shown to successfully project target-selective axonal growth and functionally organize and assemble [56–58].

Unlike the lack of efficacy from homotopic fetal VM grafts, human DA neuroblasts transplanted into the SN show much greater capacity toward reconstructing denervated nigrostriatal connectivity in 6-OHDA-lesioned mice [59]. The successful projections of DA neurons to the striatum were enhanced in several studies through the recombinant adeno-associated virus (rAAV) gene delivery of GDNF to the graft environment in both rodent and nonhuman primate PD models [59, 60].

Fascinatingly, the innate “stemness” of neural progenitor cells allows for consistent pliability of cells to differentiate in accordance to the internal milieu of the graft environment, potentially counteracting the fetal graft heterozygosity that disrupted the function of integrated cells. However, the struggle to maintain multipotency of neural progenitors pre-/posttransplantation has been difficult to overcome. Still, the outcome from early stem cell experiments showed promise in determining the proper formulation, specifically in effort to procure A9-specific DA neurons.

Fetal neural stem cells (NSC) provide the earliest mode of central nervous system multipotent cells; however, differentiation protocols have remained inadequate in the

provision of dopaminergic output in Parkinsonian models [61]. Although reports of fetal NSC grafts have shown sufficient viability, appropriately modeled differentiation within pathological zones remains a challenge [62–65]. A recent study has shown vector delivery of Lin28A in concert with neural stem cell grafts significantly enhanced the effect of progenitor networks from donor cells and concurrently improved resultant behavioral phenotypes [66]. Interestingly, undifferentiated stem cells have been shown to provide secondary support to Parkinsonian pathology (escalation of tyrosine hydroxylase and vesicular monoamine transporter-2 expression) independent of nigrostriatal dopaminergic recovery [67]. These phenomena are most likely explained through NSC provision of neurotrophic factors and signaling of astroglial activation [68, 69]. However, such compensation cannot satisfactorily counteract PD pathology pertaining to dysregulated dopaminergic tone. Thus, in order to warrant practical use in clinical PD settings, emphasis must be placed on standardization of robust DA neuron differentiation protocols prior to the administration of graft cells. By 2016, several independent groups had reported significant progress in successful generation of midbrain lineage DA neurons from pluripotent stem cells [70, 71] with subsequent behavioral recovery following grafting in PD models.

The recent wave of induced pluripotent stem cell (iPSC) research has sparked optimism in overcoming the limitations of fetal tissue and embryonic stem cells (ESC). The use of midbrain DA neural markers including, but not limited to, girk2, lmx1a, ngn2, msx1, nurr1, and pitx3 has been shown to correlate with A9-specific DA neurons [72]. Numerous other iPSC differentiation protocols have led to transplanted grafts with relative success in improving symptomatology. However, the lack of reproducibility in DA neural populations through the use of a wide range of protocols has aggravated the progress on method standardization necessary for clinical use. Additionally, some have suggested the introduction of differentiation factors to be less important than targeted manipulation of the support environment of cell grafts. For example, a recent study identified expression levels of common progenitor markers lmx1a, corin, and foxa2 to correlate poorly with selective DA neural output in transplanted cell grafts [73]. Instead, promotion of rostro-caudal organization of VM progenitors with timed FGF8b delivery served as a better predictor of graft survival and targeted differentiation [73].

The battle to control targeted progenitor populations is made even more difficult in abrogating the growth of pluripotent cells after appropriate differentiation has occurred, as continued replication in stem cell-derived neuronal populations raises concerns of tumorigenesis and uncontrolled proliferation [74, 75]. Similar to the concerns with ESC-derived dopaminergic neurons, the congruence of authentic nigral neurons and differentiated iPSC remains unclear and a field of necessary investigation [36]. Still, since iPSC-DA neuron-induced motor symptom benefit in Parkinsonian rodents was first described, numerous studies have highlighted the potential of DA-mediating allografts and autologous grafts from iPSC culture protocols [55].

The challenge of advancing stem cell technologies into a clinical setting for PD treatment lies primarily in the normalization of graft preparation methods, establishment of selection criteria for clinical studies, and delivery systems. Recent advances in the use of optogenetics have allowed for high-throughput characterization of stem

cell grafts in PD models and may accelerate necessary validation of DA-targeted stem cell electrophysiology and substrate potency [76].

In an effort to guide clinical progression and avoid the hazards encountered in fetal grafting, multiple groups have suggested variations of the following paradigm for clinical development: (1) establish clinical grade ESC or iPSC lines, (2) determine appropriate selection of neural populations while minimizing tumorigenic risk, (3) identify patient populations with optimal potential for efficacy, (4) develop outcome measures to assess therapeutic efficacy, and (5) provide alternative treatment options for unsuccessful trial subjects [77, 78].

Conclusions

Although there are still significant hurdles for stem cells in a clinical sense for PD, the potential for rapid development of protocols is promising given the recent surge in stem cell-based clinical trials in factions of other neurodegenerative pathologies. Clinical trials of disease-dependent cellular subtype transplant protocols have shown varied results in ALS, Huntington's, multiple sclerosis, and spinal cord injury [79].

Clearly FVM grafts can survive in large numbers and provide robust innervation to the host striatum. We [50, 80–83] and others [44, 84, 85] all demonstrate excellent survival and innervation, and some [50, 83] show synaptic contacts between graft-derived axons and host neural elements. However, this neuroanatomical reconstruction is still often not sufficient to produce clinical benefit [50], and this degree of neuroanatomical reconstruction might need to be combined with proper patient selection (e.g., younger and more mild patients for this approach to be successful).

An additional important note for PD pathogenesis is the existence of Lewy body pathology stemming from dysregulated α-synuclein aggregation. Evidence from multiple fetal VM grafts in postmortem patient histology has shown pathological transfer of Lewy bodies from host to graft material [84, 86, 87]. However, our recent analysis has shown this phenomenon to be time dependent, as evidenced by S129-α-synuclein (marker of pathologic inclusions) staining only seen in graft material after 10 years [50]. Data from Li et al. also featured signs of host-donor Lewy body transfer in long-term grafted neurons, although there was minimal evidence of functional impairment in affected neural populations [84]. Due to the inconsistency in graft mediation of PD symptoms, it is unclear whether the transfer of Lewy bodies will exacerbate or disrupt potential benefit from grafted cell replacement and supplementation. Several groups have shown the propensity of pathological α-synuclein to propagate from brain regions and potentially spread from enteric innervation sites in prion-like fashion [88]. What is more concerning is the potential initiation of Lewy body pathology years before the onset of PD symptomatology, limiting the therapeutic window in which cell replacement therapy may be beneficial.

An important point of consideration is the inability of current animal models to provide comprehensive pathology akin to PD. Severe models such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-OHDA rapidly induce SN neural

loss and dopamine deficiency in the striatum, contrary to actual PD pathogenesis. Such rapid modeling of Parkinsonian symptoms inadequately features non-dopaminergic pathologies and fails to address extra-nigrostriatal dysfunction seen in human PD cases. Moreover, the increasingly known clinical characterizations of immune upregulation, oxidative stress, and mitochondrial insult and dysregulation in PD patients warrant appropriate mimicry from Parkinsonian models. Additionally, as many cases of proper heterotopic graft transplantation have shown exceptional integration into host tissue without ameliorating motor symptoms, the use of rapid onset PD models fails to elucidate the temporality of pathogenesis and treatment windows. It has become increasingly clear that there is a significant unmet need for appropriate PD biomarkers to evaluate the clinical stage of pathogenesis [89].

Another challenge for both VM grafts and, potentially, stem cell grafts in the clinic will be to identify ideal substrate compositions that will allow for symptom improvement based on stratification of Parkinsonism. However, due to the ethical considerations regarding embryonic tissue, pluripotent stem cell-based strategies may be the most intriguing of potential avenues for therapeutic discovery. What remains a multimodal roadblock for cell transplantation is the optimization of cell specificity, degree of differentiation, localization, and graft composite heterogeneity upon transplantation to bolster nigrostriatal DA tone for PD models and patients [90]. Ultimately, adequate and comprehensive evaluations of graft-host interactivity and efficacy can only be performed after development of robust cell differentiation and integration protocols. However, rapid technical advances and subsequent funding in the stem cell field generate an encouraging outlook for cell-based clinical therapies for PD patients.

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Cell Therapy for Epilepsy

5

Chiara Falcicchia, Giovanna Paolone,
and Michele Simonato

Epilepsy

History

One of the first diseases to be recognized and described by mankind is epilepsy, a neurological disorder characterized by the unexpected occurrence of seizures. A glimpse at the past emphasizes the many cultural (not only medical) aspects of the disease: its sudden and dramatic manifestations led to the idea that it was caused by invasions of gods or evil spirits and that it could only be treated by supranatural powers. Babylonians of the second millennium BC provide an accurate description of what today is identified as a generalized seizure [1]. The fight against this superstitious interpretation and the foundations for a scientific concept of the disease began with Hippocrates. In his treatise *On the Sacred Disease* (400 BC), he attacked the popular superstition who termed the disease "sacred" stating, for the first time, that the seat of the illness is in the brain and, therefore, it can be treated by natural means [2]. Almost 2500 years later, the struggle against superstition and stigma is still ongoing, and the etiology of the disease is still not completely understood, even if a number of causes have been identified, from brain injury to tumors, to metabolic disorders, and to genetic alterations. Moreover, in spite of the advances in electroencephalography, neuropsychology, and pharmacology that introduced novel concepts and strategies, patients continue to experience seizures, and, in many cases, seizures increase in frequency over time and associate with cognitive decline and psychiatric disorders.

Worldwide, millions of people suffer from poorly controlled seizures and comorbidities that have a devastating impact on the quality of life, urging the need of developing new therapeutic strategies for drug-resistant patients and, more in general, for

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the wider group of patients suffering from the debilitating comorbidities of chronic epilepsy.

Definitions, Clinical Features, Therapeutic Needs, and Models

The term epilepsy refers to a collection of diseases of different etiologies characterized by the spontaneous and unpredictable occurrence of seizures, i.e., transient occurrence of signs and/or symptoms due to abnormal excessive and synchronous neuronal activity in the brain [3]. Seizures may be focal, when originating within networks limited to one hemisphere, or generalized, when originating at some point within, and rapidly engaging, bilaterally distributed networks. The etiologies of the epilepsies may be genetic, structural-metabolic (i.e., lesional), or unknown.

Epilepsy is one of the most common chronic neurological conditions. About 1% of the world population is affected by epilepsy, and up to 5% may have a seizure at some time in their lives. In most countries worldwide, the prevalence of active epilepsy ranges from 4 to 10/1000. The incidence ranges from 40 to 70/100,000 in most developed countries and is nearly double in developing countries. This discrepancy is likely due to higher risk of experiencing conditions like meningitis, malaria, pre- and perinatal complications, and malnutrition that lead to brain damage and thereby favor the development of epilepsy [4].

The age distribution of the incidence of epilepsy is bimodal, with two peaks of frequency in childhood and in the elderly. Evidence of decreasing incidence in children, with an increase in the elderly, has been reported in Western countries. This may be related to improved life expectancy associated with an increased risk for causes of epilepsy common in old age [5]. Differences in incidence rates in males and females are not statistically significant. There is no evidence of ethnic variability, although the incidence is significantly higher in low socioeconomic classes [4].

The most common form of epilepsy in adults is temporal lobe epilepsy (TLE), a condition characterized by recurrent seizures originating from the mesial or lateral temporal lobe. Mesial temporal lobe epilepsy (mTLE) arises in the hippocampus, parahippocampal gyrus, or amygdala, whereas lateral temporal lobe epilepsy (lTLE) arises in the neocortex of the temporal lobe. TLE seizures often occur after an initial insult like an infection, stroke or trauma, vascular malformation, or prolonged febrile seizures; a genetic cause is less frequent. Between the initial insult and the onset of spontaneous seizures, a silent period called “epileptogenesis” occurs, during which the patient is apparently well, even if changes in structure and physiology of the brain tissue are ongoing and will eventually lead to hyperexcitability (Fig. 5.1). The most common abnormality identified in the brain of patients with TLE is a severe loss of neurons in the hippocampus termed hippocampal sclerosis (HS) [6].

Animal models are available for many epilepsies, including TLE. An animal model for a pathology could be isomorphic, if it duplicates the disorder but not the underlying etiology, or predictive, if it does not resemble the human disorder but allows predictions about it. For TLE, the pilocarpine model, described for the first time in 1983 by Turski [7] and colleagues, is a highly isomorphic model. In this model, administration of pilocarpine induces a status epilepticus (SE) characterized by tonic-clonic generalized seizures, followed by a latent, seizure-free period and

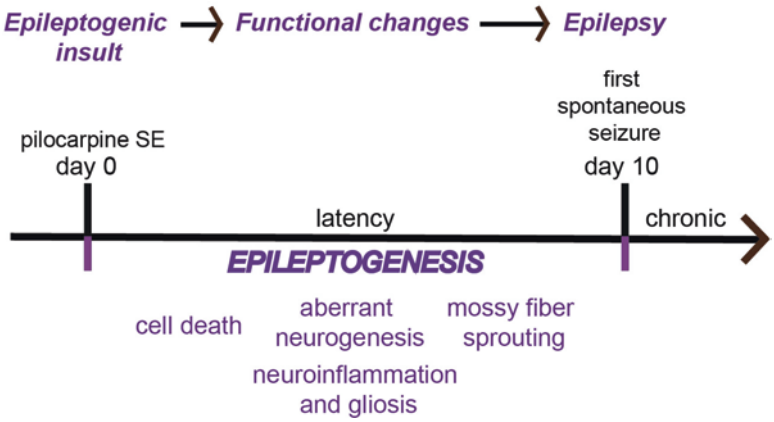

Fig. 5.1 Timeline of the major events associated with epileptogenesis

by a chronic period with the occurrence of spontaneous recurrent seizures (SRSs). The duration of the latent period varies as a function of the dose of pilocarpine, length of SE, strain, and age of the animal [8]. Cavalheiro and collaborators (1991) defined a meantime interval of 14.8 days. During the latent phase, tissue rearrangements related to epileptogenesis occur that are very similar to what is observed in humans [9]. The chronic period follows epileptogenesis, as previously mentioned, is characterized by the occurrence of SRSs. In 90% of the cases, the EEG during SRSs is characterized by hyperactivity starting in the hippocampus and spreading to the neocortex, usually lasting about 60 s [10]. Other chemicals (like kainate) and electrical stimulation of mesial temporal lobe structures represent valuable alternative models of SE followed by SRSs after a latency period.

Antiepileptic drugs (AEDs) have been and still are highly valuable for seizure control, but about 30–40% of patients present or develop resistance to pharmacological treatment [11]. This is particularly true for TLE, where continuation of seizures contributes to comorbidities like cognitive impairment and depression as well as problems in work and social activities. Furthermore, available AEDs merely suppress seizures without modifying the disease progression, with no effect on the development of epilepsy (epileptogenesis) in at risk individuals who experienced an epileptogenic insult [12, 13]. For some individuals, neurosurgical resection of the temporal focus is the only remaining option, but most of the patients are deemed unsuitable for or cannot have access to surgery. Alternative therapeutic strategies, like ketogenic diet and deep brain or vagus nerve stimulation, are not suitable for all patients because of moderate efficiency or undesirable side effects [14].

In sum, novel therapies, efficacious for either prevention or control of drug-resistant epilepsy, are highly needed. Some of these strategies envision the substitution of damaged cells or the direct, targeted, cell-mediated delivery of therapeutic compounds to the brain region that generates seizures. These cell therapy approaches are currently under investigation in preclinical models of epilepsy. There are two main avenues of research and development in this field: the direct grafting of cells in the epileptogenic tissue (generally the hippocampus) or the use of encapsulated

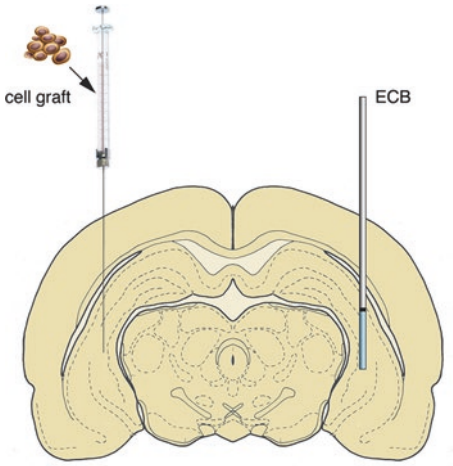

Fig. 5.2 The two main strategies for cell therapy in the epileptogenic tissue: direct cell grafting (*left*) or the use of encapsulated cell biodelivery devices (ECB, *right*)

cell biodelivery devices, ECB (Fig. 5.2). These two approaches, and the main results obtained in experimental models of epilepsy, are described and discussed below.

Cell Therapy: Direct Grafting

Introduction: Cell Types

A variety of cells have been tested in preclinical models of epilepsy for their capacity to suppress seizures following grafting into distinct regions of the brain. The donor cells examined with intracerebral grafting include hippocampal precursor cells, neural stem cells (NSCs), primary gamma-aminobutyric acid-positive (GABAergic) cells or GABAergic precursor cells from either the embryonic lateral ganglionic eminence (LGE) or the medial ganglionic eminence (MGE), GABAergic progenitors derived from mouse or human embryonic stem cells (mESCs and hESCs), and human induced pluripotent stem cells (hiPSCs) [14].

Substitution Strategies and Bystander Effects

Tissues like the skin and liver can repair themselves, because dead cells can be replaced either by proliferation of nearby cells or by activation of resident stem cells. In contrast, cell renewal in the central nervous system (CNS) is limited, rendering it particularly vulnerable to injury or disease [15]. For this reason, an appealing strategy is transplanting new cells that can replenish the damaged neural circuit.

The therapeutic effects of transplanted cells rely on their ability to survive and become structurally and functionally integrated into the brain. So far, the therapeutic potential of cell transplantation in suppressing seizures has produced mixed results. Initial studies focused on seizure-induced hippocampal cell death that is

accompanied by aberrant sprouting of the granule cell axons (mossy fiber) in the dentate subgranular zone. Shetty et al. [16] provided evidence that grafting of fetal hippocampal CA3 and CA1 neurons into the kainate-lesioned CA3 region can partially restore damaged structured and neuronal connectivity and provide a lasting inhibition of aberrant mossy fiber sprouting. Ruschenschmidt and colleagues [17] transplanted ESCs into the hippocampi of pilocarpine-treated adult rats. Although these cells developed into mature neurons, they displayed little migration away from the implantation site. However, extensive projections were observed into the host brain tissue, raising the possibility that these cells may nonetheless exert control over host brain functions.

An additional attempt of cell grafting was provided by Carpentino et al., [18] who used ESCs to produce neural progenitors that were transplanted into the hippocampi of adult mice 1 week after kainic acid-induced SE. While some of these cells produced mature-appearing neurons in the host dentate gyrus, they transformed into benign tumors when transplanted into control mice. This suggests that the hippocampus milieu after SE may provide environmental cues that favor differentiation and maturation of transplanted neural progenitors rather than transformation in tumors.

A major issue in these studies was that cell grafts did not survive for a long time. To enhance graft survival in the epileptic hippocampus, Shetty and colleagues [19] developed two preincubation strategies: one with brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and caspase inhibitor Ac-YVAD-cmk (BNC-treated grafts) and the other with fibroblast growth factor-2 (FGF-2) and caspase inhibitor Ac-YVAD-cmk (FC-treated grafts). It was found that the frequency of spontaneous seizures was highly reduced compared to those in animals receiving standard grafts or epilepsy-only rats. In addition, the yield of surviving neurons was greatly increased in BNC- and FC-treated grafts. Thus, standard grafts survive poorly in the chronically epileptic hippocampus. In contrast, BNC- and FC-treated grafts survive robustly in the chronically epileptic hippocampus, considerably reduce the frequency of seizures, and blunt the progression of chronic TLE.

A study using MSCs provided encouraging results [20]. In this case, cells were not shown to integrate in the damaged circuitries substituting dead neurons but produced bystander effects by modulating the levels of diffusible factors, in particular cytokines. MSCs were injected intravenously at 3 weeks and 10 months after pilocarpine-induced SE. In both experiments, MSC-treated rats had a marked reduction in the frequency and duration of seizures compared with saline-treated epileptic rats. Moreover, MSCs exerted neuroprotective/neurorestorative effects and anti-inflammatory effects. Interestingly, doublecortin-positive neuronal precursor cells decreased in rats receiving MSCs 3 weeks after insult, but their number increased in rats receiving MSCs 10 months after insult, compared to control rats receiving saline injections. Because abnormal neurogenesis accompanies epileptogenesis in the acute phase after insult, a reduced neurogenesis during epileptogenesis may be considered beneficial. In the chronic phase, however, neurorestorative neurogenesis may represent a physiological attempt to rescue neurodegeneration, and this may be favored by MSC injection.

Increased Inhibition Strategies

Another cell-based approach consists not just of replacing damaged hippocampal neurons but primarily of increasing inhibitory signals. Because epileptic seizures basically originate from an imbalance between excitation and inhibition in favor of hyperexcitability, the goal of these studies is to restore the balance between the two. Inhibition is largely dependent on the neurotransmitter gamma-aminobutyric acid (GABA). GABAergic neurotransmission undergoes maladaptive changes during the development of epilepsy that lead to its impairment, including the desensitization of the postsynaptic $GABA_A$ receptor and the loss of GABAergic cells [21, 22]. With the intent of restoring inhibitory neurotransmission, many therapeutic attempts have been conducted using GABAergic cells or GABAergic precursor cells from both the embryonic LGE [23] and MGE [24–26], GABAergic progenitors derived from the mESCs and hESCs, and hiPSCs [14].

Hattiangady and colleagues [23] demonstrated that bilateral grafting of appropriately treated striated precursor cells into hippocampi, 4 days after kainite-induced SE, is efficacious and considerably reduced the frequency of spontaneous recurrent motor seizures (SRMS) on a long-term basis in the chronic epilepsy period. A reduced frequency of SRMS after striatal precursor cell grafting in SE rats was associated with long-term (1-year) survival of a sizable fraction grafted cells with differentiation of a vast majority of grafted cells into GABAergic neurons. Additional analyses revealed the presence of multiple subclasses of GABAergic neurons within grafts, including neurons positive for calbindin, parvalbumin, calretinin, and neuropeptide Y.

Baraban et al. [24] used fetal neural precursors (E13.5 mice) from the MGE to treat seizures in a model of generalized epilepsy, namely, a Kv1.1 mutant mouse that has a defective potassium channel. This mutation affects primarily GABAergic interneurons, leading to generalized epilepsy with onset at 2–3 weeks of age. Neural precursors were transplanted bilaterally into the neocortex of newborn mutant mice and resulted in an 86% reduction of electrographic seizures, as compared to non-treated mice. Histological and physiological testing demonstrated the transformation of transplanted cells in GABAergic neurons that integrated into the host circuitry. Concurrently, the host brain regions implanted with MGE cells exhibited an increased level of GABA-mediated synaptic activity compared with controls. Given that transplantations were performed before the emergence of spontaneous seizures in these mice, this strategy could be classified as "anti-epileptogenic." This is a remarkable finding, considering that the genetic defect in this model is not focal and the transplanted cells were implanted in a single structure [27].

In another study, genetically engineered MSCs able to differentiate into GABAergic neurons were transplanted in the right lateral ventricle of rats 2 h after SE [28]. A clear amelioration of the disease was then observed: mortality and EEG bursts of the experimental group were reduced compared to the control group [28].

Limits of This Approach

In conclusion, the direct cell grafting into brain parenchyma may permit anatomical integration of the transplanted cells in the host tissue, and transplanted cells may remain viable long term. In cases in which the goal was not integration but simply to supplement an inhibitory molecule, adequate diffusion may be achieved. Therefore, these results are promising. However, there are several issues that must be addressed before attempting clinical translation of cell grafting for epileptic patients. These include the possibility that grafted cells induce an immune rejection response. Moreover, the migration of cells from the injection site is uncontrolled, and the cells cannot be retrieved in case of unwanted side effects.

Cell Therapy: Encapsulated Cell Biodelivery (ECB) Systems

Introduction: Description of the Strategy

Encapsulated cell biodelivery (ECB) is an attractive alternative to more direct cell therapy approaches. This technology targets diseased neurons with therapeutic biological substances that are continuously produced and secreted by genetically engineered human cells enclosed within a particular device in which cells grow on a polymer scaffold behind a semipermeable hollow fiber membrane [29]. Encapsulated cells continuously secrete a biologically active amount of a therapeutic protein directly into the localized region of the brain where the device is implanted [30]. ECB devices have the advantage of being a reversible treatment: they can be removed from the brain with a relatively simple procedure and thereby terminate their effect. The cells in the ECB devices can have long-term viability when implanted into the brain because the nutrients from the surrounding host tissue can penetrate the semipermeable membrane, while high levels of therapeutic agents can be distributed over a significant portion of the surrounding tissue. Another advantage of ECB devices is that the encapsulated cells do not alter the host cells or integrate into the host brain. Furthermore, the semipermeable membrane isolates the cells in the device from immune reactions, avoiding the need of immunosuppressant drugs. As such, this therapeutic technology platform combines the potency of de novo in situ synthesis of the treatment with the safety of an implantable, biocompatible, and retrievable medical device. These features confer a tremendous potential for treating a range of human diseases, including epilepsy.

Increased Inhibition Strategies: Galanin

Indeed, the ECB technology has already been tested in an animal model of epilepsy. Nikitidou and colleagues [31] explored the therapeutic potential of galanin-releasing ECB devices in a model of stimulus-induced seizures named kindling. The neuropeptide galanin was first discovered in porcine intestine, [32] but later it has been

found in various parts of the body, including the peripheral nervous system (PNS) and the central nervous system (CNS). Galanin has diverse physiologic functions in the normal brain, but it has also been implicated in pathophysiologic conditions, for example, depression [33], Alzheimer's disease [34], and epilepsy [35]. Several studies suggest that galanin is involved in seizure regulation and can modulate epileptic activity in the brain: during epileptic seizures, galanin is released and exerts a presynaptic inhibitory effect on the glutamatergic transmission [36].

Nikitidou et al. [31] tested two different cell clones secreting different levels of galanin in the kindling model. Intrahippocampal high-releasing galanin-producing ECB devices moderately decreased stimulation-induced focal afterdischarge duration, while the low-releasing clone did not exert any effect. No significant effect was detected on the duration of generalized seizures nor on seizure threshold when comparing animals receiving any of the treatments with controls. Therefore, despite the moderate effect of galanin, ECB devices demonstrated a certain therapeutic effect as well as potential as a valuable and safe strategy for delivering seizure-suppressant treatments locally in the brain.

Neurotrophic Factors

As an alternative to potentiation of inhibition, many others proposed to favor neuroprotection. Indeed, neurotrophic factors (NTFs) have been explored as therapeutic modalities against TLE. Specific NTFs including fibroblast growth factor-2 (FGF-2), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) have multiple, pleiotropic effects on the nervous system including not only survival but also maintenance and regeneration of specific neuronal populations in the adult and developing brain [37] and can be hypothesized to oppose most of the alterations observed in the epileptic brain [38]. In our laboratory, we are focusing in particular on two NTFs as therapeutic agents against epilepsy, namely, BDNF and GDNF. The rationale for these choices is described below.

BDNF, a member of the neurotrophin family, is widely expressed in the brain. The BDNF protein consists of a non-covalently linked homodimer and contains a signal peptide following the initiation codon and a pro-region containing an N-linked glycosylation site. It is initially produced as a proneurotrophin (pro-BDNF, ~30 kDa) that a protease cleaves to the mature form (~14 kDa; [39]). In addition to the mature form, pro-BDNF can be also released and is biologically active. Released pro-BDNF can undergo a high-affinity interaction with p75NTR, a receptor that has low affinity for the mature form, or can be transformed into mature BDNF by extracellular proteases. Mature BDNF can bind with high affinity to tropomyosin receptor kinase B (TrkB) receptors.

BDNF and TrkB exert key roles in neuronal survival, differentiation, synapse plasticity, and memory [40, 41]. It is therefore not surprising that they have been implicated in the pathophysiology of numerous diseases, including epilepsy. For instance, Kuramoto and colleagues [42], using BDNF-producing cell transplantation, provided evidence that continuous administration of BDNF at low dose can

exert antiepileptic effects. The mechanism of these effects remains obscure, but experimental evidence supports the idea that increased inhibition may be part of it. In fact, BDNF is known to modulate the expression of an inhibitory neuropeptide, neuropeptide Y (NPY). Continuous intrahippocampal administration of BDNF may exert antiepileptic effects through increased expression of NPY [43]. NPY is thought to inhibit seizure generation and is interesting to note that both kindling and kainate-induced seizures increase NPY immunoreactivity with a distribution that is strikingly similar to phospho-Trk immunoreactivity, i.e., with activated Trk receptors. This suggests that BDNF-induced TrkB activation can lead to NPY upregulation, which might subsequently limit excitability [44]. In addition, Palma and collaborators [45] demonstrated that BDNF amplifies GABA currents and prevents their run-down in Xenopus oocytes expressing $GABA_A$ receptors transplanted from surgically removed specimens of human epileptic brains.

The other trophic factor that we are investigating is GDNF. GDNF signals, through a multicomponent receptor, first bind the GDNF family receptorα1 (GFRα1) with the resulting complex recruiting the transmembrane receptor tyrosine kinase Ret or the neural cell adhesion molecule (NCAM) to initiate downstream signaling pathways [46, 47]. Although the effectiveness of GDNF in regulating the survival and maintenance of dopaminergic neurons has been known for a long time, the attempts of exploiting these properties as therapeutic tools failed when facing the relatively inaccessibility of the CNS and need of a stable availability of the protein over time [48]. More recently, GDNF has emerged as a possible new agent for epilepsy treatment, because GDNF family ligands and receptors are expressed in areas salient for seizure generation [49]. However, direct evidence of effects of the modulation of GDNF signaling in epilepsy models is still lacking.

So far, the development of effective NTF-based therapies has been hindered by the inability to deliver them across the BBB to the target site in a stable, controlled, and continuous manner. The choice of the route of administration represents a key factor for therapeutic application of NTFs, since inadequate methods might be responsible of the clinical failure. An appropriate system of supplementation might ensure a correct amount of trophic factor to the affected sites, avoiding widespread diffusion to extra-target receptors, responsible of unwanted effects. Moreover, delivery strategies based on cell grafts or viral vectors generally provide a relatively short-term treatment, whereas, by their very nature, chronic diseases like epilepsy require long-term treatments.

Delivering NTF with ECB Devices

Our preliminary studies demonstrate that ECB devices filled with cells modified to secrete high levels of BDNF or GDNF, when bilaterally implanted in the hippocampus, significantly (by about 80%) decrease the frequency of spontaneous seizures in rats made chronically epileptic following pilocarpine-induced SE (Fig. 5.3). In addition, animals implanted with devices containing unmodified cells or empty devices displayed identical seizure frequency and severity as those completely

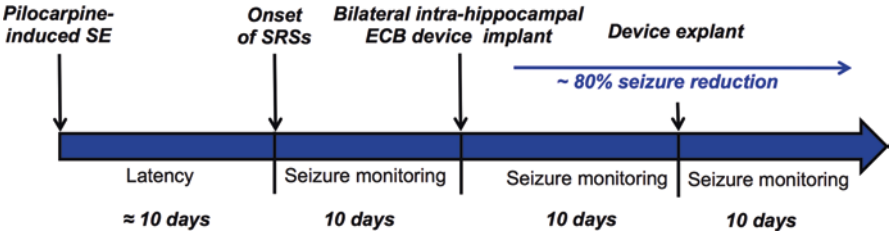

Fig. 5.3 Schematic diagram of the ECB experiments

untreated. Considering that only a subset of spontaneous seizures originate from the hippocampus in the pilocarpine model, this effect may be even greater if referred only to hippocampal seizures. Moreover, epileptic rats treated with ECB devices releasing either BDNF or GDNF performed better than those not treated in behavioral tests of memory and anxiety. These data suggest that ECB devices could potentially be an effective system for the long-term delivery of exogenous NTFs to the hippocampus and that this strategy can reduce the frequency of generalized seizures and ameliorate epilepsy-associated comorbidities. Of course, a limiting factor may be the extent of damage caused by the implant. In translational terms, however, the impact of damage would be relatively smaller in the much larger human brain. In addition, this factor did not prove critical in our experimental setup, in that animals implanted with empty devices or with devices containing parental cells displayed identical seizure frequency and severity as those completely untreated. Understanding in depth the mechanistic basis of these effects will require further studies, but these preliminary data are very encouraging.

Some aspects of the present findings are worthy of note. First, the implantation of ECB devices was performed under conditions that are perfectly compatible with the clinical situation: chronic patients with surgically treatable TLE that are planned to undergo a two-step surgery (i.e., first, implantation of a grid of electrodes to map the epileptogenic area; second, after a period of video-EEG monitoring, the resection) may be an ideal population to clinically test this approach. The ECB device may be implanted together with the electrodes, and, should it prove ineffective, it could be removed, and the patient could undergo surgery as originally planned. Second, the results are promising not only in epileptic field, but also for other neurodegenerative diseases or to test other NTFs. Indeed, the ECB technology has been already tested in other neurologic diseases, such as Alzheimer's disease, and has demonstrated good safety and tolerability [50, 51].

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Dendritic Cell-Based Cancer Therapies: Current Status and Future Directions

6

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Abbreviations

APC	Antigen-presenting cell
cDC	Conventional DC
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
iDC	Immature DC
IFN	Interferon
IL	Interleukin
KLH	Keyhole limpet hemocyanin
mDC	Mature DC
PD-1	Programmed death receptor-1
pDC	Plasmacytoid DC
TLR	Toll-like receptor

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Introduction

Vaccination is an effective method for preventing the spread of infectious diseases. Edward Jenner is credited with developing the first vaccine, which he used against smallpox in 1796 [1]. In the twenty-first century, the use of vaccines has become indispensable to the eradication of infectious diseases [2]. Vaccines can either be preventative or therapeutic. Preventive vaccines block the spread of a disease within populations by inducing the generation of specific antibodies and the formation of long-lived memory B cells. In addition, preventive vaccines can also induce cellular immunity [3]. Therapeutic vaccines, on the other hand, stimulate the immune system to help eliminate the cause of the disease in the body. Therapeutic vaccines can be used to treat infectious diseases or cancer [4].

The word "cancer" refers to a group of diseases involving abnormal growth of cells. These cells have the potential to spread to other parts of the body. Moreover, cancer cells tend to form tumors, which are complex tissues comprised of multiple types of heterogeneous neoplastic cells [5]. Cancer immunotherapy is a targeted therapy that utilizes proteins associated with the tumor for therapy. Due to the heterogeneous nature of tumors, multiple combinations of targeted therapies are useful to achieve maximum clinical success.

A recent search on www.clinicaltrials.gov for the term "cancer vaccines" yielded 1837 clinical studies as of October 31, 2016. One hundred eighty-three of these were phase III clinical trials, and 833 were phase II clinical trials. This highlights the importance of cancer vaccine studies. The activity of therapeutic vaccines depends on antigen-specific $CD8^+$ T cells, which generate cytotoxic T lymphocytes (CTLs) to reject cancer or infected cells. The CTLs generated by a vaccine need to have (1) higher affinity T cell receptor and higher T cell avidity against peptide-MHC molecules expressed on tumor cells [6], (2) higher expression of the secretory molecules perforin and granzyme [6], (3) expression of certain chemokines (e.g., CXCR3) which allow them to migrate to the site of the tumor [7], (4) persistence at the tumor site (e.g., integrins CD103 [8] and CD49a [9]), and (5) high expression of co-stimulatory molecules (e.g., CD137 [10]) and low expression of inhibitory molecules (e.g., cytotoxic T lymphocyte antigen 4 (CTLA-4) [11] or PD-1 [12]). The generation of such $CD8^+$ T cells requires antigen presentation by appropriate antigen-presenting cells (APCs) [13, 14] and the generation of $CD4^+$ T cells that produce cytokines such as IL-21, which promote $CD8^+$ T cell proliferation and differentiation [15]. Therapeutic vaccines should also induce the formation of long-lived memory $CD8^+$ T cells, which act to prevent relapse of the disease [16].

Dendritic Cell Subpopulations

Dendritic cells (DCs) were identified by Steinman and colleagues more than 40 years ago on the basis of their cytolytic features and the absence of surface immunoglobulin (Ig) molecules, thy-1, and brain antigens [17, 18]. DCs are professional APCs, whose principle function is to present antigens to resting naïve T lymphocytes in

order to induce primary immune responses. DCs have high cell surface expression of major histocompatibility complex (MHC) class I molecules, MHC class II molecules, and CD86 [19].

DCs are an essential component of vaccines, due to their ability to capture an antigen, process it, and present it to T cells [20]. In peripheral tissues, immature DCs (iDCs) can efficiently capture antigen and present it to T cells, resulting in immune tolerance due to the lack of co-stimulatory molecules [21]. Targeting DCs for vaccination requires a detailed understanding of the function of the various DC subsets in immunity. DC subtypes differ in location, migratory pathways, immunological function, and dependence on infection or other types of inflammatory stimuli. Depending on their localization in lymphoid tissues, mouse and human DCs are divided into two major subsets: plasmacytoid DCs and conventional/myeloid DCs.

Plasmacytoid Dendritic Cells

Plasmacytoid DCs (pDCs) are a rare subset of DCs that are morphologically and functionally unique from conventional DCs. At steady state, pDCs express low levels of MHC class II and co-stimulatory molecules, as well as low levels of integrin CD11c [22]. During viral infections, or upon recognition of foreign nucleic acids, pDCs produce large amounts of type I interferon (IFN) in response to toll-like receptor (TLR) 7 and TLR9 ligation [23]. pDCs have poor antigen presentation capacity and have been associated with immune tolerance in mice [24]. However, whether a similar tolerogenic function exists in human pDCs remains unknown.

Conventional/Myeloid Dendritic Cells

Conventional DCs (cDCs) have long dendrite extensions and high MHC class II expression. Although recent evidence suggests that mouse and human cDCs have functional homology, cDCs express species-specific surface markers [25]. Lymphoid tissue-resident cDCs are found in the thymus, spleen, lymph nodes, and Peyer's patches in mice. They express either CD8 alpha or CD11b. Mouse CD8 alpha$^+$ cDCs recognize and cross-present extracellular antigens to CD8$^+$ T cells, which produce IL-12 and IFN-γ upon activation, and prime Th1 and cytotoxic T cell responses. On the other hand, the CD11b$^+$ cDC subset preferentially activates CD4$^+$ T cells and induces Th2 or Th17 differentiation. In addition to the lymphoid tissue-resident cDCs, three subsets of mouse migratory/nonlymphoid tissue cDCs have also been identified. These include CD103$^-$CD11b$^+$ cDCs, CD103$^+$CD11b$^-$ cDCs, and CD103$^+$CD11b$^+$ intestinal cDCs. Migratory/nonlymphoid tissue CD11b$^+$ cDCs are functionally similar to lymphoid tissue-resident CD11b$^+$ cDCs, while CD103$^+$ cDCs share similarities with lymphoid tissue-resident CD8 alpha$^+$ cDCs, including their ability to cross-present antigens to CD8$^+$ T cells.

Skin Dendritic Cells

The skin also contains some DC subsets that can also be found in the lymphoid organs. Mouse and human epidermis contains Langerhans cells (LCs), which are characterized by the expression of langerin and E-cadherin, and are strong stimulators of $CD4^+$ T cells [26–28]. Ex vivo experimental models have shown that both mouse and human LCs are strong cross-presenting cells [29]. However, there is conflicting evidence regarding their in vivo cross-presentation capacity [30].

Inflammatory DCs

Inflamed tissues and draining lymphoid organs contain another subset of DCs called inflammatory DCs. Inflammatory DCs are generated from monocytes and express macrophage-specific markers such as F4/80, CD64, and the high-affinity IgE receptor, FcεRI [31]. Inflammatory DCs can be generated in culture from human monocyte-derived DCs (moDCs) using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [32]. Mouse inflammatory DCs function in both innate and adaptive immunity and activate $CD4^+$ T cells for polarizing into Th1-type and Th2-type immunity [33]. During bacterial infection, inflammatory DCs produce iNOS and TNF-α, and their activation is dependent on TLR4 and TLR9 [34]. The first evidence for the role of inflammatory DCs in inducing Th2-type immunity came from the study by Kool et al. [35], where they demonstrated a rapid recruitment of $CD11b^+F4/80^{Int}Ly6C^{high}$ "inflammatory monocytes" to the peritoneal cavity within 6 h after intraperitoneal injection of OVA-albumin. In mice, several infectious models have suggested that inflammatory DCs are critical for Th1-type immunity. Upon immunization or viral infection, these inflammatory DCs produce IL-12 and stimulate T cell-mediated IFN-γ production [33].

Dendritic Cell Functions

Activating Signals from DCs

Dendritic cells express co-stimulatory molecules on their surface, which are important for the induction of immune responses. T cell activation occurs through the recognition of antigenic MHC peptides on DCs by T cell receptors. However, robust T cell responses also require interactions between co-stimulatory ligands on T cells and their receptors expressed on DCs. Without this co-stimulatory molecule interaction, antigen-specific T cells become anergic. DC vaccines can be useful by upregulating co-stimulatory signals in therapeutic immunity [36, 37]. Two major families of co-stimulatory molecules are expressed on dendritic cells: the B7/CD28 family and the tumor necrosis factor (TNF) family receptors. The initiation of the cell-mediated immune response is mediated by B7/CD28 family members, whereas the

later phase of T cell activation is dependent on TNF-receptor family members. In the B7/CD28 family, the expression of CD80 (B7.1) and CD86 (B7.2) [37] on DCs is the most important co-stimulatory pathway in T cell activation. Others include ICOS-ligand (ICOSL; B7-H2, [38]), programmed death-1 ligand (PD-L1 or B7-H1), PD-L2 or B7-DC, as well as B7-H3 [39] and B7-H4, also known as B7x and B7S1 [40]. The TNF superfamily co-stimulatory molecules in DCs include CD40, OX40L, CD27, 4-1BB (CD137), TNF receptor superfamily member 4 (TNFRSF4), TNF ligand superfamily member 14 (TNFSF14), and glucocorticoid-induced tumor necrosis factor receptor (GITR) [41–43]. T cells express the activating receptor CD28 and the inhibitory receptor CTLA-4 (CD152) on their surface. The co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on DCs bind with either the activating receptor CD28 or the inhibitory receptor CTLA-4 on T cells (Fig. 6.1). Interaction of B7.1 or B7.2 with CD28 stimulates the activation of T cells. In contrast, engagement of B7.1 or B7.2 with CTLA-4 inhibits T cell-mediated immune responses. Downregulation of immune responses leads to immune tolerance and autoimmunity. Therefore, the blocking of inhibitory signals through CTLA-4, in addition to the upregulation of B7.1 and B7.2 by immunostimulants, is essential for enhancing antitumor responses [44].

DCs are pulsed with multiple ligands, including TLR agonists, CD40 ligand, and TNFRSF4 ligand, which induce the expression of co-stimulatory molecules on their surface. TLRs are expressed on most immune cells, and in some cases also on tumor cells. For example, GL261 cells express TLR2, TLR3, and TLR4 and increase MHC class I expression [45]. Activation of DCs with TLR signals induces the upregulation of co-stimulatory molecules [46]. Activated DCs secrete immunomodulatory cytokines (i.e., IL-12) and increase antigen processing and presentation to T cells and B cells. TLR agonist injection at the tumor site (intratumoral injection) produced a survival benefit in multiple tumor models in rodents [47].

DCs also talk to invariant natural killer T cells (iNKTs) through the interaction of CD40 and CD40 ligand. The synthetic iNKT agonist α-galactosylceramide (α-GalCer) promotes T cell responses to DC vaccines. DCs can acquire and present α-GalCer to CD1d molecules (an MHC class I-like molecule, highly expressed in NKT cells) and induce the expression of the co-stimulatory molecule CD40 upon interaction with iNKTs [48].

Inhibitory Signals from DCs

Certain co-stimulatory molecules on DCs, including PD-L1, PD-L2, and B7-H4, inhibit T cell-mediated immune activation [40, 49]. Antibody blocking of PD-L1 and PD-L2 on DCs improves the proliferation and cytokine production of $CD4^+$ T cells. DCs also express suppressive molecules, including zinc finger protein (ZFP) A20, DC-derived immunoglobulin receptor 2 (DIgR2), Notch ligands, and suppressor of cytokine signaling 1 (SOCS1) [50]. Targeting of these molecules could potentially increase the efficacy of DC-based therapeutic vaccines.

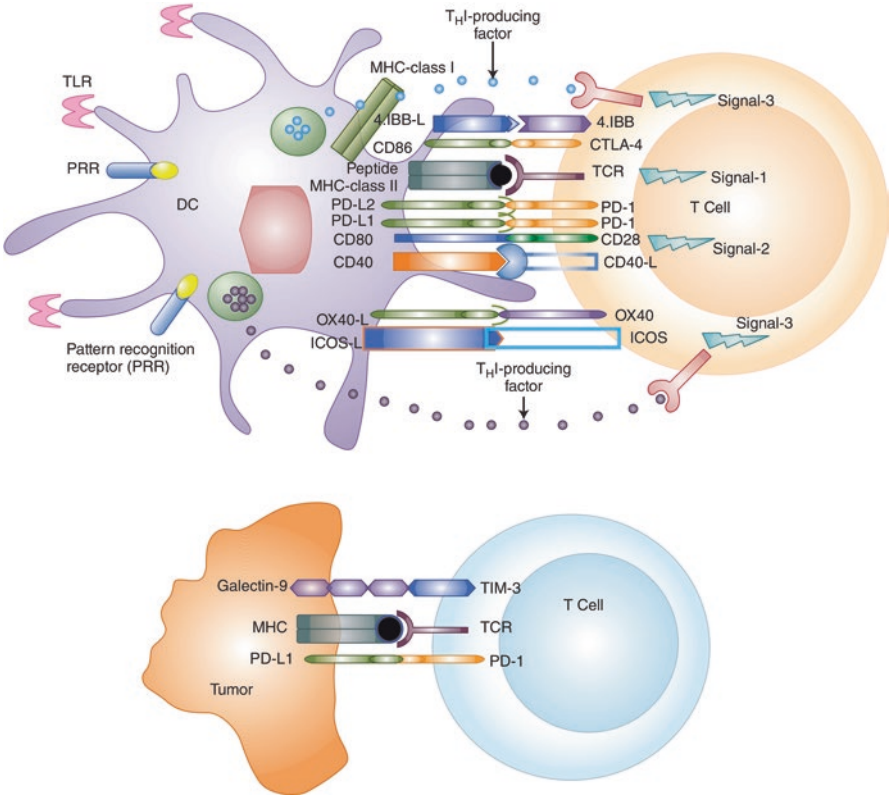

Fig. 6.1 (**a**) Signals generated between T cells and DCs. Both T cells and DCs express complex networks of transmembrane receptors and ligands, which interact with each other to enhance T cell activation. Interaction of T cell receptor (TCR) with MHC complex on DCs generates signal-1. Co-stimulatory signal (signal-2) is required for full activation of T cells, which is mediated by the interaction of CD28 (expressed on T cells) with CD80 and CD86 on DCs. For functional activation of T cells, signal-3 is important. This signal is mediated by soluble factors such as IL-12, IL-15, IL-16, or TNF-α. Generation of signal-3 also depends on the activation of PRRs by PAMPs or TFs, which activates DCs to produce T_H1- or T_H2-cell polarizing factors. ICOS, expressed on T cells upon activation, interacts with ICOS-ligand (ICOS-L: B7-H2) on DCs to regulate T cell growth, proliferation, survival, and polarization. Ligation of CD40 (expressed on T cells) with CD40L on DCs induces expression of co-stimulatory, adhesion, and MHC molecules and promotes the secretion of T cell stimulatory cytokines like IL-12. T cells also express inhibitory receptors such as CTLA-4 and PD-1, which suppress T cell activation by interacting with ligands (PD-L1, PD-L2) expressed on DCs. (**b**) Interaction of T cells with tumor. The inhibitory receptors (PD-1, TIM-3) expressed on T cells can bind with their ligands (PD-L1, Galectin-9) expressed on tumor cells. This will inactivate the T cells, providing an immunosuppressive environment for the tumor

Antigen Loading into DCs

The therapeutic efficacy of DC vaccination depends on the effectiveness of the uptake and loading of tumor-specific antigens into MHC complexes, as well as the expansion of DC subsets that can prime naïve T cells for targeted immune activation. Antigens can be comprised of RNA, DNA, proteins, peptides, tumor lysate, fusion proteins, or apoptotic cells [51]. Research on antigen presentation for DC vaccination has shown that whole tumor antigens induced greater clinical responses than single peptide antigens, in consideration of the heterogeneous properties of tumors [52, 53]. In contrast, DCs loaded with apoptotic bodies of GBMs can increase the risk for induction of tolerogenic DCs [54].

DC-Mediated Immune Responses Against Tumors

The goal of DC-mediated cancer vaccines is to induce the formation of tumor antigen-specific cytotoxic $CD8^+$ T cells, which can recognize and kill target tumor cells [55]. Recent evidence has suggested that DCs can capture tumor antigens, process them, and cross-present them to T cells in tumor-draining lymph nodes. This generates tumor-specific cytotoxic $CD8^+$ T cells, which contribute greatly to tumor rejection [56, 57]. Data from clinical trials have indicated that DC vaccination also induces natural killer (NK) cell immunity, which includes enhancing NK cell number and functional activation [58]. DCs have been used for tumor eradication in a mouse melanoma model, and the effect was completely abrogated after depletion of NK cells, indicating the strong positive role of NK cells in DC-mediated vaccination against tumors [59, 60].

DCs are present in most tumors and play an important role in the tumor microenvironment in controlling tumor progression [61]. DCs infiltrate into the tumor site and serve to recruit and activate disease-fighting immune effector cells. Recent advances in the understanding of the tumor microenvironment have led to targeting tumor-infiltrating DCs for cancer therapeutics. Altering immunosuppressive DCs to become immune-stimulatory DCs is one of the strategies employed for effective cancer immunotherapy [61, 62]. DCs can take up tumor-specific antigens by interacting with live tumor cells or by capturing dying tumor cells. Dying tumor cells not only release tumor antigen for uptake by DCs but also express many signaling molecules on their surface, which could be either stimulatory or inhibitory for DCs. For example, tumor cells can express phagocytic markers like CX3CL1, which activates DCs for phagocytic activity, or CD47, which interacts with signal regulatory protein-a on phagocytes to provide inhibitory signals that prevent phagocytosis. Using a CD47-blocking antibody in combination with rituximab (a CD20 antibody that depletes B cells) results in increased phagocytosis in tumor models in mice [63].

Tumor cells have mechanisms to suppress DC function, or to recruit immune-suppressive DCs to the tumor site [55]. The tumor can switch the differentiation of monocytes to macrophages and prevent the priming of tumor-specific cytotoxic T cells by DCs. In addition, tumor glycoprotein antigens can be endocytosed into

early endosomal compartments, which prevents efficient processing and presentation to T cells [64, 65]. Tumor cells also secrete the cytokine IL-10 [66], which can inhibit DC maturation, leading to anergy [67]. In addition, the maturation of mDCs can be altered by tumor-derived factors, leading to the development of DC cell types that indirectly promote tumor growth. pDCs can also play an important role in tumor progression by inducing naïve $CD4^+$ T cells to differentiate into IL-10-producing T cells with immunosuppressive functions [68]. In breast carcinoma, infiltrating pDCs produce little IFN-α upon ligation with TLRs, due to the inhibitory signals from ligation of immunoglobulin-like transcript 7 (ILT7) on pDCs with bone marrow stromal antigen 2 (BST2). DCs require IFN-α for cross-presenting tumor antigens to T cells. Thus, the inhibition of IFN-α signaling affects the generation of tumor antigen-specific cytotoxic T cells [57, 69].

Dendritic Cell Immunotherapy

The main goal of cancer immunotherapy is to stimulate tumor antigen-specific T cells to inhibit the malignant activity of cancer cells. Vaccines should target the cancer cells but also leave healthy cells unaffected [70]. The discovery that vaccination with DCs loaded with tumor antigens stimulates strong and broad immune responses in tumor-bearing animals has rejuvenated the field of cancer vaccine research and has led to many clinical trials. These trials have involved multiple different methods of generating DCs, introducing antigens to DCs in vitro or in vivo, as well as introducing DCs into the body.

Generation of DCs and Antigen Loading

As DCs constitute only about 1% of peripheral blood mononuclear cells (PBMCs), therapeutic DCs need to be generated from precursors. In the presence of IL-4 and GM-CSF, considerable numbers of DCs can be generated from monocytes or $CD34^+$ progenitors [71–74]. However, it is unclear whether ex vivo-differentiated DCs are the optimal source for DC-based immunotherapy. Long-term culture of DCs ex vivo, with the cytokines and mediators required for their activation and differentiation, might negatively affect the function of DCs when injected into patients. The newly injected DCs might express different receptors or lose the ability to produce some pro-inflammatory cytokines [75].

DCs loaded with tumor antigens linked to keyhole limpet hemocyanin (KLH), an immunogenic protein, produce an enhanced antitumor immune response [76]. Tumor-bearing mice immunized with DCs loaded with anti-idiotype protein produced tumor-specific immune responses [77, 78]. Helper antigens can also be coupled with tumor antigens for presentation by DCs, which can produce more robust immune responses against both the tumor target antigen and the helper antigen [79]. Antigens from whole cell lysate can also be loaded into DCs for vaccination. DCs pulsed with myeloma lysates induced myeloma-associated immunity [80]. Whole-cell RNA,

DNA, or apoptotic bodies are also used for antigen loading into DCs [81, 82]. Whole cell lysate or cancer cells fused with DCs are used to present a wide variety of tumor antigens, which can ultimately enhance co-stimulatory functions of DCs. MC38 carcinoma cells, which express MHC classes I and II, co-stimulating molecules, and intercellular cell adhesion molecule-1 (ICAM-1), when fused with DCs for vaccination, strongly induced M38 tumor-specific immunity in vivo. Multiple myeloma cells freshly isolated from patients and fused with autologous DCs induced myeloma-specific cytotoxicity [83–86]. Mycobacterium tuberculosis heat shock protein X (HspX) has been used as an immunoadjuvant in DC-based tumor immunotherapy, which has significant potential in immunotherapeutics. The activated DCs induced pro-inflammatory cytokine production, and in vivo injection into mice significantly attenuated the metastatic capacity of B16-BL6 melanoma cancer cells toward the lung [87].

In Vivo DC Targeting for Immunotherapy

A recent area for DC cancer immunotherapy is to target DCs in vivo, using antibodies of activating receptors in addition to tumor antigens [88–90]. DCs process and present the antigen to T cells in order to stimulate an antitumor response. The optimal induction of this immune response also requires the presence of adjuvants, which stimulate DC activation. If an antigen-antibody complex is presented to DCs without adjuvants, the DCs can induce tolerance rather than immunity [91]. Nonactivated (immature) DCs can present self-antigens to T cells, which leads to immune tolerance, either through T cell depletion or through the differentiation of regulatory or suppressor T cells [92–95]. Naturally occurring regulatory T cells ($CD4^+$) are important for the maintenance of immune tolerance. $CD4^+$ regulatory T cells express CD25 (IL-2Ra), CTLA-4, and the transcription factor FoxP3, and exert their immunosuppressive effect either in a cell contact-dependent manner or by producing the cytokines IL-10 and TGFβ [96, 97]. Several studies have shown that using adjuvants (TLR agonists) combined with antigen-antibody conjugates stimulates antigen-specific immunity and prevents tolerance [98]. Other studies have shown that targeting the molecules expressed on DCs for delivering the antigen elicits antitumor responses. These include endocytic receptor DEC-205 [99–101], CD11c [102, 103], C-type lectin domain family 9 member A (Clec9A), MHC class II [104, 105], lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX1), mannose receptor (MR) [106, 107], CD36 (also known as fatty acid translocase (FAT)) [108], and bone marrow stromal cell antigen 2 (Bst2 or CD317), a molecule expressed on pDC [109]. DCs upregulate surface expression of CXCL16 following in vivo injection of the glycolipid antigen α-GalCer, which interacts with iNKT cells for IFN-γ production and tumor control against the metastatic B16 melanoma model in the liver and lung [110]. T cells activated by autologous tumor antigen-pulsed DCs were used for immunotherapy in human patients with endometrial cancer, where tumor-specific cytotoxic $CD8^+$ T cells were generated [111]. Also in humans, DC-mediated idiotype vaccination was used against multiple myeloma [112] and against prostate cancer [113]. Antigen-loaded upconversion

Table 6.1 Published clinical trial results of dendritic cell-based cancer immunotherapy

Vaccine	Cancer type	Findings	Reference
Autologous DCs pulsed with tumor-specific idiotype protein	B-cell lymphoma	Antitumor immune response; partial tumor regression	[117]
DCs pulsed with tumor lysate or MHC I peptides	Metastatic melanoma	Antitumor immune response; partial regression of metastases	[118]
Mature, monocyte-derived DCs pulsed with tumor peptide and recall antigen	Metastatic melanoma	Antitumor immune response; partial regression of metastases	[119]
Progenitor-derived DCs pulsed with tumor peptides	Metastatic melanoma	Antitumor immune response	[120]
DC/glioma cell fusion	Glioma	Antitumor immune response; reduction in tumor size	[121]
DCs loaded with tumor RNA or cDNA	Multiple advanced cancers	Antitumor immune response; partial disease regression	[122–124]
Allogenic DCs fused with tumor cells	Metastatic renal cell carcinoma	Antitumor immune response	[125]
DC vaccines combined with T_{reg} depletion	Metastatic renal cell carcinoma	Antitumor immune response	[126]
Autologous DCs loaded with allogenic tumor cell lysate	Metastatic melanoma	Enhanced antitumor immune response; partial disease regression	[127, 128]
αDC1s combined with poly (I:C)	Glioma	Antitumor immune response; partial disease regression	[129]
Langerhans cells (LCs) pulsed with tumor peptide	Metastatic melanoma	LC-based vaccines resulted in greater tyrosinase-HLA-A*0201 activity than DC-based vaccines	[130]
Autologous DCs combined with stimulated T cells	Ovarian cancer	Antitumor immune response; one complete clinical response	[131]
DC/tumor cell fusion following autologous stem cell transplant	Multiple myeloma	Antitumor immune response; reduction of residual disease	[132]
Preconditioning vaccine site with Td	Glioblastoma multiforme	Enhanced DC migration and improved patient survival	[133]

nanoparticles (UCNPs) have been used to label and stimulate DCs in vivo to induce antigen-specific immune responses by producing IFN-γ and generating CTLs [114]. Polymers of lactic acid and glycolic acid (PLGA) have also been used to supply DCs with cytokines, TLR ligands, and tumor lysates in vivo, which stimulated tumor-specific T cell responses against melanoma and glioma in tumor-bearing

animals [115, 116]. Here in Table 6.1, we have summarized the results of clinical trials that used DCs as immunotherapy in multiple different tumor targets, either alone or in combination with other agents.

DC Vaccines Combined with Other Therapies

Animal studies using DC vaccines combined with IL-2 for the treatment of sarcoma and squamous cell carcinoma (SCC) showed significantly suppressed tumor growth [134, 135]. Intratumoral injection of DCs combined with systemic chemotherapy (cyclophosphamide) led to complete tumor regression in a murine CT26 colon adenocarcinoma model [136]. Recent studies have shown that DC-based immunotherapy can also be combined with suppressive signals to positively regulate anticancer immunity. T cells express PD-1, which can bind with its ligand PD-L1 (expressed on the tumor cells or cancer cells) and suppress the immune system. Targeting this inhibitory signal, either by using siRNA of PD-L1 or using anti-PD-1 and anti-PD-L1 agents, has demonstrated huge clinical success in a wide range of malignancies [137]. Ipilimumab, an anti-CTLA-4 antibody, was also used in combination with DCs for the treatment of metastatic melanoma. Adriamycin plus DC combination therapy was also used for the B16 melanoma model [136]. CpG oligonucleotides (TLR ligand) combined with mature antigen-pulsed DCs were used in a syngeneic murine colon carcinoma model [138]. Autologous DCs loaded with tumor lysate or glioma-associated antigen in combination with radiotherapy were used to treat patients with malignant glioma [139]. Patients with treatment-refractory cancers were injected intratumorally with immature DCs that had been combined with KLH and a cytokine-based adjuvant. This was followed by an IV infusion of activated T cells. This treatment course was administered before and after radiation, which killed tumor cells so that DCs could use them as a source of antigen. Half of the patients in the trial showed a complete clinical response [140]. Table 6.2 summarizes the list of current phase III clinical trials using DC-based immunotherapy combined with chemotherapy or other agents. A recent cell type-based immunotherapy called sipuleucel-T consists of APCs (including DCs) activated by a recombinant fusion protein (PA2024) consisting of prostatic acid phosphatase fused to GM-SCF. Sipuleucel-T was used to treat patients with metastatic castration-resistant prostate cancer in a phase III clinical trial. Data from the study indicated short-term survival benefits and immune activation [141–143]. These studies suggest that combination therapy of DCs with other conventional therapies could be very effective against different types of cancer.

DC Vaccine Clinical Trials

Many of the DC vaccine discoveries from animal models have been translated to human clinical trials, with varying levels of success (Fig. 6.2). The first clinical study was performed in 1996 and involved the administration of immature DCs

Table 6.2 Current phase III clinical trials of dendritic cell-based cancer immunotherapy

DC intervention	Cancer type	Status	Results	ClinicalTrials.gov identifier
Autologous DCs pulsed with tumor lysate, combined with chemotherapy	Metastatic prostate cancer	Recruiting	N/A	NCT02111577
DC vaccine, combined with proteome-modified autologous HSCs, and CTLs	Glioblastoma multiforme	Enrolling by invitation	N/A	NCT01759810
DC vaccine, combined with autologous HSCs, and CTLs	Breast cancer brain metastases	Enrolling by invitation	N/A	NCT01782274
DC vaccine, combined with autologous HSCs, and CTLs	Lung cancer brain metastases	Enrolling by invitation	N/A	NCT01782287
Autologous APCs (including DCs) loaded with sipuleucel-T	Metastatic prostate cancer	Completed, has results	Improved overall survival; longer time to first use of opioid analgesics	NCT00065442; NCT01133704; NCT00005947
Autologous APCs (including DCs) loaded with sipuleucel-T	Metastatic prostate cancer	Completed	N/A	NCT00779402
DCs loaded with tumor lysate, combined with chemotherapy and radiation	Glioblastoma multiforme	Ongoing, not recruiting	N/A	NCT00045968
Autologous DCs loaded with tumor RNA and CD40L RNA, combined with sunitinib	Advanced renal cell carcinoma	Ongoing, not recruiting	N/A	NCT01582672

pulsed with tumor-specific idiotype protein to B-cell lymphoma patients [117]. Two years later, DCs were being pulsed with tumor lysate or MHC I peptide [118]. By 1999, mature DCs were being explored [119, 144, 145], which included stimulating these DCs with IL-2 [135]. Subsequent studies revealed that immature DCs were tolerogenic [146, 147]. In the early 2000s, researchers continued to test different antigens, including MHC I and II peptides [148, 149] and tumor-derived RNA [122–124]. Researchers also generated DC/tumor cell hybrid vaccines [121], as

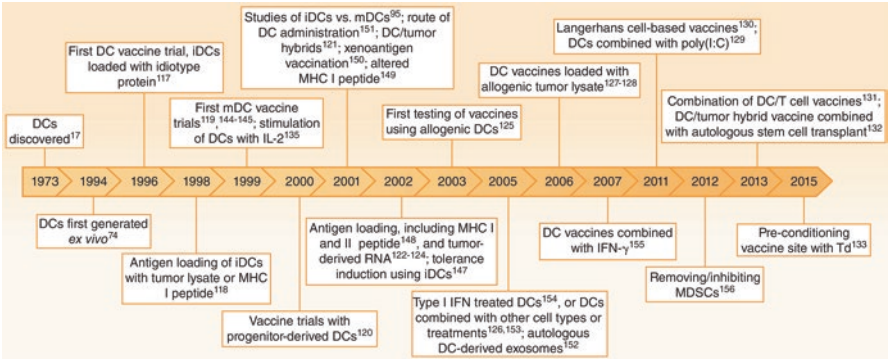

Fig. 6.2 Timeline of the development of the dendritic cell-based cancer vaccines. DCs were first discovered in 1973. However, they were not successfully generated ex vivo until 1994. This allowed researchers to begin exploring the potential of DCs for therapeutic cancer vaccines. The first clinical study was performed in 1996 and involved the administration of immature DCs pulsed with tumor-specific idiotype protein to B-cell lymphoma patients. Two years later, DCs were pulsed with tumor lysate or MHC I peptide. By 1999, mature DCs were being explored, which included stimulating these DCs with IL-2. Subsequent studies revealed that immature DCs were tolerogenic, so researchers fully shifted to mature DCs. In the early 2000s, researchers continued to test different antigen-loading conditions and different antigens, including MHC I and II peptides and tumor-derived RNA. In the second half of the decade, researchers continued to refine antigen sourcing and loading while combining DC-based vaccines with additional treatments. These included depletion of regulatory immune cells, as well as combination with other therapies such as chemotherapy and IFN-γ. This focus on combination therapies and removing inhibitory cells has generally been maintained over the last few years. Recent studies have involved combining DC vaccines with stimulated T cells and using DC/tumor cell hybrid vaccines after stem cell transplant for hematological cancers

well as DC vaccines using xenoantigen [150]. The route of DC administration was also found to be important for clinical outcomes [151]. DC exosome-based vaccines were also tested [152]. The combination of a DC vaccine with cyclophosphamide was later used in human renal cell carcinoma (RCC) patients [153]. Allogenic DCs were fused with tumor cells to produce a hybrid vaccine against metastatic RCC [125]. DCs were also activated with type I IFN [154] or IFN-γ [155] to enhance the immune response. In the second half of the 2000s, researchers began loading DCs with allogenic tumor cell lysates [127, 128]. DCs have also been combined with poly (I:C) [129] and vaccine-primed T cells [131]. Langerhans cell-based vaccines were also found to be effective at inducing immune responses to certain types of cancer [130]. DC vaccines have been used in conjunction with the depletion of regulatory T cells [126] or myeloid-derived suppressor cells (MDSCs) [156]. A DC/tumor fusion vaccine has also been tested in multiple myeloma patients following autologous stem cell transplant [132]. A recent study also revealed that preconditioning the vaccination site with tetanus/diphtheria toxoid (Td) improves DC migration and survival in human glioblastoma patients [133].

Conclusions and Future Directions

DCs have been used for cancer immunotherapy for the past 20 years with diverse clinical outcomes. In animal model studies, DCs have been effective at generating tumor antigen-specific cytotoxic T cells, which ultimately kill tumor cells and produce long-term survival. So far, data from human clinical trials has suggested that DC-based vaccines are safe, yet the clinical responses have been generally less impressive than in animal models. Even with these setbacks, the FDA approved the first therapeutic DC-based vaccine in 2010 (sipuleucel-T, Provenge®) for use in patients with asymptomatic or minimally symptomatic metastatic hormone-refractory prostate cancer [141, 157].

Researchers continue to make new discoveries that improve the efficacy of DC-based cancer vaccines. DC immunotherapy has inherent limitations due to the immunosuppressive environment in the tumor site. However, combination therapy with antibodies or other immunomodulating agents might play an important role for generating antigen-specific antitumor immunity. Hopefully, further optimization of these therapy regimens will result in improved clinical outcomes in patients.

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Encapsulation Approaches to Cell Therapy

7

Paul de Vos

Introduction

The conventional way of preventing rejection of donor tissue is by application of immunosuppressive medication. Although this is effective, it is associated with systemic suppression of immune responses and therefore with increased chances on infections and oncological issues [1, 2]. For these reasons organ and cell transplantation is now only applied as a lifesaving procedure in patients that as the consequence of severe disease or complications thereof have no other choices than accepting a donor organ or cellular transplant in combination with systemic suppression of the immune system.

In the past two decades, many approaches have been explored to allow transplantation of donor tissue in the absence of immunosuppression. Such an approach would be highly beneficial for the treatment of a wide variety of diseases and would allow application in a much higher number of patients than currently treated with transplants. Examples of technical approaches to allow transplantation of donor tissue in the absence of immunosuppression are immunomodulation [3], genetic engineering [4–6], as well as immunoisolation by encapsulation of donor cells. Especially immunoisolation has received much attention in the past decade as it is not associated with any interference in the immunity of the recipient and holds the promise of application of stem cells or cells obtained of animal sources [7–11].

Immunoisolation is a procedure involving encapsulation of living cells for protection for the effects of the host immune system by enveloping the cells in a membrane that is impermeable for hazardous components of the immune system. The technology is currently receiving much attention but was introduced already in 1933 by Bisceglie et al. [12]. Bisceglie was interested in survival of cells in the absence of

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vascularization and encapsulated cell lines in immunoisolating amnion sheets and demonstrated prolonged survival of avascular cells in the abdominal cavity of pigs [12]. Although innovative, Bisceglie did not realize the potential of this technology and it took until 1950 when Algire et al. [13] formally introduced the concept of immunoisolation for the cure of disease. Algire was also the first to recognize the importance of the application of biomaterials that should not provoke severe responses and also the need for guidelines and application of materials that can be produced in a reproducible fashion [13]. In the past two decades, the scientific community has learned to realize that this is a far from simple assignment. Since the 1950s, encapsulation devices have been produced in different conformations [14], with different types of biomaterials [15], and have been applied for the treatment of many diseases that require a minute-to-minute regulation of metabolites such as in hemophilia B [16], anemia [17], dwarfism [18], kidney [19] and liver failure [20], pituitary disorders [21], central nervous system insufficiency [22], and diabetes mellitus [23]. Although the principle applicability of encapsulation for the treatment has been repeatedly demonstrated, it is still far from clear which device should have the preference for which disease and which biomaterials qualify for human application. In this chapter we will review the different geometries, the capsule properties that we currently have identified as being crucial for functional performance, and possible research strategies to make immunoisolation of cells a realistic option for treatment of disease.

Macro- or Microencapsulation

Immunoisolation by encapsulation is currently applied in two geometries, i.e. macro- and microcapsules. In macrocapsules, the cells are enveloped in one or several relative large devices. These devices have semipermeable properties. These macrocapsules are produced as hollow fibers, flat sheets, and disks [24]. Macrocapsules can be implanted as shunts to the vasculature of the recipient as intravascular devices or implanted without direct vascular access as extravascular devices [25]. In the intravascular approach, the cells or organoids are enveloped around or in polymeric capillaries and connected by anastomosis to the circulation. The advantage of this system is close contact of the grafted tissue with the circulation allowing almost real-time exchange of metabolites, nutrients, and oxygen [26]. A major disadvantage of this system however is that it is associated with thrombosis at the site of anastomosis. As a consequence, application of lifelong anticoagulation therapy is a prerequisite until now. This is why many researchers in the field abandoned this approach as the risk of thrombosis makes this approach an unacceptable alternative for the majority of available conventional treatments [27].

Extravascular devices are not directly connected to the blood stream but implanted freely under the skin or intraperitoneally on locations where they do not do any harm to vital organs. The systems can usually be implanted with not more than minor surgery and are replaceable in case of failure of the graft. Exchange of therapeutic molecules, metabolites, and oxygen between the enveloped cells and the

surrounding tissue depends on free diffusion over the semi-membranes of the devices. Although not yet applied on a large clinical scale, there are numerous reports demonstrating the principle applicability of extravascular devices by showing functional performance in both experimental animals and humans [28–31].

Extravascular devices are produced as relatively large macrocapsules and much smaller microcapsules. In macrocapsules, groups of cells are immobilized in a matrix and brought into one or several capsules in the form of fibers, flat sheets, or disks. Many reports demonstrate successful application [32–39], but there is also a drawback of the system which is the relatively large surface-to-volume ratio. To obtain a fast and therapeutic effective ingress and outgress of the therapeutic nutrients and oxygen, an effective and strong diffusion gradient has to be built. This requires a high amount of cells which implies high consumption rates of essential nutrients such as oxygen. This can lead to hypoxia and necrosis of tissue. As a consequence, the cell density in macrocapsules cannot exceed a critical volume. The cells should not compete for nutrients such as oxygen as it will be inevitable that cells will stop functioning or will become even necrotic when the density is too high [40–42]. Within most applications, without external supply of nutrients, the cell density should not exceed 5–10% of the volume of the device [26]. This implies that if large numbers of cells are required to cure disease [26], several or very large devices have to be implanted. Another, very promising approach to enhance the cell density in the devices, is by supplying the device with an external supplier of critical nutrients. For immunoisolation of pancreatic islets for the cure of diabetes, it has been shown that oxygen is such an essential nutritional factor. Diffusion of oxygen is insufficiently low in aqueous environments to allow optimal supply to islet cells, and islets consume oxygen at a high and fast rate. By providing macrocapsules containing an external oxygen supply unit, it has been shown that not only can islets survive for prolonged periods of time in macrocapsules, but it has also been demonstrated that the seeding density in the device can be enhanced to realistic numbers. These devices have already been tested in humans and hold promises for application of macrocapsules for other diseases as well [34, 42–51].

Biocompatibility of Encapsulation Biopolymers

The tissue responses of the host and the associated functional performance of the encapsulated tissue are generally referred to as the biocompatibility of the encapsulation devices. Although generally used for explaining limitations in graft performance of encapsulation devices, it is far from easy to give an adequate description of how biocompatibility should be defined for encapsulation devices [15, 52, 53]. Biocompatibility is usually defined as “the ability of a biomaterial to perform with an appropriate host response in a specific application.” This definition was formulated for fully artificial organs, such as artificial prostheses [54]. With full biomaterial-based constructs, it is desired that a fast and strong innate immune response occurs to allow fibrosis of the prosthesis and integration into the surrounding tissue. The tissue response is needed to achieve integration of the prosthesis into the host tissue

and is therefore considered to be an "appropriate host response." For bioartificial organs that contain living cells, such as an immunoisolated cellular graft, it is much more difficult to define "appropriate host response." The grafts are protected by a semipermeable membrane that is typically not allowing free diffusion of molecules between 80 and 120 kDa [7, 51]. Most cytokines produced during a typical immune response against a prosthesis are small enough to pass the membrane and hurt the cells [26]. A full innate immune response as observed against a prosthesis would lead to massive cell death in encapsulated grafts [52]. With encapsulated cells the tissue response should be as low as possible to prevent this massive cell death, and to manage this response is far from easy [15, 52, 53] (Fig. 7.1).

A typical immune response against an encapsulated graft starts at the moment of surgery. Although it is usually not more than minor surgery, underneath the side of the surgical incision, immune cells will be activated to prepare for and delete potential pathogen infections. This leads to release of chemical signals including release of chemotactic cytokines that attract macrophages and neutrophils to the side of implantations. Normally, in case of absence of a pathogenic infection, immune signals such as IL-10 release will attenuate the activation and induce tissue repair responses. In case of implantation of a bioartificial organ, the surgeon disturbs this process. At the moment of release of chemical signals due to the implantation surgery and attraction and activation of the local innate immune system, we implant a bioartificial organ. This is an implant that produces biological signals that can enhance immune responses, and it also contains biopolymers that can be recognized by specialized receptors on the attracted immune cells [10, 15, 53, 55–57]. The role of the cells in provoking responses has been underestimated up to now, and strategies to manage this loss of cells have gained not more than minor attention.

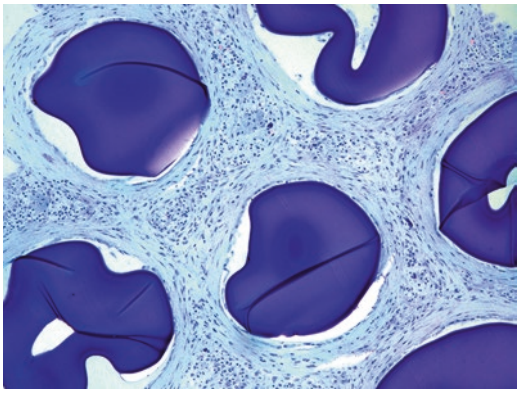

Fig. 7.1 Capsules applied for immunoisolation can elicit foreign body responses for several reasons. When pathogen-associated molecular patterns are present in the polymers or irregularities are present on the capsule's surface, severe fibrotic responses may occur. The capsules depicted here are embedded in several layers of fibroblasts after implantation in the peritoneal cavity of Albino Oxford rats. Cells in these capsules will not survive the immune attack preceding the fibrosis. GMA-embedded capsules. Original magnification × 40

During the first few days after implantation, encapsulated grafts always contain dying cells that have difficulties to adapt to the implantation side or that die due to cytotoxicity of the cytokines in the immediate vicinity [58–60]. These cells are not only lost for maintenance of graft function but also contribute actively to the destruction of the graft. All cells release specific alarm molecules when they are in the process of dying by necrosis or necroptosis. These alarm molecules are also referred as danger-associated molecular patterns (DAMPs). Typical molecules that are released by dying cells in capsules are intracellular components such as DNA, RNA, and HMGB1 that bind to specific receptors on immune cells (Fig. 7.2). The specific receptors on immune cells are called pattern recognition receptors (PRRs). Examples of PRRs are Toll-like receptors (TLRs), NOD receptors, and C-type lectins [61–65]. The DNA, RNA, and HMGB1 that leak out of the capsules are very potent stimulators of the PRRs on the immune cells in implantation side and can enhance immunity and destruction [55]. These are all recent observations [58] and demonstrate that creating "biocompatible" capsules is not all about the materials applied. The viability and the extent to which the cells in the capsules can withstand temporary hazardous circumstances in the implantation side are at least of equal importance. By designing a semipermeable alginate-based membrane that prevents entry of molecules smaller than 100 kDa, it was possible to prevent leakage

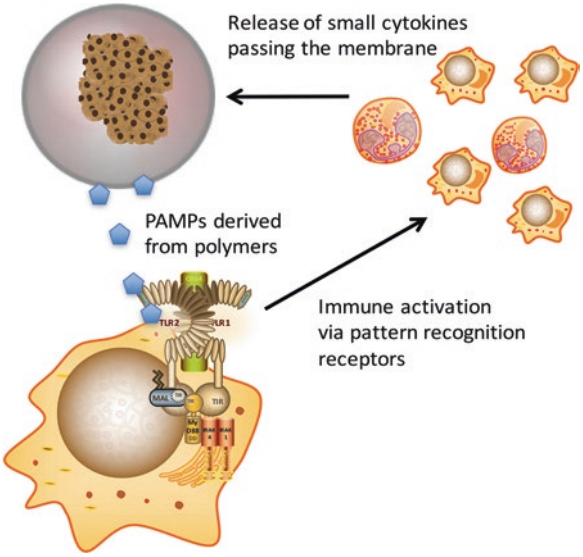

Fig. 7.2 All biopolymers applied for encapsulation contain pathogen-associated molecular patterns (PAMPS). These PAMPs can be introduced during processing to synthesize the polymers or are introduced during the encapsulation process. These PAMPs activate pattern recognition receptors on immune cells in the vicinity and will attract immune cells to the implantation site. This will lead to production of high amounts of cytokines that are small enough to diffuse through the capsule membrane and damage the encapsulated cells. We have observed that this process can kill up to 60% of the cells in the first 2 weeks after implantation. Highly pure polymers and complete PAMP-free manufacturing processes are a requirement when going into humans

of some DAMPs, but the associated immune activation could not be completely prevented as small molecular structures probably escape from the capsules and bind PRRs on immune cells in the vicinity of the encapsulated cells [55]. Conceivable approaches to overcome DAMP release and associated cell loss are by managing cell death in the capsules. The main cell death processes responsible for DAMP release are necrosis and necroptosis [55]. We recently demonstrated that DAMP release and associated cell death processes can be diminished by adding a necroptosis blocker called NEC-1. Coencapsulation of NEC-1 in immunoisolated capsules containing human pancreatic islets prevented cell death, DAMP release, as well as activation of cells in the surrounding of the capsules.

The second factor provoking activation of the innate immune system directly after implantation is the physical chemical properties of the capsules. To prevent interference with survival of the encapsulated cells, the materials should provoke no, or a minimal, cellular overgrowth to ensure free diffusion of nutrients and oxygen and to guarantee exchange of therapeutic molecules. Any enhanced activation of the already present innate immune activation may lead to anchoring of macrophages and granulocytes and cellular overgrowth of the capsules. Many groups have therefore focused in the past decades on understanding and preventing the adhesion of cells on capsule surfaces. These efforts have led to revision of the term biocompatibility in the field of cell encapsulation. The term that slowly becoming accepted in the field is "biotolerability" [52] and defined as "the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reactions" [66]. This definition not only covers the host responses of against the biomaterials but also the compatibility of the encapsulation materials with the enveloped cells. The therapeutic cells should function in the polymer network as adequately as possible and preferably as in their natural environment [67, 68].

Tissue responses against capsule materials can be provoked by many factors. Surface properties of the capsules are without any doubt the most important factor. The majority of capsules applied for cell encapsulation are hydrogels with application of hydrophilic polymers. Although hydrophilic polymers by nature are not associated with strong cell binding, they will not escape from innate immune responses when inserted in the membrane of an immunoisolating membrane. For example, a too high surface roughness will provoke strong innate responses [39, 69, 70] with strong cell adhesion around the capsules as a consequence. A stepwise analysis has demonstrated that a surface roughness below 10 nM will avoid cell adhesion when other confounding factors are absent [67]. Although we always aim on lower surface roughness, immune responses may still occur when proinflammatory chemical groups are exposed.

Device surfaces should have a chemical composition that does not lead to strong protein adsorption or activation and anchoring of immune cells. This chemistry should be optimal and the same all over the surface of the capsules. This is not a difficult task for groups with a chemical background but very challenging in groups that lack such a multidisciplinary approach. Although many chemical anomalies can provoke innate immune responses, there are a few examples that are illustrative for the current state of documentation of the surface chemistry of capsules. In many

applications of encapsulated cells, the permeability of the capsules is reduced by application of polyaminoacids such as poly-L-lysine and poly-L-ornithine that form a membrane around the capsules and prevent large molecules from entering the intracapsular space. Conceptually this procedure is simple, but getting the polyaminoacids into a correct conformation is complicated and requires a skilled chemical technician. As such the polyaminoacids are highly proinflammatory [71–75]. Polyaminoacids such as poly-L-lysine has to be forced into a superhelical core with the core material, often alginate, and into beta-sheets to prevent activation of innate immune responses [71, 74]. The majority of groups in the field have no knowledge nor the technical tools to measure the interaction between their preferred coating chemicals and core materials and have many batch-to-batch differences resulting in a variable graft outcome. Also, it is often insufficiently realized that seemingly minor modification in the cell encapsulation procedure has a profound influence on the chemistry of the membrane and therewith on the tissue responses against the capsules.

Surface properties are rarely or not documented in the cell encapsulation research field. This is one of the factors contributing to the low degree of reproducibility of encapsulation procedures and interfering with progress. A worrisome development in this respect is the introduction of new technologies to produce encapsulated cells without taking changes in surface properties into account. New technologies such as microfluids, electrospinning, and several emulsification approaches [76] are being proposed as alternative for the more conventional droplet formation technologies [77]. Although the technologies are very welcome to solve issues such as upscaling problems, it is not sufficiently recognized in the field that these new technologies produce capsules with unique and technology-dependent variations in capsule surface properties. Our group has tested many different capsule types produced by these emerging technologies and observed large differences in surface roughness and chemistry even with application of identical polymers. This has a profound impact on tissue responses and biotolerability. These observations underpin the importance of documenting capsule properties in order to allow side-by-side comparisons of success rates of capsule properties and to learn about the factors contributing to success and failure of capsules (Fig. 7.3).

In addition to surface properties, it is of pivotal importance to apply biopolymers in the construction that do not provoke immune responses or do not contain contaminations that might initiate immune responses. Many biopolymers applied in the field have been shown to elicit not more than minor immune responses, but traces of endotoxins can induce severe inflammatory responses with fibrosis of the capsules as a consequence [78–82]. Alginate belongs to the best-studied encapsulation material and has served as an illustrative biopolymer by which the essence of preventing endotoxin contaminations has been shown [83]. Despite this only rarely the purity degree of biopolymers is documented or measured, and this is may be one of the most important reasons for the high variations of tissue responses against encapsulated cells.

Commercially, some biopolymers are sold as being pure. This also applies to alginates [83]. The alginates are usually low in endotoxin lipopolysaccharide (LPS) but still provoke responses. This has led to research on identification of other

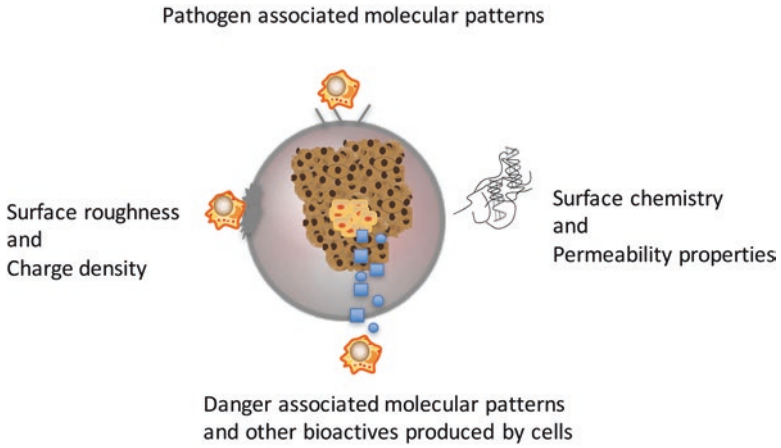

Fig. 7.3 Currently accepted capsule properties that have a profound influence on the tissue responses against encapsulated cellular graft. The minimal capsule properties that need to be documented and reported in publication are surface roughness, chemistry, and presence of proinflammatory molecules such as pathogen-associated molecular patterns in the biopolymers

contaminating factors in alginates than just LPS [53, 56, 83]. LPS is the most studied endotoxin in biopolymers but also referred to as pathogen-associated molecular pattern (PAMP). LPS as PAMP binds to Toll-like receptor 4 and can induce proinflammatory responses [84–86]. LPS is however not the only PAMP. There are many different types of PAMPs that can be present in biopolymers. These PAMPs can have the same immune-stimulating effects as LPS. In a recent study, we tested different types of alginates and other biopolymers such as polyethylene glycol and always found PAMPs even when LPS was removed in undisclosed purification procedures. In most cases we found lipoteichoic acids (LTA) and proteoglycans (PG) [83]. These are TLR-2-stimulating PAMPs and responsible for proinflammatory responses in vitro and in vivo [53, 56, 83, 87]. The reason that these PAMPs are found in purified biopolymers even after purification is that these contaminating PAMPs are difficult to remove as they often have similar chemical properties such as charge densities as the biopolymer. Novel methodologies for removal of the contaminants have to be designed. Notably however, biopolymers also have to be stored in an adapted fashion as we observed that PAMPs could be reintroduced during mandatory handlings during the encapsulation procedure [83]. We found, for example, that PAMPs can be introduced during unsterile conditions or by use of equipment or disposables that contained PAMPs.

A novel technology platform has been introduced to screen for PAMPs in biopolymers [83]. This platform applies different reporter cell lines and ELISA approaches to identify the PAMPs in a cost-effective fashion [83]. By identifying the specific PAMP, we can give recommendations for removal or prevention of the contaminations. During recent years this platform has been applied to screen more than 100 biopolymers applied in encapsulation research. Some of these biopolymers were recommended for human application. Without exception, all the commercially available biopolymers contained significant amounts of PAMPs and

induced immune responses. Without a solution to this issue, tissue responses and low reproducibility remain an issue in the field.

Improving Biotolerability

Of uttermost importance it documents capsule properties to allow side-by-side comparison of data sets and to be able to pinpoint the factors determining success or failure of encapsulated grafts. These factors have been identified by an international team of researchers in the field [88] and are:

1. Composition of the polymer
2. The presence or absence of confounding factors that induce immune responses
3. Toxicity to enveloped cells
4. The permeability of membrane
5. Mechanical properties (rigidity and flexibility)
6. Surface properties

As outlined in a preceding section, it is not as simple as it looks to produce capsules that do not provoke any responses. Only a few groups have the mandatory multidisciplinary skills and technology to produce capsules with the correct critical characteristics to prevent tissue responses [88]. However, documenting the above-mentioned critical parameters is not too complicated and would benefit the field as it will be more easy to understand why some capsule formulations do fail despite application of seemingly the same procedure [52].

As not many groups in the encapsulation field combine chemical, physical, and biomedical knowledge, it is essential in efforts to improve biotolerability to introduce technologies that can be applied in labs that lack physicochemical methodologies. Probably for this reason, many groups have tested surface-changing procedures by adding chemical groups as a mean to apply biofouling [70, 89–105] and to prevent tissue responses. A novel and in concept relative simple method to reduce tissue responses against biomaterials is by applying so-called polymer brushes on surfaces of capsules. Under normal circumstances molecules that bind to capsule surfaces bind in the format of mushroom-like structures [106–111]. By enhancing the density of the polymers, more molecules will be bound by which mushrooms cannot be formed anymore. Upon a critical density, the molecules will stretch and form a polymer brush on the surface of the capsules [110]. The advantage of this system is that brushes prevent adhesion of proteins, are versatile, can be adapted to every surface [110, 111], and can diminish responses against capsules even when, for example, some impurities are still present at the capsule surface [111].

Polymer brushes require application of relative long polymers. These polymers are usually diblock polymers. One side of the polymers has properties facilitating binding to the core capsule materials, and the other side is usually chosen to be beneficial for biotolerability. We have selected and synthesized a series of candidate molecules [112] applicable for cell encapsulation of cells that can be connected to capsule surface with relative simple procedures that are available in labs with minimal equipment.

Biotolerability from the Inside Out

Much attention has been given to biopolymers and creation of capsules that provoke minimal or no tissue responses, but even if this has been accomplished, the grafts are having a limited survival time as shown by many [15, 52, 57]. It has become more and more clear that survival of cells in capsules is not only influenced by immune responses against the capsules but also by the compatibility between the cells and the intracapsular environment. An example is application of a too rigid intracapsular milieu. Cells need a certain freedom to spread to perform their function. When the intracapsular milieu is too rigid, cells cannot perform in an adequate fashion with cellular rearrangements and finally cell death as a consequence [67]. This process of anomalies in cell rearrangements due to inappropriate mechanical properties is called mechanotransduction [67, 113–118]. When cells in capsules are exposed to high mechanical forces, the cells will activate specific biochemical pathways [67, 113–118]. This will lead to adjustments in cellular and extracellular structures and disturbances in basic functions such as proliferation, differentiation, migration, and apoptosis. The molecular processes and sensors responsible for anomalies associated with mechanotransduction are largely unknown, but integrins play a key role. With application of alginate matrixes with a high glucuronic acid content, we recently encountered mechanotransduction as a homeostasis interfering process [67]. The glucuronic acid components of alginate that link constitutive molecules in an egg-box model provide rigidity to the capsule because of its high rigidity for areas where high shear forces are to be expected such as in the brain [68]. Despite the fact that high-G alginates have been applied for many years, we found that the matrix formed by this alginate induced mechanotransduction-associated cell death in a few days after encapsulation. This mechanotransduction-induced cell death was not observed in less rigid matrices or in inner capsules with an alginate of a different composition. These observations emphasize the need to also take into account the compatibility of the intracapsular environment and the cells in longevity studies. Mechanotransduction-induced cell death is not a process that is observed within a few days. It is a chronic event that might be essential in the limited longevity of encapsulated grafts.

Engineering an Optimal Capsule Requires Intracapsular Modifications

The majority of groups in the field of encapsulation have focused in the past two decades on creating novel geometries with application of different biopolymers to enhance functional survival of cellular grafts. Although this has brought many advances in knowledge on production processes, it did not bring about the desired long-term survival of encapsulated cellular grafts. To our opinion this is due to a too strong focus on the material part of the device and associated tissue responses at the cost of research on the prerequisites for the cells that need to survive in some applications for several years.

As outlined above all cell types will produce DAMPs under stressful conditions. In encapsulated human islets applicable for treatment of diabetes, we found that directly after isolation of human islets, a significant number of cells produce DAMPs and provoke inflammatory responses. This process could not be stopped by improving the capsule formulations but could be diminished by a pharmaceutical intervention with an agent called NEC-1 [58]. This NEC-1 can be included in the intracapsular environment and prevent cell death, and at the same time, it diminishes the proinflammatory responses in the immediate period after implantation.

Another modification that we feel is required in the current intracapsular formulations is the addition of an appropriate extracellular matrix (ECM). All cells interact with specialized integrins and other receptors with ECM. The currently applied biopolymers do, to the best of our knowledge, not support interaction with integrins and other cell regulatory receptors on enveloped cells [68]. ECM serves as matrix for facilitating cellular replication [119–122] and contains many growth factors. A few groups have applied laminin sequences such as RGD in their concept of encapsulation [123, 124] and have reported long periods of grafts survival in small as well as large animal models. We recently showed that human pancreatic islets function longer and produce more insulin when they are encapsulated in a matrix containing collagen IV in combination with specific laminin sequences [125, 126]. These findings demonstrate that managing function and survival of cells by modifying the intracapsular environment might be an effective strategy to prolong survival and diminish immune responses against encapsulated grafts.

Concluding Remarks and Future Considerations

In this chapter, we have given recommendations for research strategies to improve the outcome of encapsulated cellular grafts. We have focused not only on the current approaches and technologies but also on the requirement these devices have to meet to allow long-term survival of encapsulated cellular grafts. These recommendations should not be interpreted as a suggestion that there is a lack of progress in the field. Important advances have been made in the past period with encapsulated cell therapy, but at the same time, we have learned that it is not all about the biomaterials. Many new concepts have been introduced, and many factors have been identified that determine the presence or absence of a tissue response against capsules. Clinical trials have now started [127, 128].

There are however, items that need a critical view and debate. One of these is the current regulatory trend in the USA that nonhuman primates are the ultimate models for human application of, for example, encapsulated islets grafts. As recently reviewed, this interferes with progress [56]. Nonhuman primates have innate and adaptive immune responses that are very different on essential parts from human responses against cellular grafts. Especially the above discussed innate immune pathways that are different in nonhuman primates [56]. Much more and more readily, information can be obtained from rodent studies with a humanized immune system and small clinical trials.

The step to human application has started but will need adaptations in the systems. Changing species as recipient implies many adaptations in capsule properties. Just going from mice to rats and from rats to pigs requires adaptations in mechanics, osmolarity, and permeability. Up to now these adaptations have not been adequately documented, and it is not known what the requirements are that capsules have to meet to work in humans [15, 52]. To identify the values of the critical parameters requires a systematic approach. This is not only a scientific challenge but also a regulatory challenge. Bioartificial organs such as encapsulated cellular grafts are new for regulatory agencies. The currently applied regulatory guidelines are coming from other areas such as organ transplantation. These do not all apply and some might be too loose for cell encapsulation. For example, regulations about presence of endotoxins or PAMPs are too high for the cell encapsulation field [88]. Regulation might therefore facilitate progress.

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Biomaterials and Cells for Revascularization

8

Priscilla A. Williams, Kevin T. Campbell, and Eduardo A. Silva

Introduction

Ischemic vascular diseases are the main cause of mortality worldwide, and yet current therapies only delay disease progression and improve quality of life without addressing the fundamental problem of tissue loss [1]. Insufficient vascular network formation and maintenance drives many of these clinical disorders including diabetes, arthritis, atherosclerosis, and peripheral artery disease [2, 3]. Current treatment approaches consist of lifestyle changes, pharmacological agents (e.g., antianginal drugs), or the use of invasive surgical procedures (e.g., coronary artery bypass grafting) aimed at reestablishing blood perfusion to affected areas [2, 4]. However, there still remains a major need for new approaches to treat patients that are not candidates for current therapies [5, 6]. Alternatively, the field of tissue engineering, via cell-based strategies, offers the potential to treat these patients and additionally could help to eliminate donor complications and shortages associated with whole organ transplants (Fig. 8.1). Indeed, establishing sufficient vascularization is paramount for both treating ischemic vascular diseases and for the success of tissue engineering strategies.

Therapeutic angiogenesis is aimed at treating ischemia by promoting new blood vessel formation via the delivery of angiogenic factors or cells. Typical strategies involve administering recombinant proteins and/or cells capable of orchestrating new blood vessel formation [7]. Recombinant protein strategies are hampered by both the short half-life of the protein and the amount that can be delivered [8, 9]. In addition, local cells may be unresponsive to massive doses of proteins, as prominent cell types responsible for maintaining and promoting healthy vasculature are functionally impaired under diseased states [10–12]. Cell-based therapies have the potential to be

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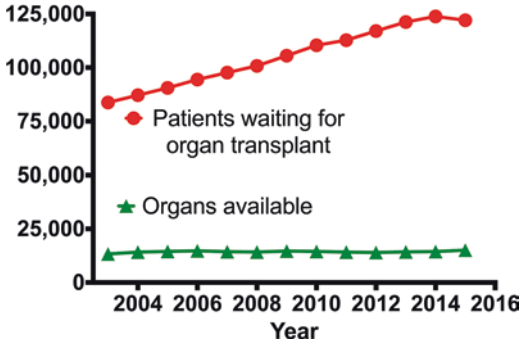


Fig. 8.1 Supply and demand of new organs. There is a dramatic supply gap between the number of patients awaiting organ transplantation and the number of available donor organs. Source: Organ Procurement and Transplantation Network (US Department of Health & Human Services)

a game changer for vascular diseases as they aim to address and not simply control or ameliorate a specific preexisting condition of the disease. Thus, an emerging concept is to exogenously deliver greater numbers of functional and stimulatory cells that promote new blood vessel formation and revascularization. Herein, cells may be harvested from donor tissue, cultured and expanded ex vivo, and finally administered to the patient via a variety of delivery methods. While clinical trials have thus far robustly demonstrated safety of cell therapy approaches for revascularization, therapeutic benefit has been lacking to date [13]. These trials, while supporting the safety of these cells, indicate that there are several unresolved aspects hampering the clinical translational potential of cell-based therapies, including the appropriate cell type, the optimal cell dose, the route of cell administration, the efficiency of cell engraftment at the target tissue, and the frequency of treatment [14].

Currently, cell-based clinical trials involve the infusion of massive numbers of cells, ranging from 20 to 800 million cells/patient, in an attempt to bypass the limited survival and poor retention of administrated cells [14]. The design of biomaterial systems for revascularization via cell therapy provides a promising approach to overcome current therapeutic challenges by providing a more favorable microenvironment to the administered cells that supports enhanced survival, localization, and integration with host tissue. A variety of naturally occurring and synthetic polymers have been used for biomaterial-based delivery of proangiogenic cells for enhanced vascularization. In this chapter, we review various cell therapy methods aimed at providing revascularization and discuss the potential for using biomaterial-based delivery strategies to enhance the future medical outlook.

Specific Barriers for Revascularization Cell Therapy

Cell therapy provides a promising approach to overcome the limitations of current therapeutic strategies aimed at promoting revascularization. While invasive procedures such as surgical bypass grafting may provide successful reperfusion of an occluded artery, results are often temporary, and there are major issues involved with either the limited supply of autologous grafts or immune compatibility with synthetic grafts. Thus, cell therapies aim to promote the development of new blood

vessels for permanent revascularization and sustained recovery of blood perfusion. Here, the current strategies being investigated clinically are explored along with both the biological and engineering challenges that are associated.

Current Therapeutic Outlook

Traditional revascularization cell therapies typically involve systemically infusing the cells as the chosen delivery method [15–17]. Clinical trials for treatment of patients with coronary artery disease [18], including the TOPCARE-AMI [19], REPAIR-AMI [20], and TOPCARE-CHD [21, 22] studies, administered heterogeneous bone marrow-derived progenitor cells via bolus intracoronary infusion and provided proof of concept with moderate yet statistically significant improvements of left ventricular function [23, 24]. Alternative bolus methods use local injection within the target ischemic tissue to decrease the distance that the cells are required to navigate in order to reach the target site [25]. While moderate therapeutic benefit has been achieved with bolus infusion (systemic or local), the lack of control over post-administered cell survival, localization, fate, and functional activity poses major challenges and severely limits the degree of therapeutic benefit and clinical success [14, 25, 26]. Therefore, the efficacy of administered cells highly depends on the mode of delivery and subsequent control over administered cell fate after transplantation. The TAC-HFT trial evaluated administration of either autologous bone marrow or mesenchymal cells using a targeted transendocardial injection via catheter to demonstrate both safety and efficacy as treatment for heart failure [27–29]. However, clinical results have thus far been underwhelming due to both biological and engineering challenges associated with cell therapy strategies.

Biology Challenges

Current cell therapy typically relies on the delivery of exogenous cells and is thus encumbered by numerous biological challenges, including the involvement of complex, time-consuming, and costly ex vivo cell manipulations (Fig. 8.2). This use of ex vivo cultivated cells typically first requires the harvest of suitable donor tissue and transport of the tissue to a good manufacturing practice (GMP) facility so that the cells of interest may be isolated, expanded, and potentially differentiated in 2D culture [30]. Not only is this approach largely restricted by the limited availability of cell sources and numbers, but it also requires labor-intensive procedures and incurs excessive costs that pose translational barriers including the inability to meet the vast medical need and anticipated issues in obtaining regulatory approval [25, 31, 32]. Furthermore, large risk involved with the manipulation of cells ex vivo prior to transplantation in vivo poses major difficulties and controversy in the field [33–37].

Most often, the cells are obtained by either allogeneic or autologous sources. Herein, the use of autologous tissue reduces problems associated with compatibility and harmful immune reactions in response to the transplanted cells [38]. However,

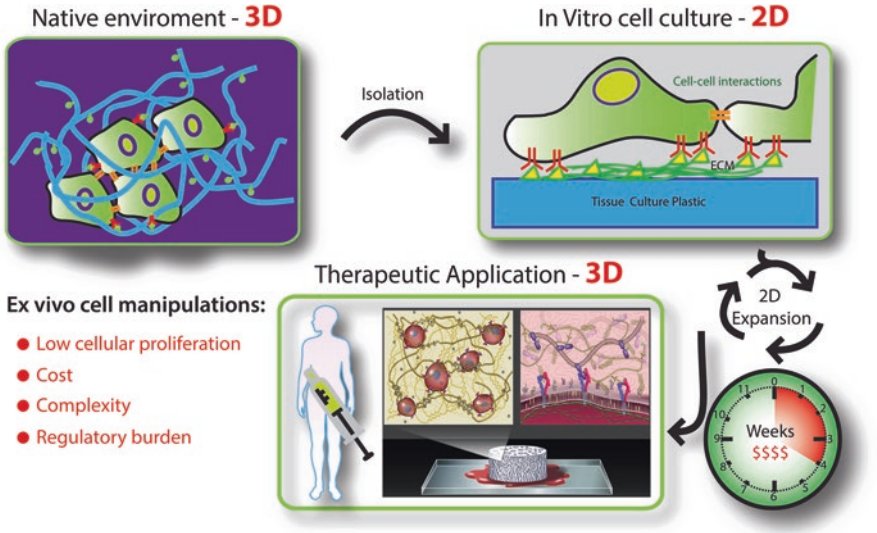


Fig. 8.2 Current cell therapy approaches require complex ex vivo manipulations that are inconvenienced by heavy cost, time, and potential regulatory burdens. Typically, cells are first harvested from their native 3D environment and are then transferred to a foreign 2D culture for in vitro expansion in a laboratory that maintains good manufacturing practices. Due to low cellular growth rates, it takes extensive time and resources to grow the cells to a large enough number useful for clinical application. The cells are then harvested and resuspended in a 3D environment either within a saline solution for intravenous/bolus administration or within a biomaterial system for subsequent transplantation

patients encumbered by disease often may not have enough or sufficiently functional cells to use [27, 29]. Allogeneic cells are thus used when autologous sources are compromised or the use of a constant cell source is desirable, but these cells pose risk of immune rejection and pathogen transmission [39]. Rejection of hematopoietic cell transplantations is typically mediated by immune recipient T-cells but can also be caused by viral infections including human herpesvirus type 6, cytomegalovirus, and parvovirus [40]. Donor cells must be thoroughly treated and the recipients must also receive immunosuppressants to reduce the risk of host rejection, though it is difficult to optimize these parameters for each patient [41].

Moreover, once enough cells (either autologous or allogeneic) are obtained from ex vivo culture, the process of cellular delivery is further encumbered with biological difficulties. Methods of cellular infusion often have limited efficacy due to high cell death and poor recruitment and/or retention within the ischemic tissue target [26, 42, 43]. In particular, the majority of systemically infused cells often get trapped in the microvasculature of other off-target organs/tissues [14, 44]. For example, pulmonary passage is a major obstacle due to both the size of the cells and adhesion to the vascular endothelium [45]. Furthermore, immediately upon transplantation, the administered cells encounter a harsh microenvironment (i.e., hypoxia, reactive oxygen species, etc.) that leads to high cell death, wherein over

90% of transplanted cells typically die within hours to days after administration [43]. Very large numbers of cells are thus required in order for enough cells to survive and home to the target tissue for therapeutic benefit [14, 45, 46]. In particular, clinical trials for heart failure involving bone marrow progenitor cells often require between 20 and 800 million cells per patient for a single treatment [14]. Given the limited expansion capacity of mature or differentiated cells, the field is thus moving toward the use of stem and progenitor cells owing to their higher clonal capacity [47]. However, the use of these cells also poses controversy particularly concerning the potential for tumorigenesis [30]. Thus, the field of biomedical and tissue engineering aims to derive biomaterial systems that ameliorate some of these issues by providing local and spatiotemporal control over delivery and regulation of post-administered cell fate [48].

Engineering Challenges

The design of biomaterial-based systems for revascularization via cell therapy further presents a number of engineering challenges that must be addressed. This includes choosing the appropriate cell type(s), the optimal cell dose, the route of cell administration, the efficiency of cell engraftment/localization at the target site, and the frequency of treatment required [14]. Furthermore, development and optimization of biomaterial scaffolds for cell delivery may take considerable time to solve [14, 30]. Many of these challenges also present potentially interacting parameters; for example, the particular pathophysiological setting may dictate the optimal cell type, which may subsequently influence the dose required, which in turn may be influenced by the delivery system employed. A few clinical studies have addressed the question of cell dose (Clinicaltrials.gov Identifiers NCT00135850 and NCT00721045) [27], but more information for other cell types and delivery routes are required.

The use of biomaterials could enhance the efficacy of cell therapy by providing the necessary matrix-related signals required for cell engraftment, survival, and function [14, 43, 49]. The optimal cell delivery vehicle should combine a large number of cells/area with chemical and physical cues capable of ensuring cell viability while protecting the desired phenotype and genotype [50]. This need of high loading efficiency to specific sites represents a great engineering challenge for the design and selection of the appropriate biomaterial systems [51–53]. In addition, another engineering challenge arises from the need for biomaterial-based delivery vehicles to provide the necessary mechanical support to counter compressive and tensile forces that exist within the in vivo microenvironment [54–58]. In certain situations, the biomaterial systems need to display a semipermeable morphology to guide integration between the native and exogenous cell populations [59–64]. A major key to success lies with the biomaterial selection, where the mechanical and physical properties of the polymeric systems may be readily adjusted through modification of several parameters including polymer molecular weight, ratio of different monomers, use of additives, charge of polymer chains, pore size and shape,

degradation rate, and presence of cell anchorage cues [65–68]. Here we present some of the most commonly used polymer systems utilized for cell-based strategies in the context of revascularization.

Cell Candidates for Revascularization Cell Therapy Strategies

Both blood and lymphatic vasculature have essential roles in maintaining tissue homeostasis (Fig. 8.3); however promoting functional new vascular networks still presents a serious challenge. These challenges have prompted interest in applying both fully differentiated and progenitor cells from autologous and allogeneic sources to participate in revascularization. Here, we provide a brief overview of both blood and lymphatic vessel formation to identify potential therapeutic cellular mechanisms to promote new vasculature. Then we discuss different types of differentiated and progenitor cells that can be used to promote new vascular networks.

Angiogenesis and Lymphangiogenesis

The designation "angiogenesis" was introduced in 1935 to broadly describe the formation of new blood vessels in the placenta [69]. Currently, the term angiogenesis is used to describe the sprouting and stabilization of new blood vessels from preexisting vessels [70]. Angiogenesis involves a cascade of events, including endothelial cell activation, recruitment, and proliferation followed by interactions with mural cells for the stabilization of the initially immature new blood vasculature. A large number of angiogenic and anti-angiogenic signals and microenvironment cues have been identified, and these factors are presented locally in a well-regulated way to control angiogenesis [71, 72]. Angiogenesis is primarily initiated through hypoxia, with hypoxia-inducible factor increasing the expression of numerous angiogenic genes and proteins including vascular endothelial growth factor (VEGF)-A, angiopoietin-1 (Ang1), angiopoietin-2 (Ang2), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) [73–75]. In a more simplistic view, angiogenesis may be described as a process first involving the vasodilation of existing blood vessels, an event that is mainly mediated by VEGF, accompanied by an increase of the vascular permeability. Associated with these processes is the degradation of the extracellular matrix (ECM), which is necessary for the subsequent endothelial cell migration. Activated endothelial cells that begin the sprouting process are commonly referred to as tip cells. Tip cell migration is controlled primarily by VEGF-A expression, and endothelial cells will proliferate to expand the blood sprout creating immature blood vessel networks [76]. These immature endothelial cell assemblies are susceptible to regression due to endothelial cell apoptosis. VEGF, Ang1, and Ang2 are key players in all steps of these processes [77, 78]. Finally, the vessels mature to form stable vascular networks via the recruitment of supporting pericytes and mural cells (e.g., smooth muscle cells

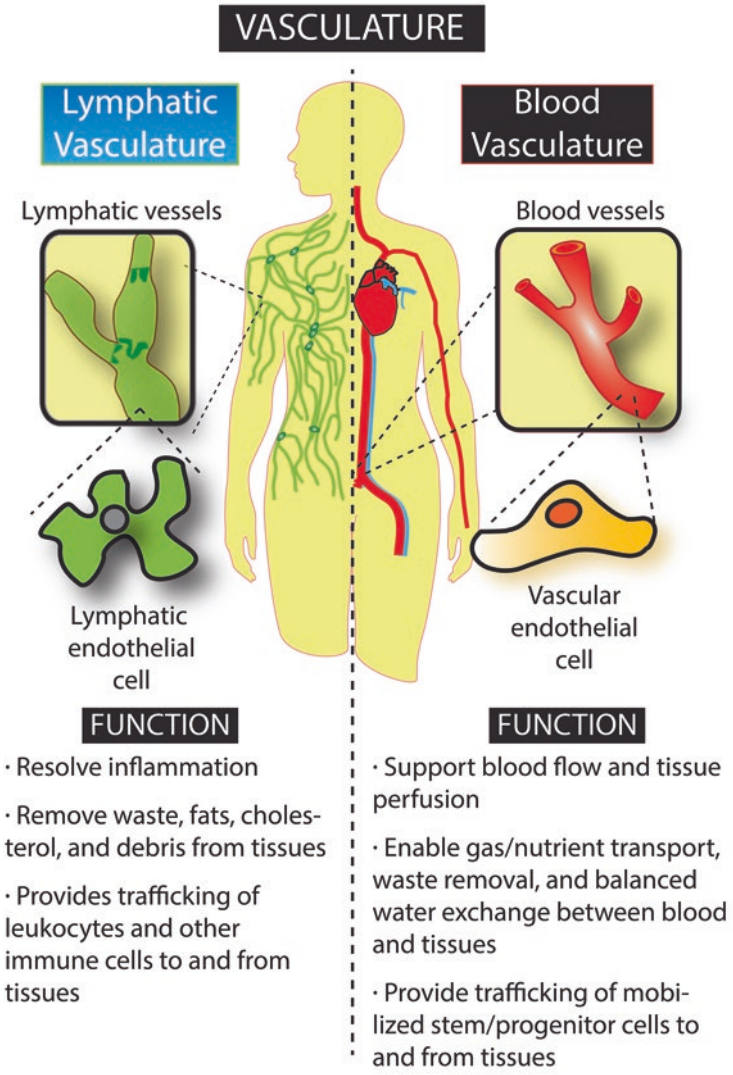


Fig. 8.3 Both blood and lymphatic vasculatures play essential roles in maintaining tissue homeostasis. The lymphatic vascular system (*left*) is heavily involved in resolving inflammation, removing undesirable substances from peripheral tissues, and providing a main route for the trafficking of immune cells such as macrophages, dendritic cells, and both T and B lymphocytes. The cardiovascular system (*right*) provides blood perfusion to the periphery thus providing oxygen and other essential nutrients, enabling water exchange between blood and tissue, and providing a main circulatory pathway for mobilized progenitor cells to traffic to and from target tissues

(SMC)), and this process is mainly regulated by platelet-derived growth factor β (PDGF-β) [70].

Angiogenesis is not only critical for virtually any organ's growth and tissue repair but is also associated with several physiologic events including menstruation [79], wound healing, and repair [80, 81]. On the other hand, misgoverned angiogenesis is also linked with more than 70 pathologic disorders, including cancer, diabetes, and multiple sclerosis [80, 82]. Therefore, an advanced control and manipulation of the key proangiogenic cell types will likely boost certain therapies in the medical field.

The process of forming new lymphatic vessels (lymphangiogenesis) is similar to angiogenesis and is responsible for resolving inflammation and removing waste, fats, and debris from tissues. Similar to angiogenesis, lymphangiogenesis is triggered by both hypoxia and inflammation [83]. Hypoxia-inducible factors and inflammatory markers including p50, p60, and members of the nuclear factor-kappaB family have been shown to upregulate lymphangiogenesis [84–86]. Conversely, the mechanism for regulating lymphangiogenesis is much different than angiogenesis. Although VEGF-receptor 2 (VEGFR-2) stimulation is involved with proliferation of lymphatic cells, VEGFR-3 stimulation is necessary for functional lymphatic vessels [87–89]. These differences are likely the result of lymphatic cells expressing prospero homeobox protein 1 (PROX-1) gene, the master regulator gene for lymphatics [90]. PROX-1, expressed in venous cells during development, is responsible for inhibiting approximately 40% blood vascular genes while activating certain lymphatic-specific genes including VEGFR-3 [91]. Indeed, VEGFR-3 stimulation by either VEGF-C or VEGF-D is required for lymphatic vessel maturity and directs lymphatic cells during lymphangiogenesis [88, 92]. Lymphangiogenesis results in interconnected, highly permeable, and blind-ended lymphatic capillaries. These lymphatic vessels are one cell thick and contain flap-like mini-valves that are permeable to larger molecules like proteins, lipids, and even cellular debris [93, 94]. Lymphatic vessels contribute to tissue homeostasis by pumping extracellular wastes through the lymphatic networks, which contain valves to prevent backflow, and by regulating immune surveillance and trafficking [93, 95].

Differentiated Cells for Revascularization

Blood endothelial cells (ECs) are fully differentiated cells naturally responsible for participating in angiogenesis and have been some of the first targets for promoting new vasculature [96]. The level of heterogeneity between different EC phenotypes is very well established and includes differences at the level of cell morphology, function, gene expression, and antigen composition [97]. Nevertheless, ECs have been incorporated into biomaterials to direct and promote blood vasculature [98, 99]. Some of the early work involved incorporating human dermal microvascular endothelial cells (HDMECs) into biodegradable poly-L-lactic acid scaffolds and transplanting them into severe combined immunodeficient (SCID) mice [98]. The

human ECs had migrated through the scaffold following the first day of transplantation, tubular structures had formed within the scaffold by day 5, and by day 7 these tubular structures started supporting murine blood flow [98]. Microvasculature inside of the scaffold was predominantly HDMECs, showing that the incorporated cells were participating in angiogenesis. Though at the peripherals of the scaffold, there were vessels which were a combination of mouse and human ECs which allowed for the scaffold microvasculature to connect to the host's cardiovascular system [98]. The transplanted cells also showed signs of forming mature vessels by recruiting perivascular α-smooth muscle actin. Within 28 days, the transplanted human cells were being replaced by mouse cells, suggesting that the host remodels the vasculature after forming functional blood vessels [98]. Additional work with transplanting HDMECs with VEGF-A via biodegradable scaffolds in SCID mice has shown a 160% increase in the number of human-derived blood vessels after 14 days [99]. Scaffolds with both VEGF-A and HDMECs lead to a significant increase in blood vessel density than scaffold with HDMECs alone [99]. However, despite the promising therapeutic value of ECs, challenges limit the efficacy of applying these cells for transplantations. For example, allogenic sources of ECs often trigger an immune response, which can impede revascularization and contribute to swelling [100–103]. On the other hand, autogenic sources of ECs would avoid this immune response, though many patients with atherosclerosis have ECs which are less likely to participate in vascularization (known as endothelial dysfunction) [104]. These limitations with ECs have prompted interest into using other sources of cells for blood revascularization including endothelial progenitor cells.

Lymphatic endothelial cells (LECs) are fully differentiated cells originating from venous cells that make up the structure of lymphatic vessels and have a prominent role in lymphangiogenesis [105]. LECs have not been as well established as their EC counterparts, though both endothelial cell types have fundamental differences at the level of cell morphology, gene expression, and function. Some of these morphological differences between LECs and ECs include LECs having a wider and irregular lumen, numerous intercellular junctions, and increased cytoplasmic vesicles [106]. Gene expression also differs, with the PROX-1 gene in LECs being responsible for upregulating VEGFR-3 and vesicle targeting and fusion proteins while suppressing approximately 40% of EC-specific genes, including CD44 and integrin α5 [91, 107]. These differences in LECs likely stem from the role lymphatic capillaries have as a unidirectional pump to uptake fat and cholesterol and modulate immune cell activation [95]. Although both endothelial cell types will upregulate key vascular promoting genes including early growth response (EGR) 1, EGR2, and epidermal growth factor receptor (EGFR) 3 in response to VEGF-A, VEGF-A alone will ultimately lead to enlarged vessels (lymphatic hyperplasia) [108–112]. VEGFR-3 stimulation will promote proliferation and migration of LECs and will allow for functional lymphatic vessels, making LECs a potent target for stimulating lymphangiogenesis [113].

LECs have also been used in cellular transplants to assist in revascularization [114, 115]. Some of the first work involved incorporating LECs into biodegradable polyglycolic acid scaffolds to participate in new vessel formation in vivo [114]. The

LECs were seeded into the scaffold and cultured in vitro for 10 days prior to be implanted in nude mice [114]. The scaffold was found to be compatible and support adhesion, growth, and spreading of LECs [114]. LECs from the scaffold organized and incorporated themselves into the tissue-engineered lymphatic vessels and expressed lymphatic genes, whereas LECs transplanted without the scaffold had collapsed lumens and were not positive for lymphatic genes after 6 weeks [114]. Additional work involved culturing and differentiating human-induced pluripotent stem cells and human embryonic stem cells into LECs in vitro prior to transplantation in athymic nude mice to promote lymphangiogenesis [115]. These differentiated LECs were transplanted into an ear wound model, where they were found to incorporate into murine lymphatic vessels and were positive for lymphangiogenic genes and factors including VEGF-C and VEGF-D [115]. Additionally, LECs derived from embryonic stem cells that were transplanted in the peripheries of a skin wound in mice accelerated wound healing [115]. However, despite the promising therapeutic value of LECs, challenges similar to those that ECs face may also limit applications of LECs for transplantation and may prompt interest into using other sources of cells for lymphatic revascularization including lymphatic endothelial progenitor cells (LEPCs).

Progenitors Cells for Revascularization

Novel discoveries in regenerative medicine involve using different cell types to augment the process of new vascular networks in patients with chronic diseases including ischemic vascular diseases. In particular, the use of stem/progenitor cells for treating and preventing vascular ischemic diseases has transformed cardiovascular research and resulted in major clinical investigation [116, 117]. These studies have afforded greater understanding of vasculogenesis and progenitor cell trafficking to maintain vasculature and tissue homeostasis. With these novel insights, current engineering approaches aim to manipulate these processes for enhancing cardiovascular repair under ischemia.

There have been many preclinical and clinical studies using bone marrow-derived stem cells due to the relatively high concentration of stem cells in the bone marrow and given the ease of procurement using simple culture techniques [14]. Hematopoietic stem cells (HSCs) are one of the most commonly used tools for cell therapy and have advantageously been extensively characterized [118]. In fact, more than 50,000 HSC transplantations are performed worldwide annually [119]. Clinical trials have shown that intracoronary or intramuscular injections of hematopoietic CD34+ cells may ameliorate vascular ischemia [120]. HSCs are commonly isolated from bone marrow but can also be obtained from umbilical cord blood and adult peripheral blood [119]. Importantly, HSCs home to sites of endothelial injury [118, 119] and can serve paracrine roles to enhance the process of neovascularization [119, 121]. However, autologous HSC effectiveness declines with both age and disease, and complications of immune rejection and lack of suitable donors for all patients represent major limiting factors for safe and effective clinical application [118].

Alternatively, mesenchymal stem cells (MSCs), also known as bone marrow stromal cells, are an attractive non-hematopoietic source of stem cells that are inherently nonimmunogenic and display beneficial immunomodulatory and anti-inflammatory effects in vivo [117, 118]. MSCs reside alongside endothelial progenitor cells (EPCs) in the bone marrow stroma, peripheral blood, and vessel walls in most tissues and organs [118]. MSCs are significant contributors to vascular regeneration via secretion of paracrine factors that stimulate vascular endothelial cells [122, 123]. Administration of MSCs in animal models of chronic heart failure has presented promising results regarding the use of MSCs for treating vascular ischemic diseases [14]. For example, direct epicardial injection of allogenic MSCs increased vascularity and improved myocardial function in a dog model of ischemic heart failure induced by ameroid constriction [124]. Such studies fueled clinical trials, such as PROMETHEUS (Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery; Clinicaltrials.gov Identifier NCT00587990), an ongoing randomized, double-blind, placebo-controlled trial using autologous MSCs for patients with chronic ischemic left ventricular dysfunction undergoing cardiac surgery [14]. Given these promising results, investigators have attempted to modify this stem cell population to enhance their therapeutic efficacy. For example, hypoxic preconditioning of MSCs enhanced retention and survival posttransplantation in the ischemic myocardium of mice and increased MSC expression of proangiogenic factors [123]. In addition, MSCs have been genetically modified to overexpress prosurvival and angiogenic factors in order to increase their therapeutic potency [122, 125]. However, the tendency for MSCs to differentiate into bone-forming osteoblasts or other off-target cell types poses challenges to their use in cell therapy for vascular repair [125].

EPCs are beneficially suited for revascularization cell therapy given their innate responsibility for natural vascular homeostasis and repair [126, 127]. In contrast to HSCs and MSCs, EPCs are particularly useful as tools for cardiovascular cell therapy given their ability to incorporate into vasculature and newly formed capillaries, participate in neovascularization, and additionally promote angiogenic activity in resident endothelial cells [96] by release of paracrine angiogenic factors including fibroblast growth factor1 (FGF1 or FGF-a), interleukin-2 (IL-2), and interferon gamma-induced protein 10 (IP-10/CXCL10) [26, 128, 129]. EPCs were first identified by Asahara and colleagues in 1997 as CD34+ mononuclear cells (MNCs) in adult human peripheral blood [116]. These CD34+ MNCs were shown to differentiate into mature ECs and home to neovascularization foci in animal models of ischemia [116]. It is now recognized that EPCs can be isolated from both adult peripheral and umbilical cord blood, and they may express both hematopoietic and endothelial cell surface markers dependent on the method of isolation [34, 116, 119, 127, 129–131]. Herein, the term has been used to represent a vastly heterogeneous population of cells, which has led experts in the field to urge discontinuing its use [116, 119, 127, 129, 132]. Outgrowth endothelial cells (OECs), or endothelial colony forming cells (ECFCs), are a specific subset of EPCs of solely endothelial lineage that directly participate in blood vessel formation in contrast to hematopoietic EPCs [26, 49, 133, 134]. Herein, while hematopoietic EPCs mainly contribute to neovascularization via

paracrine effects by cytokine production, OECs directly incorporate within native vascular endothelium and participate in blood vessel formation [26]. A hierarchy of EPCs and associated subtypes such as OECs has also been identified based upon their clonogenic and proliferative potential [135]. Herein, it has been shown that cells isolated from umbilical cord blood retain greater proliferative potential and can be expanded with higher population doublings before reaching senescence than comparable cells isolated from adult peripheral blood sources [135].

In a similar way, LEPCs have been isolated from umbilical cord blood [136–138]. Salven and colleagues were among the first to identify circulating cells positive for the stem/precursor cell marker CD133 and VEGFR-3 [139]. These CD133+ and VEGFR-3+ cells were found to form monolayers of ECs expressing CD34 and lymphatic-specific surface markers [139]. Other works isolating CD34+ and VEGFR-3+ EPCs found that these progenitors displayed high proliferation and allowed for repeated subculture, and importantly they could be differentiated into LECs by expressing lymphatic-specific markers (i.e., Prox-1) in the presence of VEGF-C [138]. Additionally, bone marrow-derived mononuclear cells were also found to incorporate into lymphatic vessels in vivo, where they expressed high levels of lymphatic chemokines [140]. Recent research has isolated two types of ECFCs from peripheral blood, one expressing high blood endothelial marker VEGFR-1 and the other expressing high levels of VEGFR-3 and Prox-1 [136]. EPCs were also isolated from umbilical cord blood and could be differentiated toward LECs in the presence of VEGF-C, further supporting EPCs with a lymphatic lineage [138].

With this astounding vasculogenic potential, the discovery of EPCs drastically changed scientific understanding of adult blood vessel formation and postnatal vasculogenesis [129]. Thus, the field of EPC biology generated substantial enthusiasm over the past decade in reparative cell therapy for a variety of human clinical disorders [118, 119]. Systemic infusion by intracardiac injection of culture-expanded EPCs derived from peripheral blood has been shown to enhance blood perfusion and somewhat prevent limb loss (50% recovery) in murine models of hind limb ischemia [141]. Here, 56% of blood vessels in the ischemic tissue contained transplanted human EPCs, demonstrating these cells' reparative function. Many preclinical and clinical studies have capitalized on the potent therapeutic potential of these cells, as previously reviewed [119, 142].

Biomaterials

Cellular-based therapies are currently limited by low survival rates, difficulty recruiting cells to ischemic tissue, and challenges in incorporating the administered cells into new vasculature (Fig. 8.4). Biomaterials have been used to bypass some of these limitations arising from the systemic delivery of cells [143]. Biomaterials have the advantage of safely incorporating cells while also having the ability to localize exogenous cells to a particular tissue of interest. Additionally, biomaterials

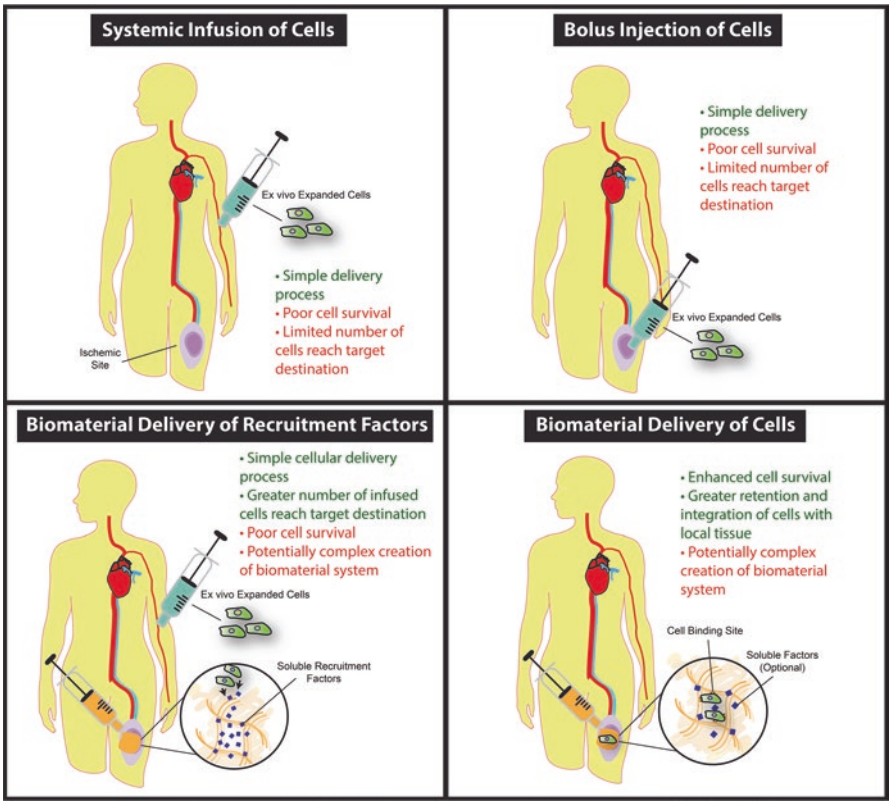


Fig. 8.4 The use of biomaterial systems enhances efficacy of cell-based therapy for revascularization. Current cell therapy approaches typically provide cells via either systemic (*top left*) or bolus (*top right*) delivery. While these approaches provide ease of delivery, they are encumbered with numerous biological challenges. Biomaterials may be used to provide spatiotemporally controlled delivery of soluble factors that promote recruitment of administered cells and overcome some of these limitations (*bottom left*). Furthermore, incorporation of cells within the biomaterial system provides further control over administered cell fate and greater therapeutic efficiency (*bottom right*)

can be loaded with other therapeutics to increase the viability of incorporated cells and promote these cells to participate in vascularization.

The design of biomaterials for cell-based approaches could get inspiration from the natural ECM. The ECM plays a number of critical roles in regulating tissue regeneration including cell anchorage via specific receptors, conveyance of mechanical signals, and presentation of growth factors and cytokines. These combinations of soluble and insoluble cues presented by the natural ECM vary in a temporally dynamic manner due to constant remodeling of the ECM. One particularly important function of the ECM is providing a foundation for cell attachment, as cell anchorage plays an important role in the regulation of cell growth, differentiation, and apoptosis. The adhesion of cells to native ECM is mediated by specific cell

surface receptors, such as integrins, that interact with short amino acid sequences presented in the ECM molecules [144]. This signaling is frequently mimicked in material system architecture by the presentation of cell-binding motifs from the polymers used to fabricate the synthetic ECMs. The amino acid sequence arginine-glycine-aspartic acid (RGD), a ubiquitous cell-binding domain derived from fibronectin and laminin, is the peptide used most frequently to promote cellular attachment to synthetic ECMs [145–147], and the density of these ligands presented to cells from the material has been shown to regulate the cellular response in vitro and in vivo [144, 148–150].

Cell adhesion peptides can be linked to materials via different techniques including physical immobilization and covalent coupling [151, 152]. Covalent coupling is an appealing approach due to the presence of terminal amine groups on the adhesion peptide chains, and carbodiimide chemistry has been utilized to couple RGD sequences to different biomaterials [153, 154]. Conversely, several biomaterials do interact nonspecifically with proteins present on ECM and serum and therefore do allow cell anchorage without the need of specific bioconjugations.

A variety of polymeric materials have been previously utilized as cell delivery vehicles, including naturally occurring and synthetic polymers. This chapter will focus on some of the commonly applied types of naturally occurring and synthetic polymers used for cell therapies in the context of revascularization strategies (Table 8.1).

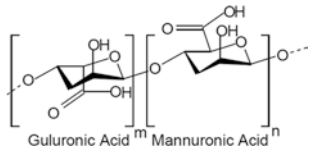
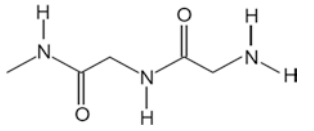
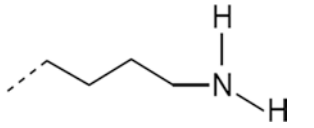
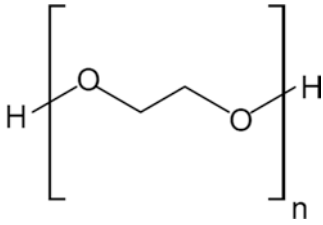
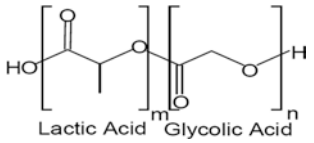
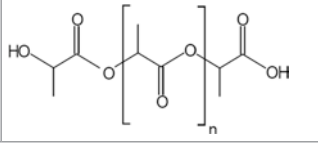
Types of Biomaterials

Naturally Occurring Polymers

Naturally occurring polymers are isolated from plant, animal, or human tissues and display many physical and chemical characteristics that are advantageous for delivery of cellular therapies. These polymers typically provide structural roles within the ECM, and subsequently they often mimic key characteristics of the native tissues enabling gentle incorporation of cells into their polymer matrices while guiding tissue repair and remodeling. In general, naturally occurring polymers display a range of favorable properties and characteristics including their biocompatibility (i.e., low immunogenic response), relative abundance and availability, and ease of processing. However, naturally occurring polymers also exhibit certain disadvantages for cell-based therapies including cost and batch-to-batch physiochemical property variability. Nevertheless, a variety of naturally occurring polymers have been used for cell-based approaches in the context of revascularization, but this chapter will focus on three biomaterials including alginate, fibrin, and gelatin (Table 8.2).

Alginate is used to describe a naturally occurring (linear unbranched) polysaccharide comprised of a α-L-guluronic (G-block) and β-D-mannuronic (M-block) acid sugar residues that is produced by brown algae and bacteria [174]. Despite being produced by both brown algae and bacteria, to date, all commercially available alginates have been extracted from algae, mainly by *Laminaria hyperborea*,

Table 8.1 Properties of naturally occurring and synthetic polymers commonly used for revascularization strategies

Natural	Structure	Net charge	MW (kDa)	Mechanism of degradation
Alginate		Anionic	32–400 [155]	Nondegradable unless modified Oxidized: hydrolysis [155]
Fibrin		Anionic	~ 340 [156]	Enzymatic [157] (Fibrinolysis)
Gelatin		Type A: cationicType B: anionic	20–100	Thermal [158] Enzymatic [159]
Synthetic				
PEG		Cationic	1–100	Nondegradable unless modifiedCombined with PLGA: hydrolysis [160]
PLGA		Anionic	10–100 [161]	Hydrolysis [161]
PLLA		Anionic	10–100	Hydrolysis [161]

Macrocystis pyrifera, *Laminaria japonica*, and *Laminaria digitata*. Therefore, the composition of alginate varies according to the seaweeds used. In addition, commercially available alginates display molecular weight polydispersity, mainly due to depolymerization phenomenon during extraction. Consequently, available alginates have an average molecular weight of approximately 250 kDa. Importantly, the chemical and physical properties of the alginate copolymer are intrinsically related with the ratio and sequence of the two monomers. Alginate has a long history of use for transplantation and cell therapy given the excellent biocompatibility and favorable mass transport features [175–177]. However, alginate is biologically inert

Table 8.2 Selected examples of cell-based strategies delivered with naturally occurring polymers to promote revascularization

Biomaterial type	Cells delivered	Concentration cell/mL scaffold	Predicted mechanism of revascularization	Intended goal	Reference
Natural					
Alginate	Human umbilical vein ECs	Unspecified	Vessels/cells incorporate with native vasculature	Prevascularized matrix accelerates anastomosis	[96]
	Fibroblasts				
	Microvascular ECs	7.2×10^7	Cells incorporate with native vasculature	VEGF-A and ECs promote vascularization	[99]
	Neonatal cardiac cells	7.0×10^7	Vessels/cells incorporate with native vasculature	Revascularize/ repair damaged heart muscle	[162]
	Endothelial progenitor cells	6.3×10^7	Paracrine secretion	Depot of cells participate in vascularization	[26]
	Outgrowth ECs		Cells incorporate with native vasculature		
	Mesenchymal stem cells	$6.6–7.7 \times 10^7$	Paracrine secretion	Reperfusion of ischemic tissue	[163]
			Cells incorporate with native vasculature		
	Adipose-derived stem cells	1.0×10^6	Paracrine secretion	Enhance vascularization and bone mineralization	[164]
Fibrin	Human umbilical vein ECs	Unspecified	Vessels/cells incorporate with native vasculature	Prevascularized matrix accelerates anastomosis	[165]
	Fibroblasts ECs	Unspecified	Cells incorporate with native vasculature	Promote neovascularization in myocardium	[166]
	Skeletal myoblasts	1.0×10^8	Cells incorporate with native vasculature	Induce neovasculature after myocardial infarction	[167]
	Mesenchymal stem cells	2.0×10^7	Paracrine secretion	Promote neovascularization in myocardium	[168]
	Embryonic stem cells	3.6×10^6	Paracrine secretion	Revascularize the heart in a patient	[156]

(continued)

Table 8.2 (continued)

Biomaterial type	Cells delivered	Concentration cell/mL scaffold	Predicted mechanism of revascularization	Intended goal	Reference
Gelatin	Fetal heart cells Adult heart cells	4.4×10^6	Formed cardiac tissue in heart	Repair and revascularize heart tissue	[169]
	ECFCs Mesenchymal stem cells	2.0×10^6	Vessels/cells incorporate with native vasculature	Modulate cell behavior and vascularization	[170]
	Mesenchymal stem cells	7.0×10^6	Paracrine secretionCells incorporate with native vasculature	Promoting angiogenesis for spinal cord injury	[171]
	Adipose-derived stem cells	1.0×10^4	Cells incorporate with native vasculature	VEGF-C and cells promote lymphangiogenesis	[172]
	Adipose-derived stem cells	1.0×10^5	Paracrine secretion	bFGF and cells for muscle revascularization	[173]

(i.e., lack of bioadhesivity) and nonbiodegradable. These features have motivated the extensive lines of research aimed to chemically modify alginate [26, 49, 150, 154, 178, 179]. Some of these modifications include covalently attaching peptides which can bind integrin receptors present on cells to the backbone of alginate polymers, including RGD, DGEA (Asp-Gly-Glu-Ala), and YIGSR (Tyr-Ile-Gly-Ser-Arg) [180–182]. Alginate can also be partially oxidized with sodium periodate to allow for the alginate polymer backbone to be susceptible to hydrolysis [179].

Alginate scaffolds have been used to incorporate cells to promote new blood vessel formation. Alginate scaffolds with PLGA or collagen delivered ECs with angiogenic factors like VEGF-A or monocyte chemotactic protein-1 and have been demonstrated to significantly increase the number of functional blood vessels in the murine ischemic hind limb, which was not observed for bolus EC injections [96, 99]. Alginate scaffolds have also been used for repairing and revascularizing infarcted rat hearts. Neonatal cardiac cells with prosurvival and angiogenic factors were encapsulated into alginate scaffolds and implanted into infarcted rat hearts [162]. The incorporated factors enhanced vascularization in the scaffold, improved myocardiac function and inhibited ventricular dilatation in the rats [162]. Other works showed that transplanting OECs and EPCs in alginate scaffolds significantly increased the density of blood vessels and reperfusion of the murine ischemic hind limb, whereas bolus injections of cells had little improvement on revascularization [26]. Scaffolds with both OECs and EPCs were found to have a synergistic effect

and lead to the most reperfusion, with OECs providing direct interactions with native ECs and EPCs contributing to cytokine production [26]. Another distinctive strategy uses a nonvascular progenitor cell population that can participate indirectly on vascularization by acting as local producers of vascular-inductive cytokines. For example, MSCs seeded in alginate microbeads and delivered into the ischemic hind limb of mice led to an increase in murine genes associated with angiogenesis, including VEGF-A, endoglin, and sphingosine kinase 1 [163]. These MSCs also led to an increase in capillary and arteriole density, which was not observed with the bolus injection [163]. In addition, adipose-derived stem cells (ADSCs) also excrete angiogenic chemokines including VEGF, fibroblast growth factor, and hepatocyte growth factor and can participate in both vascularization and bone repair when encapsulated into alginate microbeads and injected into the dorsum of mice [164].

Fibrin, and its precursor fibrinogen, is a naturally derived non-globular protein involved in the process of blood clotting. Fibrinogen that is derived and purified from humans is FDA approved as a sealant, though other commercial sources provide baboon-, bovine-, and murine-derived fibrinogen. Fibrinogen is also a very large glycoprotein (350 kDa) that is composed of three main structural units and is predominantly produced by hepatocytes in the liver [183, 184]. Fibrin cross-linking spontaneously occurs in vivo when fibrinogen is cleaved by the serine protease thrombin in response to tissue injury and will be degraded by plasmin following tissue repair [185, 186]. Cross-linking can be accomplished in vitro by mixing fibrinogen with thrombin, and aprotinin can also be added to inhibit fibrinogen proteases. The chemical and physical properties of fibrin can be tailored by altering the ionic strength, pH, and concentration of fibrinogen during polymerization [187]. Some of the major advantages of fibrin scaffolds are that the polymers are biocompatible and bioresorbable and contain adhesion biomolecules for cells. Fibrinogen naturally contains the RGD amino acid sequences, which allows for cells to attach [188]. Additionally, the fibrin network supports infiltration from neutrophils, fibroblasts, and macrophages which can secrete ECM components including collagen and fibronectin, providing additional cellular adhesion sites [189].

Transplanting cells using fibrin scaffolds has been used for both in vivo and in clinical revascularization applications. Fibrin scaffolds have been used to seed ECs to participate in new vasculature. Human umbilical vein ECs and fibroblasts transplanted in fibrin scaffolds in the dorsal region of mice lead to the exogenous ECs forming blood vessels and anastomosing with the native vasculature [165]. ECs were also delivered with fibrin to sheep hearts after prolonged ameroid constriction leading to neovascularization and improved myocardial blood flow [166]. Work has also been done with fibrin scaffold to deliver cells to revascularize the heart, including delivering skeletal myoblasts to replace damaged cardiomyocytes in rats [167]. The seeded scaffolds were found to increase skeletal myoblast survivability, induce microvessel formation, and reduce scar size. Fibrin scaffolds have also been used to deliver stem cells that secrete angiogenic factors to promote new vascularization. MSCs delivered in fibrin scaffolds to chronically infarcted rat hearts showed a significant increase in VEGF secretion and blood vessel density, which was not observed for the bolus-delivered cells [168]. Additionally, for the first time,

allogenic embryonic stem cells seeded on fibrin scaffolds have been used in a patient with severe heart failure [156]. These stem cells were committed to a mesodermal/cardiac lineage via exposure to specific factors and were delivered into the infract area of a 68-year-old patient who symptomatically improved after 3 months [156].

Gelatin is a natural component of the ECM that is derived from denaturing collagen by acid and alkaline hydrolysis. Gelatin is typically derived from different animal sources, including bovine and porcine, where it can be extracted from skin, tendon, and bone tissues. The source and process of extracting the gelatin can lead to significant variation in the size of gelatin molecules. Regardless of the source or processing, gelatin molecules tend to share a Gly-X-Y motif with both polar and nonpolar amino acids [190]. Gelatin polymers have excellent biocompatibility, have been generally recognized as safe (GRAS) by the FDA, and are commonly used in food products [191]. Additionally, gelatin naturally contains adhesion peptides like RGD to support cell adhesion and is readily chemically modified due to its diverse and accessible functional groups [192]. However, the thermo-reversible cross-linking of gelatin scaffolds is easily broken at physiological temperatures, requiring the use of additional cross-linkers like glutaraldehyde prior to in vivo use [193].

Gelatin scaffolds have been used to successfully deliver cells committed for the revascularization process. For example, neonatal heart cells seeded in gelatin scaffolds lead to new blood vessels, arterioles, and venules when implanted into resected rat hearts [169]. In addition, gelatin scaffolds have also been used to co-transplant different types of stem cells. Scaffolds containing both ECFCs and MSCs were found to form extensive vascular networks and had rapid anastomosis when implanted in the subcutaneous space in the back of mice [170]. MSCs, besides their supporting role in the secretion of angiogenic cytokines, were also able to assist in vascularization by differentiating into perivascular cells and therefore providing the necessary maturation for the new established capillaries [170]. Other works with gelatin scaffolds seeded with MSCs have also assisted revascularization when implanted into transected rat spinal cords [171]. These MSCs were found to upregulate HIF-1α and express VEGF, form perivascular cells around new blood vessels, and consequently promote angiogenesis in the injured area of the spinal cord [171]. Alternatively, ADSCs are another type of stem cell commonly seeded in gelatin scaffolds that have been found to promote both lymphangiogenesis and angiogenesis in vivo. Gelatin scaffolds containing both ADSCs and VEGF-C have been shown to increase lymphatic density in the footpad of lymphedema mice [172]. The combination of both VEGF-C and ADSCs increased the lymphatic potential of ADSCs and lead to the most lymphatic vessel formation. ADSCs have also been combined with bFGF in gelatin scaffolds to promote revascularization and repair damaged muscle fibers in mice with a lacerated gastrocnemius [173].

Synthetically Occurring Polymers

Synthetic biomaterials are manufactured materials that provide many physical and chemical properties which are advantageous for cellular delivery. The polymer properties of synthetic biomaterials are manufactured reproducibly on a large scale

and have mechanical properties which can be readily controlled and manipulated. Synthetic materials also avoid some potential immunogenic responses that can arise from deriving polymers from animals. However, synthetic materials lack ECM constituents, especially those responsible for cell recognition and adhesion. Nevertheless, a large variety of synthetic polymers have been used for cell-based approaches for revascularization applications, but this chapter will only focus on three biomaterials including poly(ethylene glycol) (PEG), poly(lactic-co-glycolic) acid (PLGA), and poly(l-lactic acid) (PLLA) (Table 8.3).

Poly(ethylene glycol) (PEG) is a synthetic polymer and one of the most commonly used polymers for biomedical applications, including for gene and drug delivery, medical devices, and tissue engineering scaffolds. Generally, PEG displays many favorable characteristics for biomedical applications including high hydrophilicity, bioinertness, and remarkable biocompatibility. The process of anionic ring-opening polymerization to form PEG can create a wide range of polymer weights, allowing for a variety of different mechanical properties and pore sizes for the scaffolds [204]. Multiple types of polymerization methods exist to form PEG to further control mechanical properties and typically involve either chain-growth or step-growth polymerization [205]. PEG is water soluble, and the cross-linked PEG chains (PEG hydrogels) are typically prone to aqueous hydrolysis and are therefore seen as biodegradable systems. The biodegradable characteristics are related with the molecular weight of the PEG chains and require that the chains be below the necessary molecular weight threshold to allow renal elimination of the individual polymer chains. In addition, PEG can be combined with PLGA to further increase their degradation by hydrolysis [160]. PEG is also nontoxic and nonimmunogenic and has been approved by the FDA for a variety of clinical uses including medical implants and drug delivery depots. However, PEG scaffolds lack sites for cell adhesion. Derivatizing amino reactive PEGs containing both cell-binding peptides and peptide substrates for proteases has been shown to be a particularly promising strategy for creating mimics of the natural ECM and thereby allowing for cell interactions with PEG [206, 207].

PEG scaffolds have shown promise for revascularization therapies and have recently been used to transplant cells to promote vascularization. Modified PEG scaffolds have supported vascular structures when seeded with ECs and can also be combined with angiogenic factors like thymosin beta 4 (Tβ4) and VEGF-A for additional EC response in vitro [208, 209]. In vivo work using PEG scaffolds to encapsulate brain-derived microvascular ECs and neural progenitor cells into the backs of mice has led to significant vascularization within the scaffold [194]. The new vessels were found to anastomose with the host vasculature and support blood flow, with the most vascularization occurring in scaffolds containing both cell types [194]. More recent work has encapsulated human umbilical vein ECs and lung fibroblasts into PEG scaffolds to deliver into the dorsal flank of mice [195]. The PEG scaffolds were able to support the seeded cells and allow them to participate in new vasculature in vivo [195]. PEG scaffolds have also been shown to support EPC and stem cell encapsulation. EPCs, human umbilical vein ECs, and smooth muscle cells adhered, degraded, and formed 3D vasculature in matrix metalloproteinase

Table 8.3 Selected examples of cell-based strategies delivered with synthetic polymers to promote revascularization

Biomaterial type	Cells delivered	Concentration cell/mL scaffold	Predicted mechanism of revascularization	Intended goal	Ref
Synthetic					
PEG	Brain-derived microvascular ECs	5.0×10^7	Paracrine secretion	Promote stabilized microvasculature	[194]
	Neural progenitor cells	5.0×10^6			
	Human umbilical vein ECs	1.0×10^7	Cells incorporate with native vasculature	Promote vascularization	[195]
	Normal human lung fibroblasts				
	Induced pluripotent stem cells	Unspecified	Cells incorporate with native vasculature	Revascularization and restored cardiac tissue	[196]
PLGA	Microvascular ECs	7.2×10^7	Cells incorporate with native vasculature	VEGF-A and ECs promote vascularization	[99]
	Human dermal microvascular ECs	2.7×10^7	Cells incorporate with native vasculature	Cells participate in vascularization	[98]
	Aortic ECs	$0.83–1.6 \times 10^7$	Paracrine secretionCells incorporate with native vasculature	Generated new blood and lymphatic structures	[197]
	Aortic smooth muscle cells				
	Skeletal muscle cells				
	LECs	2.4×10^8	Unspecified	Generate new lymphatic vessels	[114]
	Marrow stromal cells	2.6×10^8	Paracrine secretion	Transfected cells secrete VEGF-A for vessels	[198]
	Mesenchymal stem cells	Unspecified	Paracrine secretion	Promote and participate in vascularization	[199]
	Kidney ECs		Cells incorporate with native vasculature		
	Embryonic stem cells	4.0×10^7	Vessels/cells incorporate with native vasculature	Preimplanted vascular network	[200]

(continued)

Table 8.3 (continued)

Biomaterial type	Cells delivered	Concentration cell/mL scaffold	Predicted mechanism of revascularization	Intended goal	Ref
PLLA	Human dermal microvascular ECs	2.7×10^7	Cells incorporate with native vasculature	Cells to participate in vascularization	[98]
	Differentiated embryonic stem cells	1.0×10^8	Cells incorporate with native vasculature	Promote vasculogenesis	[201]
	Aortic smooth muscle cells	$1.0–4.9 \times 10^7$	Paracrine secretion Cells incorporate with native vasculature	Differentiate and support new vasculature	[202]
	EPCs	1.2×10^7	Paracrine secretion Cells incorporate with native vasculature	Localize EPCs promote vasculogenesis	[203]
	Embryonic stem cells	4.0×10^7	Vessels/cells incorporate with native vasculature	Preimplanted vascular network	[200]

(MMP)-degradable PEG scaffolds [210]. PEG/fibrinogen scaffolds seeded with induced pluripotent stem cells have been implanted into ischemic mice hearts to increase perfusion and restore cardiac function [196]. These PEG/fibrinogen scaffolds were able to help induce cardiac differentiation of the stem cells and also lead to significantly more revascularization and improved fractional shortening and systolic/diastolic functions than bolus injection of cells [196].

Poly(lactic-co-glycolic) acid (PLGA) is another very commonly used polymer for biomedical applications, including implants, drug delivery devices, and tissue-engineered scaffolds [211]. Generally, PLGA has favorable properties for biomedical applications due to their biocompatibility, adjustable degradation, and hydrophilicity. PLGA scaffolds are comprised of varying ratios of lactic and glycolic acid, allowing for scaffolds to have a variety of adjustable mechanical properties [212]. Herein, we will refer to all scaffolds containing any ratio of glycolic to lactic acid as PLGA, with the exception of all PLLA scaffolds. PLGA synthesis occurs through ring opening of the cyclic dimers of glycolic and lactic acid, which can create a wide range of polymer weights and additionally attenuate the mechanical properties of the scaffold [213, 214]. Degradation of PLGA occurs through the aqueous hydrolysis of the ester linkages of the polymers, and the by-products are removed from the body via respiration [211, 215]. PLGA is also nonimmunogenic, has acceptable toxicity, and has been approved by the FDA for a variety of clinical uses including drug delivery [216]. However, PLGA has acidic degradation

products, limited number of functional groups available for chemical modification, and poor cellular adhesion properties [211]. Covalent techniques can be used to immobilize biomolecules to PLGA which can attenuate cellular adhesion to PLGA scaffolds [217, 218].

PLGA scaffolds have been widely used to deliver cells to promote new vasculature. As mentioned earlier, PLGA and alginate scaffolds have been used to deliver ECs and angiogenic factors to promote vascularization [99]. PLGA scaffolds loaded with HDMECs were used to transplant these ECs into the dorsal region of mice where the cells formed into new microvasculature [98]. Aortic ECs, skeletal muscle cells, and aortic smooth muscle cells seeded into PLGA scaffolds were able to participate in vascularization when implanted in the subcutaneous space in rats [197]. While all three cell types were found to lead to increased capillary and lymphatic structures, only aortic ECs had parallel capillary arrays and large thin-walled vessels [197]. PLGA scaffolds have also been used to incorporate and deliver LECs into the caudal area of mice [114]. PLGA scaffolds were found to be both compatible with LECs and allow for LECs to form vessel-like structures in vivo [114]. More recent work described the use of PLGA scaffolds to deliver paracrine secreting cells for revascularization applications. Marrow stromal cells that were transfected with adenovirus to express VEGF-A were seeded into PLGA scaffolds and transplanted into bone defects in rabbits [198]. These marrow stromal cells both promoted vascularization within the bone defect and also differentiated into new bone [198]. MSCs and kidney vascular ECs seeded into PLGA scaffolds were implanted into rat thighs and lead to increased vascularization [199]. Additionally, embryonic stem cells were allowed to grow into vessels in PLGA/PLLA scaffolds in vitro prior to being implanted into the dorsal region of mice where they were able to anastomose with native mouse vasculature [200].

PLLA is also commonly used for biomedical applications, including drug delivery and medical implants. PLLA is a synthetic biodegradable polyester that is produced via a ring-opening polymerization of dilactide to create lactic acid polymers [219]. Generally, both PLLA and PLGA have many similar properties, including outstanding biocompatibility, due to them both containing lactic acid. Additionally, the ring-opening polymerization to form both PLLA and PLGA creates a wide range of polymer weights, allowing for a variety of different mechanical properties [204]. However, all three forms of lactic acid, including l-lactide, d-lactide, or *meso*-lactide, are more hydrophobic than their glycolic acid counterparts [161]. Although PLLA will undergo hydrolysis of the ester linkages of the polymers and the by-products are removed via respiration, this degradation is typically slower than an equivalent scaffold containing polyglycolic acid [161]. PLLA also has FDA approval and has been used in several biomedical devices including drug delivery and bioresorbable vascular scaffolds. However, PLLA also has acidic degradation products and poor cellular adhesion properties [211]. A number of techniques can be used to increase cellular adhesion to PLLA including immobilizing biomolecules and incorporating other polymers [217, 218, 220].

The transplantation of cells using PLLA scaffolds has been extensively used to promote vascularization. PLLA scaffolds have been used to transplant HDMECs

into the dorsal region of mice where the ECs formed blood-carrying microvasculature invested by native smooth muscle cells [98]. Other works with ECs found that embryonic-derived ECs in PLLA scaffolds formed network-like microvessels in a pattern more consistent with embryonic vascularization when implanted into the dorsal region of mice [201]. PLLA scaffolds have also been seeded with human aortic smooth muscle cells and subcutaneously implanted into mice for the purposes of guiding and supporting new vasculature [202]. Stem and progenitor cells have also been successfully delivered with PLLA scaffolds to participate in vascularization. For example, EPCs seeded in PLLA scaffolds were able to participate in wound regeneration in mice [203]. These PLLA scaffolds greatly increased the amount of surviving EPCs transplanted in the mice compared to bolus, and these seeded EPCs led to increased vascularization [203]. Similarly, PLLA/PLGA scaffolds loaded with embryonic stem cells that had vessels grown in vitro showed that the vessels could anastomose with native vasculature when transplanted into the dorsal region of mice [200].

Conclusions and Future Directions

Revascularization cell therapy holds tremendous therapeutic and curative potential for vascular ischemic diseases [23, 46]. The further development and optimization of biomaterial-based systems boasts a growing field within tissue engineering and regenerative medicine that promises to enhance the future clinical outlook of cellular delivery. These biomaterials may be used to either enhance cellular adhesion, survival, and function upon transplantation or to appropriately provide soluble growth and migratory factors that enable enhanced cellular recruitment. This may further involve investigation of potentially combining multiple cell types and/or soluble cues that synergistically promote functional and localized neovessel formation. However, such cell therapy approaches remain hampered by the limited supply of donor cells and challenges associated with ex vivo cellular manipulation. Herein, a recently growing area of research aims to design strategies that enhance the functional capacity (i.e., homing efficiency, angiogenic activity, stimulatory effect) of endogenous stem and progenitor cells [25, 46]. A greater understanding of the mechanisms involved in progenitor cell homing and function under diseased states is thus important for elucidating strategies that may either bypass or enhance approaches involving exogenous cellular transplantation.

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Cell Reprogramming for Cardiac Regeneration and Rare Disease Modeling

9

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Introduction: Cell Reprogramming

The cell differentiation process was once believed to be irreversible. First scientific evidence of the opposite hypothesis was given by Dr. Gurdon in 1962, which performed novel experiments of somatic cell nuclear transfer. By injecting the nucleus of a tadpole intestinal cell into ultraviolet-light-irradiated oocytes, not only tadpoles but also adult frogs were derived. At that time however, the molecular mechanisms were not well understood and could not be reproduced in larger species, being the whole process questioned and even considered as an artifact. It was not until almost 40 years later when the first mammal, the sheep Dolly, was cloned by following similar experiments and results, as expected, genetically identical to the somatic cell donor sheep [1]. Although this experiment was quite inefficient with one successful cloning in 227 trials, the impact of the results was enormous, as they confirmed the reprogramming capacity of somatic cells, including those ones from large species. Furthermore, cell fusion experiments reinforced this concept too, showing the modification of cell genetic profile when fused with other cell types. Thus, somatic cells could activate tissue-specific genes when fused with specific cell lines or even express pluripotency genes after fusion with embryonic stem cells (ESC) [2]. In any case, although all the described experiments gave key insights about the plasticity

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of the cells, it was not until 2006 when the whole cell reprogramming field arose. At that year, the laboratory of Dr. Yamanaka reported for the first time the generation of embryonic stemlike cells from somatic cells. These cells were termed as induced pluripotent stem cells (iPSC), and their finding resulted, together with Dr. Gurdon's cloning discovery, in 2012 Nobel Prize [3]. Their generation, features, and main applications will be described in the next sections.

Induced Pluripotent Stem Cells (iPSC)

iPSC Discovery and Derivation

iPSC were first derived from adult mouse fibroblasts being cell dedifferentiation induced through retroviral transduction of several factors involved in pluripotency and self-renewal of ESC. Initially, a total of 24 genes were selected and overexpressed in various combinations in order to identify those that might participate in cell reprogramming, which was ultimately found to depend on only four of the factors: *Oct3/Oct4*, *Klf4*, *Sox2*, and *c-Myc* (OSKM) [3]. One year later, human iPSC were also generated, using either the same combination of transcriptional regulators [4] or a different set of factors (*OCT3/OCT4*, *SOX2*, *NANOG*, and *LIN28*) [5].

Reprogramming of somatic cells toward ESC-like was evidenced by several features that have become the rule for identification and characterization of iPSC. Formation of compact colonies with well-defined edges and rounded or flat shape (depending on their mouse or human origin, respectively) is the first approximation for iPSC clone selection. If these colonies can be properly expanded in an ESC-defined media, which indicates self-renewal properties, more specific assays need to be performed in order to identify them as truly reprogrammed cells. Thus, endogenous expression of pluripotent genes like *OCT4*, *SOX2*, and *NANOG* and exogenous gene silencing are expected after cell dedifferentiation. Also, reprogramming is accompanied by an extensive remodeling of epigenetic marks and demethylation of pluripotency genes as result of their reactivation. In mouse, activation of both X chromosomes is also observed as it is established for mouse ESC. Not only at the gene but at the protein level, expression of the pluripotent transcription factors and alkaline phosphatase activity is also detected in iPSC. Importantly, as a pluripotent cell type, three-lineage differentiation must be confirmed. In vitro and in vivo protocols have been established based on ESC existing protocols. Thus, formation of teratomas with the presence of all types of three-lineage-derived somatic cells can be detected after iPSC subcutaneous injection in immune-deficient mice [6]. Furthermore, contribution of iPSC to chimeras after injection into the embryos and germ line transmission is considered the best proof for iPSC correct reprogramming. Being even stricter, tetraploid complementation can be used in order to demonstrate total contribution of the derived iPSC to the developed embryo. All these features have been proved for mouse iPSC, demonstrating their similarities to mouse ESC.

Interestingly, although iPSC main characteristics like the expression of pluripotent genes and in vitro and in vivo differentiation through teratoma formation are

common for mouse and human lineages, there are several other features in which both species-derived cells differ. Distinct molecular features including the expression of several differentiation markers and the inactivation of one of the X chromosomes are observed in human iPSC. Low chimera contribution capacity has been also shown for nonhuman primate iPSC. These aspects indicate a lower potency of the human/nonhuman primate cells (termed as primed state) similar to the one exhibited by mouse epiblast cells and in contraposition with rodent iPSC, which present a more potent or so-called naïve state [7]. Following these initial discoveries, efforts to simplify and increase the efficiency of the reprogramming process have revealed that a reduced set of reprogramming factors is sufficient to generate iPSC (reviewed in [8]). Even overexpression of only one factor can reprogram certain cell types. For example, neural stem cells can be reprogrammed when *OCT4* is overexpressed, being facilitated by the endogenous expression of *SOX*2 and *c-MYC* of this neural cell population [9]. Furthermore, the overexpression of many other factors and combinations has been also successful for iPSC reprogramming, demonstrating the striking plasticity of the cells (reviewed in [8]).

In addition, due to safety concerns surrounding spontaneous reactivation of viral transgenes or possible oncogene activation via lentiviral insertion, many alternative iPSC generation strategies have been developed and tested. These have involved the use of adenoviruses, RNA-based Sendai viruses, episomal vectors, DNA plasmids, excisable vectors, mRNAs, microRNAs, or even proteins, being many of these systems already commercialized for experimental purposes. Moreover, the use of genetic factors, chemical inhibitors, and signaling molecules that can either replace core reprogramming factors or enhance reprogramming efficiency has also been investigated (reviewed in [8]).

Finally, many cell types have been also successfully used to produce iPSC, including somatic cells (β-pancreatic cells, neurons, keratinocytes, hepatocytes, etc.) and also stem cells (hematopoietic, neural, and adipose tissue-derived cells). Interestingly, although cells can be reprogrammed independently of their origin and differentiation stage, the efficiency for their reprogramming varies, being usually greater when adult stem cells rather than somatic cells are reprogrammed (reviewed in [10]).

Also, despite that the efficiency of the protocol developed for iPSC generation still remains quite low, it has been proved to be quite reproducible. Indeed, iPSC with common pluripotent features have already been derived from many species, including humans, nonhuman primates, pigs, and rodents. However, like ESC, these iPSC can display differential phenotypes, morphologies, and/or culture requirements depending on the species of origin and consequence of their differences in their pluripotency stage. Thus, culture of mouse iPSC in the presence of LIF [11] is required for the maintenance of the self-renewal and pluripotency capacity of the cells, whereas bFGF is indispensable for human iPSC culture [12]. In this regard, recent modifications in the composition of the culture media adding chemical inhibitors that target stem cell self-renewal and differentiation pathways have improved the efficacy for deriving and maintaining pluripotent cells. The 2i so-called media, composed of the molecules CHIR99021 (a GSK3 inhibitor) and PD0325901 (a

MEK inhibitor), has allowed not only a stabilized growth of the pluripotent cells but also, and more importantly, to direct them to a more primitive state, directing human and primate primed pluripotent cells to a naive-like state (reviewed in [7]). As a consequence of that, chimera contribution of primate naive-like stem cells has been achieved [13].

iPSC Differentiation Potential

Numerous protocols, most of them based on previous ESC techniques, have been used to in vitro differentiate iPSC into diverse cell types. In vivo, together with uncontrolled differentiation through teratoma formation or chimera formation, contribution to specific organs in tissue-specific-deficient mice has been also shown (reviewed in [8]). For example, injection of wild-type mouse-derived iPSC into embryos from a knockout mouse for the kidney-related gene *Sall1* totally contributes to the formation of the kidney of the embryos and adult mice [14]. Interestingly, interspecies contribution was also demonstrated for the first time when rat iPSC were injected into mouse blastocysts that were deficient for *Pdx1*, an essential gene required for pancreas development, generating a functional rat pancreas [15].

Additionally, the in vivo differentiation potential of implanted iPSC in adult tissues/organs has been confirmed in many animal models for hepatic, cardiac, or neural diseases, among others. However, the total contribution of the implanted undifferentiated iPSC to tissue-specific cells has been minimal and uncontrolled, due to the lack of a proper tissue environment to direct the right differentiation process, many times forming teratomas. Also, a limited contribution has been detected when in vitro pre-differentiated cells have been injected, consequence also of the low retention and survival of the implanted cells. Interestingly, functional improvement has been observed in the treated tissues after iPSC-derived cells, being partially related to their trophic effect. Many approaches like combination of iPSC-derived cells with bioengineered approaches or in vivo reprogramming are being studied in order to overcome these issues.

Partial and Direct Cell Reprogramming

Even though methods for safe and efficient iPSC-derived differentiated cells are being rapidly developed, their residual tumorigenic potential remains as a major limitation for clinical applications. Overcoming this problem requires careful optimization of methods for differentiation, isolation, and/or characterization of the cells to be transplanted. In this regard, differentiation of somatic cells into another cell type through induction of a transitory undifferentiated state or even by direct reprogramming to the required somatic cell could be a safer, quicker, and more efficient manner for specific cell-type derivation. This phenomenon in fact is not a new concept. Already in the 1980s, it demonstrated the direct transdifferentiation of fibroblasts toward myoblasts when the MyoD gene was overexpressed [16]. Similarly, several hematopoietic lineages could be differentiated to other hematopoietic lineages by overexpression or inhibition of different factors [17].

Going a step further, the laboratory of Dr. Ding has made major contributions to this research field by demonstrating how partial reprogramming can significantly facilitate somatic cell differentiation. In this reprogramming method, the same reprogramming factors as for iPSC generation (OSKM) are used. However, shortly after transduction, taking the advantage that the reprogramming cells acquire a transient "plastic" or partial undifferentiated state (demonstrated to be pluripotent state later [18, 19]), the exogenous supplementation of pluripotency pathway inhibitors and tissue-specific differentiation growth factors can induce specification into a particular progenitor or differentiated cell type [20].

Ultimately, in vitro direct reprogramming from somatic cells has been also assayed. By this strategy, overexpression of tissue-specific factors, usually involved in tissue specification in the embryo development, can directly direct differentiation of one somatic cell into a different lineage somatic cell. Direct transdifferentiation of fibroblasts toward cardiomyocytes (CM), endothelial cells (EC), various subtypes of neurons, hepatocytes, skeletal muscle cells, hematopoietic progenitors, and β-pancreatic cells has been already demonstrated by this method [21].

Once proven the transdifferentiation capacity of the cells, in vivo studies have been also performed with regenerative purposes. Overexpression of key genes or miRNAs by viral approaches has been assayed for treating several neural, kidney, and heart pathologies. For example, conversion of endogenous glial cells into functional neurons has been successfully achieved for treating injured or diseased brain and spinal cord injury (reviewed in [22]). Still, long-term functional effects of neural reprogrammed circuits need to be in-depth assessed. Similarly, in vivo fibroblast transdifferentiation toward CM has been also shown in a mouse model of myocardial infarction (MI) with a functional improvement detected [23, 24]. This last issue will be reviewed in detail in section "In Vivo Reprogramming of the Resident Cardiac Fibroblasts".

Application of Cell Reprogramming Technology

The scientific and therapeutic potential of cell reprogramming is unquestionable. After only 10 years of iPSC discovery, more than 10,000 scientific reports have been already published in this field, and the application of reprogrammed cells in regenerative therapy widely studied. Thus, the first clinical trial has been recently reported in Japan treating a single patient suffering from age-related macular degeneration with autologous iPSC-derived retinal pigment epithelium cell sheets. Although this patient suffered "no serious adverse effects," the clinical trial has been suspended due to a change in the therapeutic strategy, now focused in using partially matched donor cells rather than the individual's own cells [25]. Furthermore, the parallel development of the bioengineering field can greatly reinforce the potential of the stem cell therapy. Biocompatible biomaterials and novel techniques for 3D scaffold production are being fast developed, making possible the creation of tissue patches and organoids that will boost their therapeutic action.

In addition to their regenerative capacity, iPSC constitute an important tool for disease modeling (reviewed in [26] and discussed in section "Rare Disease Modeling"), allowing to study the molecular mechanisms involved in many pathologies and to test specific drug targets. In fact, the generation of iPSC from patients with genetic cardiac and rare disorders among other pathologies has already been shown (reviewed in [27, 28]). Using these cells as a model, studies have analyzed the potential therapeutic efficacy of drugs or small molecules for correcting disease phenotypes (section "Therapeutic Applications of iPSC Models of Genetic Diseases"). Thus, important advances are likely to stem from the use of iPSC-related disease models, which can be utilized to study mechanisms of pathogenesis, to identify toxic effects of drugs, and to characterize the protective effects or optimal doses of the therapeutic agents.

Taken together, reprogramming of somatic cells either to iPSC or to different lineage somatic cells offers exciting new tools that can be used to gain molecular insight into diseases and should contribute to the development of novel therapies. In this chapter, we will next review the experimental research and future perspectives of cell reprogramming for cardiac regeneration and rare disease modeling, focusing on the cell reprogramming procedures used to obtain functionally relevant cell types for heart regeneration and the novel developed techniques for rare disease modeling.

Cell Reprogramming for Cardiac Regeneration

Mammalian Cardiac Regeneration Capacity

Urodele amphibians, such as salamanders and newts, and teleost fish, such as zebrafish, have an extraordinary capacity to regenerate injured tissues, including the heart, even in adulthood. Recent evidence suggests that heart regeneration in these animals likely occurs via partial dedifferentiation of mature CM followed by proliferation [29, 30].

Until recently, it was generally assumed that shortly after birth, CM were terminally differentiated blocking cytokinesis and causing binucleation; thus, it was believed heart growth should occur entirely by CM hypertrophy during preadolescence. Recent evidences have challenged this dogma [31, 32]. Moreover, the analysis of carbon-14 integration in CM determined that human CM are capable of renewal during adulthood at a low rate, 1 or 0.45% per year calculated at the age of 25 or 75, respectively. This low rate could be explained by the fact that mammalian CM are mostly multinucleated and/or polyploid, and in most of the studies, the cycling CM were mononucleated [31, 33]. Alternatively, other authors postulate that newly formed CM derive from resident cardiac progenitor cells (CPC) [34]. Both mechanisms could be involved in de novo CM regeneration, and this remains a subject of debate.

Unfortunately, in adult mammals, the resident cardiac cells are not able to regenerate heart tissue and restore efficiently the cardiac function in response to injury.

Cardiac injury such as acute MI is most often caused by plaque rupture with thrombus formation in a coronary vessel, resulting in acute reduction of blood supply (ischemia) to a portion of the myocardium. The ischemia induces irreversible damage and death of the tissue, and it is calculated that a patient loses approximately one billion CM, which is substituted by a nonfunctional scar. The subsequent electrical uncoupling to the remaining myocardium and the unfavorable remodeling of ventricular walls, being hypertrophy the most relevant compensation for the loss of CM, eventually lead to heart failure [35].

Current Therapeutical Approaches for Heart Regeneration

Despite the advances in medical and catheter-based therapy for acute MI, the 1-year mortality remains as high as 13%, and the 5-year mortality for patients with heart failure remains as high as 50%. Pharmacological treatment is still a palliative therapy not capable to repair the massive loss of CM, being heart transplantation the only real option for severe cases. Unfortunately, there are not enough heart donors available for transplantation. This acute shortage of human organs has prompted significant research and development into alternatives in recent years such as xenotransplantation [36], defined as the transplantation of organs between different species; however, to be successful in humans, xenotransplantation must overcome issues of transplant rejection, cross-species infection, and ethics. Other alternatives include cell-based and cell-free approaches capable of regenerating the damaged heart, recently reviewed in Sahara et al. [37].

Briefly, cell-free approaches are focused primarily on the activation of resident CPC or the proliferation of pre-existing CM. Ligands such as periostin or neuregulin-1, inhibitors of p38, or certain miRNAs can promote the proliferation of adult CM. Moreover, thymosin β4 and VEGFA can stimulate epicardial-derived progenitor cell proliferation and their differentiation mainly toward vascular cell lineages, including vascular EC and smooth muscle cells (SMC), which in turn leads to neovascularization.

On the other hand, a number of groups have been focused on the use of the stem cells to repair cardiac tissue. Adult somatic stem cells can be isolated from different tissues and can spontaneously differentiate in vivo in response to endogenous cues. Based on this idea, skeletal muscle cells, adipose tissue-derived cells, and cardiac-derived stem cells have been explored in the treatment of acute MI and chronic ischemic heart failure, but the largest clinical experience has been acquired with intracoronary delivery of bone marrow stem cells (BMSC). A detailed description of somatic stem cell transplantation therapies for heart diseases used in clinical trials has recently been reviewed [38, 39]. Unfortunately, the results of the collected clinical data of the last decade using BMSC are ambiguous and do not prove substantial long-term benefit, particularly in improving life expectancy in patients [38, 40]. The current consensus is that any improvement in cardiac function after BMSC transplantation is likely to be the result of a paracrine action [41]. Thus, success of cardiac cell therapy will be determined by both, the generation of cells capable to

regenerate the damaged tissue and the development of methods to improve cell survival, retention, and engraftment [40].

Cell Reprogramming for Regeneration of Damaged Heart Tissue

Ideally, an optimal cell type considered for cardiac regeneration should satisfy the following items: (1) ensure safety, (2) be expandable or scalable, (3) be immunocompatible within the donor heart or at least immune tolerant, and (4) be functionally integrable with host myocardium tissue increasing either the force of contraction (by coupling with host CM) or the survival of the host tissue (by promoting neovascularization) or both.

Human cardiac and vascular cells (including CM, SMC, EC, or CPC) could be considered optimal candidates from a functional point of view. In any case, large amounts of starting material need to be readily available for manipulation and transplantation. Thus, which are the available sources to obtain these cardiac and vascular cell types? Human host tissue is an option only in the case of CPC where there are ready-to-use and well-stablished protocols to isolate CPC from heart biopsies and be enriched via cardiosphere culture. Despite this, in contrast to embryonic or neonatal CPC, the myogenic potential of adult CPC is limited [42].

Cell reprogramming strategies have offered the possibility to obtain human cardiac and vascular cells, creating novel cellular sources with higher potential for cardiovascular therapies than adult somatic stem cells. Here, we review the recent reprogramming methods used to generate functionally relevant cell types such as CM, SMC, EC, or CPC for cardiovascular therapy. The current described methods to obtain cardiac cells through different cell reprogramming approaches are summarized in Fig. 9.1.

Cardiac and Vascular Cells from Differentiated iPSC

The fact that CM can be derived in vitro from pluripotent stem cells when cultured as suspension aggregates called embryoid bodies was obvious in 2000 [43]. Since then, a multitude of CM differentiation approaches have been developed and are reviewed elsewhere [44]. The most successful methodologies to derive cardiac cells from pluripotent stem cells are those that have recapitulated the pathways that control the establishment of cardiac lineage in the early embryo. These protocols are serum-free and are based on the addition of specific growth factors, including BMP, Activin A, bFGF, WNT proteins and inhibitors, and VEGF, along the differentiation process. Whatever method is used, careful consideration must be given to concentrations and the timing of addition or removal of growth factors, especially the ratio of BMP4/Activin A [45] and the embryoid body size or density of monolayer cultures [46]. Although numerous methods for cardiac differentiation have been described, outcomes vary substantially between laboratories and human pluripotent stem cell lines. Moreover, since there is no consensus on the presentation of CM differentiation efficiency (such as the number of CM generated per input pluripotent

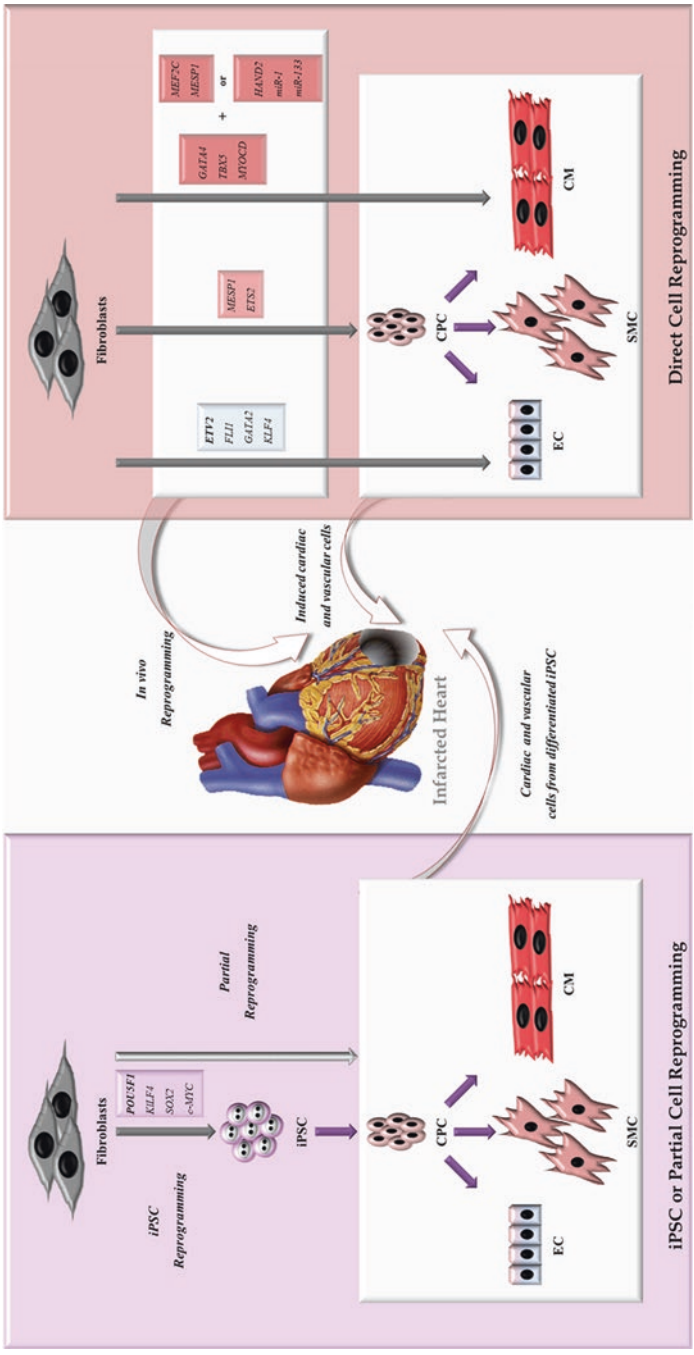

Fig. 9.1 Schematic representation of different reprogramming strategies used to obtain human cardiac and vascular cells

stem cells), it is not possible to make a thorough comparison of published methods.

CM enrichment protocols have enabled to yield, independently of the efficiency of differentiation, up to 99% pure CM. Different strategies, some of them reviewed in Sahara et al. [37], have been used to purify CM such as: (1) Percoll gradients followed by centrifugation [47]; (2) FACS separation based on surface marker expression (SIRPA, VCAM1 [48]) or the mitochondria-specific fluorescent dyes, since CM are enriched for these markers or have high mitochondria content; (3) genetic selection strategies in which cells are genetically modified with a CM-specific promoter driving the expression of a drug-resistant gene [49]; and (4) culture of CM in a glucose-depleted and lactate-enriched medium.

To date, there is no clinical test of human pluripotent stem cell-derived CM transplantation into human patients, but the first clinical-scale transplantation of human pluripotent stem cell-derived CM into a nonhuman primate has been reported [50]. One billion in vitro-derived CM were produced and injected intramyocardially after MI. Interestingly, transplanted CM remuscularized substantially the damaged monkey heart, and the new muscle grafts were perfused by host vasculature and electromechanically coupled to host myocytes. However, transplanted CM showed progressive but incomplete maturation and ventricular arrhythmias were registered.

Furthermore, the use of VEGF and other pro-angiogenic cytokines to enhance ischemia-mediated angiogenesis in humans has thus far been almost entirely unsuccessful. Thus, the transplantation of cells with angiogenic potential, as EC, could improve the survival of the resident and/or other transplanted cells in ischemic cardiomyopathy. As for CM, iPSC differentiation into EC follows the same process as ESC differentiation and vasculogenesis. EC differentiation has been successfully performed by culturing iPSC on OP9 feeder cell layers [51] or in suspension as embryoid bodies [52] and supplementing the differentiation media mainly with VEGF. EC can be easily identified by the expression of specific surface markers such as VEGFR2/KDR/Flk1, VE-cadherin/CD144, or PECAM-1/CD31 [51, 52], and their potential can be tested in vitro or in vivo, using assays such as the tube formation on a 3D matrix and the uptake of acetylated LDL or the Matrigel plug assay, respectively [52, 53]. The recent preclinical studies using iPSC-derived EC in animal models of MI indicate that there is an increased capillary density in iPSC-derived EC-treated myocardium and although the transplanted EC may be contributing to neovascularization, the major action of these cells seems to be mediated through paracrine mechanisms [54].

Due to the potential of CPC to proliferate and differentiate into the main cardiovascular lineages (CM, EC, and SMC), CPC could be an ideal cellular source for cardiac regenerative therapy. Multipotent human CPC, with tri-lineage cardiovascular differentiation potential, can be derived from differentiating human pluripotent stem cells in vitro based on the expression of specific transcription factors (Mesp1, Isl1, Nkx2.5) or surface markers (SSEA1+, KDR+/PDGFR-α+, GFRA2+) [55]. CPC undergo a rapid transition from multipotency to commitment which difficults the study and isolation of these cells. Wnt signaling is essential for vertebrate

heart development, and several groups have used Wnt signaling activators (Wnt3a or GSK3 inhibitors) to promote pluripotent stem cell-derived CPC expansion [55].

Interestingly, in 2015 the first clinical case using human pluripotent stem cell-derived CPC was reported after obtaining encouraging functional and safety data in nonhuman primates [56]. These clinical-grade SSEA1+ CPC were induced with BMP2 and FGF inhibitor, sorted immunomagnetically, and embedded into a fibrin scaffold. In rats, SSEA1+ CPC-derived grafts were only detected shortly after transplantation and were not present after 4 months. Once more, the fact that SSEA1+ CPC-derived grafts were not observed 4 months after transplantation [57] together with the observation of functional recovery of mice with chronic heart failure using CPC-derived extracellular vesicles [58] suggests that any functional improvement observed after CPC transplantation is mainly mediated by a paracrine rather than a regenerative action.

Partial Reprogramming to Obtain Cardiac and Vascular Cells

The partial cell reprogramming approach can generate different cardiac cell types including CM, EC, and CPC cells in a fast and efficient manner.

In 2011, using this reprogramming method, CM were obtained with three transcription factors (*Oct4*, *Sox2*, and *Klf4*) and the supplementation of the media with BMP4 [20]. First contracting colonies were observed as early as 12 days after viral transduction. This reprogramming method generated mostly atrial-like CM. In 2014, this type of cardiac reprogramming was achieved using only *Oct4* transcription factor together with four small molecules (SB431542, CHIR99021, parnate, and forskolin).

In the course of the reprogramming experiments mentioned above, intermediate precursors such as Flk1+ and Nkx2.5+ cells [20] and Isl1+ cells [59] were observed. Moreover, SMC or EC could be generated when media was switched to either SMC or EC differentiation medium, respectively [60, 61]. Interestingly, Zhang et al. recently reported that these CPC can be expanded with a cocktail called BACS (BMP4, Activin A, CHIR99021, and SU5402). The authors were able to expand these CPC and produce billions of cells, without losing potentiality to differentiate into CM, EC, or SMC. Interestingly, 2 weeks after the transplantation of one million CPC, these cells spontaneously differentiated into CM, EC, and SMC and significantly reduced adverse remodeling after MI.

Direct Cell Reprogramming to Induce Cardiac and Vascular Cells

Alternatively, to the previous cell reprogramming approaches in which pluripotent intermediates are produced, CM, EC, or CPC can be induced (iCM, iEC, or iCPC, respectively) through direct cell fate conversion approaches.

In 2010 Dr. Deepak Srivastava laboratory demonstrated that mouse cardiac fibroblasts, and less efficiently tail-tip fibroblasts, could be directly converted into CM-like cells with *Gata4*, *Mef2c*, and *Tbx5* (GMT) transcription factors without passing through a progenitor cell state. For the screening of the cardiac reprogramming factors, cardiac fibroblasts isolated from α-myosin heavy chain (αMHC) promoter-driven enhanced green fluorescent protein (GFP) transgenic mice were

used. Although 15–20% GFP+ cells were registered 7 days after GMT transduction, only 5% expressed the CM-specific marker cTnT, and only 0.01–0.1% of the starting population contracted spontaneously after 4–5 weeks in culture, suggesting an incomplete cardiac reprogramming. Following this discovery, other groups also achieved direct reprogramming of mouse fibroblasts into iCM by expressing a cocktail of GMT and *Hand2* (GHMT), or *MyocD* and MT (MMT) transcription factors, or by using a combination of miRNAs 1, 133, 208, and 499. Although the miRNAs were not sufficient to reprogram tail-tip fibroblasts into iCM, the addition of *Hand2* to GMT increased the reprogramming efficiency, and the generated iCM from both cardiac and tail-tip fibroblasts manifested calcium transients, action potentials, and spontaneous contractions. Moreover, it was recently demonstrated that efficiency of conversion can be increased with inhibitors of pro-fibrotic signaling (TGF-B or Rho-associated signaling pathways) [62].

The direct reprogramming of human fibroblasts into iCM has also been achieved; however, the combination of factors used in mouse was not sufficient in human to trigger the induction of CM, additional factors or miRNAs were required, and the cardiac reprogramming was less efficient than in mouse fibroblasts. Interestingly, it was very recently demonstrated by S. Ding laboratory that the reprogramming of somatic fibroblasts into iCM without genetic manipulation is possible, with a combination of nine small molecules: CHIR99021, A83–01, BIX01294, AS8351, SC1, Y27632, OAC2, SU16F, and JNJ10198409 [63]. These iCM were capable of contracting synchronously and resemble human CM in their epigenome and transcriptional and electrophysiological properties. Consequently, this latter method may have important implications in cardiac regenerative therapies.

In 2012 human amniotic fluid-derived cells were transdifferentiated into EC with three factors (*ETV2*, *ERG*, and *FLI1*) and a TGFβ inhibitor; however, this approach did not work for postnatal cells. In 2014, using a transgenic mouse with a EC-specific reporter (Tie2-GFP), five factors (*Foxo1*, *Er71*, *Klf2*, *Tal1*, and *Lmo2*) were selected out of 11 candidates to efficiently reprogram skin fibroblasts into Tie2-GFP+ cells [64]. These iEC showed an epigenetic and transcriptomic profile similar to primary EC and were functional in vitro and in vivo in a murine model of hind limb ischemia. It was recently described that human neonatal fibroblasts can be transdifferentiated into functional EC in vitro using four factors (*ETV2*, *FLI1*, *GATA2*, and *KLF4*) [65]. Interestingly, it was recently reported that a single factor, *ETV2*, could induce direct reprogramming of human fibroblasts into endothelial progenitor cells with low efficiency, which can be increased sevenfold when the reprogramming procedure is performed under hypoxic conditions [66].

In 2012 the direct reprogramming of human fibroblasts into KDR+/Nkx2.5+ iCPC was achieved with *ETS2* and *MESP1* factors [67]. This approach was based on the idea that the generation of CPC in ascidian *Ciona* is dependent on the activation of these two factors. However, these iCPC were not extensively characterized, and their function in vivo was not analyzed since these cells spontaneously differentiated into immature CM. Recently it was demonstrated that the ectopic expression of at least five cardiac factors (*Mesp1*, *Tbx5*, *Gata4*, *Nkx2.5*, and *Baf60c*) in combination with LIF and the BIO (a GSK3β inhibitor) can reprogram adult mouse

fibroblasts from different tissues (cardiac, lung, and tail-tip) into proliferative and multipotent iCPC [68]. These iCPC were able to differentiate into SMC, CM, and EC in vitro. However, the iCPC-derived CM did not contract spontaneously and only started contracting when co-cultured with mESC-derived CM. The potential of these iCPC to differentiate into three cardiovascular lineages in vivo was shown when 1–1.5 million iCPC were injected in a mouse model of MI, and the survival of the animals receiving iCPC significatively improved. But one of the most remarkable findings in this study, as in the study described by Zhang et al. mentioned above, was to define culture conditions able to maintain and expand these iCPC, generating billions of iCPC without losing their differentiation potential [55].

In Vivo Reprogramming of the Resident Cardiac Fibroblasts

In 2012 retroviral strategies were used to successfully convert cardiac fibroblasts into iCM in vivo in mouse models of MI [23, 24], considering that hypoxia induces cardiac fibroblast proliferation and retrovirus only infects dividing cells. For fibroblast lineage tracing, the promoters of fibroblast-specific protein 1 (Fsp1) and the fibroblast-enriched gene periostin were used to drive Cre recombinase expression in LacZ reporter mice. Retrovirus encoding GMT [23] or GHMT [24] transcription factors was used. In these studies, 2–6% [24] or 35% [23] of the CM in the border zone of the infarct were newly generated iCM derived from resident cardiac fibroblasts. The functional integration and coupling with host CM was confirmed. Remarkably, the developmental maturity of iCM was confirmed since iCM showed similar contracting patterns and calcium transients to host CM. The functional benefit of cardiac transdifferentiation in vivo was demonstrated by trichrome staining and magnetic resonance imaging analyses, observing reduced fibrosis and improved heart function in animals receiving retrovirus encoding the cardiac transcription factors, even 3 months after injury. In another study a polycistronic retrovirus expressing GMT was used to improve the transduction and reprogramming efficiency; however, the reprogramming efficiency with GMT used separately was strikingly lower than previously reported. These differences may be attributed to variables such as different mouse strains or levels of expression of the transcription factors achieved after transduction. Moreover, the co-administration of GMT with thymosin β4 or VEGF can increase the functional recovery, by enhancing the delivery of the factors or the neoangiogenesis, respectively.

The in vivo conversion of cardiac fibroblasts into iCM also has been achieved by the direct administration of lentiviral miR-1, miR-133, miR-208, and miR-499 into mouse infarcted hearts, although the reprogramming efficiency was low (1%) and functional parameters were not assessed.

Most of these in vitro and in vivo direct cell conversion approaches toward iCM have been recently reviewed in Muraoka et al. [69] and Yamakawa et al. [70]. From these studies one may conclude that the iCM derived in vivo are more fully reprogrammed and more similar to endogenous CM than the ones derived in vitro, suggesting that the cardiac microenvironment may have factors absent in the tissue culture dish.

Rare Disease Modeling

Definition and Epidemiology

Rare diseases are defined as pathologies that affect a small percentage of the population. However, there is not a single and widely accepted definition for rare diseases. Some definitions rely solely on the number of people living with a disease, like in the United States, where rare diseases are defined as any disease or condition that affects less than 1 in 1500 people [71]. In the European Union (EU), the European Commission includes other factors in the definition, such as the severity of the disease and the inexistence of adequate treatments. Thus, in EU, rare diseases are life-threatening or chronically debilitating diseases with a prevalence fewer than 1 in 2000 people that require special combined efforts to be addressed by them [72].

At least 80% of rare diseases are genetic and therefore chronic. Those genetic disorders are caused by a change in the DNA sequence away from the normal sequence and can be classified as monogenic disorders (caused by a mutation in a single gene), multifactorial inheritance disorders (caused by a combination of small variations in genes), and chromosome disorders (caused by an excess, deficiency, or structural changes within chromosomes). However, not all rare diseases are genetics; there are also very rare forms of infectious diseases, autoimmune diseases, and rare cancers. To date, the causes for many of those rare diseases are still unknown.

According to last estimations, more than 300 million people worldwide are affected by any of the 7000 different rare diseases that have been identified, and 50% of them are children, making rare diseases the major cause of death and disability for children worldwide. Despite the strong commitment of funding agencies through different research programs to invest in rare disease's research, aiming to lead new diagnostics, treatments, and/or cures, currently 95% of all rare diseases do not have treatment. According to the European Medicines Agency (EMA), fewer than 1000 diseases benefit from even minimal amounts of scientific knowledge, with less than 400 approved therapies worldwide. Thus, there is a considerable interest in translating the advances in the understanding of molecular mechanisms of the diseases into targeted therapies. In this section we will focus on the recent advances in disease modeling of rare diseases by cell reprogramming.

Cell Reprogramming for Rare Disease Modeling

In vitro and in vivo disease models are indispensable tools for understanding the molecular mechanisms that drive pathogenesis and enable the development of novel therapies. In general, the study of the mechanism underlying human genetic diseases has been derived from their study in mouse models, considered more informative than cell-based in vitro approaches. Mouse models have several advantages including that can be manipulated genetically, that mice are phylogenetically close to humans, and their low cost compared to large animal models. However, these advantages can be offset for disease modeling due to species-specific differences

between human and mice. Furthermore, in some cases mice generated by disrupting the gene underlying the disorder do not reliably reproduce the human phenotypes.

Cell reprogramming and in particular iPSC generation opened a new avenue for disease modeling. The discovery that human fibroblasts could be reprogrammed directly to iPSC by forced expression of only four transcription factors [4] provided a new approach to disease modeling. iPSC can be derived from multiple somatic cell types obtained directly from individuals with the desired disease or from cell repositories. The ability of iPSC to model human diseases in vitro has revolutionized the ways in which monogenic, complex, or epigenetic disorders are studied, offering an advantageous system for the development of novel therapeutics and their use in drug screening [73, 74].

Considerations for iPSC Disease Modeling Development

As with any other modeling system, several issues should be considered before derivation of iPSC-based models. In addition to the appropriate reprogramming cocktail, the delivery method, culture conditions, or the cell type (reviewed in [75, 76]), other considerations include:

- Absence of relevant mouse models. iPSC models will have a great potential in diseases for which no good mouse systems are available. Furthermore, iPSC models can be a valuable complement for further validation of the observations made in mice.
- Differentiation protocols. iPSC should be differentiated into the appropriate cell type to study the phenotype of the disease, and this aspect can be only affordable if a robust differentiation protocol for the desired cell type is available.
- A basic requirement is the ability of differentiated cells to reproduce the specific phenotype of the disease as seen in patients. In some cases, the phenotype of the disease is observed in a different tissue/organ than the affected one. As an example in primary hyperoxaluria type I (PH1), a metabolic disease of the glyoxylate pathway produced by deficiencies in the liver-specific enzyme alanine-glyoxylate aminotransferase (AGT) induces progressive kidney deterioration due to calcium oxalate deposition. Thus, iPSC from PH1 patients [77] could be a good option to develop new drugs impairing oxalate production, but they will be not suitable to study kidney deterioration.
- Certain disorders might affect the efficiency of reprogramming making the derivation of iPSC more difficult or even not possible. In some diseases, like Fanconi's anemia [78] or Sanfilippo syndrome type B (mucopolysaccharidosis IIIB, a specific lysosomal storage diseases) [79], it is necessary to correct the genetic defect for successful reprogramming and iPSC generation.

Reprogramming to Patient-Specific iPSC

Patient-specific iPSC are ideal when studying genetic diseases and in particular monogenic disorders. The first report describing patient-derived human iPSC was

performed by Park and colleagues in 2008 [80]. In that study iPSC from patients with a variety of genetic diseases were generated. In the last few years, the number of iPSC generated from patients with many different rare diseases is continuously increasing (review in [27, 28]).

One of the first iPSC-based disease models was generated for spinal muscular atrophy (SMA), characterized by a degeneration of the motor neurons as a result of loss-of-function mutations in the *SMN1* gene [81]. The first report of an in vitro iPSC model of a cardiac disease was developed from the LEOPARD syndrome [82]. LEOPARD syndrome is a rare autosomal-dominant inherited RAS pathway disorder, mainly caused by missense mutations in the *PTPN11* gene. After in vitro cardiac differentiation, LEOPARD syndrome patient-specific iPSC provided a new system for the study of disease pathogenesis, showing increased cell size, sarcomeric organization, and nuclear NFATC4 localization, correlating with the hypertrophic state observed in LEOPARD syndrome patients. Other examples of cardiac disease modeling with iPSC are the long QT syndrome types 1 and 2, caused by mutations in *KCNQ1* and *KCNH2*, respectively, both of which encode essential voltage-gated potassium channels [83, 84]. iPSC-derived CM recapitulated the disease phenotype in vitro, allowing the evaluation of existing and new therapeutic agents. Hepatic disorders like Wilson's disease [85] or alpha-1-antitrypsin deficiency (A1AT) [86] and hematopoietic acquired blood disorders [87] have been also modeled using iPSC.

Although more than 80% of the diseases modeled using iPSC are monogenic disorders, complex diseases are being successfully modeled using human iPSC. For example, iPSC models of amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, a disease that causes the death of motor neurons due to mutations in SOD1, demonstrated that motor neurons derived from three ALS patients recapitulate pathology observed in postmortem tissue from one of the same patients from which the iPSC were derived [88]. Other complex genetic diseases like cancer [89] or chromosomal disorders [90, 91] are also under study using iPSC-based models.

Whereas most inherited diseases are linked to genetic defects, some rare disorders can be defined as epigenetic, and iPSC models could also represent an alternative and attractive tool for the study of these diseases. The immunodeficiency, centromeric instability, and facial anomaly (ICF) syndrome type I, caused by lossof-function mutations in *DNMT3B*, is a good example where differentiated cells form patient-specific iPSC exhibit DNA hypomethylation in pericentromeric and subtelomeric regions, as well as altered gene expression profiles [92]. Functional studies in patient-specific iPSC models of Prader-Willi syndrome (PWS), a neurobehavioral imprinting disorders, revealed that the long noncoding RNA IPW, which is absent in PWS, is involved in regulating the *DLK1-DIO3* imprinted locus [93].

In summary cell reprogramming and especially patient-specific iPSC are well suited and offer a valuable tool for generating disease models for rare genetic and epigenetic disorders.

Rare Disease Modeling by Gene Editing

Each patient may harbor a substantial number of genetic variants, and finding reproducible differences in pathology using iPSC from different patients might be not conclusive due to the effects of modifying background mutations. Furthermore, getting access to patient's samples from particularly rare genetic disorder might be difficult or even unavailable. To solve these questions, genome editing can be applied to iPSC-based disease models enabling the creation of isogenic iPSC lines that differ only at specific loci. Thus, the effects of a chosen gene or mutation can be dissected from the modifying effects of the genetic background.

Sequence-specific nucleases (SSNs) enable the precise and specific modification of the genome introducing site-specific double-strand breaks in the DNA that are repaired either through error-prone nonhomologous end joining or precise homology-directed repair (reviewed in [94]). Experiments using zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) provided the first evidences of genome editing in pluripotent stem cells allowing the generation of gene knockouts, the insertion of transgenes to generate cell-type-specific lineage reporters, or the insertion or repair of disease-relevant point mutations (reviewed in [95, 96]). However, this extensive work with ZFNs and TALENs, demonstrating the power of genome editing, highlighted the necessity of a universal, cheaper, and simpler platform for genome editing. This aspect was fulfilled applying the bacterial clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) as SSN [97]. CRISPR/Cas9 is a simple-to-use system with a key advantage over previous systems: a small RNA containing 20-bp homology to the target region is enough to guide the Cas9 endonuclease activity to a specific genome locus. Thus, laborious engineering of DNA-binding proteins is not required, and the CRISPR/Cas9 system has largely replaced previous SSN technologies.

The most common application of gene editing in disease modeling has been to correct a disease-causing mutation in iPSC generated from a patient with a monogenic disorder, allowing the proof of principle that observed phenotypes are caused by a specific genetic mutation. For example, in A1AT deficiency, the hepatocytes derived from A1AT-corrected iPSC produced A1AT protein in vitro and in vivo [86], suggesting that this approach could be used for cell therapy by transplanting these corrected iPSC-derived hepatocytes into patients. Another study in SMN1 mutant iPSC from a patient with SMA suggests that correction of iPSC could be used to optimize possible therapies. Using genome editing strategies, the paralogous SMN2 gene was converted into an SMN1-like gene, correcting the phenotype of differentiated neurons from these iPSC. Transplantation of genetically corrected neurons into a mouse model of SMA led to extended life span [98].

As commented above, the accessibility to samples from patients with extremely rare disorders could be not available, hindering the generation of desired iPSC. A solution would be to use genome editing to introduce disease-causing mutations into iPSC from healthy donors. Ding and colleagues demonstrated for 15 different

disorders that introducing a single disease-linked mutation in a gene was sufficient to induce disease-associated phenotype [99].

Moreover, genome editing provides the first opportunity to evaluate the potential contribution of individual mutations to polygenic disorders. iPSC can be derived from patients with complex disorders, even when the disease-causing mutations are unknown. Then, a specific locus can be modified using SSNs to determine the relative contribution of a particular genetic variant to a particular phenotype. Furthermore, CRISPR/Cas9-mediated genome editing has been described as an efficient method to induce specific chromosomal rearrangements in vitro in iPSC [100] as well as in vivo in mice [101].

Therapeutic Applications of iPSC Models of Genetic Diseases

One of the main applications of iPSC models of genetic disorders is the development of new therapies that enable their treatment, absent in most cases. iPSC-based models offer a unique platform for high-throughput screening (HTS) of small molecule libraries that in animal models, when available, is usually not feasible. The first large-scale screening study in iPSC-based models used iPSC-derived hepatocytes from a patient with A1AT deficiency and allowed the identification of approved drugs that could be tested in clinical trials as novel therapeutics [102]. A larger screening was performed in models using ALS-specific iPSC, testing their ability to protect motor neurons from degeneration, with several novel hit compounds identified [103]. The main limitation of HTS in iPSC models is the requirement of an automatically measurable and quantifiable phenotype (readout). This is useful when dealing with cell survival rates or with protein expression that can be easily measured with reporter genes, but it is less beneficial when the disease modeled has a complex phenotype. Other applications include the use of patient-specific iPSC models of a particular disease to test in vitro the efficacy of a specific compound. Observed results could be correlated with the genotype to identify mutations that could be used to stratify patients, so those patients who are likely to better respond to the drug would be selected for treatment in clinical trials.

Finally, the ultimate goal of developing patient-specific iPSC in regenerative medicine would be the generation of immune-compatible cells and tissues for autologous transplantation. Although at present the clinical translation of iPSC-based cell therapies seems to be more futuristic than the in vitro use of iPSC for research and drug development, several studies have provided the proof of principle that iPSC-based regenerative medicine can become a reality [104, 105]. In a recent study CRISPR/Cas9-mediated genome editing technology was combined with patient-specific iPSC to develop a promising exon skipping approach to treat Duchenne muscular dystrophy (DMD). In this work Young and colleagues [106] described a therapeutically relevant CRISPR/Cas9 approach to modify *DMD* gene in patient-derived iPSC, inducing the excision of exons 45–55 to reframe dystrophin into a modified protein that is stable and functional. Modified dystrophin expression in iPSC-derived CM and skeletal myotubes restores the membrane functionality

and the dystrophin glycoprotein complex in vitro. Additionally, after injection of skeletal muscle cells derived from reframed iPSC into the tibialis anterior mdx mice, a correctly localized dystrophin and β-dystroglycan were observed.

Challenges and Concluding Remarks

Cell reprogramming has open up a great opportunity to generate functionally relevant cell types to treat or model certain diseases. However, before moving toward a realistic clinical application of these cells, many critical issues need to be addressed. The major concern of the use of human iPSC-derived cells is the risk of tumorigenicity; thus, the identity and purity of the cells to be transplanted will be mandatory. Another concern is the risk of arrhythmias caused by the different electrophysiological properties between the transplanted and host cardiomyocytes; thus the maturity and subtype of CM should be considered. Undoubtedly, it is imperative to develop in any case non-integrative gene transfer approaches to obtain the reprogrammed cells. Studies in large animal will be necessary to evaluate the potential efficacy and long-term effect of the reprogrammed cell product before clinical trials. Transplanted reprogrammed cells have the potential to regenerate the damaged heart tissue either through their integration or induction of endogenous cells. Knowing by which mechanism these cells contribute to the generation of new CM and improve cardiac function would be essential to evaluate if a cell product is needed or otherwise cellular derivatives (as microvesicles) or growth factors/cytokines are sufficient to achieve the same functional effect. A cost- and time-effective large-scale production of reprogrammed cells will be required for both clinical and disease modeling applications, but more importantly, the major limitation observed when using those cells in vivo, that is, the low retention of the transplanted cells, should be circumvented. In this sense, some strategies to improve long-term cell engraftment in the ischemic heart are under investigation, including genetic modification of the transplanted cells, cardiac tissue engineering, or regression of fibrotic scar.

On the other hand, despite the promising future of iPSC-based models, there are still substantial issues to be addressed, like the accessibility to samples from patients suffering rare disease conditions. It is expected that in the future tissue and/or iPSC repositories would be available enabling the modeling of practically any genetic disease, whether monogenic, chromosomal, or complex. Moreover the ability to model specific rare disorders depends on efficient and robust differentiation protocols that are still absent for some cell types. To overcome this issue, cell culture optimization as well as improved functional characterization of differentiated cells will be required for therapeutic applications. This aspect will have special relevance in some rare disorders lacking appropriate animal models where the therapy will be implemented directly from the dish to the patient. Although further research and work is still required, recent advances have confirmed the value of iPSC in rare disease modeling.

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Engineering Organoid Systems to Model Health and Disease

10

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Introduction

The ability to propagate cells in vitro revolutionized biology and medicine in the mid-twentieth century enabling the study of individual cell types and cell clones independent of their complex in vivo environment. Cell culture also proved immensely useful in the reproducibility of results, as cell lines could be generated and shared among laboratories. Perhaps most famously, the HeLa cell lines ushered in a new paradigm for cancer research and drug discovery [1]. While critical to the advancement of science to date, in vitro systems have not been without significant shortcomings. Discoveries in cell culture have been notoriously difficult to successfully translate to in vivo animal

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Table 10.1 Differences among 2D and 3D culture systems

Variable	2D	3D
Transport	Gradients absent	Diffusional transport limitations
Focal adhesions	Basal surface only	Distributed in 3D
Cell junctions	Integrin-ECM	Integrin-ECM
		Adhesion junctions
		Gap junctions
		Tight junctions
		Desmosomes
Mechanical properties	Single stiffness, typically high kPa	Potential for variable stiffness
Organization	Uncontrolled organization	Spatially distinct zones
Platforms	Culture flasks	Scaffolds
	Transwell coculture inserts	Scaffold-free speroids
	Matrix coated 2D	Fluidics systems
	Fluidic systems	Spinning flask bioreactors
		Organotypic explant culture
		Micropatterned surface microplates
		Microcarrier culture
		Gels
Polarity	Forced apical-basal polarity	No prescribed polarity
Cell interface	50% cell-surface (plastic/matrix)	80% cell-cell and cell-matrix
	50% cell-liquid	20% cell-liquid

models [2, 3]. What is beneficial to a specific cell type in culture may prove toxic to other cell types in the body. Furthermore, the pharmacokinetics of drugs cannot be appreciated with simple in vitro systems as they are dependent on the metabolism of drugs by the liver and the kinetics of drug excretion [3]. In addition, the artificial nature of cell culture systems in which cells are rapidly dividing and bathed in growth factors and nutrients can create biological scenarios that would rarely, if ever, be observed if the cell were in its native environment. Without discounting the immense utility of cell culture systems, it is important to understand the similarities and differences between the in vitro and in vivo environment.

Multicellular organisms are by nature systems in which each cell interacts and impacts the fate and function of the surrounding cells. Such systems quickly grow in complexity as they form distinct tissues, organs, and regulatory networks. Embedded into the fiber of every model system is an assumption or allowance of approximation to reality. As novel technologies are developed, this allowance of approximation must necessarily decrease as our models more closely approach the true value of the subject being modeled. The environment of a cell in traditional in vitro culture systems is distinct from its natural in vivo environment in multiple aspects as outlined in Table 10.1. In vivo environments exist in three dimensions, are exposed to endocrine signaling from distant tissues, thrive in low oxygen tensions, and receive paracrine signaling from both similar and distinct cell types in their local vicinity. In contrast, purified cultures in vitro lack the physical architecture and

signaling from their neighbors and are instead sustained by growth factors and metabolites supplemented in culture media. Such signaling sustains the viability of the cells in culture allowing the culture to be propagated and studied in a highly controlled fashion. However, the in vitro phenotype of cells can easily be altered causing in vitro cultures to fall short of recapitulating in vivo biology.

While in vitro systems lose the systems-level regulation seen in vivo, they also provide critical advantages over in vivo systems that make them of immense utility for both research and the development of new therapies. Cell culture systems are cheaper and more accessible than animal or human studies. For example, cell culture systems enable high-throughput screening of hundreds or thousands of drugs to identify new candidates to treat cancer. The analogous experiment in an in vivo system, whether small animal or human, would be both cost-prohibitive and ethically questionable. In addition, many culture systems are amenable to culture expansion, in which billions of cells can be generated from a common source. Thus, the cells in each well of an experiment or even in experiments performed on opposite sides of the world can be of a common source. Such expansion potential makes culture systems highly reproducible and distributable. Finally, cell culture systems are highly controllable, as their environment is completely controlled by the researcher. Virtually every aspect of the cell's environment including its extracellular matrix, pH, and exposure to oxygen, glucose, nutrients, growth factors, and cytokines can be tightly monitored and controlled. This control enables robust identification of specific pathways and mechanisms that control cell phenotype and has led to the discovery of protocols that drive the differentiation of progenitor cells and pluripotent stem cells. Such strategies are now being leveraged to generate large pools of cells that can be used for drug screening as well as the development of cell-based therapies. Two-dimensional in vitro cell culture has been a vital tool cultivated in research labs to probe and model questions of cellular biology without the complexity of the multivariate in vivo environment. However, 2D cell culture scenarios are rife with assumptions that make translation of scientific discoveries to medical therapies that much more complicated, motivating the development of the next generation of in vitro models.

Organoid systems under development today seek to combine the advantages of in vitro systems (e.g. reproducibility, scalability, and cost) with the systems-level communication cells are accustom to experiencing in vivo. Herein we will highlight progress to date in organoid development in key fields including mesenchymal stem cells (MSC), mini-brains, insulin-producing cells, and intestinal stem cells and look at the future prospect of organoid systems in research and therapy.

Spheroid Systems to Enhance MSC Potency for Cellular Therapies

While organoid systems typically include multiple cell types, the strategies used to create 3D niches and the advantages they provide have also been leveraged to enhance cells for cell therapies, specifically mesenchymal stem cells. Mesenchymal stem cells, also commonly referred to as multipotent mesenchymal stromal cells or simply MSCs, have emerged as the feature stem cell in many therapeutic applications worldwide, due to their ease of isolation and remarkably diverse functionality

[4–7]. Among their numerous tissue rescue/repair mechanisms, MSCs can differentiate into and replace multiple mature cell types, secrete bioactive factors with powerful trophic and/or immunomodulatory activities, and even transfer organelles to neighboring cells [4]. To date, hundreds of clinical trials using MSCs for treatment of numerous intractable conditions, ranging from genetic connective tissue disorders to autoimmune disease and ischemic tissue injury, have been reported [8, 9]. Ex vivo expansion has been particularly important for the development of therapies using human bone marrow MSCs, which comprise a rare yet powerful subset of all mononuclear cells [5].

At the turn of the twenty-first century, it was progressively realized that ex vivo expansion of MSCs alters their plasticity [10, 11], phenotype [12–14], and potentially other important cellular characteristics/functions [14–18]. Indeed, some clinical trials capitalizing on the immune-modulating abilities of MSCs have at times produced disappointing results, particularly when using cells expanded extensively in culture [8, 17, 18]. Together, these findings raised concerns about the negative impact of conventional 2D plastic-adherent growth conditions on cell quality. Perhaps just as important, concerns mounted that experimental data obtained from MSCs prepared on the rigid substrata of tissue-culture plastic could be misleading and even flawed, especially with regard to their true biology [19]. In general, it was recognized that native MSC behaviors are poorly represented in 2D cultures, which overlook the importance of signaling pathways activated by cell-cell and cell-matrix interactions in the 3D microenvironment of tissues [19, 20].

Over the last decade, an encouraging amount of research using 3D MSC cultures has been conducted. One very simple yet increasingly important 3D system is the multicellular aggregate or cluster, often referred to as a spheroid due to its spherical shape [21]. Multicellular spheroids have long been utilized to study embryonic development and tumor biology and reportedly provide biologically relevant conditions in vitro to mimic endogenous cell-specific behaviors [19–22]. Essentially, when adherence to a planar surface is restricted, MSCs in suspension will self-assemble, initially into loose cellular aggregates that with time, typically 24–48 h, coalesce and condense into a single MSC spheroid (Fig. 10.1a) [22, 23]. Cells in spheroids are not distributed randomly but appear to organize into distinct concentric compartments (Fig. 10.1b–d) [21]. The MSC spheroid interior is largely composed of small round cells embedded in self-synthesized extracellular matrix (ECM), while the sphere surface is comprised of elongated, spindle-shaped cells [23].

Early studies used MSC spheroids to enhance chondrogenic differentiation of the cells [24, 25] or to exploit the high cell-to-medium ratio and hypoxic conditions for concentrating proangiogenic factors in conditioned medium [26]. Other early studies viewed MSC spheroid cultures as an opportunity to replenish important MSC characteristics that were diminished or even lost following expansion [23, 27]. At the same time, it became apparent that MSCs primarily heal injured tissues without long-term engraftment (i.e., through a “hit and run” scenario) [9, 28, 29] and through secretion of immunomodulatory and trophic factors, in response to signals from injured tissues [4–7]. In parallel, studies progressively

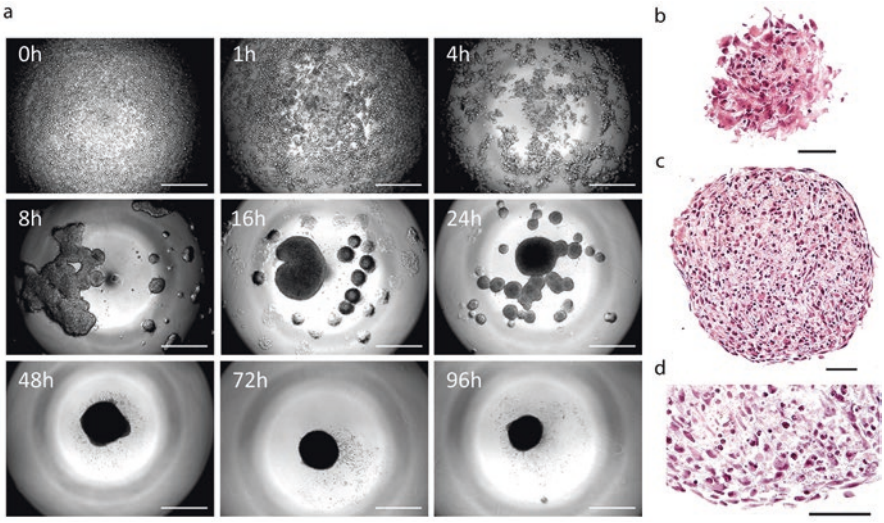

Fig. 10.1 Maturation of MSC spheroids over 96 h in hanging drop cultures. (**a**) 25,000 MSC seeded in hanging drops coalesce into a single unified spheroid by 48 h in culture. After 72 h in hanging drops, spheroids were sectioned and stained with H&E. MSC on the (**b**) surface of the spheroid have a distinct morphology from MSC in the (**c**) center of the spheroid. Side-by-side comparison of the distinct zones of within the spheroid can be appreciated in (**d**) (Adapted from Bartosh et al. [23])

showed that MSCs aggregate in vivo shortly after they are injected [16, 23, 30] and are subsequently activated to secrete anti-inflammatory and other factors [31, 32]. Taken together, it was suggested that spheroids could be utilized to pre-activate or prime MSCs and enhance the therapeutic window by reducing lag time for their activation in vivo [23].

Regardless of rationale for use, it was rapidly appreciated that spheroid formation promotes radical transformation of the gene expression profiles [22, 23], many of which are known to enhance a range of therapeutic cellular functions, and many of which appeared to recapitulate biological activities of MSCs that form aggregates in vivo after injection of the cells into mice [31–33]. Indeed, studies showed that sphere formation enhances the anti-inflammatory [23, 31, 34, 35], angiogenic [26, 36, 37], and tumor-suppressive [23, 27] effects of the cells and also improves cell survival after transplantation [37, 38]. Moreover, it delays replicative senescence and promotes expression of pluripotency marker genes, including Oct4, Nanog, and Sox2, indicating enhanced overall stemness and regenerative capacity [22, 36].

Soon after these initial reports were published, it was realized that MSC phenotype in 3D cultures is highly dependent on the source of MSCs as well as the type of culture medium used, suggesting that extrinsic factors are involved [39, 40]. While this sensitivity to medium type could limit some applications of the cells, it also explains their plasticity and powerful tissue-healing response to various injury

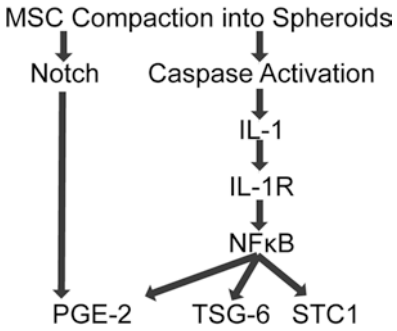

Fig. 10.2 MSC compaction in spheroids triggers intracellular (caspase) and paracrine signaling (Notch and IL-1) that initiates an anti-inflammatory gene program in MSC and production of immunomodulatory factors including prostaglandin E2 (PGE-2), tumor necrosis factor alpha-induced protein 6 (TSG-6), and stanniocalcin 1 (STC1) [Adapted from 31]

stimuli. Clearly, numerous signaling pathways that are silent in 2D MSCs are set in motion when MSCs interact in 3D spheroids. Intrinsic cell-matrix and cell-cell interactions, mediated by integrins and cadherins, respectively, guide sphere formation and likely influence cellular properties beyond those observed in 2D cultures [40–42]. Moreover, the self-aggregation process appears to initiate a caspase-dependent interleukin-1 (IL-1) signaling loop that drives expression of key anti-inflammatory and other potentially therapeutic factors (Fig. 10.2) [31]. In addition, oxygen diffusion limitations [21], mechanical/elasticity forces [43], and even epigenetics [44] are thought to influence some of the cellular changes accrued when MSCs assemble into spheroids.

Overall, cultures of MSC spheroids, although a simplified portrayal of complex 3D tissues, provide some distinct benefits over standard 2D growth conditions, which are highly artificial and less physiological. Moreover, the simplicity of multicellular spheroid cultures makes them amenable to high-throughput screening [45]. In parallel, they could provide building blocks for more complex tissue engineering applications that utilize biomaterials to recreate intricate tissue architecture [45, 46] and to construct 3D MSC microniches for therapeutic applications [47]. Importantly, research on MSCs in 3D cultures has been timely and coincided with the shift in focus from early feasibility studies to optimization of therapeutic efficacy. Moreover, it has coincided with the concept that it is of high importance to move past the convenience of 2D cultures, which have often failed in the clinic, and focus on improving cell functionality, even if it requires more involved culture systems. Although much remains unknown about the effects of 3D culture on MSCs, it is apparent that research on MSC spheroids has merit and could be extremely valuable for advancing our knowledge of innate MSC biology, as well as for accelerating the translation of experimental findings into therapies.

Multicellular Organoids

While spheroids composed of a single cell type can dramatically alter the phenotype of the cells, 3D culture systems can also replicate in vivo anatomical relationships of different types of cells. These multi-cell-type assemblies are called organoids, and techniques to develop them have been developed to aid in the study of many fields including neuroscience and stem cell niches. Multicellular organoids are inherently more complicated, as they require different cell types to be supported by a common culture media and to be arranged in such a way that the spatial relationship of different types of cells are maintained. In this section, we highlight advances over the last decade in the development of in vitro organoid systems.

Development of a Mini-brain in a Dish

Neurons and glial cells that give rise to the brain heavily rely on an orderly composition of mechanical, biochemical, and spatial cues to coordinate differentiation, migration, survival, gene expression, and synaptic transmission. Given the complexity of the brain, studying neuronal development and disease has proven to be challenging in living organisms. These important structural and microenvironment cues become dismantled when neurons are grown in 2D planar cultures, making it difficult to accurately characterize neuronal behavior. Driven from the need to develop better in vitro models capable of recapitulating brain tissue, neuronal spheroids have emerged as a novel research platform to interrogate neuronal development, drug transport, and pathogenesis of neuronal disease.

Initial development of 3D neuronal constructs relied on scaffold-based structures composed of collagen, Matrigel, alginate, and silk derivations [48–52]. More sophisticated strategies which utilize composite biomaterials constructed of alternating mechanical properties allowed for use of a stiff scaffold to provide neuronal anchoring, while softer gel matrixes promoted axonal connectivity. This strategy demonstrated that modular 3D architectures are capable of being created and can be used to mimic relevant biomechanical stimuli needed to support neuronal development. Despite this, biomaterials still present degradation and structural challenges that can limit the size, complexity, and viable time window an individual organoid can be studied. Alternative approaches to neuronal organoid development include biomaterial-free architectures that promote spontaneous neurogenesis, akin to central nervous system (CNS) processes that naturally occur during development. At this time, there are a number of emerging methods to generate ordered 3D tissue using neural progenitor cell aggregates [10, 53–57]. The serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) method has been widely used to generate discrete CNS regions such as cerebral cortex [57–60], pituitary [61], and retina [57, 62]. Similar methods relying on poly(dimethylsiloxane) (PDMS) microwells to facilitate aggregation of neural progenitor cells have demonstrated functional connectivity and transmission of electrical stimulus propagated through a 2D array of bundled spheroids [63]. Future implementations of a

microchanneled network could conceivably tether discrete CNS organoids in an interconnected and modular 2D array. Linking neural organoids in this manner could allow for sophisticated models for drug screening and neurophysiology.

Importantly, Lancaster et al. have also reported a method to generate self-organized organoids composed of discrete, yet interdependent, multiregional subunits (Fig. 10.3) [54, 64]. Both the SFEBq and former organoid methods show high similarity in the development of pallium tissues, yet they maintain distinct differences in media formulations and developmental timings. Notably the method developed by Lancaster et al. incorporates a 3D Matrigel to facilitate growth of embryoid bodies into continuous neuroepithelial tissue, whereas adaptations to the SFEBq approaches have attempted to utilize addition of dissolved extracellular matrix proteins to expand growth of this tissue [58, 65–67]. Using a spinning bioreactor, suspended 3D Matrigel constructs were able to spontaneously generate cerebral organoids with discrete hippocampus, forebrain, choroid plexus, dorsal cortex, prefrontal lobe, retina, and cortical interneurons after 2 months (Fig. 10.4). Furthermore, the cerebral organoids could be maintained for over a year and grew up to 4 mm in diameter [64]. Next-generation cerebral organoids might employ engineered vascular networks capable of delivering nutrients to the inner cell mass to improve survival and maturation of these structures.

While numerous groups have reported on the structural similarities of these neuronal organoid systems, Camp et al. identified close genomic similarity of cortical processes like progenitor cell proliferation, production of extracellular matrix, migration, adherence, delamination, and differentiation between structured fetal neocortex and their organoid culture system counterparts [68]. Given their functional and near-physiologic structural similarities to brain tissue, cerebral neuronal organoids are emerging as an essential tool for basic neuroscience investigations in neuronal development [57, 58, 62, 68].

Intestinal Organoids

The intestine is covered by a single layer of epithelial cells which form crypt-villus structures. This epithelial lining has a remarkable ability to repair and renew itself, undergoing rapid cell turnover in fewer than 5 days. The existence of multipotent cells in intestinal epithelium has long been accepted given the remarkable renewing and regenerative potential of intestinal epithelium [69], and two stem cell models—the +4 label retaining cells (LRCs) and crypt base columnar (CBC) cells—were proposed since then. Although there are some controversies regarding the existence of two populations of stem cells in the intestinal epithelium, the CBC cells are regarded as intestinal stem cells that actively renew and maintain the homeostasis of intestinal epithelium. Recently, using elegant lineage tracing experiments, Barker et al. showed that a Wnt target gene, leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), marks stem cells in the crypt base corresponding to the CBC cells [70]. Using a Lgr5-EGFP-ires-CreER mice, they showed that Lgr5 is predominantly expressed in CBC cells. By lineage tracing, they also showed that

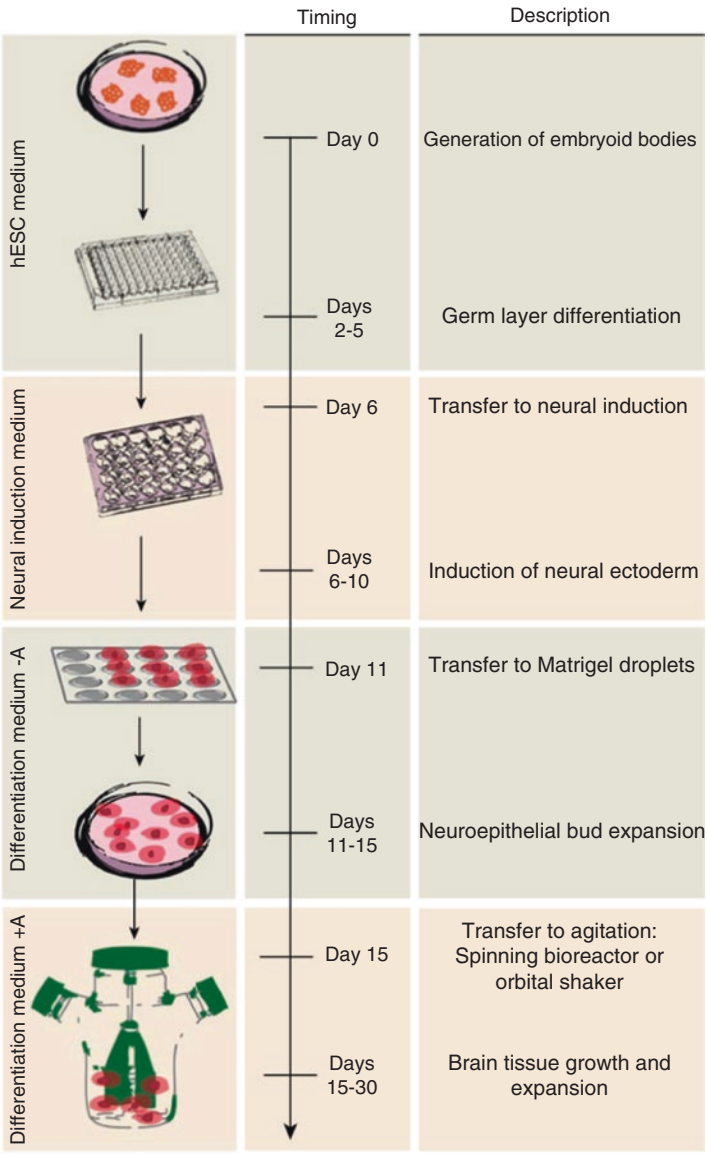

Fig. 10.3 Schematic diagram of the cerebral organoid production method and timing. Figure adapted from Lancaster et al. [64] (With permission from the Nature Publishing Group)

Lgr5+ cells have the ability of long-term self-renewal and multiple lineage differentiation to generate all the cells in the crypt-villus axis, both characteristics of stem cells.

In vivo, Lgr5+ ISCs are actively renewing at the bottom of crypts. They can also generate rapidly dividing, transit-amplifying (TA) daughter cells which further

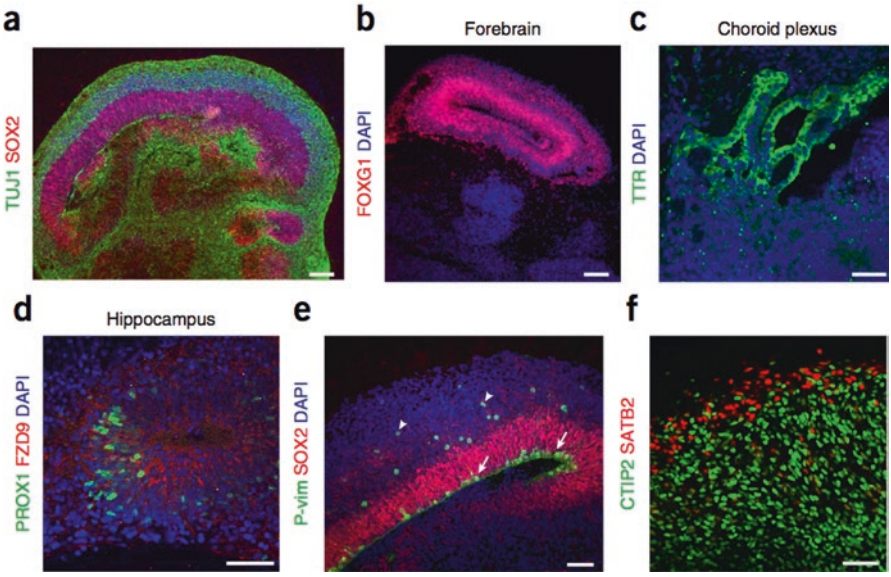

Fig. 10.4 Staining for brain regions and neuronal cell identities in *in vitro*-grown mini-brains. Organoids grown in the lab can be stained to reveal numerous distinct regions of brain ranging from (**a**) cortical tissue composed of neurons (TUJ1, *green*) and progenitors (SOX2, *red*), (**b**) forebrain identified by positive staining for FOXG1 (*red*), (**c**) choroid plexus identified by TTR staining and convoluted cuboidal epithelium, and (**d**) hippocampal regions identified by PROX1 (*green*) and FZD9 (*red*) staining. (**e**) Staining for mitotic radial glia (P-vimentin (P-vim), *green*) in a cortical region reveals inner radial glia undergoing mitosis at the apical membrane (*arrows*), whereas outer radial glia undergo mitosis outside the ventricular zone (*arrowheads*). All radial glia are marked by SOX2 (*red*). (**f**) Staining for cortical layer identities of advanced organoids (75 days). Later-born superficial-layer identity (SATB2, *red*) neurons populate more superficial regions of the organoid, whereas early-born deep-layer identity (CTIP2, *green*) neurons populate deeper regions of the organoid. DAPI in **a–e** labels nuclei (*blue*). Samples in **a–e** are 30–35 days after initiation of the protocol. Scale bars, 100 μm (**a**, **b**) and 50 μm (**c–f**). Figure and description adapted from Lancaster et al. [64] (With permission from the Nature Publishing Group)

differentiate to functional mature cells including absorptive enterocyte and secretory cells such as goblet cells, Paneth cells, enteroendocrine cells and tuft cells. These cells form a single layer of epithelium anchored on the basement membrane, a thin layer of extracellular matrix separating the epithelium from the underline mesenchyme [71].

The self-renewal and differentiation of Lgr5+ ISCs are tightly controlled by biochemical and biophysical signals surrounding the cells, collectively called the stem cell niche. The Lgr5+ ISC niche consists of signals generated from both the epithelium and underlying mesenchyme, as well as anchoring signals from extracellular matrix in the basement membrane. Four major signaling pathways—Wnt, Notch, BMP, and EGF pathways [72]—are present in the intestinal crypt-villus axis in a well-controlled gradient and synergistically control the self-renewal and fate determination of intestinal stem cells. While Wnt is the key pathway to maintain stem

cell self-renewal and the proliferation of stem and TA cells [73], simultaneous activation of Notch (which is provided by Paneth cells) is necessary to maintain stem cells in their undifferentiated state. Wnt is also essential for the differentiation of Paneth cells, and its inactivation is necessary for generating enterocytes and goblet cells. Meanwhile, Notch lateral inhibition controls the enterocyte-secretory fate determination. In this way, they cooperatively control the self-renewal and differentiation of the stem cells [74]. EGF signaling exerts strong mitogenic effects to promote the proliferation of stem cells and TA cells [75]. BMP antagonists at the bottom of crypts create a crypt-permissive environment for the self-renewal of intestinal stem cells [76] and likely, the differentiation of Paneth cells, while BMP in the villus is essential for terminal differentiation of epithelial cells, especially secretory cells [77, 78].

These niche signals are supplied to the stem cells at multiple levels (Fig. 10.5). Specifically, the basement membrane provides ECM support for the anchoring and survival of all the epithelial cells, while the underlying mesenchyme secretes Wnt and BMP antagonists near the crypt bottom and BMPs along the crypt-villus axis [79]. Inside the epithelium, Notch ligand and EGF are expressed by Paneth cells surrounding the stem cells [80].

In vitro propagation of primary intestinal epithelium has been pursued for a long time with limited success [82]. The identification of the ISC niche and Lgr5+ stem cells have paved the way for the establishment of in vitro cultures that maintain ISCs. The role of Wnt, BMP, and EGF pathways in maintaining intestinal stem cells and promoting the proliferation of intestinal epithelial cells provided valuable insights in establishing in vitro cultures for intestinal epithelium. Specifically, Wnt is the pivotal pathway in maintaining intestinal stem cells, and R-Spondin 1, which is a Wnt agonist, induces potent proliferative effects on intestinal crypt cells [81]. It was later found that R-Spondin 1 is a ligand of Lgr5, which acts by neutralizing Rnf43/znrf3 to amplify Wnt signaling in the presence of Wnt ligand [83]. In addition, BMP pathway is active in the villus compartment and the inhibition of BMP induces ectopic crypt formation [76]. Furthermore, the requirement of anchoring of epithelial cells to the basement membrane (which are enrich in Collagen IV) to prevent anoikis also suggests the importance of extracellular matrix to maintain the survival of intestinal epithelial cells. Essentially, the key step in establishing an in vitro intestinal epithelium culture lies in the ability to maintain the self-renewal and to a lesser extent the differentiation of ISCs, through mimicking the in vivo ISC niche. This has been achieved by providing all essential niche factors for ISCs, at multiple levels [46].

Ootani et al. reported that an air-liquid interface model can be used for culturing intestinal fragments in vitro for several months, in a collagen gel in the presence of serum [84]. These fragments contain both epithelial and mesenchymal components (and essentially, the basement membrane between them). Intestinal epithelial cells expand as sphere-like organoids with proliferation and multilineage differentiation. Although responsive to Wnt signals such as R-Spondin 1, this system does not require exogenously added factors such as Wnt and BMP antagonists, owing to the presence of mesenchyme components.

Further, Sato et al. established an organoid culture system starting from isolated intestinal crypts or single Lgr5 stem cells [85]. By embedding these cells in Matrigel and supplementing with essential growth factors (i.e., EGF, Noggin, and R-Spondin 1), the cells develop into "mini-gut" containing crypt-villus structures and all cell types in the intestinal epithelium. This elegant organoid system takes advantage of the self-organizing property of intestinal stem cells (and their progeny) as well as previous efforts of deconstructing the intestinal stem cell niche, and reassembling essential niche components together with the stem cells in a manner which closely resembles the in vivo situation. Meanwhile, the organoids also closely resemble the in vivo intestinal epithelium. The cells form highly polarized epithelium with basolateral side oriented toward the surrounding Matrigel and apical side toward the lumen. In addition, the cell distribution mimics the in vivo epithelium, where Paneth cells and stem cells cluster at the crypt-like domains, while goblet cells and enterocytes localize at the villus domains. Here, Matrigel is an important component and a clear example of a material capable of mimicking and integrating multiple niche

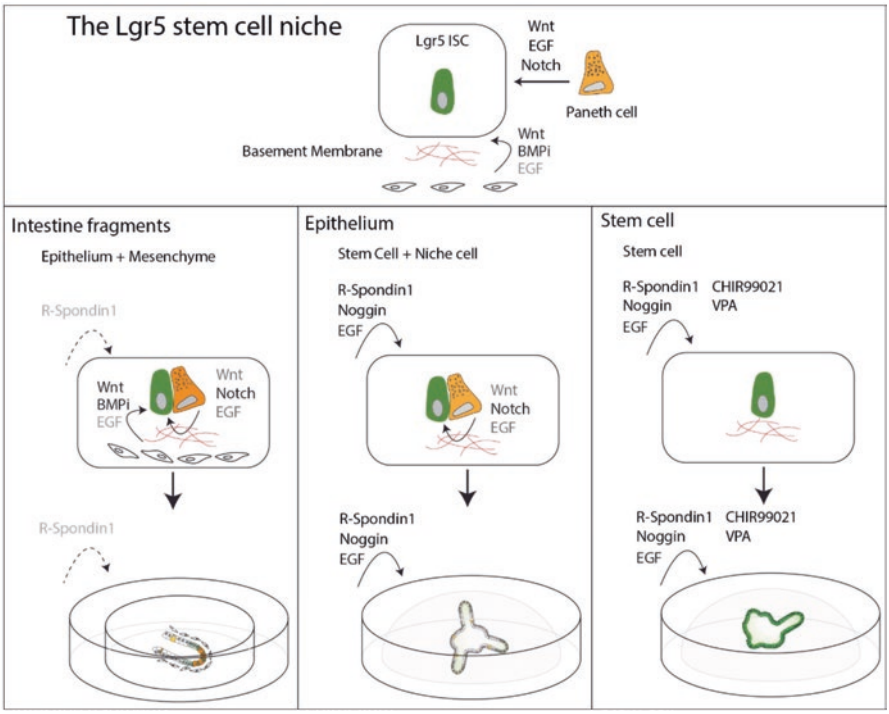

Fig. 10.5 The Lgr5 stem cell niche is sustained by signaling from neighboring cells and basement membrane presented cues. While intestinal fragments placed into a dish retain all signaling necessary to maintain the intestinal stem cell niche including Paneth cells, ECM, and mesenchymal cells, isolated intestinal epithelium requires exogenously supplied growth factors and Matrigel to maintain the niche [81]. Recently, CHIR99021 and VPA have been identified as small molecules capable of fulfilling the role of Paneth cells in supporting the Lgr5 intestinal stem cell niche [74] (With permission from the Nature Publishing Group)

components together in vitro. It is used to replace the function of the basement membrane, which provides structural support as well as a mode of presenting certain survival signals (e.g., through integrin binding) to the epithelial cells. It is not only a platform that allows cells the freedom to grow and extend out three-dimensionally but also permits the diffusion of growth factors and gradient formation, both critical requirements for the generation of organoids. Other components, like the growth factor-secreting mesenchyme, are substituted with direct addition of growth factors, including EGF, BMP antagonist Noggin, and R-Spondin 1, while the Notch signal is provided by Paneth cells generated by stem cells. An important feature of organoid formation is the presence of growth factor gradients, which allow for self-renewal and differentiation of the stem cells to occur within the same system. This is seen here, through the unique property of Wnt ligands within the Matrigel matrix. Wnts are secreted proteins that harbor a covalent palmitate lipid modification. This lipid group renders the Wnt hydrophobic and tethers it to the cell membrane or its cognate receptors [86]. In addition, exogenously added R-Spondin 1 acts by amplifying Wnt activity only in the presence of Wnt proteins, which are secreted by Paneth cells in organoids. Thus, a localized Wnt-activated niche is created in the Matrigel, which maintains a limited number of stem cells inside while permitting the differentiation of cells leaving the niche.

In this system, exogenously supplemented niche factors (e.g., ECM and growth factors including EGF, Noggin, and R-Spondin 1) work cooperatively with niche factors generated by stem cells themselves (e.g., Paneth cells, and factors generated by Paneth cells including Notch, EGF, and Wnt proteins). By further dissecting the stem cell niche, small molecules which provide Wnt and Notch signals have been identified, such as CHIR99021 and valproic acid, respectively [74]. The two small molecules essentially replace Paneth cells and replenish missing factors that can now be provided exogenously (Fig. 10.5). Importantly, the efficiency of colony formation from single stem cells is greatly increased in the presence of small molecules mimicking the role of Paneth cells [80]. Overall, elucidating specific cues and substituting them with small molecules, rather than culturing an additional cell type, presents a useful strategy to simplify in vitro systems while still mimicking the natural environment.

Pancreatic Organoids for Insulin Production

Development of mulitcellular organoids represents an exciting cell replacement tool that could modernize organ transplantation for degenerative diseases like type 1 diabetes (T1D). Pancreatic islet transplants based off the “Edmonton protocol” relied on transplantation of allogenic islets to restore insulin independence. Despite the relative success of this therapy, serious issues including the massive loss of islet post-transplantation due to instant blood-mediated inflammatory reaction and a limited donor supply have limited the therapeutic reach of this procedure. Pancreatic organ donations alone are insufficient to meet the demand of patients waiting for a

curative treatment for T1D. This has prompted numerous groups to develop alternative sources of insulin-producing cells.

The pancreas is a glandular organ serving both exocrine and endocrine functions. The endocrine functions of the pancreas are primarily mediated through beta, delta, alpha, and PP cells resident in the islets of Langerhans. The embryonic cells that generate the islets of Langerhans are traditionally characterized by the presence of the transcription factors PDX1, PTF1a, SOX9, and HNF1b [87]. 3D self-organization and commitment of these cells to a terminal duct or acinus fate require a highly regulated series of intra- and intercellular signaling events compounded with spatial-temporal environmental cues during foregut development. Differentiation capacity and origin of adult pancreatic stem cells, however, remains an openly debated topic. In fact, several sophisticated linage tracking studies have argued for and against the idea of adult multipotent stem cells capable of being sourced from ductal and acinar cells [88–93]. Although several adult and iPS-derived sources have been employed, the generation of 3D organoids in culture has primarily been accomplished using embryonic progenitors.

The earliest instances of pancreatic organoid research utilized neonatal rat pancreatic endocrine cells which were found to reorganize into a smooth-contoured 3D structure on a collagen gel [94]. This reorganized structure also resembled topographical patterns of the islets of Langerhans seen in vivo. Since then, multiple culture conditions have been reported to successfully generate pancreatic organoid-like structures from disassociated E10.5-E11.5 embryoid progenitors, including spheres, clustered ductal networks, and mini-pancreatic tissues. Similarly, a number of culture conditions for pancreatic endocrine spheroids expanded from adult mouse and human ductal sources have been developed to generate pancreatic endocrine spheroids [95–97]. For a comprehensive review on the developmental techniques and culture conditions used to generate many of these pancreatic organoids, we refer the reader to the excellent review described by Greggio et al. [98]. Matrigel has been widely employed as the material substrate of choice to facilitate the spontaneous generation of a multicellular pancreatic environment. Laminin is a major component of Matrigel and has been shown to play an important role in facilitating endocrine differentiation of adult and embryonic derived cell types. Similarly stiffness of these hydrogels has been shown to play an important role in pancreatic organoid clustering, as only hydrogels with low modulus of elasticity (~250 Pa) were capable of maintaining these structures [99]. While gel composition and structure appear to be playing clear roles in the engineered pancreatic niche, advancement of differentiation protocols will likely require development of new materials with tunable structural properties and integrated lineage directing cues to better dictate cell fate in pancreatic organogenesis.

While embryonic cells remain the standard for generating insulin-producing cells (IPC), ethical and immunogenic HLA barriers surrounding these cell sources have put increased pressure on identifying alternative sources of insulin-producing cells (IPC). Mouse fibroblast-derived induced pluripotent stem cell (iPS)-derived insulin-producing cells have been shown to effectively reverse hyperglycemia in a diabetic mouse model [100]. Human iPS-derived IPC were also shown to form a

vascularized organoid when injected under kidney capsules of $Rag2^{-/-}$ $\gamma c^{-/-}$ streptozotocin-induced diabetic mice [101]. No biomaterial scheme was implemented in this model, yet transplanted IPC still showed tissue adhesion at the sight of injection and neovascular development. This strategy showed that iPS-derived IPCs secreted insulin and were able to reduce resting serum glucose levels over the course of 150 days. However, it is unclear whether the spontaneous development of these iPS-derived islet-like structures in vivo results in organized or randomly distributes endocrine beta, alpha, and delta cells.

Corporate and academic interests alike have already begun to work together on pancreatic organoid technology transfer. For instance, the consortium "LFM4LIFE" brings corporations and academic institutions from six European countries for the development of large-scale organoid therapies in T1D. The group's primarily aim is to source adult human progenitor cells from pancreatic ductal cells. Injury by partial ligation has been shown to be naturally associated with Wnt pathway activation and $Lgr5^{+}$ cell emergence during ductal regeneration [96]. Facilitated by a Wnt agonist RSPO1, pancreatic organoids produced in these culture conditions allows for unlimited expansion of ductal fragments; capable for use in large commercial scale-up applications.

Alternative adult sources for renewable beta cells include intestinal and stomach epithelial tissue reprogramed for ubiquitous expression of Ngn3, Pdx1, and Mafa [102, 103]. While islets showed highest levels of insulin response and secretion, stomach antrum-derived tissue showed highest reprogramming efficiency and more closely mimicked beta cell functionality than cells derived from the colon, ileum, or duodenum [103]. Using a Matrigel support, reprogramed stomach antrum spheroids where capable of reversing hyperglycemia after transplantation. This work highlights the potential of engineered gastric- and intestinal-derived tissues to serve as novel sources of insulin-producing cells.

Applications of Organoids

Propagation of Difficult to Culture Cell Types

Pluripotent stem cells and a variety of other adult stem cells can be differentiated into a vast number of cells to form organoids. The use of stem cells provides a new level of control over genetics of tissues allowing researchers the ability to study many diseases in vitro [104]. With the advent of induced pluripotent stem cell (iPSC) technology, many organ systems can be developed without embryonic stem cells and often derived from a specific patient, resulting in an organoid system with genetic defects identical to those of the donor. Additionally, cells can be harvested from a variety of tissues and used to create organoids by partially digesting organs into single cell suspensions and then aggregating the cells making it possible to derive organoids for biopsies [104]. This has the advantage of allowing for the patient's specific tissue to be studied enabling the development of treatment regimens that are tailored to the patient.

Many tissue systems contain primary cells that are difficult to culture and normally cannot be expanded for long-term use without genetic alterations to immortalize the cells. Even with immortalization, the loss of spatial relationships and cues from neighboring cells often leads to dedifferentiation of cells and loss of their native phenotype. Organoids overcome this limitation by retaining the spatial relationship between different cell types allowing cells to retain their phenotype in vitro. For instance, stratum corneum, keratin 5/14+ stem cells, and granular cells have been combined to create lymphatic tissue in the mouth known as lingual tissue [105]. Similarly, groups have created organoids which contain Lgr5+ stem cells, enteroendocrine cells, goblet cells, and enterocytes that work collectively to produce colon-like tissue [104]. Lgr5+ stem cells, mucous neck cells, pit cells, and enteroendocrine cells recapitulate the pylorus of the stomach [104, 106]. CD24/CD29+ stem cells, cytokeratin 7/18+ duct cells, and aquaporin 5 expressing acinar cell combinations have been utilized to produce salivary organoids [104]. Maimets et al.'s use of organoids to produce salivary tissue found that EpCAM+ cells from salivary ducts respond to WNT/β-catenin signaling allowing for over 50 population doublings of the ductal cells [107]. Similarly, Huch et al. were able to culture Lrg5+ liver cells, intestine, stomach, and pancreas cells for over a year [108]. They noted that their liver organoids were genetically stable over time indicating that these ex vivo samples were able to maintain a mature phenotype more closely related to the original tissue than traditionally cultured liver cells [108]. Therefore, unlike standard 2D culture systems, organoids allow for long-term culture and formation of much more complex structures and interactions between multiple cell types.

Disease Models

While healthy organoid function can provide useful information about normal organ function, advances in gene editing such as CRISPR/Cas9 and viral transduction in addition to the availability of disease tissue samples have allowed for in vitro modeling of many diseases while retaining much of the structural relationships seen in vivo. Perhaps the most well-developed diseases in organoid systems are various forms of cancer. Precancerous transformations such as Barrett's esophagus, *helicobacter pylori* infection, and irritable bowel disease allow for interrogation of mechanisms of cancer formation. Cancerous lesions such as lingual carcinoma, stomach, intestinal, colon, pancreatic, and prostate have been modeled in organoid systems [104]. Not only does this give insight into progression of cancerous lesions and potential therapeutic screens, but these models also allow for interrogation of the etiology of each malignancy in the context of its 3D multicellular environment. Such systems are critical as our understanding of cancer moves beyond the cancer cells themselves to understanding the role of the tumor stroma that supports the survival and propagation of the cancer cells [109].

Other disease models developed using organoid techniques include models of cystic fibrosis (CF). Dekkers et al. developed a CF intestinal organoid model where they can screen for CFTR function in CF by measuring organoid swelling due to

forskolin treatment, a molecule which causes chloride influx in non-CF tissues. Demonstrating the potential utility of organoids, CF organoids treated with CF correcting drugs had normal swelling characteristics after treatment [110]. Another disease model using organoid techniques is a model of hyposalivation, a condition where patients' salivary glands do not produce adequate amounts of saliva. It has been reported that isolated stem cells highly expressing CD24 and CD29 could restore function to irradiated salivary glands in mice [111]. Bacterial and viral infection models in both the stomach and intestines have been developed to study formation of ulcers and understand bacterial interactions with epithelial cells of the gut [104]. Enteric bacteria normally do not pose a problem to their human host, but there is a complex regulation of bacterial colonization by the gut which when disrupted can result in severe sickness. Wilson et al. and Zhang et al. have studied the antimicrobial α-defensins produced by Paneth cells and bacterial disruption of epithelial tight junctions, induction of inflammation, and depopulation of regenerative stem cells, respectively, in intestinal organoid systems [112, 113]. Huch et al. demonstrated that organoids derived from patient samples with α1-antitrypsin (A1AT) deficiency undergo similar pathology to the original biopsied samples as demonstrated by A1AT aggregation in cells, decreased secretion of A1AT, decreased elastin blocking activity, increased ER stress, eIF2α phosphorylation, and increased cell apoptosis [108]. Cerebral organoids like those described in detail above are also being used as analytic tools to probe disease mechanisms in autism [114] and Zika virus-associated microcephaly [54, 115, 116]. As in the brain, 3D ductal pancreatic organoid systems are emerging as a promising tool to model disease development, function, and as drug screening tools. While these platforms have been used to model pancreatic abnormalities in cystic fibrosis, the primary application has been in modeling pancreatic cancer [117–121]. 3D tumor organoids promise to be an incredibly powerful tool in cancer research. Biopsies of human pancreatic neoplasms can be used to generate organoids that maintain differentiation status, tumor architecture, and retain patient specific physiological changes (Fig. 10.6). Once these cancer organoids have been grown in culture, they can be transplanted into mouse models and screened against drugs to better understand patient specific disease progression and treatment options. Transcriptional and proteomic analysis of these transplanted neoplastic organoids have uncovered important genes and pathways involved in the development of the disease [120]. Use of these models may help uncover patient specific biomarkers and characteristics involved in malignancy. Overall, organoid systems provide superior in vitro models of many diseases compared to conventional 2D cultures and continue to gain prominence as a research tool.

Drug Screening

Having an in vitro system where structure and complexity are maintained is highly useful when screening for drugs that could potentially be used to treat a host of diseases and conditions. Currently, drug companies invest over $350 million to

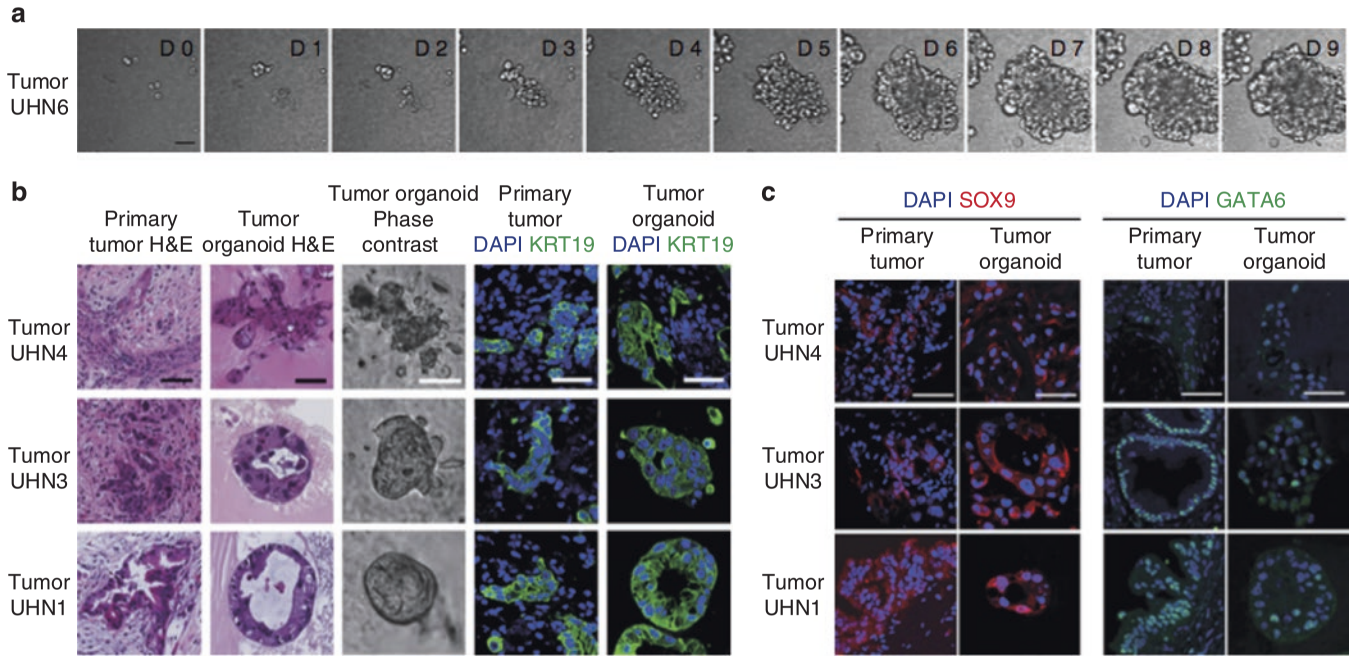

Fig. 10.6 Establishment of tumor organoids that conserve patient-specific traits. (**a**) Time-lapse imaging sequence of UHN6 organoids. Scale bars, 50 μm. (**b**) H&E, phase, and immunofluorescence images of KRT19 (*green*) and DAPI (*blue*) staining of tumor organoids and matched primary tumors. Scale bars, 50 μm. (**c**) SOX9 (*red*) and GATA6 (*green*) staining in primary tumors and tumor organoids. Scale bars, 50 μm. (Reproduced from Huang et al. [121]; with permission from the Nature Publishing Group)

bring a new drug to market often taking 12 years before approval by the FDA [122, 123]. Proving safety and efficacy is the cornerstone of approval, and identifying promising drugs and off-target effects early is critical to reducing the cost and time invested in drug development. Therefore, more efficacious tools for drug screening are important to ensure therapeutics are more likely to advance toward successful clinical translation.

Huang et al. used an organoid model of pancreatic cancer derived from iPS cells to examine if cancerous mutations in organoids corresponded to clinical outcomes seen in patients. The researchers found that SOX9 was localized to the cytoplasm, a previously known prognosticator in breast cancer, in organoids expressing the $TP53^{R175H}$ mutation. In patients with pancreatic cancer, it was seen that those with the TP53 mutation also had cytoplasmic SOX9 localization and poorer survival rates compared to those without the mutation. Additionally, the group used organoids to screen drugs targeting epigenetic regulators and successfully identified a drug combination to reduce tumor growth [121]. Drug toxicity screening in the kidney is another area of interest in drug development. Morizane et al. used nephron organoids to test the toxicity of gentamicin, a drug known to cause damage in the proximal tubules of the kidney, and cisplatin, a drug known to be toxic to both the proximal and distal tubules. They found that KIM-1, a marker of kidney damage, was upregulated in the LTL+ proximal tubules of the organoid, but not the E-cadherin + distal tubules after gentamicin treatment, whereas cisplatin damaged both the proximal and distal tubules as demonstrated by increased KIM-1 expression [124]. These experiments demonstrated that organ-specific damage caused by drugs could be predicted in vitro through the use of organoid model systems. While the application of organoids to disease modeling and drug screening is still in its infancy, the ability to create in vivo phenotypes and spatial relationships of different cell types in a dish has been proven, and the potential for more efficacious and predictive drug screens motivates the continued development and refinement of organoid systems.

Therapeutics and Injectable Products

Organ damage and loss of function are serious concerns in many diseases such as cancer, kidney disease, and cystic fibrosis. Another application of organoids is their use as therapeutics to repair damaged organs. Maimets et al. removed EpCAM+ cells from salivary glands inducing organoid formation and treating with WNT for seven cell passages. To examine if salivary gland organoids could restore function, mice were irradiated, resulting in salivary production dropping to 20% of normal. Animals receiving organoids transplanted back into the salivary gland recovered 80% of normal salivary production. These transplanted cells formed ducts and acini within the irradiated gland aiding in restoration of tissue function [107]. Xinaris et al. transplanted kidney organoids derived from mESCs into immunocompromised rat kidneys treated with vascular endothelial growth factor (VEGF), to promote integration of the organoid graft and maturation of the organoids. They noted that these organoids matured and formed tubular structures as would be expected in

kidney tissue. Additionally, these engrafted organoids treated with VEGF showed Bowman's capsule lined with parietal cells mature podocytes in the glomeruli in a similar distribution to that seen in normal mouse kidney. These engrafted organoids were shown to concentrate albumin and dextran and produce erythropoietin [125]. Promising, ongoing work in the application of organoids is focused on integrating the organoid-derived tissue into the native tissue allowing for the full functional benefit of the graft to be realized.

Future Directions

Human on a Chip Drug Screening Platforms

Closely related to organoid development is the development of human organs on a chip. Organs on a chip refine the approximation of an in vitro model to in vivo by reintroducing multiple layers of complexity, including spatially appropriate cell-cell interactions, differential extracellular fluid exposure, and biomechanical stretch and strain [126, 127]. Researchers from the Wyss Institute at Harvard University pioneered organ on a chip systems when they introduced an on-chip model of the lung. In addition to recreating the complex interface between capillary endothelium and the delicate, simple, squamous architecture of the alveolar epithelium through two centrally adjoined microfluidic chambers separated by a porous membrane, Huh et al. designed a chip with two laterally placed microfluidic inlets [128]. When vacuum was cyclically applied to the lateral chambers, it caused deformation of the central chambers akin to negative pressure expansion of the alveoli during inspiration. Incorporation of physiologically relevant deformation pressures more accurately modeled in vivo alveolar response to lung irritants, inflammatory cells, and pathogenic bacteria, highlighting both the novelty and necessity of accounting for biomechanical pressures in complex, multicellular in vitro modeling [128].

To date, human lung [128], kidney [129], liver [130, 131], intestine [132], heart [133], fat [134], bone marrow [135], vasculature [136], blood-brain barrier [137], and more have been modeled using on-chip technology (Fig. 10.7). In addition to more reliably recapitulating normal physiology, on-chip technology has been employed to study complex multicellular pathophysiology including metastatic spread of cancerous cells [138], epithelial-to-mesenchymal transition in renal tubular cells exposed to high proteinaceous loads [139], microvascular clot formation leading to thromboembolic damage [136], and gastrointestinal disease caused by enteroinvasive bacterial species in the setting of commensal bacteria cocultures with enterocytes grown in villus formation [132]. On-chip technologies modeling both physiology and pathophysiology in these settings have delineated the role of biomechanical forces in both health and disease at a fraction of the cost incurred during whole animal modeling.

A number of production strategies are currently in place to create human organs on a chip. A common strategy, convenient for its lower expense, involves fabricating a polydimethylsiloxane (PDMS) stamp by pouring PDMS over an etched silicon

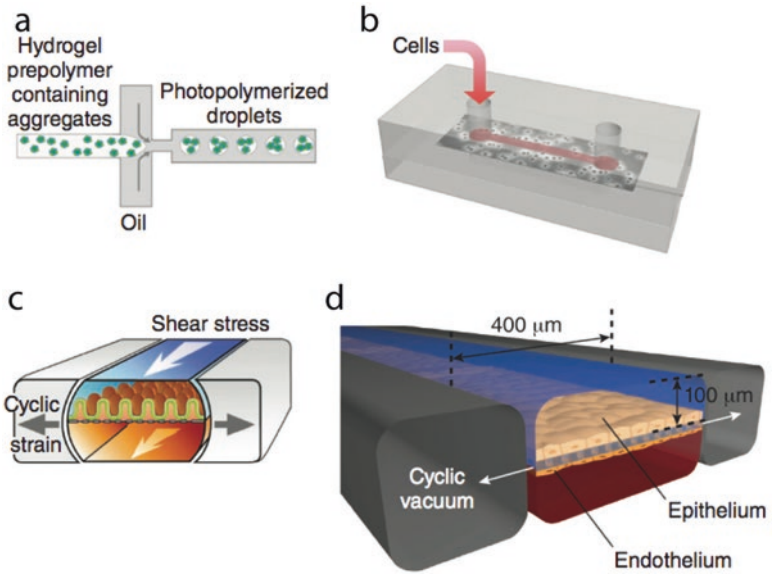

Fig. 10.7 Examples of increasingly complex organ-on-chip designs. (**a**) A liver-on-a-chip in which hepatic microtissues composed of microscale hydrogels containing hepatocytes and fibroblasts are microengineered in one microfluidic system and then used to populate another chip for culture and real-time multiplexed analysis [140]. (**b**) A kidney-on-a-chip in which human kidney proximal tubular epithelial cells are cultured on the top of a porous membrane separating two channels, enabling analysis of transcellular transport, uptake, and secretion [129]. (**c**) A gut-on-a-chip in which human Caco-2 intestinal epithelial cells are cultured on top of an ECM-coated, porous PDMS membrane separating two channels. Application of cyclic suction to side chambers mimics peristalsis [141, 142]. (**d**) A "breathing" lung-on-a-chip that recapitulates the alveolar-capillary interface. Human alveolar epithelial cells are cultured on top of a flexible, porous, ECM-coated membrane and human capillary endothelial cells on the bottom. Air is passed through the upper channel to create an air-liquid interface with the alveolar epithelium, and culture medium is flowed through the vascular channel, with or without human immune cells [128, 143]. Breathing motions are mimicked by applying cyclic suction to full-height side chambers that rhythmically distort and relax the flexible PDMS side walls and attached porous membrane. (Adapted from Bhatia et al. [126]; with permission from the Nature Publishing Group)

wafer to create fluidic channels followed by sealing this stamp to a glass substrate. PDMS has the additional advantages of being gas permeable and transparent which allows experimenters access to phase-contrast and fluorescent microscopy of microfluidic channels, as well as reliable replicative ability in a low-cost material [126, 144, 145]. Ongoing development in organ on a chip systems includes the integration of multiple organ systems in parallel to create a "human on a chip" platform that can recreate the systems-level communication and interactions of in vivo biology in an in vitro system. In parallel, recent advances have been made in integrating sensors into organ on a chip systems, enabling repeat and continuous monitoring of organoids in situ [146].

Human Organ on a Chip for In Vitro Drug Discovery

Metabolism and clearance of pharmaceutical agents are complex processes that are highly dependent upon the chemical characteristics of the compound, including its blood/gas and oil/gas partition coefficients, serum half-life, bioavailability, and pKa, as well as the primary organ or organs responsible for its clearance. Much of the drug clearance in vivo is mediated by biotransformation reactions in the liver and excretory pathways in the kidney. These pharmacokinetic properties of a compound are collectively referred to as absorption, distribution, metabolism, and excretion (ADME) [147]. Improper clearance of drug metabolites can cause acute liver or kidney injury, resulting in devastating long-term consequences for patients. Given the multi-organ involvement of drug activity, metabolism, and clearance, preclinical testing of new pharmaceutical compounds typically occurs in animal models. The ability of on-chip technology to readily mimic organ-level complexity at a lower cost than animal models while using human-derived cells makes it an incredibly enticing platform for in vitro drug discovery and testing [126, 127].

Of central concern to on-chip technology being able to mimic the complex processes of drug metabolism and clearance is the ability to link multiple organ chips together in a sequence that reflects in vivo drug exposure [126, 148]. In 2004, researchers at Cornell University used a four-chamber microfluidic chip with a different tissue type in each chamber to demonstrate bioaccumulation of hydrophobic compounds in the presence of adipocytes. This was the first instance of multi-organ modeling of drug metabolism in an on-chip platform [134]. Since that time, "human-body-on-a-chip" designs have become more advanced in demonstrating the efficacy of using multi-organ chip models for drug discovery. Recent iterations of body-on-chip systems have linked human intestine, liver, skin, and kidney organ chips [149], as well as gastrointestinal and liver organs for analysis of homeostatic balance between the tissues over extended culture periods and tracing of nanoparticles for drug delivery [131], respectively. The combinatorial variety of a modular body-on-chip system has the potential to allow more rigorous testing of preclinical pharmaceuticals at a highly reduced cost to investigators [126, 148].

Conclusion

Just as the advent of in vitro systems catalyzed the last century of biology, 3D organoid systems that recapitulate and maintain mature phenotypes of a large variety of cell types will drive discoveries over the next few decades. Success in organoid systems have demonstrated the importance of moving past the convenience and simplicity of 2D cultures, which often fail to recapitulate relevant biology, and turn our focus instead toward creating in vitro systems that maintain phenotype and functionality, even if it requires more challenging techniques and technologies. While strategies to create organoids for different tissue types have their own key

characteristics, there are commonalities. Most systems employ either a scaffold-free-induced aggregation technique, as seen in MSC spheroid formation and neuronal SFEBq strategies, while others employ scaffolds, most commonly Matrigel, to facilitate 3D development and outgrowth of the organoid. Another key factor driving organoid protocol development is the identification of signaling factors that drive and maintain cell fate. These cues take a variety of forms including mechanical cues from the substrate, presentation of factors from neighboring cells (Notch) or the substrate (ECM), and soluble secreted factors. Once identified, these factors can be engineered into the system, through inclusion of the producing cells themselves, or by substituting the cues with small molecules and recombinant factors that stimulate the same pathways. Thus, as more is discovered about the pathways that regulate and maintain cell fate decisions in different tissues, more precise and predictable organoid systems can be designed and made available for research and therapeutic uses.

The applications of organoids are many, from understanding development, to generating therapeutic cells to repair damaged organs, to creating drug discovery systems that recapitulate multisystem human physiology. The future success of organoid systems will likely depend on collaboration between biologists and engineers from diverse backgrounds from both academia and industry in order to implement basic biology discoveries into scalable and predictable systems. If the early years of organoid development are any indication, the future of engineered organoid systems is bright.

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3D Printing for Cell Therapy Applications

11

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Stem Cells Used in Regenerative Medicine Applications

Regenerative medicine is a very promising and rapidly expanding area of translational research dealing with replacing, engineering, or regenerating damaged tissues and organs to restore normal function. It is an interdisciplinary field which draws input from both clinicians and basic science researchers with biomedical and engineering expertise. There are four main areas of research within regenerative medicine: medical devices and artificial organs, tissue engineering and biomaterials, cell therapies, and clinical translation [1]. Tissue engineering holds great promise of fabricating on-demand tissues and organs in the laboratory that may obviate the need for allogenic organ donation and its requisite immunosuppression. Donor shortage is an expanding public health concern as the number of patients with end-stage organ failure far exceeds organ supply. Therefore, regenerative medicine is an exciting and developing field in the treatment of organ dysfunction and tissue loss. Three-dimensional (3D) bioprinting is a new technology which integrates the abovementioned research areas for tissue engineering. 3D bioprinting technology utilizes biomaterials, growth factors, and cells for in vitro tissue creation [2]. Stem cells can benefit this process as they offer

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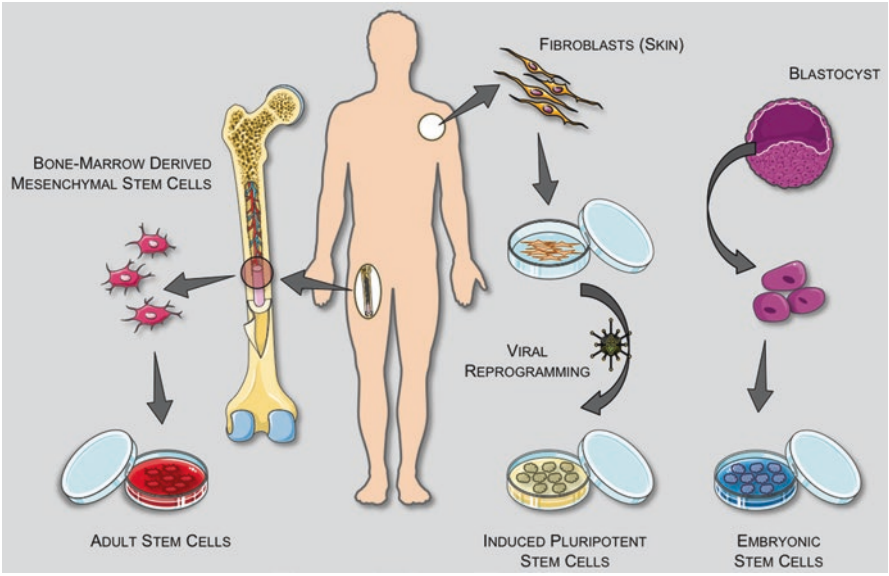

Fig. 11.1 Stem cell sources [Reproduced with permission from Christopherson GT and Nesti LJ stem cell applications in military medicine [4]

self-renewal and the ability to differentiate into multiple cell types across endo-, ecto-, and mesodermal lineages. They can be classified into four distinct groups based on their origin: embryonic, fetal, induced pluripotent, and adult stem cells (Fig. 11.1) [3].

Embryonic Stem Cells

Embryonic stem cells (ESCs) give rise to approximately 220 different cell types across all germ layers during embryonic development. They were first isolated in 1998 from the inner cell mass of in vitro fertilized embryos in the blastocyst phase [5] and cultured with growth factors on an irradiated mouse fibroblast layer. However, the use of fertilized embryos had generated fierce ethical debates leading to alternative strategies such as dead embryo utilization [6], single cell biopsy [7], and somatic cell nuclear transfer [8]. By utilizing dead embryos, researchers can avoid planned destruction of an embryo and associated moral dilemma [6]. Single cell biopsy is a described technique in which a single blastomere could be extracted from an embryo without adverse sequela to embryonic growth [7]. Somatic cell nuclear transfer involves the transfer of a somatic cell nucleus into an enucleated oocyte. It is also referred to as "therapeutic cloning" and led to the creation of a cloned sheep in 1996 [9]. Despite these scientific breakthroughs, all these techniques are limited in their scope of availability and federal funding in the United States (US).

Fetal Stem Cells

Fetal mesenchymal stem cells (MSCs) can be isolated from the fetal blood, bone marrow, and tissue [10]. Unlike fetal hematopoietic stem cells which give rise to all types of blood cells, fetal MSCs can give rise to a variety of cell types, such as osteocytes, chondrocytes, adipocytes, and stromal cells which support vascular formation. Mesenchymal stem cells were first described in the 1960s as non-hematopoietic cells in the bone marrow that adhered to plastic [11]. Since then these multipotent stem cells have been isolated from many tissues, with Campagnoli demonstrating their retrieval from first-trimester human fetuses in 2001 [12]. Although fetal MSCs offer less developmental potential than ESCs, they have greater multipotency and proliferative capacity than adult stem cells [10], while limiting immunogenicity. In one study, fetal MSCs did not elicit alloreactive T-cell proliferative responses from adult lymphocytes suggesting that they may have a significant advantage in transplantation [11]. Fetal MSCs are only obtained from terminated fetuses from which the tissue would be otherwise discarded. Therefore, the utilization of fetal MSCs may not offer any improvement in ethical concerns when compared to ESCs. This has led to new stem cell sources being explored, notably from adults.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are adult cells that are reprogrammed to have pluripotent capabilities and were developed as an embryo-free strategy. Induced pluripotent stem cells were successfully obtained from mouse embryonic fibroblasts in 2006 and the following year from adult human fibroblasts. They were generated by introducing four transcription factors into skin fibroblasts: Oct3/4, Sox2, c-Myc, and Klf4 [13], which had been found to be important in embryonic stem cells. These enabled the conversion of pluripotent stem cells from differentiated adult cells [14]. While iPSCs obviate ethical concerns, they do demonstrate lower differentiation capability when compared to ESCs and an increased risk of teratoma formation [14, 15]. As the risk of teratoma formation limits clinical translation, researchers have developed chemically induced pluripotent stem cells in mice [16], which do not rely on transcription factors. While these labor-intensive engineered cells have demonstrated increased differentiation potential, genetic instability still exists [16]. This has steered researchers to explore other sources of stem cells from adults.

Adult Stem Cells

Adult stem cells are located in various locations throughout the body including the bone marrow (BM), blood, skeletal muscle, liver, dental pulp, and adipose tissue. They maintain and repair tissue and have effectively been utilized in bone marrow

transplantation mainly for leukemia [17]. The bone marrow contains an abundant amount of hematopoietic stem cells and a smaller population of MSCs. Bone marrow MSCs have been extensively researched for regenerative medicine applications and have required less in vitro manipulation than ESCs and iPSCs [18]. In addition, when compared to iPSCs, malignant transformation has diminished [18]. However, bone marrow stem cells are limited by painful harvest and reduced differentiation ability with increasing age [19, 20]. A less intrusive MSC source was identified in 2001 when adipose-derived stem cells (ADSCs) were isolated from lipoaspirates [21]. These multipotent stem cells are found in large numbers in white adipose tissue [22]. When compared to bone marrow mesenchymal stem cells, they offer a longer lifespan and faster growth rates [23]. Their ubiquity and differentiation potential position them as an autologous stem cell source for bioprinting applications.

Bioprinting

Bioprinting is an innovative technology, which uses a 3D additive manufacturing process to precisely deposit cell-laden biomaterials for the creation of functional tissues and organs [24]. Tissue creation via bioprinting occurs under sterile conditions in three stages: pre-organ printing, organ printing, and post-organ printing. Several types of bioprinters exist including droplet, extrusion, and laser-based bioprinters (Table 11.1), each with their own advantages and disadvantages [25].

Droplet-Based Bioprinting

Droplet-based bioprinting (DBB) comprises inkjet, acoustic-droplet-ejection, and micro-valve bioprinting [27]. Inkjet bioprinting was the first bioprinting technology developed in 2003 and is the most commonly used type of DBB-based bioprinter. It is based on standard 2D inkjet printing (Fig. 11.2a), and a traditional printer can

Table 11.1 Types of bioprinters and their respective characteristics [data has been derived from Ref. [26]

	DBB	EBB	LBB
Cost	Low[a]	Medium	High
Viscosity	<15 mPa/s	<6 × 10^7 mPa/s[a]	<300 mPa/s
Cell density	<10^6 cells/mL	High, spheroids[a]	<10^8 cells/mL
Print speed	Medium	Slow	Fast[a]
Resolution	50–100 μm	100 μm	20 μm[a]
Common bioinks	Agarose, alginate, collagen, fibrin, methacrylated gelatin, polyethylene glycol	Alginate, hyaluronic acid, polyethylene glycol, agarose, collagen, gelatin, pluronic, matrigel, fibrin[a]	Alginate, collagen, gelatin, matrigel
Cell viability	>85%	80%	95%[a]

[a]Denotes best for that characteristic

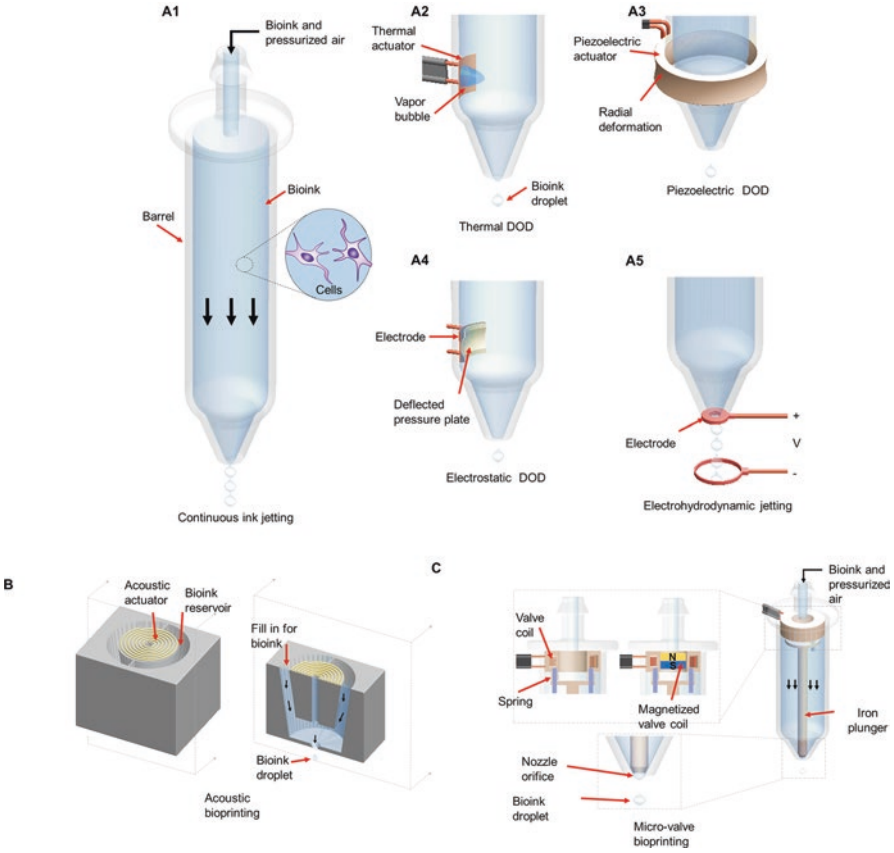

Fig. 11.2 Mechanisms of droplet-based bioprinting. (**a**) Inkjet bioprinting techniques: (**a1**) continuous inkjetting relies on Rayleigh-Plateau instability, which breaks bioink jets into droplets; (**a2**) thermal drop-on-demand bioprinting employs a thermal actuator to locally heat bioink solutions to generate droplets; (**a3**) piezoelectric drop-on-demand bioprinting depends on radial deformation of a piezoelectric actuator to generate droplets; (**a4**) electrostatic bioprinting relies on deflection of pressure plate to generate droplets; and (**a5**) electrohydrodynamic jetting uses an electric field, resulting from the electric potential difference between the printhead and the substrate, to pull a stream of bioink droplets through the printhead orifice. (**b**) Acoustic droplet ejection relies on a gentle acoustic field generated by an acoustic actuator to eject droplets from an open pool of bioink solution. (**c**) Micro-valve (solenoid) bioprinting operates with an electromechanical valve to dispense droplets

be modified by simply adding a controller to the print heads to create a 2D bioprinter [28]. The bioink is stored in a cartridge, which is connected to the print head and then manipulated to form droplets using gravity, atmospheric pressure, and fluid mechanics. Inkjet bioprinting can be further broken down into three types: continuous, drop on demand, and electrohydrodynamic [27]. In continuous inkjet (CIJ) bioprinting, pressure is used to force the bioink through a nozzle (Fig. 11.2a1). Drop on demand (DOD) generates droplets when required, making control easier, and therefore it is preferred over CIJ. Thermal, piezoelectric, or electrostatic mechanisms are

used to create pressure pulses, which are introduced to a fluid chamber where the ink is stored (Fig. 11.2a2–4). The droplet is dispensed when the surface tension is overcome. However, DOD requires high pressures to eject droplets through a nozzle, which can be harmful to cells whereas electrohydrodynamic (EHD) jet bioprinters utilize an electric field to pull the bioink through (Fig. 11.2a5) limiting shear stress-induced cell damage.

Acoustic-droplet-ejection bioprinting applies an acoustic field to eject droplets from a pool instead of a nozzle (Fig. 11.2b) [29]. It typically consists of a single or group of 2D microfluidic channels, and the bioink is held in place by surface tension. A piezoelectric substrate and gold rings are used to generate acoustic waves with a focal point at the interface near the exit channel. Droplets are ejected when the force from the waves overcomes the surface tension. As a result of this mechanism, cells are not exposed to the stress of inkjet printing; however, the moving heads can disrupt the acoustic field leading to poor control of deposition.

In micro-valve bioprinting, an electromechanical valve is used to generate droplets (Fig. 11.2c) [30]. The bioink is in a pressurized fluid chamber and gated by a micro-valve consisting of a solenoid coil and plunger blocking the orifice. The coil generates a magnetic field to pull up the plunger when voltage is applied. Bioink is dispersed in a continuous manner or drop on demand depending on the pressure and gating time. This process limits cell damage but creates larger droplets (50–300 μm) leading to a lower resolution.

Highly complex constructs can be created with all types of droplet-based bioprinters. [31]. These printers tend to be affordable and user friendly since they are very similar to commercial 2D printers [27]. When selecting a hydrogel for bioink preparation, ejectability and compatibility must be considered to minimize nozzle clogging while still allowing for cell admixtures at relevant concentrations [31].

Extrusion-Based Bioprinting

Extrusion-based bioprinting (EBB) in general utilizes shear-thinning behavior of bioink materials that has progressed significantly in the past decade. The first extrusion-based bioprinter was reported in 2002, and since then the price has dramatically reduced. Bioink is deposited from a fluid dispensing system under the control of a computer and can be dispensed in cylindrical lines rather than droplets. Fluid deposition is driven by a pneumatic, mechanical, or solenoid system. A pneumatic system uses pressurized air (Fig. 11.3a), whereas mechanical uses a piston or screw (Fig. 11.3b). Piston-driven provides more control of the flow through the nozzle, whereas screw-driven has better spatial control and is optimal for inks with high viscosities. However, a screw-driven mechanism creates more pressure, which can damage cells. A solenoid system utilizes electrical pulses (Fig. 11.3c). With all types of EBB, cell viability after printing is usually around 80% but can be as high as 97% with optimization of process parameters [32]. Cell survival is decreased with increasing pressure, larger nozzle gauges, and elevated shear stress from increased cell densities. Computer-aided design (CAD) software is easily incorporated, and the

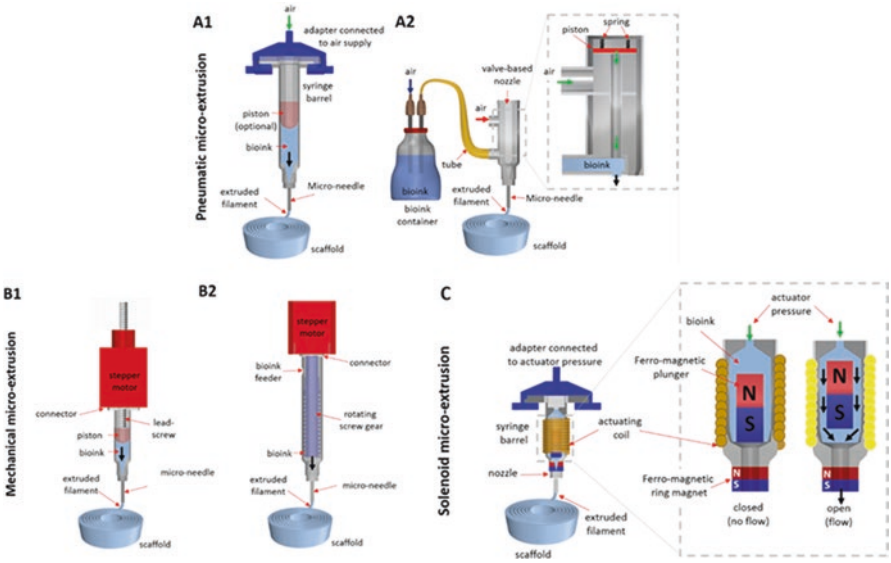

Fig. 11.3 EBB systems: (**a**) pneumatic micro-extrusion including (**a1**) valve-free and (**a2**) valve-based; (**b**) mechanical micro-extrusion including (**b1**) piston- or (**b2**) screw-driven and (**c**) solenoid micro-extrusion

continuous deposition improves structural integrity [33]. EBB also offers greater printing speed facilitating scalability and clinical translation. Unlike droplet-based bioprinting, a larger variety of inks are able to be used; however, they are still limited by the need for rapid gelation. Resolution is typically limited to 100 μm [34].

Laser-Based Bioprinting

Laser-based bioprinting (LBB) was first used at the Naval Research Laboratory in 2004. LBB requires a donor layer comprised of a ribbon structure, which contains an energy-absorbing top layer that responds to laser stimulation (i.e., titanium or gold) and a bioink bottom layer (Fig. 11.4). A laser pulse vaporizes the donor layer creating a bubble at the interface and propelling the bioink to form a droplet that is collected on the receiving substrate and subsequently crosslinked [35]. Direct contact with the printer and ink is avoided, which reduces mechanical stress on the cells resulting in a high viability of >95% [31]. Additionally, highly viscous materials can be printed, and resolution is the highest of all bioprinting methods with a droplet size of 20 μm. It is also highly precise and enables cells to be placed within 5 μm of the template [36]. Despite these benefits, the cellular effects of laser exposure are not known, and lasers are expensive compared to the other systems. The systems are large and complex; therefore, there are fewer laser-based printers and less research in this area.

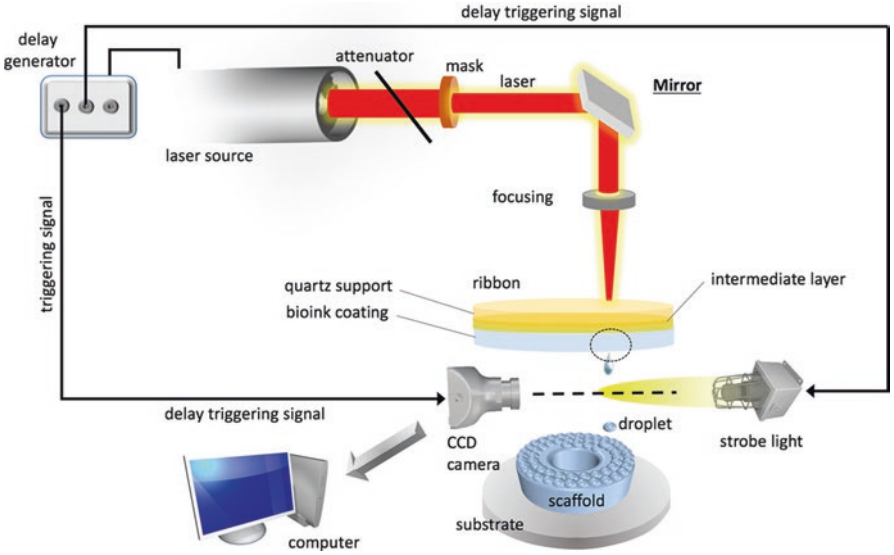

Fig. 11.4 Schematics of laser printing setup based on laser-induced forward transfer: the upper donor slide is coated underneath with a thin laser energy-absorbing layer and a layer of biological material to be transferred. The donor slide is placed above a second collector slide. Laser pulses are focused on the donor slide, evaporate the absorbing layer, and generate vapor pressure propelling the cell-containing hydrogel toward the collector slide

Stages of Bioprinting

Pre-organ Printing

Pre-organ printing refers to the process of preparing samples for the printing stage either through a scaffold-based or scaffold-free approach. Scaffold-based constructs utilize exogenous biomaterials, whereas scaffold-free constructs are made purely of cells which are first formed into neotissue aggregates (i.e., spheroids, cell pellet, and tissue strands) [37].

Scaffold-Based Bioprinting

In the scaffold-based approach, a bioink is prepared which consists of cells mixed with hydrogels, decellularized extracellular matrix (dECM) components, and/or micro-carriers that help creating a proper environment for cell proliferation while also serving as a structural component. Either differentiated cells or stem cells can be utilized in bioink, with stem cells commonly used in regenerative medicine applications. Ideal bioinks are accurate when printed, crosslinkable, maintain their properties after polymerization, biocompatible, and undergo controllable degradation and by-product production. Two types of hydrogels exist: natural and synthetic. Natural hydrogels include extracellular matrix (ECM) components and polymers such as collagen, fibronectin, and alginate. Synthetic polymers are artificial, and

therefore their properties are more controllable than natural polymers, but their long-term effects on cells are unknown. Common synthetic hydrogels include methacrylated gelatin, Pluronic, and polyethylene glycol (Table 11.2). Hydrogels are extremely hydrophilic; therefore, suspended cells are often rounded in shape when compared to their native morphology [38]. Researchers often combine various hydrogels and other components to improve bioink characteristics. Decellularized matrix components are a newer bioink source obtained by chopping tissue into small fragments, lysing the cells, and extracting the remaining ECM. However, only a small amount of dECM is obtained, and it loses its mechanical and structural integrity during processing; therefore, a supportive frame is required during bioprinting [32]. Micro-carriers have recently been used in bioprinting to enhance scaled-up tissue fabrication. They are particles designed to promote cell attachment, survival, and expansion. They can easily be suspended in culture media due to their low density [38], and micro-carrier/cell complexes can be embedded in hydrogels for use in bioprinting. Both chondrocytes and osteoblasts cultured in micro-carriers have shown greater regenerative potential than standard two-dimensional (2D) cultured cells [38, 39]. However, there are limitations associated with micro-carriers including nozzle clogging during printing, ensuring contact within the construct, and poor degradation.

Scaffold-Free Bioprinting

Unlike in scaffold-based constructs where cells in lower numbers are placed in a bioink to proliferate, cells in scaffold-free constructs are printed without a supporting hydrogel. Cells are placed in exceptionally high concentrations triggering them to deposit their own ECM, which provides support and facilitates maturation [32]. A scaffold-free approach is only compatible with extrusion-based bioprinting. Cell aggregates are typically arranged into spheroids, pellets, and strands. Tissue spheroids are sphere-shaped cell conglomerates formed by self-assembly of cell suspensions. Typically, agarose-based molds are used to facilitate this assembly. Spheroids are printed in close proximity and allowed to fuse during maturation (Fig. 11.5a–b). Tan et al. printed tissue spheroids containing endothelial cells and smooth muscle cells that fused into a toroid shape post-printing [40]. Several challenges exist with spheroids including the requirement of a delivery medium (sacrificial ink) for extrusion, premature fusion causing nozzle clogging, and gaps between printed spheroids leading to leaky tissues. Cells can instead be bioprinted into an inert hydrogel mold as a pellet and triggered to aggregate [41]. Final tissue size is limited by mold dimensions, which prevents easy human clinical translation. To overcome this, tissue strands were developed. Using a custom nozzle, long strands can be printed without the use of a mold. Yu et al. printed cartilage tissue strands to fabricate articular cartilage (Fig. 11.5c) [42]. Scale-up tissues fabricated from cell aggregates will require vascularization as hypoxia occurs at diameters greater than 400 μm [43]. Despite advances in scaffold-free printing, most researchers still use scaffold-based approaches due to their simplicity, scalability, ease of bioprinting, and required cell numbers [32].

Table 11.2 Types of hydrogels with their source, type, crosslinking modality, advantages, and disadvantages

Hydrogel	Source	Natural or synthetic	Crosslink	Advantages	Disadvantages	Refs
Agarose	Seaweed	Natural	Thermal	High mechanical strength, low cost	Low cell adhesion	Gu et al.[a] Daly et al.[a] Campos et al.[a]
Alginate	Brown seaweed & bacteria	Natural	Ionic	Fast gelation, low cost, good stability	Poor cell attachment, easily clogs at high concentrations	Ahn et al. Gu et al.[a] Markstedt et al.[a] Daly et al.[a]
Chitosan	Deacetylation of chitin	Natural	Ionic	Antibacterial and antifungal	Slow gelation, poor mechanical properties	Ye et al. Campos et al.[a] Gu et al.[a]
Collagen I	Fibrous tissue	Natural	pH or thermal	Promotes cell attachment, good printing abilities, have RGD sequence	Poor mechanical stability, slow gelation, easily clogs	Skardal et al.[a] Campos et al.[a]
Fibrin	Blood	Natural	Enzymatic (Ca^{2+}, thrombin)	Promotes angiogenesis, fast gelation	Poor mechanical stability, easily clogs	Song et al.[a] Skardal et al.[a]
Gelatin	Hydrolysis of collagen	Natural	Thermal	Reversible, promotes cell adhesion	Unstable/fragile, poor abilities without modification	Gaetani et al.[a]
Hyaluronic acid (HA)	Connective tissue	Natural	Ionic	Promotes proliferation and angiogenesis, fast gelation	Rapid degradation, poor mechanical stability	Gaetani et al.[a] Patel et al. Jakus et al. Zhou et al. Malda et al. Zhang et al.[a]

Matrigel	Mouse sarcoma cells	Natural	Thermal	Promotes differentiation	Clogs easily, made from tumor cells	Fedorovich et al.
Gelatin methacrylate (GelMA)	Gelatin with methacrylate added	Synthetic	UV	Easily degradable, high mechanical strength	Slow gelation, requires UV (cell damage)	Hu et al.[a] Daly et al.[a] Ma et al. Kolesky et al.[a] Zhou et al.[a] Malda et al.[a]
Pluronic	Copolymer, FDA approved for use in burns and wounds	Synthetic	Thermal	Reversible (good sacrificial ink)	Poor mechanical stability, rapid degradation, requires thermal control to prevent loss of shape	Wu et al. Kolesky et al.[a]
Polyethylene glycol (PEG)	Laxative and excipient for medicines, FDA approved for internal use	Synthetic	UV	Good when combined with other components	Low cell proliferation and adhesion, poor mechanical strength, requires UV (cell damage)	Daly et al.[a] Gao et al.

[a]Denotes the researchers combined hydrogels or other components

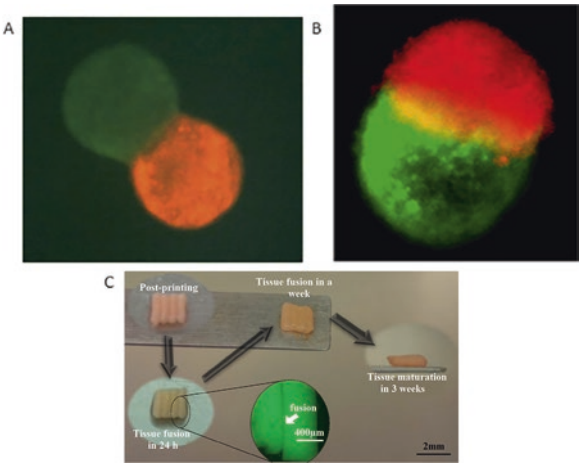

Fig. 11.5 Initial deposition (**a**) and equal amounts of tissue fusion (**b**) of bioprinted spheroids seen on an envelopment assay [Reproduced with permission from Hajdu et al., 2009 [44]. (**c**) Evolving tissue fusion and maturation of printed tissue strands [Reproduced from Yu et al., 2016 [42]

Organ Printing

Computed tomography (CT) or magnetic resonance imaging (MRI) scans can be used to obtain a digital blueprint of the desired organ [33]. Computer-aided design is then used to create a model which is converted to a stereolithography (.stl) file and sliced into layers to create a toolpath for the bioprinter. A G-code is then generated which controls the bioprinting system. Layers are deposited and sequentially added to form a 3D object with printer resolution determining layer thickness. Following deposition the bioink is crosslinked either physically (i.e., thermal energy or ionic interactions) or chemically (i.e., alginate and Ca^{2+}). Physically crosslinked gels are unstable after a period of time and therefore are often used as sacrificial inks, whereas chemically crosslinked gels are stable for prolonged periods. Cells must be able to survive printing shear stress and the crosslinking process, with stem cells more affected than somatic cells [45]. The extrusion rate and bioprinting speed can be adjusted to maximize cell viability and function post-printing. A schematic of the 3D bioprinting process is detailed in Fig. 11.6.

Post-organ Printing

After the printing process is complete, constructs must undergo several weeks to months of tissue maturation prior to being ready for implantation. During maturation, tissues transition from a fluid- to solid-like state with formation of adhesions between cells and ECM production [44]. Scaffold-free constructs undergo a different series of events than scaffold-based constructs. Cells in scaffold-free constructs adhere to each other to minimize energy [47]. Over time, they produce their own ECM and form neotissues. In scaffold-based constructs, fusions start immediately with cell migration and ECM deposition into intervening spaces. As cells proliferate, the surrounding hydrogel matrix degrades. Additionally, if endothelial cells are incorporated into the bioink, small vascular networks begin to form via angiogenesis [32]. All of these changes lead to the morphology and physiology progressing

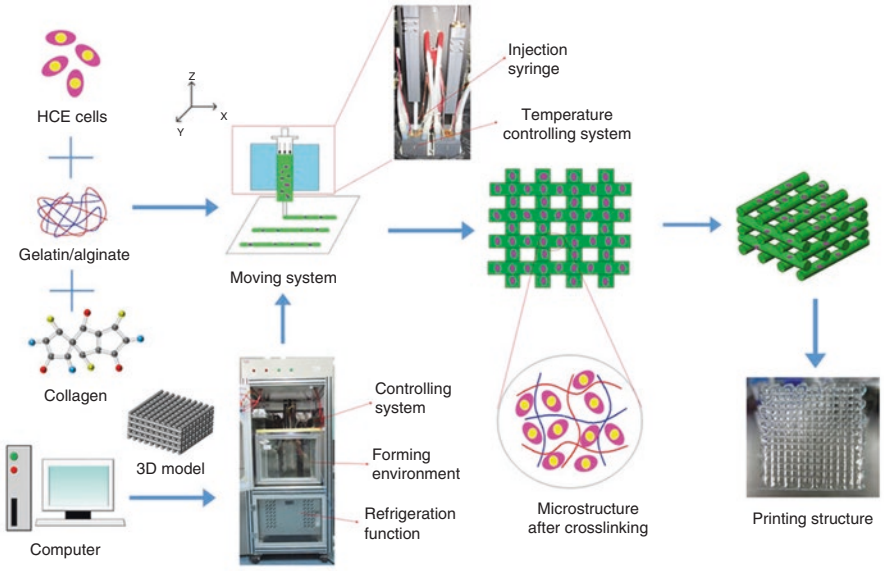

Fig. 11.6 Schematic illustration of the 3D bioprinting process and optical images of the printing setup and constructs [Reproduced from Wu, Z et al. 2016 [46]

towards a native-like state. However, the ultimate evolution to a functional tissue or organ still holds many unknowns. Tissue assessment during maturation must be performed. Measuring the extent of tissue fusion, which is suggestive of maturation, can be performed using an envelopment assay [44]. During an envelopment assay, cells are labeled with fluorescent markers, and then the treated aggregate diameter covered (enveloped) by the control is measured.

Recent Achievements in Stem Cell Bioprinting

Bioprinting has the advantage of enabling precise control over cell placement and the ability to develop complex constructs, which traditional regenerative medicine applications lack [48]. Novel bioprinting techniques have been used to overcome limitations, and there have been many achievements in this rapidly expanding field. Researchers have successfully printed various tissue constructs including but not limited to the vasculature, bone, cartilage, and nerve [49]. Promising research on more complex organs, such as the cardiac and liver tissue, has also been performed.

Vasculature

Much focus has been placed on vascular network formation as this is a limiting factor in creating larger-scale tissue constructs. Cells require oxygen and nutrient delivery as well as waste removal. Larger constructs require vasculature as the maximum diffusion distance is 200 μm [50]. Native vessels contain three histological layers

with various cell types in each. Engineered vessels must at least contain a hollow lumen which is lined with an intimal layer containing endothelial cells and pericytes [45]. All of the printing methods have difficulty fabricating complex hollow structures that do not collapse. One solution for this is the use of a sacrificial material to create channels, which is called indirect bioprinting. Scaffold-based printing of microvasculature involves printing a network of rigid fibers in a hydrogel matrix then selectively removing the fibers to form perfusable channels and seeding the channels with endothelial cells to create vessels. In 2009 a group used agarose to form fibers, added GelMA or PEGMA, and utilized a vacuum to remove the agarose forming channels [51]. Since this may compromise channel structure, other groups have used sacrificial bioinks that can be liquefied by altering the temperature. For example, Pluronic F127 returns to a solution below 4 °C. Kolesky et al. used Pluronic to print channels then endothelialized them with human umbilical vein endothelial cells [52]. On the other hand, Dolati et al. used direct bioprinting with a coaxial nozzle to print scaffold-free perfusable vascular conduits reinforced with carbon nanotubules [53]. The constructs were mechanically strong, cell viability was high, and they produced ECM post-printing. It is currently not feasible to print capillaries due to their small size; therefore, microvascular (~100 μm) channels are printed alongside adjacent endothelial cells with angiogenesis creating the interconnections (Fig. 11.7a) [54].

Bone

Trauma and tumor resections can lead to large amounts of bone loss. Bone grafts are limited to defects <5 cm in size, cause donor morbidity, and often fail due to repetitive stress [58]. Therefore, an engineered improvement would be welcome among clinicians, and various methods have been described. Human nasal inferior turbinate MSCs were added to 3D printed polycaprolactone/poly-lactic-co-glycolic acid/β-tricalcium phosphate scaffolds which showed improved osteo-inductive and osteo-conductive properties in rat calvarial defects when compared to plain scaffolds [59]. Gao et al. used an inkjet printer to print acrylated peptides and PEG with simultaneous photopolymerization using bone marrow MSCs, which showed significantly enhanced osteogenic differentiation with minimal clogging of the print head [60]. The same group also used PEGMA and hydroxyapatite (HA), which showed increased alkaline phosphatase activity with the addition of HA, specifically [58]. Hydroxyapatite has been shown to significantly increase osteogenic differentiation and ECM production [61]. Jakus et al. combined HA microspheres with graphene nano-flakes which significantly upregulated osteogenic gene expression after 14 days [62]. Another group has printed bone matrices loaded with either osteoblasts or MSCs that produce alkaline phosphatase [63]. Recently, a polylactic acid (PLA)-HA composite with β-tricalcium phosphate (β-TCP) and partially demineralized bone matrix seeded with rat bone marrow MSCs were used to fabricate a bone substitute which was then implanted into a critical size calvarial defect in a rat model [64]. The scaffolds showed good osteogenic activity demonstrating their potential application in bone regeneration.

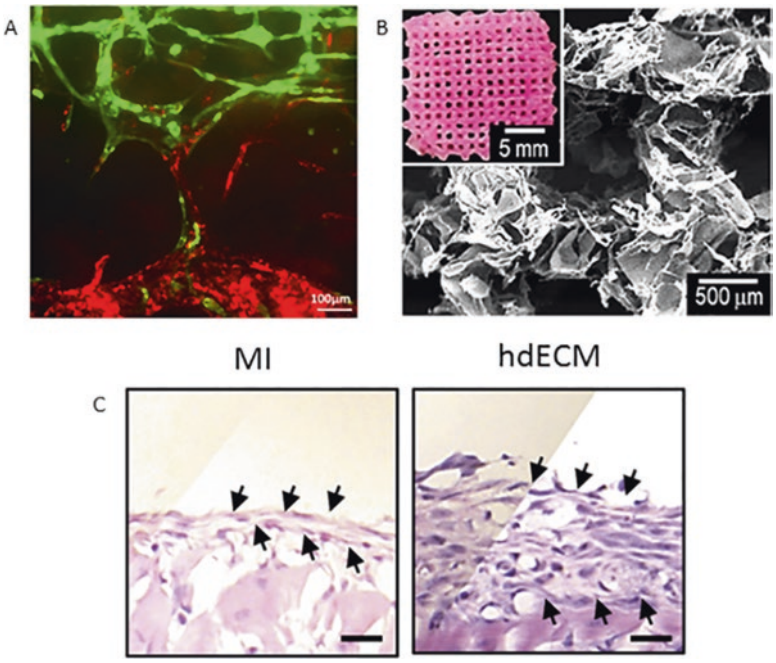

Fig. 11.7 (**a**) An immunohistochemistry image showing integration of large sprouts from the parental vascular channel (*red*) to the capillary network (*green*) [Reproduced and adapted with permission from Lee et al. 2014 [55]. (**b**) Optical and SEM images of the cell-laden mesh. [Reproduced and adapted with permission from Ahn et al. 2015 [56]. (**c**) Representative histological sections of the epicardium following hematoxylin and eosin staining (scale bars, 20 mm, *black arrows* show the epicardial epithelial cell layer at the epicardium tissue) [Reproduced and adapted with permission from Jang et al. 2017 [57]

Cartilage

Cartilage has also been a large area of interest, and many groups have printed chondrocytes. As with other types of tissues, bioink preparations are a major challenge. Daly et al. compared several bioinks and showed that alginate and agarose better support the development of hyaline-like cartilage, whereas GelMA and PEGMA supported fibrocartilage [65]. They also showed that compressive strength was increased and similar to articular cartilage with the addition of polycaprolactone. Cui et al. demonstrated that integration of bioprinted human chondrocyte implants with surrounding native tissue is crucial for long-term stability and enhanced functionality in cartilage repair [66]. Yu et al. used a scaffold-free technique to bioprint cartilage strands in which bovine chondrocytes were injected into long tubes that were then printed using a coaxial micro-extrusion system [42]. Chondrocytes showed minimal damage, and fusion was completed by 7 days

post-printing, representing the possibility for scale-up fabrication. In addition, cells produced their own ECM and underwent further remodeling when implanted into bovine osteochondral tissue defects.

Nerve/Brain

Nerve injuries normally occur after trauma and can have debilitating effects. Their treatments each have their own disadvantages. Owens et al. reported on a novel approach to biofabricate fully biologic nerve grafts composed exclusively of bone marrow MSCs and cell-secreted materials [67]. Hu et al. seeded a GelMA conduit with ADSCs which supported reinnervation of a 10 cm nerve gap in a rat sciatic nerve model achieving functional results similar to an autograft [68]. Gu and colleagues successfully printed functional neural tissue which was GABAergic and responded to calcium [69]. Another group demonstrated improved neurologic function in a zebra fish embryo traumatic brain injury model after the injection of polyurethane hydrogel loaded with neural stem cells [70].

Pancreas

Diabetes is a rapidly growing chronic disease resulting from inadequate insulin production from beta (β)-cells or insufficient response. It affects more than 30 million people in the USA [71] and, when not properly treated, leads to debilitating and life-threatening complications. β-Cell replacement therapies are at the forefront of diabetes research. One group printed ESCs with laminin in a circular patch that were successfully differentiated into pancreatic endoderm-like cells, which expressed β-cell markers PDX1 and NKX6.1 [72]. Yang et al. seeded mouse pancreatic progenitor cells in a 3D printed RGD (arginine-glycine-aspartate)-patterned polystyrene substrate, which resulted in the formation of confluent cell clusters that maintained their pluripotency [73]. More recently, Song et al. fabricated a degradable fibrin gel with PLA housing β-cell clusters derived from human ESCs [74]. The insulin-secreting clusters functioned for 12 weeks when transplanted into mice representing a novel platform for pancreas regeneration.

Liver

Liver transplantation is the only cure for liver failure; however, there are more people waiting for livers than there are donors, leading to many deaths while waiting for a transplant. Faulkner-Jones et al. differentiated iPSCs into hepatocytes after bioprinting, which showed that stem cells maintain their pluripotency during the printing process [75]. Ahn et al. printed a multilayer porous mesh structure made with alginate and ADSCs, which they successfully differentiated into a hepatogenic lineage expressing liver-specific genes (Fig. 11.7b) [56]. This study demonstrated a

new crosslinking system in which the crosslinking agent flowed through a shell and the hydrogel through a core leading to improve gene expression profiles.

Cardiac

Cardiovascular disease is the leading cause of morbidity and mortality with cardiac replacements being developed for years. Some groups have used human cardiomyocyte progenitor cells, which showed increased viability and proliferation while maintaining the commitment to cardiac lineage [76]. Gaebel et al. used polyester urethane urea seeded with human endothelial cells and MSCs to create a cardiac patch which when transplanted into infarcted rat myocardium showed increased vascular formation and functional improvement [77]. Another group also showed functional improvement in a rat myocardial infarction model after implanting an ECM hydrogel with cardiac progenitor cells to create a patch (Fig. 11.7c) [57]. More recently, a group developed a thermally responsive sheet which facilitated easy transfer of cardiac cells after coculture, eliminating the use of proteolytic enzymes, which can affect cell viability and the surrounding ECM [78].

Skin Tissue

Large nonhealing wounds and extensive burns also cost the USA billions of dollars per year [79, 80]. Bone marrow MSCs and amniotic fluid-derived stem cells (AFSCs) mixed with a fibrin and collagen hydrogel have been printed directly into full thickness skin wounds in mice. The defects with AFSCs showed increased wound closure rates and angiogenesis compared to bone marrow MSCs, which was determined to be due to the release of growth factors by AFSCs [81]. Other groups have used human fibroblasts to bioprint skin constructs in both scaffold-free and scaffold-based constructs [82, 83]. Research is also being performed on vascularization of tissue-engineered skin [84].

Challenges and Future Perspective

To date there has only been limited clinical experience with 3D bioprinting. In 2013, emergent FDA approval was obtained for a printed acellular tracheal splint to be placed into an infant with severe tracheomalacia [85]. The incorporation of stem cells will likely increase future therapeutic applications; however, the lack of uniformity of the bioprinting process, cell sources, and bioink preparations requires correction prior to clinical translation. For example, currently no standards exist for the culture and differentiation of stem cells leading to difficulty in comparing study results. Differentiation of stem cells can require prolonged culture periods increasing the risk of microbial contamination despite the use of antibiotics and antimycotics, which adds another concern. Culture media typically require the use of animal

sources, which can predispose patients to zoonosis, as well as raising religious concerns. Ideally standardized culture and differentiation protocols need to be developed which are based on serum-free media. While the introduction of ADSCs can allow for an autologous stem cell source, it is still unknown if prolonged in vitro expansion and differentiation will alter the immunological framework. Bioprinting of clinically relevant tissue volumes continues to be plagued by its own challenges including vascularization, precise patterning of cells, and the need for biocompatible materials [86]. Currently bioprinting research focuses mostly on the concurrent deposition of only a small number of cell types whereas tissues and organs are complex, containing up to 30 cell types. Despite this, bioprinting is a promising technique for regenerative medicine. In the future, we will be able to better treat degenerative diseases and replace diseased/injured tissues with engineered constructs that afford less morbidity than current treatments.

Conclusion

Regenerative medicine is changing the way we treat disease. Stem cells are an important component, with research on ADSCs leading to favorable results. Bioprinting is a promising new technology, which will likely play a large role in regenerative medicine. Many technical challenges still need to be overcome including limitations in resolution, cell distribution, vascularization, and innervation. However, this technology is poised to alleviate the treatment limitations of end-stage organ dysfunction and failure. The use of stem cell therapies and bioprinting in clinical practice will continue to emerge in the upcoming years.

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Cell Therapy for Ophthalmic Diseases

12

Konrad Kauper and Arne Nystuen

Ocular Disease Prevalence

An estimated 180 million people worldwide have ophthalmic disease causing visual impairment. Of these, between 40 and 45 million people are blind [1]. The leading causes of blindness are glaucoma, macular degeneration, cataract, optic nerve atrophy, diabetic retinopathy, and retinitis pigmentosa (RP). These causes account for 74% of all cases of blindness [2]. In the United States, it has been reported that 43% of the population has some form of disease causing impairment of vision, with the greatest percentage, 15%, afflicting those 75 and older [3]. The economic impact of vision impairment is estimated to cost $35 billion USD in health costs in the United States alone [4]. As medical advances continue to extend the lifespan of the world's population and the prevalence of worldwide vision loss increases as a result, new treatment options for eye diseases, such as those based upon cell therapy, must keep pace in an effort to minimize the health-associated economic impact and social burden.

Anatomy and Physiology of the Eye

Capable of perceiving light and distinguishing nearly ten million colors, the human eye is a unique sense organ. As an individual focuses upon an object, the light that enters the eye is refracted and then received by highly specialized retinal cells which translate the stimulus into a nerve impulse. These electrical signals are then transmitted to the brain which interprets those signals.

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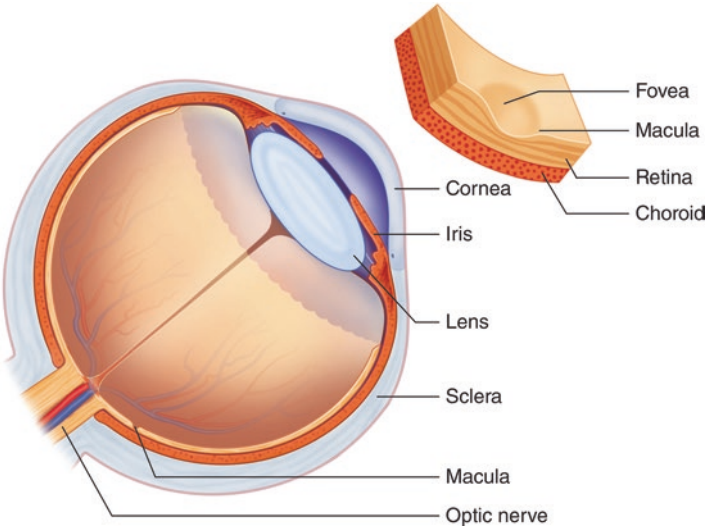

Fig. 12.1 Major structures of the eye with cutaway view of the retina

The structure of the eye can be divided into two main parts: anterior segment and posterior segment (Fig. 12.1). The anterior segment of the eye occupies approximately one-third of the globe, while the remaining portion is considered the posterior segment. Tissues such as the cornea, conjunctiva, aqueous humor, iris, ciliary body, and lens are located in the anterior portion. Structures in the posterior segment of the eye include the sclera, choroid, retinal pigment epithelium, neural retina, optic nerve, and vitreous humor. The anterior and posterior segments of the eye are affected by various vision-threatening diseases. Diseases affecting anterior segment include, but are not limited to glaucoma, allergic conjunctivitis, anterior uveitis, and cataract, while age-related macular degeneration (AMD) , later-stage glaucoma, and diabetic retinopathy are the most prevalent diseases affecting posterior segment of the eye.

Proceeding from the vitreous cavity to the choroid are the well-organized and synaptic layers of the retina including the internal limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), ellipsoid zone containing the rod and cone, inner and outer segments (IS/OS), and retinal pigment epithelium (RPE).

Simplistically, light refracting through the lens and the vitreous of the eye and penetrating the layers of the neural retina initiate a sequence of chemical and electric processes (called phototransduction) which ultimately trigger nerve impulses that are sent to the brain and translated into visual images. The neural retina is composed of three layers of neural cells (photoreceptor cells, bipolar cells, and ganglion cells) within the retina, while the entire retina refers to these three layers plus a layer of the RPE cells [5]. Under normal physiologic conditions, the RPE lies adjacent

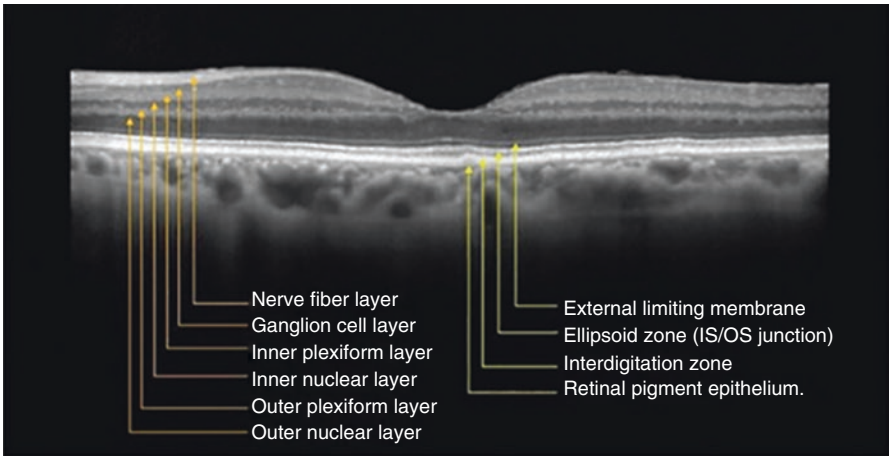

Fig. 12.2 Optical coherence tomography image of a normal retina with anatomical layers listed from inner (vitreous) to outer (choroid)

and firmly attached to the photoreceptor complex and contributes to the proper maintenance of the photoreceptor complex. Tight junctions formed by the RPE allow only selective exchange of nutrients between the choroid and the neural retina. In addition to the formation of the blood–retinal barrier to transport, the RPE is also responsible for phagocytosis of photoreceptor outer segments, retinoid transport, and production of growth factors and pigments [6, 7]. The neuronal cells directly responsive to light are the rod and cone cells of the photoreceptor cell complex. The rods, and associated rhodopsin pigment molecules, and cones, and the associated opsin pigment molecules, absorb the light photons and convert these into biochemical messages [8]. Closest to the inner surface layer of the eye exist the retina ganglion cells which are responsible for processing and sending the impulses received from the photoreceptors to the brain through the optic nerve fibers. It is this complex relationship and interaction between the neural anatomy and vision physiology that is affected by the diseases of the posterior segment and retina.

For the purposes of this chapter, the remaining discussion will focus on posterior segment and in particular the retina of the eye (Fig. 12.2).

Diseases of the Retina

In many ocular disorders, loss of vision is due to a progressive degeneration of neurons in the retina. Therefore, therapeutic strategies are typically aimed at either prevention of the degenerative process or regeneration of retinal neurons. Over 250 genetic disorders affecting vision typically resulting in a debilitating, permanent loss of vision have been identified [9]. Advances in the understanding of molecular disease mechanisms reveal a multitude of different degenerative pathways underpin the genetic heterogeneity of vision loss and have led to the identification a novel

drug targets. Degeneration of the cone or rod photoreceptors can occur by a variety of means including but not limited to defective protein trafficking to the outer segment, defective phototransduction machinery, and metabolic defects. Currently, many of the diseases that result in photoreceptor loss lack therapeutic options. In some of these cases, the therapeutic target, such as a mutant gene, is known; however, the mechanism of action and the delivery of therapeutic have not yet been established. The following are a few examples of ocular disease that will be discussed in the chapter as major targets of cellular and gene-based therapeutic development.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in people 50 years of age and older in the developed world, and the incidence and prevalence are expected to increase substantially as the population ages. In the United States, an estimated 8.5 million people over 40 years of age have some form of AMD. The hallmark of wet AMD, which accounts for about 10% of the total AMD cases, is choroidal neovascularization (CNV). Breaks in the retinal pigment epithelium and Bruch's membrane allow naturally occurring vessels in the choroid to grow aberrantly into the subretinal space. These choroidal neovascular vessels typically leak and bleed, causing exudative or hemorrhagic retinal detachments. Without treatment, the process often evolves into a fibrous scar and resulting severe, irreversible loss of vision. Therapies targeting VEGF have revolutionized the treatment of neovascular AMD; however, there is still a large unmet need for the treatment of dry AMD. Prior to the introduction of anti-VEGF therapies, patients could expect an inexorable loss of vision. With the introduction of drugs such as ranibizumab and aflibercept, improvement in and stabilization of vision loss are expected [10, 11].

Macular Telangiectasia

Macular telangiectasia (MacTel) is a retinal disease with two distinct forms. Type 2 MacTel is the more prevalent form affecting up to 0.1% of the population [12]. Idiopathic macular telangiectasia (MacTel) type 2 is a bilateral degenerative condition of unknown etiology with characteristic neurosensory atrophy and perifoveal telangiectatic vessels which leak on fluorescein angiography. Type 2 MacTel is a bilateral disease characterized by pathological alterations in the juxtafoveal blood vessels and concomitant retinal atrophy. Blood vessels as observed by fundus fluorescein angiography can be dilated and blunted vessels typically without edema. Type 2 MacTel is a late-onset disease with clinical findings that can also include loss of retinal transparency, crystalline deposits in the retina, and lamellar holes. Upon analysis with SD-OCT, a progression of IS/OS lesion break area is apparent and correlated with a loss of visual field sensitivity [13]. Diagnosis typically occurs after

a loss of visual acuity. Type 2 MacTel is a degenerative disease as studies have demonstrated the loss of Müller cells and cones in the macula [14, 15]. Currently, there is no effective treatment for either type of MacTel.

Glaucoma

Glaucoma is genetically a heterogeneous group of diseases characterized by the selective loss of retinal ganglion cells (RGCs), optic nerve degeneration, and associated reduction in the visual field. Progressive visual field loss is associated with elevated intraocular pressure (IOP), although IOP elevation is not necessary for the disease. All currently approved glaucoma medications are directed toward lowering intraocular pressure (IOP); however, degeneration can still occur when IOP is well controlled. The optic nerve comprises of axons of retinal ganglion cells which line the inner retina and serve as the sole output of the retina to the lateral geniculate nucleus of the brainstem. The RGC axons traverse the retina unmyelinated; therefore, conduction of nerve impulses requires a significant load of metabolic energy. The high metabolic demands on the RGCs are thought to place RGCs at risk for degenerative processes. When the optic nerve degenerates, the ability to relay visual information to the brain is impaired. Although IOP has traditionally been used as an endpoint in clinical trials, it is an imperfect surrogate for the clinical outcome (loss of vision) of the disease. Many patients' diseases can progress despite low IOP levels, and others remain stable despite having IOP measurements that are considered high [16–18].

Ocular Drug Delivery Barrier

The tissue barriers of the eye limit the access of drugs to their targets [19]. The corneal and conjunctival epithelial barriers cover the ocular surface. The blood–aqueous barrier, composed of the uveal capillary endothelia and ciliary epithelia, limits the access of compounds from the systemic circulation to the anterior chamber, whereas the blood–retina barrier limits the drug diffusion from the systemic blood to the retina and vice versa. The barrier has two components, outer and inner blood–retina barriers that are formed by the retinal pigment epithelium (RPE) and the tight retinal capillary walls, respectively.

Theoretically, drugs can be delivered to the posterior ocular segment from the systemic blood circulation. Drugs with high permeability characteristics can cross the blood–retina barrier to reach the retina and vitreous [20, 21]; however, only a small fraction of the systemic blood flow circulates through the posterior ocular segment. Due to the limited localized circulation, it is obvious that high drug doses would be necessary to provide therapeutic concentrations at the site of the target tissue, and, as a result, systemic adverse effects are common. A systemic approach to providing drug therapy to the back of the eye is, therefore, not feasible for potent drugs with narrow therapeutic indices. Localized targeting of therapeutics provided

by cell therapy products to specific areas such as the vitreous or directly to the choroid, RPE, ganglion cells, Müller cells, or photoreceptors of the neural retina, however, is an appealing approach.

Ocular Immune Privilege

The limited exchange between the systemic system and the ocular environment, modulated by the tissue barriers of the eye, plays a crucial role in conserving ocular immune privilege by limiting the entry of bloodborne factors and cells into the chambers of the eye. Immune privilege of the eye is thought to be an evolutionary adaptation designed to limit the intraocular expression of immunogenic inflammation [22]. Similar to the anterior chamber of the eye, the posterior segment, which includes the entirety of the vitreous body, enjoys an immune privilege and is subjected to downregulation of immune responses resembling anterior chamber-associated immune deviation (ACAID) [23–25]. Decades of studies have verified that the unique anatomical, cellular, and molecular mechanisms of the eye lessen the chance for graft-destroying immune responses within the eye and, as a direct consequence, provide an acceptable environment for the long-term transplant survival of allogeneic and xenogeneic cell tissue grafts normally rejected at extraocular sites [26, 27].

Intravitreal Administration of Recombinant Proteins

In order to overcome the barriers to delivering therapies to the posterior segment of the eye, direct intraocular injections (Fig. 12.3) have been recently employed for a number of therapeutic recombinant proteins with varying degrees of success. Although not considered as a direct cell-based therapy, the use of recombinant proteins derived from the genetic engineering of an animal or human cell source is, by the very nature of the process, a cellular therapy. Over the past several decades, the identification and subsequent manufacturing development of genetically engineered cell proteins (Table 12.1) have been investigated in dozens of clinical trials with multiple classes of cell-derived proteins demonstrating safety and therapeutic benefit with many of those gaining FDA approval for commercial use in the treatment of eye diseases [28].

Innovations in the design of selective inhibitors of angiogenesis have resulted in the development of unique cell-produced anti-VEGF proteins which have proven effective in the treatment of patients with age-related macular degeneration. The monoclonal antibody bevacizumab (Avastin™) originally designed for oncology but recently used off-label to treat neovascular diseases of the eye [29] and the fragment antibody ranibizumab (Lucentis™), specifically developed for retinal disease therapy, are both administered monthly by intraocular injections in order to bind and sequester vascular endothelial growth factor (VEGF) in the clinical management of the exudative form of age-related macular degeneration and diabetic macular edema.

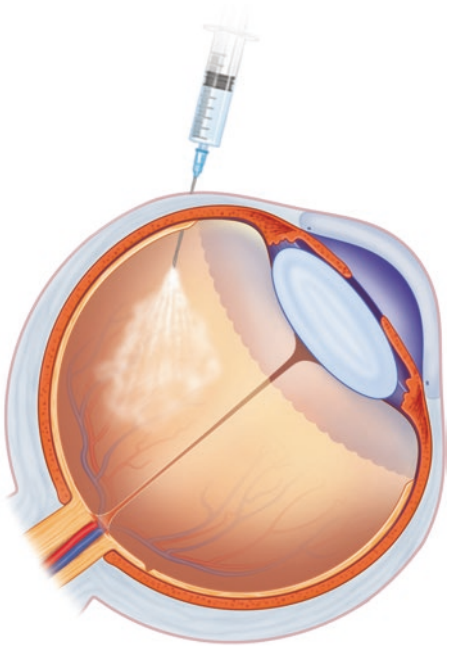

Fig. 12.3 Intraocular injection through the pars plana of an anti-angiogenic, mammalian cell-produced protein

Both ranibizumab and bevacizumab were constructed from the mouse antihuman VEGF monoclonal antibody (mAb) and subsequently humanized [30–32]. Ranibizumab, a 46 kDa fragment of the full-length antibody, is produced and purified from *E. coli* (Genentech USPI), while bevacizumab, a 149 kDa full-length antibody, is produced and purified from Chinese hamster ovary (CHO) cells. Recently, reported outcome improvements to the standard-of-care treatment for AMD patients using a novel VEGF antagonist were introduced by Regeneron Pharmaceuticals (Tarrytown, NY). Aflibercept (Eylea™), a fusion protein comprised of the selected domains of the human VEGF1 and VEGF2 receptors and coupled to constant region (Fc) portion of a human IgG molecule [33], was recently approved for neovascular AMD. The compound has a reported higher binding affinity for all isoforms of human VEGF and potentially longer half-life possibly resulting in fewer requirements for injection frequency. Aflibercept is also a cell-based therapeutic commercially manufactured from a genetically engineered mammalian CHO cell line.

The successes of anti-VEGF recombinant proteins in the management of retinal neovascular diseases have spurned research of cell-based therapeutic targets in diseases currently without treatment. Complement inhibition, for instance, has been proposed as a treatment option following the understanding of the role of the alternative complement pathway in AMD pathogenesis [34, 35]. Factor D is a key component involved in the alternative pathway, and recent data has shown that AMD patients have increased serum levels of factor D [36]. In hopes of developing a treatment for patients with progressive retinal geographic atrophy associated with AMD, Genentech has developed lampalizumab, an antigen-binding fragment

Table 12.1 Select list of investigational or commercial recombinant proteins, produced in mammalian cell lines and used to treat diseases of the eye

Therapeutic class	Therapeutic protein	Manufacturer	Protein design	Treatment indication
Anti-angiogenic factors	Bevacizumab	Genentech/Roche	Monoclonal IgG anti-VEGF antibody	CNV, DME, RVO, PDR
	Ranibizumab[a]	Genentech/Roche	Monoclonal FAB anti-VEGF antibody	CNV, DME, PDR
	Aflibercept	Regeneron/Bayer	Soluble VEGF receptor, Fc fusion protein	CNV, DME, PDR
	RO6867461	Roche	Bispecific monoclonal IgG anti-Ang2/VEGF antibody	CNV, DME, PDR
	NT-503	Neurotech	Soluble VEGF receptor, Fc fusion protein	CNV
	NT-506	Neurotech	Soluble PDGF receptor, Fc fusion protein	CNV
Anti-inflammatory	Infliximab	Janssen/J&J	Anti-TNF chimeric Mab	Uveitis
	Etanercept	Amgen	TNFR receptor, Fc fusion protein	Uveitis
	ESBA105	ESBATech	Anti-TNF α FAB	Uveitis
	EBI-031	Eleven Bio	Monoclonal IgG anti-IL-6 receptor antibody	Uveitis, DME
	Tocilizumab	Hoffmann–La Roche	Monoclonal IgG anti-IL-6 receptor antibody	Uveitis
	Sarilumab	Regeneron/Sanofi	Monoclonal IgG anti-IL-6 receptor antibody	Uveitis
Immune modulator	Lampalizumab	Genentech/Roche	Monoclonal FAB anti-factor D	GA

(continued)

Table 12.1 (continued)

Therapeutic class	Therapeutic protein	Manufacturer	Protein design	Treatment indication
Neurotrophic factors	NT-501	Neurotech	Ciliary neurotrophic factor	RP, GA, macular telangiectasia, glaucoma
	NT-502	Neurotech	Pigment epithelial-derived factor	DME

CNV choroidal neovascularization, *DME* diabetic macular edema, *RVO* retinal vein occlusion, *PDR* proliferative diabetic retinopathy, *GA* geographic atrophy, *RP* retinitis pigmentosa, *IgG* immunoglobulin G, *VEGF* vascular endothelial growth factor, *PDGF* platelet-derived growth factor, *TNF* tumor necrosis factor, *Mab* monoclonal antibody, *FAB* fragment antibody, *Fc* fragment crystallizable

[a]Manufactured in *E. coli*

derived from a humanized monoclonal antibody to factor D. A Phase II clinical trial of lampalizumab provided additional proof that inhibition of factor D can potentially arrest AMD progression. In this study a decrease of approximately 20% in the growth of the size of the atrophy lesion in treated patients was reported with an improvement of up to 44% reduction when the analysis was performed on a subgroup population of the patients with specific genetic markers [37]. A Phase III trial is currently ongoing, and there is a strong possibility that an effective treatment for AMD, using a recombinant protein produced from mammalian cells, could be available within a few years.

Over the past several decades, cytokines, successfully produced by cells using recombinant protein engineering, have been extensively studied as potential neuroprotective agents in retinal disorders. Many preclinical studies have demonstrated the promise of neurotrophic factors and cytokines for the potential neuroprotective effects in diseases such as retinitis pigmentosa, glaucoma, and uveitis [38, 39]. Ciliary neurotrophic factor (CNTF) is one of the most widely studied neuroprotective agents and reported to be one of the most effective at reducing the progressive loss of photoreceptors in animal models [40]. The biologic signaling to responsive neuronal cells occurs following cell membrane binding to CNTF alpha receptors, LIFR and gp130, which in turn activates the Jak/Tyk kinases and Jak/STAT pathways [41]. CNTF can be produced by *E. coli* bacteria and by standard transfection techniques in a variety of mammalian cell lines using plasmids encoding for CNTF. Expression levels are controlled by the amount of gene integration and, in the case of mammalian cell lines, by the further selection of polyclonal or clonal cell lines demonstrating high expression and stability of cytokine production such as CNTF. Unfortunately, the short half-life of CNTF and typically most other cytokines, generally on the order of minutes [42, 43] and the local adverse effects resulting from the high systemic dose requirements necessary to deliver therapeutic concentrations across the blood–retinal barrier, initially prevented clinical development of an effective therapy in treating retinal degenerative disorders using neuroprotective agents such as ciliary neurotrophic factor.

To circumvent the relatively short half-lives of therapeutic proteins and provide a steady-state therapeutic and safe concentration following single intraocular application, novel cell therapy technologies have been developed and advanced into human clinical studies. The following describes the development and promise of treating retinal disorders by distinct intraocular cell targeting or cell-based technologies: gene therapy, cell transplantation, and cell encapsulation.

Therapeutic Gene Delivery to the Retina

Genetic therapeutic strategies for retinal disease are an area of intense research. The broad knowledge base of the genetic causes of vision loss, readily available and well-studied preclinical retinal disease models in various species, and the relative ease of access of the eye have facilitated the development of genetic therapeutic strategies. Therapeutic approaches that deliver exogenous genetic information to the retina, most often targeting defects in photoreceptors (PRs) or retinal pigment epithelial (RPE) cells, in order to replace a defective gene have been studied for a few congenital retinal disorders over the past two decades. A major appeal of gene therapy is the opportunity to directly replace the causative defect in the target cell in order to treat the underlying cause of the disease as opposed to treating a symptom. However, given the genetic heterogeneity of diseases of the retinal and the diversity of affected cell types, the development of approvable therapeutic strategies for each of these disorders will likely require that each mechanism is targeted by specific interventions with separate development strategies. Gene therapy strategies target diseases in different ways either direct replacement of the defective gene, co-opting the patient's cells to produce a therapeutic or genetic knockdown, or editing of a defective or toxic function. To date, the furthest developed strategy is replacement of defective gene function to specific cells.

Gene Delivery Using Viral Vectors

Adeno-associated virus (AAV) is a helper-dependent parvovirus with a ssDNA linear genome [44]. Several characteristics of this vector make it well suited for cell therapy applications. Most importantly in its apparent lack of pathogenicity, AAV has an excellent track record of safety [45, 46]. Secondly, AAV is capable of efficiently transducing nondividing, terminally differentiated cells such as mature photoreceptors. Finally, despite the fact that the vector typically remains episomal, long-term expression (>1 year) of the exogenous DNA has been demonstrated [47, 48]. One limitation is the carrying capacity of the AAV capsid which is approximately 5 kb of ssDNA. Lentiviral vectors hold particular appeal for eye diseases because they integrate permanently and transduce nondividing cells. They also appear to have a low inflammatory potential. Lentiviral vectors do have higher capacity for DNA, which is critical for diseases that require large expression cassettes. Lentiviral vectors have been used successfully in preclinical trials using the

mouse Stargardt model, by transducing a 7 kb ABCA4 expression cassette [49]. The majority of focus has been on AAV vector due to their ability to transduce both photoreceptors and RPE cells efficiently, while in lentiviral vectors, efficient transduction is limited to the RPE.

Several gene therapy programs targeting retinal disease using rAAV are in clinical trials. rAAV2 delivery of RPE65 for Leber congenital amaurosis (LCA2) has been in four clinical trials including a completed Phase III trial. This disorder is characterized by a severe, early-onset retinal degeneration caused by mutations in RPE65. The prevalence of the disease is less than 1 in 1,000,000. Clinical trials have benefited from a preponderance of data from multiple mouse and dog preclinical studies. In the naturally occurring dog model of LCA2, rAAV-RPE65 treatment results in partial restoration of visual function measured by ERG and pupillary response [50]; importantly improvements in ERG were sustained out to 3 years posttreatment. Improvements were also noted histologically with near-normal morphology in mutant dogs restricted to treated areas. This effect seemed to depend on the surgical procedure with the extent of retina receiving benefit being variable. A detailed 3-year follow-up of an open-label Phase I study of 15 LCA2 patients concluded that the treatment was safe and visual function improvements were notable. Visual function measured by full-field stimulus testing (FST), pupillometry, and mobility performance in an obstacle course showed statistically significant improvement in the treated eye. A summary of the results of a randomized Phase III trial performed on a cohort of 31 patients was recently released. Statistically significant improvements in mobility testing were reported in the treated group at 1 year. Secondary endpoints of FST also showed statistically significant improvement; however, visual acuity measured by standard eye chart did not show significance.

Genetic therapies are typically delivered using a subretinal injection, which necessitates retinal detachment. Intravitreal injection is a potential alternative; however, preclinical models have shown that subretinal injection is superior to intravitreal injection in treatment efficacy. AAV is capable of transducing retinal ganglion cells upon intravitreal injection; however, the transgene expression levels are relatively low [51, 52]. In dogs, intravitreal injection of rAAV-RPE65 failed to show improvement in visual function compared to subretinal injection [53]. Despite the improved efficacy, the therapeutic effect does not appear to diffuse far from the site of subretinal injection especially evident in larger animals. AAV DNA and reporter gene expression was not found in other quadrants of the retina that had not been injected [54]. Biodistribution studies have shown that subretinal delivery of rAAV-GFP results in reporter gene expression in the contralateral lateral geniculate neurons, but not glia, and optic radiations [55]. This would suggest that viral DNA found in the contralateral brain is capable of being transported transsynaptically.

The need to deliver the therapeutic gene close to the site of the neurons that are to be treated is complicated by the seemingly high sensitivity of the fovea to detachment as sub-foveal blebs have resulted in the loss of visual acuity. Loss of visual acuity in the fovea may result in the drift of visual fixation by the establishment of a pseudo-fovea. In a 3-year follow-up of several patients that had a subretinal injection involving the fovea, macular volume measured by OCT was lost,

and extra-foveal fixation was reported [56]. BCVA showed varying degrees of significance in all LCA2 trials. For this reason, the use of improvement in visual acuity as a clinical endpoint in gene therapy trials may not reflect efficacy accurately.

Implications of the State of the Target Cell for Gene Delivery

The age of onset and speed of progression of degeneration are key considerations that can impact the effectiveness of the genetic therapy. In both the dog and mouse *CNGB3* achromatopsia models, the effectiveness of gene therapy was diminished when treatment is delivered to older animals [57]. In the dog AAV-h*CNGB3* model, treatment of older dogs (>1 year old) resulted in a significantly reduced level of effectiveness in restoring cone function when compared to treatment of younger dogs (<28 weeks old). Transgene expression in older animals was equivalent to that of younger animals, suggesting that the older photoreceptor in more advanced stages of the disease was not capable of properly integrating the therapeutic hCNGB3 protein into the deteriorating photoreceptor. A similar effect has been noted in the mouse *CNGB3* model where tests of visual acuity showed that mice older than 6 months were similar to untreated controlled mice, while mice treated under 1 month of age showed nearly wild-type acuity [58]. One possible strategy to increase the effectiveness of the gene delivery would be to augment gene therapy with trophic molecules that are thought to dedifferentiate the photoreceptors. For example, bolus intravitreal injection of ciliary neurotrophic factor (CNTF) induces alterations in gene expression of key phototransduction genes, leads to a shortening of the outer segments, and causes functional changes in the retina [59]. The theory was advanced that CNTF-induced changes in the photoreceptor reflected a "deconstructed" state, similar to that of postnatal photoreceptor development.

This theory was tested in studies of the mutant *CNGB3* dog model. Transient deconstruction of the photoreceptor driven by CNTF showed an increased effectiveness of gene transfer [60]. In dogs co-treated with CNTF and rAAV-h*CNGB3*, gene transfer was effective in all older dogs (n = 7) compared to low 10% efficacy in rAAV-h*CNGB3* + PBS-treated animals (n = 10). In both groups the transgene is effectively transduced and expressed in the photoreceptor; however, cone outer segment markers such as CNGA3 and GNAT2 did not localize properly in the PBS co-treated group. Functional rescue measured by ERG was dependent on coadministration of CNTF. This co-treatment strategy potentially mitigates some concerns surrounding the age of treatment. In limited studies, age appeared to have no effect on LCA2 treatment in dogs [61]; however, in LCA2 clinical trials, younger patients had visual function improvement in objective measures such as pupillary light reflex [62].

Cellular Transplantation for Retinal Disease

As retinal degeneration progresses and defective genes providing therapeutic function ultimately die, gene therapy becomes less of an option due to the loss of host cells, and replacement of photoreceptors using a cellular transplant becomes a more viable strategy. A potentially effective approach to cell transplantation uses pluripotent cells to repopulate a damaged cell layer. Pluripotent stem cells that can differentiate in RPE or photoreceptor lineages have been derived from embryonic (ESC) sources as well as induced pluripotent stem cell (iPSC) sources. The main therapeutic characteristics of these cells are their ability to differentiate into the cells of the retina and their ability to self-renew. However, these characteristics are also a major safety concern due to their ability to form teratomas and differentiate into undesired cell fates. Rejection by the immune system is also a concern, although the eye is considered an immune privileged site; certain disease states may compromise the blood–retina barrier allowing transplanted cells to be detected by the immune system. Finally, of critical importance, particularly for photoreceptor replacement, would be that even if the stem cells evade the immune system, differentiate properly, and are non-tumorigenic, they still need to reestablish meaningful synaptic connections in a mature retina. Despite these concerns, stem cell treatment in the eye has some advantages that make evaluation of safety for the treatment of ocular disorders relatively less complicated compared to other organs. Firstly, the eye is accessible both for treatment with subretinal or vitreal injection and for postsurgical monitoring of the transplanted cells. Secondly, the numbers of transplanted cells are relatively small, and therefore the likelihood of systemic adverse effects is low. Finally, many retinal disorders where photoreceptors are lost leave the inner retina intact, and therefore functional reconnection would not require the formation of multiple synaptic connections.

Sources of Stem Cells for Therapeutic Delivery in Retinal Disease

Several clinical trials are underway to evaluate the safety of stem cell therapy in ocular disease. These trials have been supported by years of promising preclinical work in model animals. Stem cells can be classified as multipotent or pluripotent, with multipotent being more limited as to the types of cells into which they can differentiate. Multipotent cells include fetal retinal progenitor cells. Cells have progressed along a retinal fate but have not yet committed to given cell type. Preclinical studies have shown that these cells have the ability to integrate into the host and provide some functional benefit long-term. Multipotent retinal progenitors injected in the *rho* mutant mouse showed long-term expression of rhodopsin and partial rescue of light sensitivity measured by pupillary response [63]. Functional rescue was also shown using the *Gnat1*−/− mouse mutant where transplantation restored structural photoreceptor connections and resulted in light responsive cells [64]. These studies have prompted human clinical trials using fetal retinal progenitors to treat patients with retinitis pigmentosa. Two trials in particular have demonstrated

the lack of severe adverse events due to the therapy [65, 66]. In one trial of RP and AMD patients, a short-term visual acuity benefit was observed at 12 months, which subsequently was lost. Multipotent cells from other lineages have also been investigated. In the rat RCS model, umbilical cord (UC) tissue-derived stem cells have been effective at slowing vision loss by multiple measures [67]. This result prompted Phase I studies to treat RP which were ultimately terminated; however, a new Phase I/II study using transplanted umbilical tissue-derived stem cells to treat AMD is currently ongoing. In the rat model, there was no evidence that the transplanted UC tissue-derived stem cells differentiated into photoreceptors; therefore, it is likely that the preservation that was seen out of 100 days was an effect of secreted trophic factors protecting the retina. In a Phase I/II trial treating Stargardt's macular dystrophy and AMD using RPE derived from human embryonic stem cells, there was no evidence of adverse events due to the transplanted tissue, and long-term survival of grafted cells survival was observed. There was some indication of visual improvement in the best-corrected visual acuity [68].

Induced pluripotent stem cells are generated by reprogramming a differentiated adult cell by forced expression of Oct4, Sox2, Klf4, and c-myc [69]. These cells can then be cultured under various conditions to guide them to a retinal lineage much like ESCs. Photoreceptor and RPE cells have been differentiated from iPSCs [70–72]. In fact three-dimensional eye tissue with functional light-sensitive photoreceptors can be derived from iPSC and cultured in vitro [73]. Preclinical trials of RPE cells derived from human iPSC in rodents have shown measurable improvement in visual function by ERG in the *rd12/rd12* mouse mutant [74]. Clinical trials involving autologous iPSC transplantation have been initiated. Regardless of the type of cell, preclinical studies in some cases have shown a likely benefit from secreted factors from the cells. Collectively, these results point to a strategy, whereby protective factors produced by allogeneic or xenogeneic cells may be delivered in situ to slow retinal degeneration without the need to replace the diseased cells of the patient.

Encapsulated Cell-Based Delivery of Therapeutic Molecules

To avoid the issues of discordant graft acceptance by the host and inefficient dose control and potential safety using gene therapy, and in an effort to overcome the relatively short therapeutic half-lives of protein drugs, an implantable cellular factor was developed. Cellular encapsulation technology was designed to permit therapeutic cells to survive in diseased conditions and to replace or assist the function of the failing target cells or tissue [75, 76]. Initially, microsphere encapsulation delivery platforms, dating back to the 1960s, demonstrated promise in treating systemic diseases such as diabetes and neurodegenerative disorders with allogeneic or xenogeneic cell sources. However, the associated gradual decline in therapeutic potency due to proteolytic degradation of the microsphere material and decreasing cell survival with poor nutrient influx and eventual destruction by the immune system

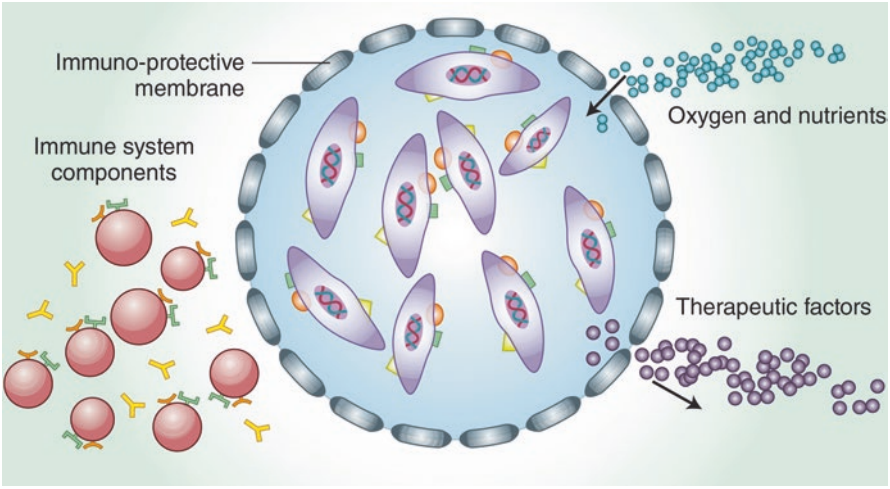

Fig. 12.4 Schematic cross section of encapsulated cell technology (ECT) implant. Thermoplastic hollow fiber membrane encapsulates human retinal pigment epithelial cells genetically engineered to allow exchange of therapeutic factors and nutrients while preventing encapsulated and host cell contact

emphasized the technical complications with this form of technology [77–79]. Thermoplastic cell encapsulation in hollow fiber membranes, called encapsulated cell technology or ECT, produced by Neurotech Pharmaceuticals (Cumberland, RI, USA), overcomes many of these obstacles by providing a fully sealed and self-contained, nondegradable, and retrievable device (Fig. 12.4) capable of supporting survival of a genetically engineered human cell line providing in situ production of a wide class of therapeutic proteins in nutrient-limited conditions such as the human vitreous.

ECT is a novel delivery system consisting of an immortalized, human retinal pigment epithelial cell line that is genetically engineered to endogenously express a selected therapeutic protein at a regulated delivery rate. The adult retinal pigment epithelial-19 (ARPE-19) is a spontaneously arising RPE cell line derived from donor [80]. The ARPE-19 cell line was purified by selective trypsinization of confluent cultures to yield an attachment-dependent, highly epithelial culture of RPE cells. ARPE-19 cells grown in culture have a regular shape of cell and nucleus and have an organized cobblestone appearance. The cells form a monolayer and show contact inhibition and well-defined tight junctions. The cell line has been experimentally shown to be non-tumorigenic in the nude mouse model. This cell line is highly amenable to genetic engineering by stable expression of transgene vectors. Secreted proteins, or proteins that are designed to be secreted by the addition of a signal peptide, can be expressed at relatively high levels from these cells. A variety of proteins have been successfully produced and secreted including trophic factors, fusion proteins, DARPins, Fab fragments, and inhibitory monoclonal antibodies.

Following cell engineering and the establishment of cell lines producing therapeutic protein expression levels, the cells are encapsulated in semipermeable polymer membrane capsules. Each encapsulated cell implant is constructed of a semipermeable polymer outer membrane, medical-grade sealant, and a titanium anchor at one end of each device to facilitate suturing to the sclera following implant through the pars plana and into the vitreous of the eye. Each implant contains an internal polyethylene terephthalate (PET) yarn scaffold that supports human cells. The membrane is porous and manufactured using traditional polymer-phase inversion techniques optimizing the pore size to prevent host cell contact with the encapsulated ECT cells while allowing optimized diffusion of the therapeutic factor produced by the encapsulated cells to the target site. Through a careful selection and combination of polymer chemistry and optimization of processing steps, the membrane is also manufactured to resist protein fouling by serum proteins such as those found in the vitreous [81].

There are several distinct advantages to ECT. Foremost, it offers the potential for any gene encoding a therapeutic protein to be engineered into a cell and therefore has a broad range of applications. In addition, the therapeutic protein is freshly synthesized and released in situ; thus, a relatively small amount of the protein is needed to achieve a therapeutic effect in comparison to pulsatile dosing. Stable, endogenous secretion of the protein assures that the availability of the protein at the target site is not only continuous but also long term. Furthermore, the output of an ECT implant can be controlled to achieve the optimal dose for treatment. Finally, treatment by ECT can be terminated if necessary by simply retrieving the implant. Thus, ECT is a potentially effective means of long-term delivery of proteins and polypeptides to the retina.

Pre-Clinical Neuroprotection Studies of Ciliary Neurotrophic Factor (CNTF) Delivered by ECT

As previously described, there is a large body of evidence to support the use of CNTF as a potential therapeutic for retinal degenerative diseases [82, 83]. Histopathologic studies in naturally occurring and genetically engineered animal models of photoreceptor dysfunction and death, phenotypically modeling retinitis pigmentosa (RP), have indicated the promise of CNTF as an effective therapeutic agent for reducing photoreceptor loss associated with degeneration of the cells of the outer retina. CNTF is one of several neurotrophic factors that are produced endogenously by neurons or Müller cells. In 12 animal models of retinal degeneration of various forms, including environmental light stress and genetic dysfunction models with resulting phenotypes similar to retinitis pigmentosa and geographic atrophy, CNTF has been demonstrated to be highly effective in retarding photoreceptor neuron loss.

During pre-clinical development of ECT, Neurotech confirmed the role of CNTF and retinal neuroprotection in two distinct animal model studies effectively demonstrating that CNTF-producing cells have a protective effect on diseased photoreceptors in the outer nuclear layer (ONL). In the first study using a transgenic rat model

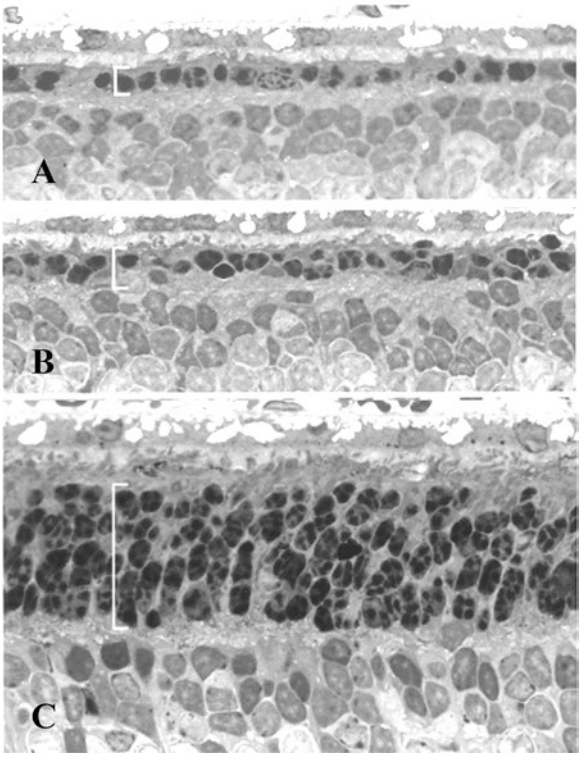

Fig. 12.5 Photomicrograph of retinal layers from S334ter rhodopsin mutation rat. Panel A is the untreated eye demonstrating 1–2 remaining rows of photoreceptor nuclei present. Panel B is the representative eye from the NTC-200 group which did not express CNTF, also showing only 1–2 remaining rows of nuclei. Panel C is the NTC-201-treated group showing a clear effect from delivery of CNTF resulting in protection of 5–6 rows of nuclei in the ONL

of rapid retinal degeneration, S334ter-3 [84], animals were treated by intraocular injection into one eye with either CNTF-producing cells (NTC-201) or wild-type cells (NTC-200) which did not express CNTF. The contralateral eye was not treated and served as a control. Histological evaluation of the retina was used as an end-point in this study and showed severe retinal degeneration in both the untreated and the wild-type (no CNTF) treated eyes with only 1-2 rows of nuclei remaining in the outer nuclear layer (ONL). The CNTF treated eyes, however, showed a statistically significant level of protection of 5-6 rows of nuclei maintained in the ONL. (Fig. 12.5).

In a second study conducted in the *rcd1* dog model, which carries a mutation for the *PDE6B* gene and which is well characterized and recognized as a model for RP, the NT-501 device delivering CNTF demonstrated a statistical protective effect on the photoreceptors in the ONL (Fig. 12.6). Over the course of 14 weeks of continuous treatment, a dose-dependent protection was observed with a maximum effective dose of ≥5 ng/day/eye resulting in the protection of 5–6 rows of the ONL compared to 2–3 rows in the untreated eyes [85].

These studies confirmed that continous delivery of CNTF by encapsulated cells could protect against photoreceptor degeneration in a nimal models and provi ded the scientific data to justify preliminary investigations of ECT delivery of CNTF in controlled human clinical trials

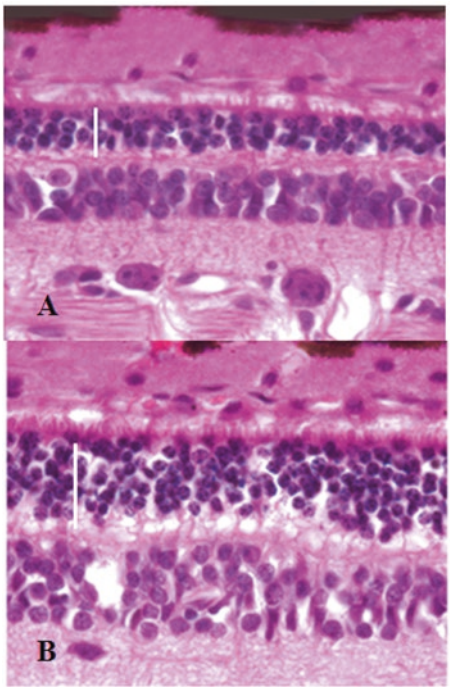

Fig. 12.6 Photomicrograph of photoreceptor layers in the RCD1 dog model of retinitis pigmentosa. Panel A shows the loss of rows of photoreceptor nuclei in the ONL at 14 weeks without treatment, while Panel B shows the results of protection of 5–6 rows following implant of over a 14-week period of NT-501 devices expressing CNTF

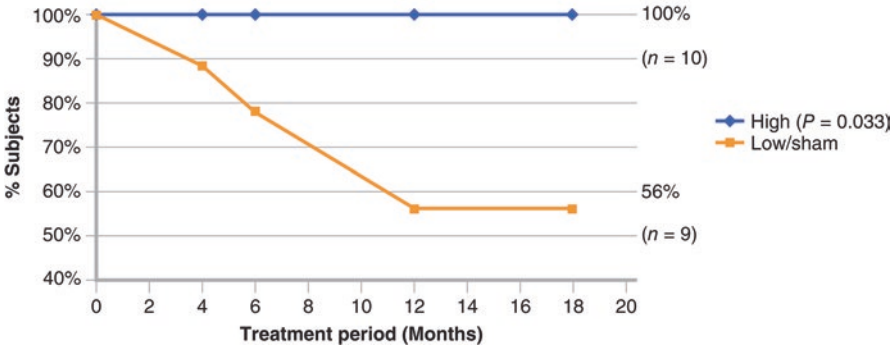

Fig. 12.7 NT-501 treatment stabilizes vision in GA patients. A subgroup analysis of patients who started the trial with a visual acuity of 20/63 showed that 100% of patients receiving the high-dose NT-501 lost less than 15 letters over the course of 18 months, while 43% of those patients in the low-dose and sham group lost greater than 15 letters over the same period

Clinical Evaluation of ECT in Geographic Atrophy and Retinitis Pigmentosa

Consistent with the CNTF levels found to protect photoreceptors in the S334-ter and *rcd1* dog models, patients with geographic atrophy (GA) associated with age-related macular degeneration (AMD) and treated with high-dose ECT implants

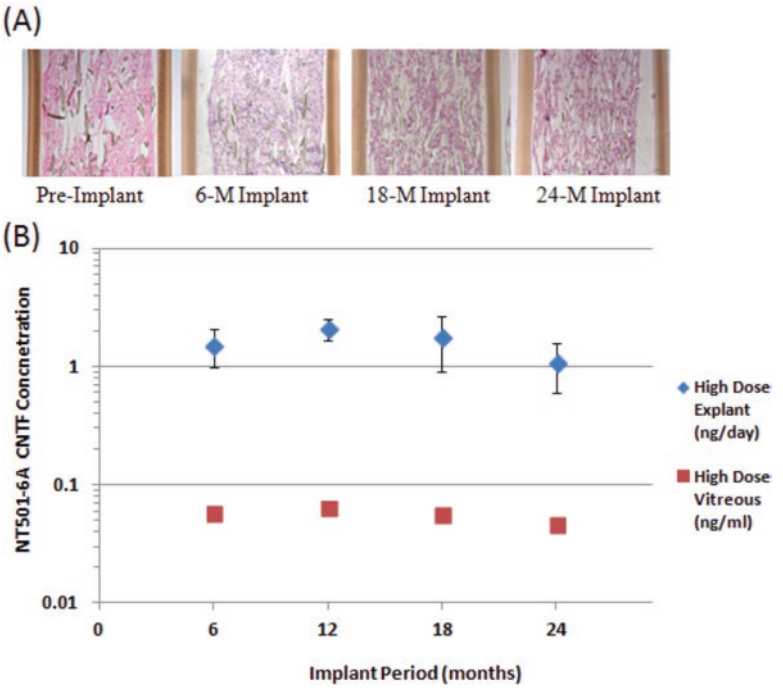

Fig. 12.8 NT-501 stability over the course of 2 years of intraocular implant in human patients. (**a**) Photomicrographs of hematoxylin and eosin-stained histological sections of explant device. Cross sections reveal high cytoplasm to nuclear ratio and high density of cell population over the course of 24 months. (**b**) CNTF levels detected in human vitreous over 6, 12, 18, and 24 months from high-dose implants compared to corresponding explanted device CNTF production rates. The high-dose implant mean rate of production over the course of 24 months was 1.6 ± 0.7 ng/day, and the corresponding mean levels of CNTF detected in the vitreous were 0.051 ± 0.018 ng/mL

(NT-501) producing approximately 20 ng/day of CNTF in vitro, demonstrated structural and also functional improvements in a Phase II clinical trial (www.clinicaltrials.gov, NCT00447954) [86]. In study patients treated with the high-dose NT-501 implant, a statistically significant increase in macular volume was documented between 4 and 12 months (study completion) using optical coherence tomography (OCT). The macular increase was also associated with a trend in stabilization of vision (loss of less than 15 letters) from baseline over 12 months. In a subgroup analysis of patients who started the trial with a visual acuity of 20/63 or better (Fig. 12.7), the high-dose treatment group ($n = 10$) gained a mean of 0.8 letters over the 12-month treatment period compared to the controlled group ($n = 9$), which lost a mean of 9.7 letters ($P < 0.033$).

Over the course of several CNTF clinical trials, the duration of CNTF delivery from the NT-501 intraocular implants was evaluated from device expression and from a small volume of patient vitreous fluid, each collected during an elective explant procedure [87]. The results of these evaluations demonstrated that CNTF

was continuously delivered over the course of a 2-year implant period (Fig. 12.8b) at rates approximating steady-state. In addition, encapsulated cells from the explanted capsules were qualitatively assessed to be viable, containing high cytoplasm to nuclear cell ratios and similar density of cell mass compared to "freshly" manufactured, pre-implant, encapsulated NT-501 devices (Fig. 12.8a). Evaluation of additional follow-up patient explants from the GA trial and from the trial investigating NT-501 treatment for patients with RP (www.clinicaltrials.gov, NCT00447980/NCT00447993) revealed that the devices maintained steady-state expression of CNTF and supported good viability of encapsulated cells for up to 5.5 years following intraocular implant [88]. Based upon the data suggesting that CNTF continuously delivered over the course of a prolonged implant period can protect human cone photoreceptors from degeneration, adaptive optics scanning laser ophthalmoscopy (AOSLO) was used to show that NT-501 treatment for RP patients maintained cone density and individual cone preservation compared to sham-treated eyes [89]. Collectively, these results demonstrate that sustained intraocular delivery of CNTF has the potential to protect the retina from degeneration in humans and further provides evidence for the therapeutic potential of CNTF delivery by encapsulated cell technology.

Potential Role of ECT and Neuroprotection in the Treatment of Glaucoma

Glaucoma treatment strategies are starting to shift from only treating increased intraocular pressure (IOP) to treating the underlying damage to the retina and optic nerve. Attempts to control the pressure in front of the eye require daily applications of IOP-lowering medications; however, the degeneration is actually happening in the posterior segment. The fundamental problem with vision loss and vision restoration in glaucoma is that there is no retinal ganglion cell (RGC) regeneration after optic nerve injury [90]. The cells die, and there is no endogenous replacement. The approaches now being investigated reflect an improved understanding of the underlying pathology of glaucoma. The first step is characterized by axon transport failure following increased IOP. The resulting axonal damage manifests as axonal thinning and dendritic changes leading to retinal ganglion cell death later in the process. Simplistically, optic nerve diseases have an irreversible effect on vision because they cause death of RGCs and loss of their axons. Similar to all other neurons, once death of the RGC occurs, it is irreversible because mammalian neurons do not ordinarily replace themselves. CNTF is a member of the IL-6 cytokine category and shown to be released by retinal glial cells in response to injury [91]. In addition to the investigated protective effects on photoreceptor survival, many years of laboratory research have also shown that CNTF promotes retinal ganglion cell survival and protects retinal ganglion cells from degeneration. In addition to a role in neuroprotection, there is evidence that CNTF may also promote regeneration of diseased cells. Initial in vitro studies attempting to identify compounds that overcome inhibition of embryonic cortical neuron growth identified CNTF as a potential

regenerative factor; subsequent evaluations in optic nerve crush models and axotomized ganglion cell models suggest that CNTF promotes optic nerve and ganglion cell regeneration [39, 92].

To evaluate the possible neuroprotective effects on ganglion cell loss associated with primary open-angle glaucoma (POAG) , a study was initiated in 2012 to evaluate NT-501 as a possible treatment. The Phase I, open-label study enrolled 11 patients with advanced POAG, carefully controlled by IOP medications, yet experiencing optic nerve degeneration and loss of visual fields (www.clinicaltrials.gov, NCT01408472). The results of the 18-month study demonstrated a biological effect following controlled intraocular delivery of CNTF by ECT associated with maintenance of visual field and evidence of increased or regenerated retinal nerve fiber layers compared to untreated controlled eyes. The biological effect from the treatment group in this study was evidenced as early as 1 month post NT-501 implant. A follow-up study of 60 patients randomized 1:1 receiving either a NT-501 implant secreting CNTF or sham implant (suture to the conjunctiva) is currently enrolling patients.

Potential of ECT and Neuroprotection in the Treatment of Macular Telangiectasia

Based upon the accumulating evidence in both animal and human studies confirming CNTF involvement in cell neuroprotection and suggestions of possible therapeutic benefit in the treatment for retinal diseases, NT-501 delivery of CNTF is currently being investigated in macular telangiectasia patients.

Idiopathic macular telangiectasia (MacTel) type 2 is a bilateral degenerative condition of unknown etiology with characteristic neurosensory atrophy and perifoveal telangiectatic vessels which leak on fluorescein angiography [93]. The spectral-domain optical coherence tomography (OCT) assessments show disruption of the photoreceptor inner segment–outer segment junction line (IS/OS line) or ellipsoid zone (EZ) and hypo-reflective cavities in both the inner and outer retina (Fig. 12.9a). “En face” imaging of the OCT retina (Fig. 12.9b) indicates topographic location of lesions, resulting in growth of the lesion as disease progresses. The decline in visual acuity is a direct result of the atrophy of the foveal photoreceptors. Marked functional impairment may occur in very late disease stages with large central areas of photoreceptor atrophy or due to the development of a larger neovascular complex [94, 95]. Currently, there are no treatments for MacTel.

In preclinical studies using animal models with disease pathologies similar to macular telangiectasia, a likely relationship was demonstrated linking hypoxia and oxidative stress, Müller cell loss, subsequent cone-dominated neuronal degeneration, and the potential beneficial role of neuroprotective factors. A transgenic mouse model with conditional retinal Müller glial (RMG) cell ablation was used to establish a link between Müller cell loss and retinal vessel telangiectasia. The selective killing of RMG cells in adult mice led to photoreceptor apoptosis, vascular telangiectasia, blood–retinal barrier breakdown, late intraretinal neovascularization, and

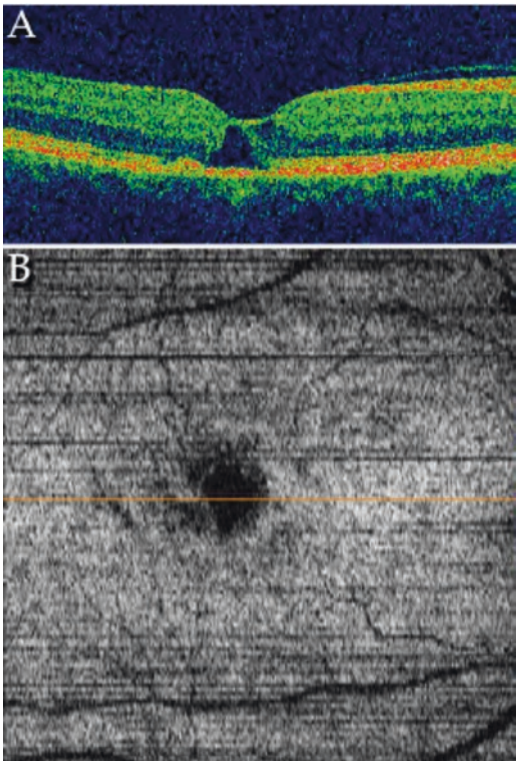

Fig. 12.9 IS/OS lesion in a macular telangiectasia patient. (**a**) Optical coherence tomography B-scan of patient's retina showing the hyporeflective dead area at the IS/OS junction. (**b**) "En face" image of the IS/OS area from (**a**) and black space indicating photoreceptor atrophy

pathologies similar to macular telangiectasia [96, 97]. In a series of separate studies, the effects of hypoxia and energy starvation were explored in the Vldlr−/− mouse model. Lipid uptake and lipid β-oxidation are curtailed in Vldlr−/− retinas leading to decreased retinal glucose uptake and decreased levels of the Krebs cycle intermediate α-ketoglutarate. Consequently, hypoxia-induced factor 1a (Ffar1) is stabilized, and vasoendothelial growth factor A is secreted by Vldlr−/− photoreceptors, giving rise to pathologic neo-vasculature and cone-dominated neuronal degeneration in this model. Intravitreal injections of glial fibrillary acidic protein (GFAP) promoter–driven serotype 2 adeno-associated viruses (AAV-2s) to the Müller glia in the Vldlr−/− mouse delivered neurotrophic factors to photoreceptors located in the outer retina via the retinal spanning Müller cell processes [98]. Targeted delivery of neurotrophic factors to the Müller glial cells significantly reduced photoreceptor degeneration and protected against visual dysfunction in the Vldlr−/− mice.

As a consequence of the increased pathologic understanding of macular telangiectasia and the developing evidence of the potential relationship between photoreceptor dysfunction and neuroprotective factors, NT-501 delivery of CNTF was investigated in a Phase I, open-label study of MacTel patients comparing treated to untreated eyes over a period of 4 years (www.clinicaltrials.gov, NCT01327911). After a total of 4 years, visual acuity data from the open-label exploratory study in

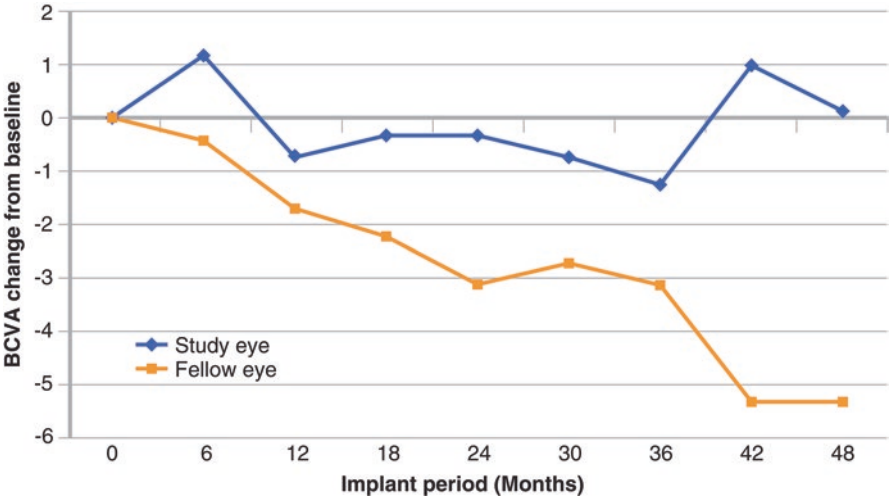

Fig. 12.10 Visual acuity change in Phase I MacTel patients treated with NT-501. At 48 months a mean benefit of approximately five letters (ETDRS) was reported comparing the treated and untreated eyes

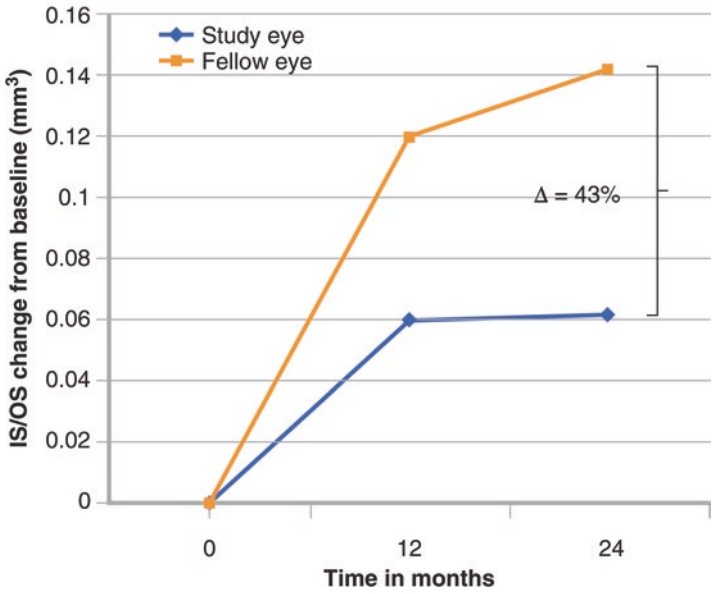

Fig. 12.11 IS/OS lesion change in Phase I MacTel patients treated with NT-501. At 24 months a difference of 43% increase in the size of the IS/OS break was reported in the untreated eyes compared to the controlled eyes

seven subjects demonstrated a five-letter improvement (Fig. 12.10) in the treated eye compared to the untreated, fellow eye, suggesting that NT-501 treatment may slow the rate of progressive visual function loss. Additionally, data showed that progression of the IS/OS break, representative of photoreceptor atrophy, was reduced by 43% in the treated compared to the untreated eyes (Fig. 12.11).

MacTel is a slowly progressing disease with vision loss of approximately one letter (ETDRS) per year as reported in the natural history studies of this disease [99]. Using visual acuity change to determine the clinical effectiveness of NT-501 in macular telangiectasia would therefore require greater than 5 years to establish a 5–10-letter difference in visual effect. In order to reduce the clinical trial duration in MacTel, natural history studies of the disease suggest that the reduction in the progression of IS/OS break is highly correlated with visual function and therefore IS/OS break area could be considered as primary clinical endpoint.

As previously described, clinical signs of MacTel type 2 using OCT imaging include hypo-reflective spaces in the inner and outer retina and a discontinuity (break) in the line commonly attributed to the junctions of the photoreceptor inner and outer segments (EZ) . The correlation of EZ abnormalities with retinal function loss and, thus, the value of the IS/OS line as a sign of photoreceptor integrity and a predictor of visual acuity has been demonstrated in hereditary and idiopathic retinal diseases [13, 100]. Additionally, structural change as a clinical endpoint in ophthalmic trials has precedence in the use of geographic atrophy progression in populations with atrophic (dry) age-related macular degeneration [101].

Using progression of macular lesions as a primary clinical endpoint, a Phase II prospective, multicenter, single-masked sham-controlled study of NT-501 treatment for macular telangiectasia was initiated, and enrollment of 67 subjects occurred over approximately 12 months (www.clinicaltrials.gov, NCT01949324). Results from the 2-year study evaluating NT-501 effect on the progression of visual acuity, reading speed, microperimetry, and area of IS/OS break will be available midyear 2017.

ECT Delivery of Anti-angiogenic Proteins

Available treatments for wet AMD have greatly improved over the last decade with the advent of anti-VEGF injections. However, these treatments are administered monthly to every 6 weeks and must continue for an indefinite period of time in order to manage the disease. This creates a significant burden on the physician, creating crowded waiting rooms and the need for additional support staff to handle patient volume. Additional burdens, mostly economic and practical, are placed on patients, their caregivers, and the healthcare system as a whole. Repeat injections can also cause general eye health to decline, with some patients experiencing increased rates of endophthalmitis (a severe eye infection) and general “injection fatigue” and pain. Moreover, as the disease progresses over many years, most patients eventually become undertreated and lose the vision benefits they may have gained [102–104].

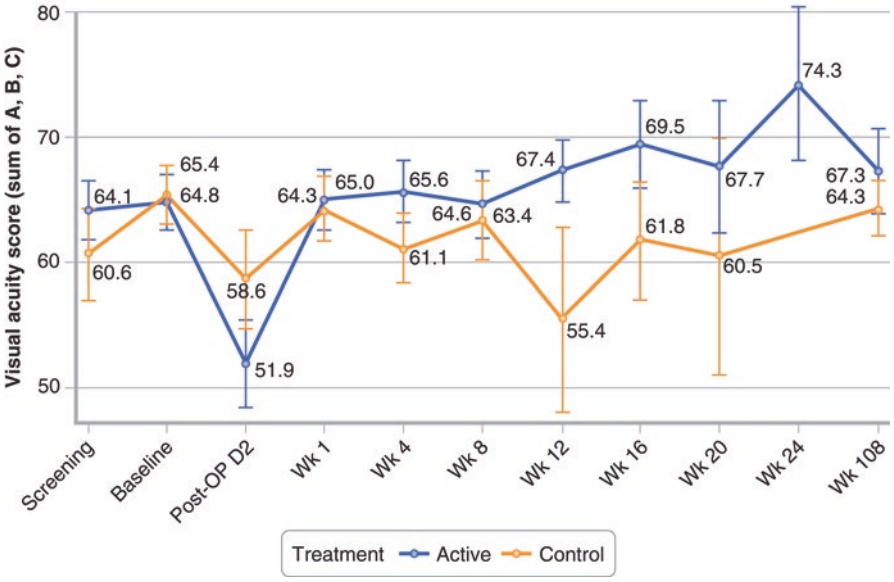

Fig. 12.12 NT-503 delivery of sVEGFR treatment in patients with choroidal neovascularization. Data shows treatment effect on visual acuity using absolute visual acuity score (mean ± SEM) for each visit comparing NT-503-3 expressing sVEGFR at a rate of approximately 2.5 μg/day/eye to control patients dosed with aflibercept every 8 weeks

In an attempt to provide longer duration benefit of anti-VEGF agents, an ECT product, designated NT-503, was developed to continuously produce a soluble VEGF receptor (sVEGFR) fusion protein for the treatment of wet AMD and other neovascular diseases of the retina. In vitro, NT-503 ECT-produced sVEGFR protein binds with picomolar affinity to human VEGFA, similar to the reported values for aflibercept (Eylea®, Regeneron) and with approximately 700 times greater efficiency than ranibizumab (Lucentis®, Genentech) [105]. Following extensive preclinical safety studies, a dose-escalation study of NT-503 ECT was conducted to evaluate three different doses and two ECT configurations. In addition to a dose-dependent reduction in the injection frequency rate, the results from the high-dose group, designated NT-503-3, producing sVEGFR at an intraocular rate of approximately 2.5 μg/day to the eye, resulted in a clinically meaningful improvement in visual acuity and reduction in macular thickening in some patients for at least 20 months. Additionally, the safety of the NT-503 product was established and the implant and procedure well tolerated. A Phase II randomized, active-controlled, masked, multisite study was subsequently conducted to evaluate the safety and efficacy of NT-503 high-dose product compared to bimonthly aflibercept injections provided to patients who previously were treated and responsive to anti-VEGF therapy. Although patients in the NT-503 arm required a greater number of supplemental injections of aflibercept than ideally planned, these patients maintained equivalent or improved stability in visual acuity (Fig. 12.12) than the standard of care and

controlled arm, overall requiring approximately 55% fewer injections following a single ECT implant [106]. Steady-state sVEGFR production levels from the NT-503 implant, assuming a vitreous elimination half-life of 9 days, were verified by mathematically correlating the steady-state implant rate (2.5 μg/day/eye) to the maximum concentrations (27–30 μg/eye) of sVEGFR levels assayed from the vitreous samples of those patient's not requiring supplemental injections.

While evident that the NT-503 ECT product was safe and capable of delivering a soluble VEGF antagonist for greater than 1 year, in order to effectively eliminate the injection burden and effectively treat patients requiring treatment frequency equal to or greater than standard of care to manage their disease, an increase in the amount of sVEGFR currently produced by NT-503 will be required. The efforts to develop a higher producing cell line capable of encapsulated delivery of sVEGFR at rates greater than a minimum effective dose of 4 μg/day, necessary to maintain vision gains in CNV patients [107], are currently ongoing.

Conclusions

Despite the fact that diseases of the retina are complex in their etiology and pathology, basic and clinical research has continued to make incremental progress in the pursuit to identify, treat, and potentially cure the underlying genetic dysfunctions and contributory environmental insults to physiologic vision. Historically, the development of potential therapeutic options for chronic retinal diseases such as macular degeneration, diabetic macular edema, retinitis pigmentosa, glaucoma, and macular telangiectasia has been hindered by the need to overcome the complexities imposed by the ocular anatomy and physiology that normally support optimal vision function. Over the past decade, however, technical and clinical progresses have been achieved in potential therapeutic technologies such as cell manufacture of protein drugs, gene therapy, cell transplantation, and cell encapsulation. These cell-based technologies were identified and then developed to overcome the therapeutic challenges unique to the eye and retina. While further technical development and clinical validations of vitreoretinal surgical techniques, cell, biomaterial, and protein engineering as well as identification of new and improved therapeutic targets will be necessary to ultimately optimize potential cell therapy products, the growing body of data highlights the great promise of cell therapy and foreshadows the eventual widespread acceptance and use of cell therapy products for the treatment of ophthalmic diseases.

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Cell Therapy: Approaching a Partnership with Pharma

13

Vincent Ling

The Drug Discovery Landscape: Circa 2017

It is traditionally held that the pharmaceutical industry is driven by drug discovery. Recently, symptoms of a troubled pharmaceutical economic ecosystem are emerging, as evidenced by regular reports of dubious financial engineering by drug companies: pharma companies are consolidating in megamergers that yield no lasting science productivity benefit [1], pursuing tax inversion strategies by relocating corporate headquarters to foreign territories [2], drug price gouging especially in generic monopolies [3], and diversifying resources into other industries and consumer goods [4]. If handsome financial returns were based on new drug discoveries, would such obtuse corporate maneuvers be considered? One interpretation surfaces: decades of inefficient returns on research investment have culminated in pharma sliding to alternate modes of revenue.

The cost of developing a drug has increased many folds in the past 30 years. Currently it is estimated that an average of $2.6 billion is spent on developing a drug, accounting for clinical failures [5]. A sobering reality that is reflected in this staggering cost is that the clinical failure rate hovers stubbornly at 90%, remaining unchanged during this time period. This non-improvement is occurring despite immense technological advances in biotechnology, availability of genomic data, and increased computation power. From the vantage point of business sustainability, the large pharma industry is under immense pressure to find new medical platform therapies that can sustainably generate multibillion dollar revenues. It is within this challenging economic backdrop that early-stage experimental technologies are being considered as "high-risk/high-return" business propositions. Could a cell-based therapy provide a safer and more potent medical approach to disease? The intrinsic biological nature of cell-cell interactions provides intriguing opportunities

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to explore new therapeutic modalities. With the recent drive to discover innovative biological approaches to medicine, various cell therapies are emerging as the next frontier for corporate investment.

Cell therapy technologies live in an innovation ecosystem dominated by venture-groomed biotech start-ups. Cell therapy competes in the same space with other glamorous high-risk biotechnologies such as gene editing, viral gene therapy, and immuno-oncology. How does a courageous researcher blaze the path from start-up and traverse the research phase "Valley of Death" to the clinic? Generally speaking, a cellular-based start-up company "Cell NewCo" that seeks initial funding attempts to produce a convincing data package worthy of seed stage investment for preclinical research. This data package with business plan should be a sufficient investment aimed at the ultimate goal of achieving proof of medicine in the clinic. Sequential investment beyond preclinical research may range from several million dollars for a phase 1 study to several tens of million dollars for phase 2 studies and to hundreds of millions for phase 3 studies.

Nothing runs without capital investment for research. The first step to obtaining funding is to find investors, and venture capitalists are the primary launch point for funding. Typically the cell technology is discovered in an academic lab and spun out as a biotech, using angel or venture capital funding. It is very common that most, if not all, of the executive teams (CEO, VP business development, etc.) are recruited and controlled by the venture partners. The voice of the founding scientist may be marginalized. In such a setup, it is very clear that the small biotech will be primarily focused on return of VC investment dollars, using the founder's science as an asset vehicle. Even if venture funding occurs, rarely does the biotech start-up receive sufficient funding from the beginning that can approach a clinical study without a large pharma partnership that will pay for and own a piece of the research program. Obtaining pharma partnerships is exceptionally difficult, and the vast majority of new start-ups never complete clinical trials. The VC's responsibility is to then provide access to the major decision-makers in the pharma industry, playing the role of the "matchmaker," dressing up, and introducing investment project opportunities into the purview of pharma executives.

Cell-based new companies (Cell NewCos) fall into three broad categories, each with their unique challenges and business models: regenerative cell therapies, adult cell therapies, and recombinant cell therapies.

Model #1: Regenerative Cell Therapy (The Promise of the Infinite)

It is alluring to dream of a universal therapy based on cellular replacement, widely touted by advocates of regenerative medicine. In this dream, the promise of replacing diseased cells with regenerated healthy cells captures the imagination of the public eye, investors, and many scientists. In reality, there are extreme technical challenges to producing healthy transplantable target cells from primordial ES and iPS cell lines. To date, there is no such clinically validated therapy that is based on

iPS or ES cell lines, due to enormous difficulties associated with the targeted differentiation necessary for target cell production. The production of differentiated target cells is protracted and circuitous, requiring sequential steps of complex differentiation and necessary enrichment of the target cell population product. Such cellular replacement therapy would require defining the growth and differentiation conditions to reproducibly and precisely manufacture the target cell type, and then implanting the cells to the patient for integration, in sufficient quantities to treat disease at the lesion site. This scenario is further followed by the notion that the cell will assume the correct biological function, and therapeutic cellular activity will replace damaged tissue to restore natural function. Such therapies have been proposed for regenerated islet cells for diabetes and cardiac cells for heart diseases and nerve regrowth for spinal injuries, among others. Regenerative stem cell therapies remain vastly challenging in science, complex manufacturing methods, and clinical implementation. Despite these enormous challenges, the advertisement of the "promise" of a universal cellular replacement system continues to draw speculators into this early-stage technology [6].

Model #2: Adult Cell Therapy (Practical Outcomes)

The most established cell therapies are those based on adult cells, because principles used for adult cell transplants are derived from the accumulated clinical knowledge of routine surgical organ transplant. Adult stem cells have a restricted potential to differentiate, and steps that lead to the final desired cell type are far fewer. Hematopoietic stem cell therapies, including bone marrow or cord blood transplantation, have been practical adjunct treatments for cancer chemotherapies for decades, and patient management is well understood. The process of producing hematopoietic stem cells, either by cytokine mobilization from the bone marrow into the blood stream or by bone marrow aspiration, has been effectively used as medical protocol. Successful engraftment of hematopoietic stem cells results in the eventual repopulation of a new hematopoietic system in the patient containing regenerated lymphoid and myeloid cells. Although bone marrow cell transplants are common practice, improvements in efficacy and expansion are still being explored to increase success rates and to minimize graft-versus-host disease that accompanies such procedures.

Mesenchymal stem cells have been tested in 100s of clinical studies for decades. Mesenchymal stem cells are generally derived and expanded from the bone marrow, adipose tissues, and other sources. Upon implantation, these cells can differentiate to a limited set of derivative cells, but generally the biological activities are attributed to growth factor release of the cells and not so much as cellular replacement. Mesenchymal stem cells are reported to have a fantastic number of applications, sometimes with near mystical properties in addressing vast numbers of diseases [7]. These cells have been tested in hematological disease, graft-versus-host disease, organ transplantation, diabetes, inflammatory diseases, and diseases in the liver, kidney, and lung, as well as cardiovascular, bone and cartilage, neurological, and autoimmune diseases. Mesenchymal cell therapy continues to face challenges in

offering a reproducible, robust benefit to disease treated in clinic. Recent clinical advances in mesenchymal stem cell application include the successful phase 3 trial for the treatment of perianal fistulas in Crohn's disease [8].

Model #3: Recombinant Cell Therapy (Implant Bioengineering)

Recombinant cell therapies offer a different biological approach than regenerative or adult stem cell therapies, in that fully differentiated adult cells are genetically altered and subsequently used as a therapeutic vehicle to affect diseases. Recently, CAR-T cell therapy has emerged as one such experimental system, where genetic elements are added to T cells expanded from the cancer patient [9]. The added genetic elements encode chimeric antigen receptors that effectively "reeducate" the T cells toward eradicating specific cancer targets. Early indications show that CAR-T cell therapy offers a powerful cellular therapy to ablate cancers, but recent clinical challenges have emerged, and deaths have occurred related to the generation of an unintended "cytokine storm" as a side effect [10]. Another challenge of commercializing CAR-T cell therapy is the manufacture of personalized T-cell expansion systems with realistic cost structures and reliable scalability [11].

Recombinant ARPE-19 cell line-based systems, which have been in development for the past two decades, offer an alternative approach in cell therapies. ARPE-19 cells are retinal epithelial cell lines, unique in that they can be readily genetically modified and, recently, have been shown to be able to secrete high levels of recombinant proteins, approaching levels similar to biomanufacturing CHO production cell lines [12]. Because ARPE-19 can be clonally expanded to form cell lines under manufacturing standards, and also survive well in nutrient-limiting environments, these cell lines have been used in hollow fiber encapsulation devices for implantation into diseased sites. Thus, the recombinant encapsulated ARPE-19 cells may function as an implanted bioreactor. As the ARPE-19 cells are simultaneously immortal and non-tumorigenic, the implanted encapsulated ARPE-19 device becomes a long-term, localized mini-bioreactor system for chronic diseases. The ARPE-19 encapsulated cell therapy system has been clinically tested in phase 1 and phase 2 studies and applied to eye disease (retinitis pigmentosa [13], wet AMD [14]), neural disease (Alzheimer's disease [15]), and as an immuno-oncology adjuvant (GM-CSF [16]). None of these trials have yet reached proof of medicine. A technical challenge of the ARPE-19 system is the coordinated development of an implant consisting of main components: a biomaterial housing device plus the production of a bioengineered cell line with good stability and high secretion levels in vivo.

Technology Considerations

The vast majority of cell therapy concepts emerge from academic laboratories where the lead principle investigator may have limited exposure to industrial research. Such academics often minimize the need for the depth of knowledge necessary to create a convincing and rigorous framework required for drug development. Frequently, academics and some technology transfer offices overvalue the worth of initial experimental results and naively assume that experimental laboratory observations are valued as a billion dollar drug. Such notions neglect to factor in the >$100M risk involved with development, clinical costs, and time involved for research, not to mention the overarching 90% failure rate in the clinic. The practical reality is that biological technologies that generate experimental observations are seldom exactly reproducible when transferred to other laboratories. In other instances, the technologies proposed are found to be intrinsically unscalable in nature. Medical and surgical procedures used in the successful experimentation on mice may not be practical in clinical settings. Indeed, independent contract research organizations are frequently used by potential investors to verify the claims of the bench researcher of their technology. The problems of experimental irreproducibility and the need for a drug product focus are generally known to the nonclinical academic. But under the right conditions, these challenges can be surmountable, given appropriate funding and capable talent.

To start the process, the inventor generates scientific publications and simultaneously files patents on the novel technology. Afterward, public awareness of the technology is disseminated by the technology transfer office to garner attention from the business world. If the professor is well connected to the venture world, as is often the case from major universities, introduction to capital resources is a simple matter.

Team Considerations

In order to develop a medical product for commercialization, academic technologies are typically spun out into start-up biotech companies or licensed directly by a pharmaceutical company. Typically academic discoveries are too early for pharmaceutical companies to directly invest in, so creation of a start-up company to bridge the science gap is common. Start-up companies are necessary because for-profit investors cannot invest in drug development within a nonprofit academic setting. A company structure is needed to gather the sufficient funds necessary to conduct preclinical and early clinical research and may reach up to $10M or more depending on complexity of the therapeutic candidate.

A founding team is created for the start-up in order to incubate the technology idea into a “pitch” to venture capitalists and other investors. The investment pitch reflects the technology, the experience of the founding team, the development path, and a business projection of the end product. The PI inventor usually becomes scientific advisor or chief scientific officer to the spinout, while the business team

publically promotes the technology to venture and pharma representatives. The best spinout teams consist of individuals that are experienced in business, knowledgeable in the field, command respect from previous successes, and have a very broad network of financial contacts in the investment and pharma world. During launch of the new company, the founding members create a capitalization table detailing ownership shares, based on the degree of participation and economic contribution of each of the team members.

It is very common that during the start-up journey, the scientific vision of the founder will diverge with the CEO's business vision. The scientific founder/PI generally guides the company through technological development and overcomes scientific hurdles encountered and gains a feeling of deep ownership of the project. In reality, the intellectual property rights are assigned to the company upon start-up formation; the scientific founder no longer owns the science, regardless of emotional connection to the project. Thus, it is critical that the academic founder has an aligned vision with the selected CEO. In many cases, altruistic academic investigators are not financially focused, and have a noble desire to see his or her discovery become a medicine that treats the sick and disabled. Meanwhile a CEO primarily thinks about how to morph that same vision into a business proposition that generates maximum revenue for the next 10 years or more. Investors enter this internal dynamic primarily with a profit motive, and thus economics becomes the driving force behind the operation. As the CEO has primary fiduciary responsibility to the investors before all else, business decisions often are made on the investor's behalf, before the needs or benefits of the scientific founder or employees are addressed. At times the direction of the company may diverge or "pivot" from the original intent of the founders. From the investor's perspective, the decision to invest is greatly influenced by the character and ability of the CEO to manage the people and understand the need for investment return. It is understandable under these circumstances that venture capital firms tend to replace founding teams with trusted members from their own circle of entrepreneurs-in-residence, once an investment is made to the spinout company.

One example of "scientific value" versus "business value" where conflict is frequently encountered, is when an academic founder disagrees with a research strategy imposed by the investor-backed CEO. The founder may think the technology is most scientifically suited for a disease "x," while the CEO drives the application of the technology to a more potentially profitable disease "y." These situations may arise, for example, when the CEO discovers the investment case for cell therapy for "x" disease is weak due to factors like small disease population size or low likelihood of medical reimbursement. The CEO may find that the investment case is far higher for disease "y" with a much larger patient population, despite facing other competing technologies. In the mind of the scientific researcher, the case for "y" may not have yet been established, or worse, the actual data is not compelling for the treatment of disease "y." Nevertheless, the CEO will pivot research toward disease "y" to attract investment money. Without investment money for research, no progress can be made toward any therapy, and the biotech NewCo withers. If progress stalls, the value of the intellectual property may slip away, employees may

leave, and the biotech will become a distressed asset—another carcass in the biotech Valley of Death. The best CEOs can finely balance the scientific realities with potential investors, all the while promoting a positive image of the start-up in the public eye.

Another concept that is generally foreign to academics is the valuation of a technology asset. Once in the business dimension, the scientific technology is converted to an economic asset and used for further business modeling and expansion. What is the technology worth? This question is important because if the technology cannot be sold, it can simultaneously be interpreted as "priceless" to some or "worthless" to others. The valuation of an asset will be very different between venture companies and pharma companies simply because the objective of each organization is fundamentally different. For venture and other early-stage investors, the object is return on investment—to polish the asset and "flip" the technology for sale to large pharma for maximum return on investment. Usually the time constraints to return profit are linked to the duration of the VC fund on which the investment is based, and the VCs are obliged to return value to investing limited partners within that time period. Such investment fund duration may be a few to several years in length, but generally the faster the sale and "exit" the better. Frequently, venture capitalists will evaluate the technology and, if they decide to fund, will obtain majority equity ownership in return for investment and then inject their own operational team which may have deeper experience. The new team, in turn, creates an economic "buzz" of greater value than the original founding team. Thus, the founding team, including the inventing researcher and original CEO, may be transitory in the life of the technology. Once Cell NewCo is established with seed stage funding, the CEO and business members reach out to pharmaceutical companies interested in investing in hot new technologies, while the scientific staff labors to conduct experiments to bolster the technology claims that increase company value.

Many academic founders are frequently disappointed that the "quality of the science" is not as large a factor in business development as they initially supposed. Science should sell itself and scientific merit should be obvious, no? In reality, the quality of science may not correspond to perceived value of a NewCo. The root cause of this cognitive disequilibrium may lie in differences in perceived realities between scientists and investor-run operations. The growth of Cell NewCo may be primarily a social endeavor that rests with skilled financial negotiators during bargaining. A brilliant negotiator may perform better with a weaker asset compared to a mediocre negotiator with a strong asset. Scientific acumen and technological knowledge frequently occupy a secondary role within such negotiations between Cell NewCo and potential pharma partners. Up-front payments, equity distribution, milestones, and royalties coupled with the cost of practical implementation, legal framework, and market risk may be the overriding topics during a business discussion. The voice of science and medical need of patients speaking for themselves may be quite distant, or even imperceptible, in such situations.

Entry of a new medical modality, such as cell therapies, will require partnership of Cell NewCo with a large pharma that embraces novelty and risk. The success of this NewCo/pharma partnership is borne by internal project champions of the new

technology within the pharma. Large pharmas with individuals comfortable with risk will overcome the resistance to perceived technological shortcomings and, once in line with internal guidance, may engage with Cell NewCo from different corporate vantage points. Pharmas have professional business development groups that continually scout for the next opportunity for acquisition or partnership. Typically business development representatives attend conferences and investor meetings throughout the year, such as JP Morgan Healthcare or Bio. BD representatives may also be approached by contacts in the industry or prior interactions with members of the Cell NewCo business development group or the company CEO. In other cases, interest in a new therapeutic modality may come from scientific meetings and scientist-to-scientist contacts. As pharma scientists are usually not directly connected to the pharma BD functions, the path of initial business discussions may be more circuitous by percolating upward from the scientific track.

The best method of approaching pharma business development is through a direct connection of the Cell NewCo CEO to a C-level executive at a large pharma. Relationships that develop between executive-level comrades have the highest possibility of continued business negotiations, as the acquisition directive will originate from the C-level of the pharma. Generally such partnerships receive far fewer roadblocks in gaining internal acceptance within the pharma. Sometimes objective analysis of the Cell NewCo may be a perfunctory glance over, compared to true formal due diligence processes required for routine deals. In normal circumstances, business negotiations from science level upward may take up to a year to consummate or just die in committee during due diligence. Regardless of the route taken, fundraising is a full-time effort for Cell NewCo business development.

Without C-level buy-in from a pharma partner, a key necessity for a successful business entry into a large pharma is presence of an “inside” pharma champion for the NewCo’s technology. Such pharma champions are rare, because they bear reputational risk in becoming the internal spokesperson for a novel “risky” technology to their organization. This champion will embrace and drive the novel technology within the company and represent it in the fairest and most favorable light in order to garner support for pharma investment. Such a champion may be a scientist that becomes personally enthralled with the novel therapy or may be a business person mandated to find assets to fill gaps in the pharma portfolio. The motivation behind the champion is also driven by the need and urgency of the pharma. For example, in executive-led partnership initiatives, the assigned champion may agree to represent a high-risk investment in a start-up with shaky data, just because the champion will not face much opposition in driving the executive project forward. Initiatives that are motivated from grassroot individuals may face more daunting scrutiny as the asset may be rejected for myriads of reasons, because vocal opponents may blanche at any minute hypothetical risk. Thus, the road to pharma partnership is filled with multiple hurdles and unpredictable outcomes based on the ever-shifting internal focus and personalities along the journey.

Timing Considerations

As the biotech funding is market based, availability of investments will be linked to the appetite for market speculation. The market is cyclical and biotech investment follows this trend. CEOs of large pharmas tend to rotate out of the company every 4–5 years, and research investment directions may change overnight based on the whim of the incoming CEO through the pharma rotating door. Certain older technologies, once out of favor, may gain resurgence in activity. For example, gene therapy initially encountered early clinical failures that held back further investments in this field for over a decade. Gene therapy is now experiencing comeback in investment and may be attributable to more sophisticated viral vector engineering, specific disease targeting, and perhaps coupled with a new generation of pharma executives that did not experience the first wave of gene therapy failures in the clinic. Likewise, cell-based therapy may also enjoy renewed interest due to the interest in CAR-T technology.

Many times it is glaringly obvious that a Cell NewCo is too early in fund-raising, as their data is incomplete, or key information is not forthcoming. Another hint of possible exclusion from further consideration is the lack of a stable financial footing with respect to the age of the start-up. If a certain type of cell therapy is decades old, why would the pharma representatives think that this technology has changed sufficiently to substantiate a current investment? Are there other non-cell therapy options that now compete in the same disease space that could be considered? For example, regenerated islet cell replacement therapy may easily be a decade or more away from market, factoring in the anticipated hurdles in manufacturing, regulatory considerations, and clinical optimization. Closed-loop mechanical insulin pumps such as the Medtronics MiniMed 670G is slated to be on the market to address diabetes imminently. As the sophistication of electronic-based mechanical devices can improve much more rapidly than biological experimentation, it may be that a first generation islet cell therapy approach for human use will compete against a marketed mechano-electronic insulin system that may be in its fifth generation as a medical device. In such an environment, what is the level of risk justification that will be needed for major pharma investment for discovery and development for a cellular technology that spans 10 years? Another example of competing technology displacing an older technology is the rise of CRISPR-related gene modification. In such gene-editing technologies, original investment and interest in gene-editing zinc finger technologies quickly migrated to spectacular investments in the broader ranging CRISPR technology, all over the space of single year. Will a quickly adaptable technology replace cell therapies in the near future?

Due Diligence Considerations

From the standpoint of the Cell NewCo team, there should be an internal belief that the Cell NewCo's technology is the most outstanding among its peers. Indeed for a start-up to exist, the inventors and business leaders must have unwavering belief in

the supremacy of their technology over competitors. From a pharma's point of view, the technology that is brought to bear is rarely completely unique. Competing companies may have sought attention from the same pharma as well. It is common to attend partnering meetings where multiple variations of cell therapies may be presented to a pharma over the course of a few days or even within a few hours of the same day. The Cell NewCo CEO or head BD representative will passionately pitch that their technology is unique and superior to all others and show "enticing" data hinting that its game-changing data points the way to the next billion dollar therapy. From the standpoint of a typical pharma BD person who is inundated with similar themes in presentations, it is difficult to technically assess the gap between the bullet point slides and true pharmaceutical merit in those presentations.

Assuming follow-up meetings with the pharma are successful, and there is continued interest to follow up, a process called due diligence is performed to assess the reality of the technology, under a signed confidentiality agreement. Corporate due diligence is initiated by a project manager of the pharma (who should not be the project champion) in order to create an unbiased report presenting the strengths and weaknesses of the technology examined. To start this activity, the project manager canvases employees of the pharma for expertise in the technical areas reflected in the technology. A large pharma may have 1000s of employees, so the individuals can usually be found who are knowledgeable in certain aspects of the proposed technology. For cell therapies, the due diligence project manager may find people who are trained in cell biology, cell line manufacturing, or have experience in cell therapy trials. Large pharma discovers scientists involved in due diligence typically focus on the robustness of the data supporting the interaction of the treatment to the molecular target linked to the disease. Such early-stage scientists called to join may be biochemists and assay development specialists. For downstream development due diligence, a representative from manufacturing would be invited, as well as animal model specialists trained in pharmacokinetics, biodistribution, and toxicology. If the cell line asset is approaching IND filing, regulatory specialists would be present to determine IND submission risk to the FDA, clinical specialists to examine clinical trial design and plans, and commercial analysts to examine projected growth and market access. It is not uncommon to have 20 experts or more involved with a formal due diligence effort, each with their own historical bias in drug failure or success, which may lead to positions that are sympathetic or apathetic to the technology proposed.

For Cell NewCo diligence, basic biology questions may arise for the need of cellular immune isolation, predicted longevity of implanted cells, and quantification of treatment potency. How would the cell therapy be effective in the presence of an adaptive immune system that would reject the implanted cells? Can the cell therapy be tested in a xenogeneic animal disease testing model, assuming the cell transplants are of human origin? Once implanted, how long will the cells survive? Hours, days, or months? How will the cells be tracked within the host/patient? How will the potency of the implanted cells be measured? Is there a biomarker that is relevant to cell activity, or is clinical improvement the main endpoint? Typically large pharma, accustomed to biologics or small molecule platforms, expect to receive

straightforward answers to such pharmacokinetic and pharmacodynamic questions, but these questions are notoriously difficult for cell therapy systems to address. For long-term cell therapy, one would need to consider immune avoidance by either an encapsulation system or chemical immunosuppression. Currently immunosuppressants are administered for cadaveric islet cell transplants but typically only when associated with kidney replacement due to organ failure. How would such limitations affect the potential of successful treatment in the broader disease population?

One very important gauntlet of questions comes from the manufacturing segment of a pharma, the CMC (chemical and manufacturing control) representatives. Most academic researchers are not even aware of the existence of CMC and federal regulation involved in the manufacture of a drug. Strict guidelines exist so that production criteria can be established within the specifications of the final stated drug product. Systems need to be in place for quality management to document (good manufacturing practice and good laboratory practice) cell production parameters that can be scaled to serve tens (or hundreds) of thousands of patients a year. The scalability issue for cell therapy may be daunting for certain types of technology. For some regenerative, CAR-T, and MSC cell applications, many rely on autologous administration of cells derived and expanded from the patient. Such individualized therapy will enter the domain of personalized medicine and would need to follow general guidelines where individual rooms or isolated chambers are dedicated to the expansion of single patients. This precaution of cell sample isolation will limit the possibility that cells may be inadvertently cross contaminated with each other during culture, especially when the possibility of pathogens may be carried from one patient to another. The maximum number of patients treatable at any one time would be limited by the number of expansion facilities available for the procedure. In stark contrast, in a pharma setting, a bulk manufacturing production run of a normal drug could produce hundreds of thousands, if not millions, of doses of drug. Current efforts in mechanizing the isolation and expansion of cells are being conducted, but such efforts could never compete in scaling of a chemical or biological drug. The cost of goods (and service) resulting from such cell expansion therapies would be more equivalent to patient-specific surgical device interventions.

For cell therapies that do not require autologous transplant, manufacturing concerns are remarkably different. For such allogeneic cell therapies, a single source of cells may be used for expansion. This strategy has been entertained for certain regenerative cell therapies, MSCs, and recombinant ARPE-19. In such cases, a cell line is selected, expanded, and used to produce a master cell bank in which all derivative “working” cell banks are made for therapeutic use. Guidelines for creating such cell banks require that the initial cells in the master cell bank perform as well as those found in the working cell banks and after expansion to final cells used in duration of the treatment. Unlike chemical drug production, expansion of cells in vitro is associated with naturally arising genetic instabilities, mutation, and selection for rapidly subpopulations within the culturing process. Frequently one rapidly growing mutant cell type within the master cell bank may dominate the final culture. If the output of the desired protein(s) from the cell treatment is altered from the

initial stock cells, that would be a concern for use in a transplantation setting. Thus, expansion of cells for banking necessitates testing for biotherapeutic protein output stability, genetic stability, and lack of tumorigenicity.

Economic Considerations

The business strategy pertaining to cell therapies may differ between treatment types. For some cell-based strategies, cells are administered temporally, and the bioactive factors released or cellular activity invoked, and then cells are naturally eliminated. In such cases, regimens must be established for periodic dosing if the treatment is recurring. For example, allogeneic MSCs are administered to release anti-inflammatory factors at the disease lesion, and natural immune processes from the patient react within a few days to clear the foreign cells from the body. In another example, for regenerative islet implantation and recombinant ARPE-19 cell therapies, application of these cells is generally meant for single, permanent implantation with continual bioactivity for chronic indications, with the expectation that re-dosing may not be desired or necessary.

The cost of treatment depends on many factors such as patient population size, insurance reimbursement authorization, and government policy. Current high-end biologic protein injections routinely cost $2000–$100,000 per dose, and it should be expected that cell therapies, due to their increased complexity in administration, could easily equal or exceed these costs. The pricing model will then depend on the cost of goods, the service procedure necessary to implant and monitor the outcome, and the number of times this process is needed before treatment ends.

Marketing Considerations

Cell therapies require labor and time-intensive development timelines, and sometimes such therapies compete against new mechanical medical device technologies. For example, regenerated islet research has been ongoing for about 15 years, still without positive late phase clinical results. Meanwhile mechanical technologies are being developed that compete with cell therapies for insulin delivery. For example, closed-loop mechanical pump systems are being developed to address diabetes and are often referred to as “artificial” or “bionic” pancreas technology. Instead of using cell therapies, reservoir cartridges of existing insulin and glucagon are loaded into an external pump/sensor device that attaches to the body through minimally invasive tubing. The sensors allow continuous glucose detection and automatically administer insulin injection through micronized fluidic pumping. These mechanical systems can be developed and marketed far more rapidly than a cellular implant system and does not require cellular differentiation or genetic engineering to achieve its effect. Indeed the first generation artificial pancreas system, Medtronic MDT gained FDA approval in September 2016 and sets the first bar for future diabetic cell therapy systems.

Likewise, cell therapy for wet age-related macular degeneration aims to eliminate or reduce the number of recurring intraocular injections of anti-angiogenic biologics. In reality recombinant encapsulated cell implantation is a surgical technology that competes against biological half-life of current injectables. If intraocular injection of anti-angiogenic biologics achieve 60–90-day efficacy before readministration, a patient or doctor would need to choose between a simple drug injection quarterly per year and an invasive surgical procedure that implants an intraocular device into the vitreous. Mechanical pumps with external reservoirs for intraocular delivery are being developed but are much further behind insulin pumps.

Cell Therapy Outlook: A Personal View

When I speak to college-age students interested in pursuing a career in industrial biology, I frequently try to imagine what the future of the pharma ecosystem would look like and what advice I would give to my own children. Given that most industries have adopted the rotating "5-year" executive cycle, and the notion of short-term research appears firmly entrenched as twenty-first century business practice, I generally advise students to aim for learning practical and transferable skills in any field they plunge into. Transferable skills may be those they can use to be employed by another industry or start an unrelated project, in five years time. I also measure cellular therapy research in this light. In many cases new technologies launch from euphoric market bubble cycles, but in reality biological advances generally take 15 years or more to mature to have medical value. Monoclonal antibody drugs as a platform also needed over a decade and a half to develop as medicines. Cellular therapies have already been pursued for over 20 years and have progressed through multiple scientific "generations." Will there finally be a marketed, clinically useful cell therapy that could emerge within the next 5 years? The ever-alluring premise of cellular therapies rests on the notion that cells offer a unique living medical treatment that affords a living, responsive biological advantage other nonliving treatment modalities cannot match. It is this distinction (challenge?) that sets cell therapies apart from small molecules, protein drugs, and drug-delivering medical devices. When cell therapy ideas are centered around using cell types not related to a disease to treat the disease condition (i.e., MSCs to treat neurological maladies), complications that arise may elicit questions regarding treatment regimens and potency issues. However, with the rise of facile cellular engineering with CRISPR technologies, we may entertain that someday we will be able to fine-tune the cellular machinery to generate an appropriate therapeutic response to a specific disease situation. For example, one can imagine a cell therapy where a cell line is engineered to respond to an environmental signal, perhaps the presence of TNF for inflammation. When activated, the TNF-responsive engineered cell can release anti-inflammatory biologics, such as IL10 to counteract the TNF trigger. This circuitry of a living on-demand response could theoretically revolutionize the field of smart drug delivery. In conjunction, advances must be attained in the field of biomaterial engineering that can withstand immune surveillance and attack as a foreign object.

Only then can the use of encapsulated cells as living bioreactors be applied in a therapeutic setting.

These problems are immense, and cell therapy is littered with decades of failure and frequently underestimated by those in drug development and investment circles. It is these ambiguities associated with such biological systems that make it scientifically challenging and inspire new generations of scientists to continue research. I encourage college students intent on pursuing this study, that biology is oftentimes unpredictable and thus never boring. Within this framework, one must gauge their career expectations on what can be accomplished within a 3–5-year industrial cycle, and if lucky, one could be working on the breakthrough of their lifetime.

Disclaimer The opinions expressed in this essay are those of the author and do not reflect the views of the author's past/present affiliations, employers, clients, or educational organizations.

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Stem Cell Therapy in Duchenne Muscular Dystrophy

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Mirella Meregalli, Marzia Belicchi, and Yvan Torrente

Skeletal Muscle Tissue

Architecture of Skeletal Muscle

Skeletal muscle is the most abundant tissue in the vertebrate body, constituting approximately 50% of the body weight. Skeletal muscle is extremely organized at the microscopic level, and skeletal muscle structure is correlated to its function. Skeletal muscle is composed of parallel elements that can be divided into myofibrils (basic rodlike unit of a muscle cell), muscle fibers (a cylindrical, multinucleate cell composed of numerous myofibrils that contracts when stimulated), and fascicles (bundle of skeletal muscle fibers surrounded by perimysium). Skeletal muscle fiber is surrounded by a plasma membrane (sarcolemma), which contains sarcoplasm, the cytoplasm of muscle cells. A muscle fiber is composed of many fibrils, which give the cell its striated appearance. Every muscle fiber makes up from the assembly of 1000 myofibrils, which are composed of different types of proteins: myosin and actin, the contractile proteins; tropomyosins and troponins, the regulatory proteins; and other proteins forming the sarcomere, including the accessory giant proteins titin and nebulin. Myosin (MyHCs) constitutes the thick filaments of myofibrils, and different muscle types are characterized by the composition of different myosin isoforms. Every myosin is composed of two heavy chains that intertwine to form a long spiral tail with two globular heads and four light chains. The heavy and light chains of several myosins intercalate in order to form a tubular rigid structure with

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the elastic heads on the extremity that protrude and function as a hinge during muscle contraction. Actin is a globular protein (G-actin) that forms the thin filaments of the myofibril. Several molecules of actin polymerize to form long filaments (F-actin), and two polymers of F-actin wrap one onto the other to form the thin filaments. The thick and thin filaments are parallel and are interconnected by transversal bridges represented by the heads of the myosins. Every G-actin has a single binding site on one head of the myosin. The disposal of thin and thick filaments in the myofibril gives origin to the alternation of light and dark bands from which derives the term "striated muscle." Every single repetition of band constitutes the sarcomere, the basic unit of striated muscle tissue. The sarcomere structure shows the following features:

- The central bipolar thick filaments are primarily composed of myosin, with each thick filament surrounded by six parallel thin filaments originating from the Z-disc at both ends of the sarcomere.
- Thin filaments from adjacent sarcomeres are cross-linked by α-actinin at the Z-disc (or Z bodies or Z lines).
- Six giant titin proteins lie along the entire length of the thick filament and beyond, spanning the center of the sarcomere to the Z-disc.
- The dark A-band corresponds to the thick filament, and the light I-band is spanned by thin filaments and titin only. The central region of A-band is called H zone.
- The site of attachment for thick filaments is M line. It is the equivalent of Z-disc for the thin filaments.

The average length of a sarcomere is about 2.5 μm (contracted ~1.5 μm, stretched ~3 μm). The unit responsible for contraction is in fact made by a group of fibers and the motor neurons innervating them. Multinucleated contractile cell diameters range from less than 10–100 μm to several centimeters (may be up to 35 cm) in length. Slow oxidative muscle fibers have the smallest diameter, fast oxidative fibers are intermediate in size, and fast glycolytic fibers are the largest. Skeletal muscles boast an abundant supply of blood vessels and nerves. This is directly related to the primary function of skeletal muscle, contraction. Before a skeletal muscle fiber can contract, it has to receive an impulse from a nerve cell. The following steps are involved in the muscle contraction process:

1. Calcium ions and energy-supplying ADP cause rearrangement of the thin filaments.
2. The myosin heads bind to the actin.
3. The myosin heads pull the thin filaments toward the center of the sarcomere.

Once contraction is complete, ATP binds to myosin, the myosin-actin bond is broken, and the myosin head rotates and binds to a new molecule of G-actin to the starting position until the cycle repeats. This cycle simultaneously occurs in many muscle fibers present in the entire muscle tissue. When the myosin head moves, it drives the F-actin to the center of the sarcomere. Myosin releases ADP and returns

to be strictly linked to actin. The cycle is ready to start again. Skeletal muscle fibers are connected to the extracellular matrix (ECM), composed of highly aligned cables of collagen with nanoscale feature sizes which provides structural and functional support to muscle fibers. The connective tissue offers support and protection for delicate cells and allows them to withstand the forces of contraction. It also provides pathways for the passage of blood vessels and nerves. Skeletal muscle fibers may be oriented parallel to the muscle's force-generating axis (known as "longitudinal" architecture), at a fixed angle relative to the force-generating axis (known as "pennate" architecture), or at multiple angles relative to the force-generating axis (known as "multi-pennate" architecture).

Skeletal Muscle Fiber Organization

Muscle fibers are the individual contractile units within muscle, and their type classification improves over the years. The original distinction of fiber type is based on color, red (high myoglobin) and white (less myoglobin), and correlates with speed of contraction (slow vs. fast, respectively) and fatigability (fatigue resistance vs. fatigable, respectively). Muscle fibers can be classified into different types which are correlated to characteristic movement rates, response to neural inputs, and metabolic features [1, 2]. In addition, different classification schemes are based on contractile properties and histologic staining for metabolic enzyme activities as slow oxidative (SO), fast oxidative glycolytic (FOG), and fast glycolytic (FG). Skeletal muscle fibers are generally classified as "slow-twitch fibers" (type 1) and "fast-twitch fibers" (type 2). Furthermore, based on differential myosin heavy chain (MYH) gene expression, there is further classification of fast-twitch fibers into three major subtypes (types 2A, 2X, and 2B). Human skeletal muscles do not show MYH4-expressing type 2B fibers [1]. Hybrid MYH expression in different fibers of a muscle group permits even more subtypes (1/2A, 2A/2X, and 2X/2B), resulting in an almost continuous range of ATP usage and muscle contraction speeds, from the fastest (type 2B) to the slowest (type 1) [1, 3].

Moreover, it is known that, in addition to standard adult MYH, different MYHs are expressed during development and are characteristic of specific muscle groups [1, 2]. A muscle fiber can express a single MyHC isoform (i.e., pure fiber) or co-express multiple isoforms (i.e., hybrid fiber) [4]. The frequency of hybrid fibers increases under various stimuli and relates to high degree of muscle plasticity, with exercise and disuse being prime determinants of muscle fiber type transition [5, 6].

Skeletal muscle fibers also vary in energy production. Type 1 and 2A fibers primarily use oxidative metabolism, and type 2X and 2B fibers primarily rely upon glycolytic metabolism. However, sometimes energy usage is not a strict predictor of fiber type. In addition to MYH expression and cellular metabolism programs, other factors contributing to fiber type identities include multiple components of the sarcomere contractile machinery, such as fast and slow tropomyosin isoforms [7]. Type 1 fibers suited for endurance and slow to fatigue because of ATP-generating oxidative metabolism. Type 2 fibers are efficient for short bursts of speed and power and use both oxidative metabolism and anaerobic metabolism depending on the particular

subtype. These fibers are quicker to fatigue. Recent bioinformatic analyses have revealed that structural proteins often use alternative splice forms in different fiber types [8–10]. Other bioinformatic analyses have identified numerous microRNAs preferentially expressed in slow or fast muscle, providing potential regulatory mechanisms to impart fiber type identity [11, 12]. Finally, the coordinated regulation of fiber type-specific biochemical and physiological systems gives each fiber type unique functional properties. In mammalian skeletal muscles, various fiber types are generally intermixed within a single muscle group, and different muscle groups have variable proportions of fiber types. The proportions of different fiber types are plastic, and muscle fibers have the capacity to modify their phenotypes in order to support muscles adapt to different functions [1–3]. Indeed, fiber type diversity is associated with functional diversity; alterations in muscle fiber types affect contractile, metabolic, and biochemical properties of the muscle. On the other hand, disease conditions, such as muscular dystrophy, involve altered proportions of fiber types.

Exercise-induced changes in fiber type transition are determined by frequent nerve stimulation resulting in an increased duration of elevated cytosolic free Ca^{2+}. It is believed that calcineurin, a calcium- and calmodulin-dependent serine/threonine protein phosphatase, has an effect over fiber type-specific gene regulation. Precisely, selective upregulation of calcineurin promotes type 1 fiber gene products, while inhibition of calcineurin promotes type 2 fiber-specific gene activity [13]. Recent studies have shown that calcineurin activity and the subsequent nuclear translocation of NFAT (nuclear factor of activated T cells) preferentially respond to continued, low-amplitude elevations of $(Ca^{2+})_i$ [14, 15]. In fast myofibers, resting $(Ca^{2+})_i$ is maintained at levels of only 50 nM [16], and the high-amplitude ($\sim$1 μM) calcium transients are too brief to induce calcineurin-stimulated signaling. Chronic stimulation at 10 Hz of the motor nerve innervating fast myofibers results in sustained elevations of $(Ca^{2+})_i$ and fosters fast-to-slow fiber change [17]. Several reports demonstrate that the $(Ca^{2+})_i$ is elevated in mdx mice and DMD human fibers [18].

Webster et al. showed that in human muscles type 2 fibers (2B and 2X) are the first fibers to degenerate and are eventually missing in DMD patients, whereas type 1 fibers are affected relatively late [19]. The type 1 fibers remaining in DMD patients do not show normal features because they can co-express embryonic or fetal MYHs along with slow MYH, which indicates that those fibers have undergone degeneration and regeneration, but these effects in type 1 fibers are not as severe as those observed in type 2 fibers [20]. Moreover, DMD fetal muscle tissues show a delay in maturation of type 2 fibers [21].

Duchenne Muscular Dystrophy (DMD)

Diseases affecting skeletal muscle are defined as myopathies and are caused by a structural or functional alteration of the muscle. Myopathies can be either hereditary or acquired. In particular, the muscular dystrophies (MDs) [22] are genetic disorders characterized by progressive muscle wasting, leading to a variable limitation of

patient's motor capacity, including confinement to a wheelchair and, in most severe forms such as Duchenne muscular dystrophy (DMD), heart and/or respiratory failure. Among myopathies, Duchenne muscular dystrophy is an X-linked recessive genetic muscle disorder [23] caused by mutations in the DMD gene encoding the dystrophin protein, or duplications/deletions of its exons, which give the disease progressive characteristics [24]. Dystrophin is a rod-shaped cytoplasmic protein (427 kD) that imparts structural stability to the plasma membranes of myofibers, so that they are better able to withstand the contraction/relaxation cycles and force generation required of muscle tissue. Dystrophin is primarily involved in stabilizing interactions among sarcolemma, cytoskeleton, and extracellular matrix (ECM) proteins of skeletal and cardiac muscles [25, 26], via the transmembrane dystrophin-glycoprotein complex (DGC) composed of dystroglycan, sarcoglycan, sarcospan, dystrophin, syntrophin, and α-dystrobrevin [27]. It is now clear that the absence of dystrophin compromises the dystrophin-associated proteins (DAP), targeting the membrane and causing a disruption of the link between cytoskeleton and extracellular matrix. This process leads to cell membrane fragility which is eventually damaged during eccentric muscle contraction; in addition, it involves dysregulation of membrane proteins, especially ion channels. Consequently, DMD myofibers show an important perturbation of calcium handling [28, 29]. Moreover, the absence of dystrophin in DMD myofibers and related muscular degeneration progressively lead to contraction-induced membrane damage with release of cytoplasmic contents and stimulation of innate immunity, cycles of myofiber degeneration/regeneration, decrease of the amount and size of myofibers, age-related replacement of muscle by fibrofatty tissue which leads to connective tissue, muscle weakness, and, finally, death. In this sense, it is known that in the early stage of disease, muscle satellite cells could compensate muscle fiber loss, but these progenitors get exhausted in a short time and are no more able to regenerate damaged muscles [30]. This leads to muscular fibrosis, where lost muscle tissue is replaced by fibrous and fatty connective tissue with progressive muscle weakness and atrophy [22]. Unfortunately, DMD patients are in most cases confined to a wheelchair before the age of 12 and eventually die from heart and respiratory failure [22]. There is actually no cure for muscular dystrophies, with the exception of corticosteroids which can slow down the disease in the short term but cause several adverse effect [31]. In the natural evolution, DMD patients mostly die before the age of 20 because of pulmonary insufficiency associated with cardiac complications; however, in latest years, the improvement in patient survival is significant, thanks to development in clinical research and progress in supportive care which have permitted several patients to reach the age of 40. As actual clinical treatment for muscular dystrophies, and in particular in Duchenne muscular dystrophy, is encouraging but still very ambitious and challenging, important progress has been made through the involvement of materials science and stem cell technologies. Stem cells are able to differentiate into several cell types and to generate different tissues [32, 33]. Thus, a variety of stem cell types, described in this chapter, may be used for muscle regeneration.

Human Stem Cell Therapy Candidates: Preclinical and Clinical Studies

Stem cells could be defined in many different ways, but they have two important characteristics: (1) the ability to continuously renew themselves and (2) the ability to differentiate into many cell types [34]. Embryonic stem cells (ESCs), derived from the inner cell mass of blastocysts, are capable to differentiate into all the tissues of the body [35]. Ethical and political issues surrounding ESCs, however, make their use controversial. Yamanaka and co-workers generated a new class of stem cells, called induced pluripotent cells (iPS cells), from adult cells, in particular fibroblasts, by introduction of embryogenesis-related genes [36]. Tissue-specific adult stem cells can be isolated from various sources at different developmental stages; they maintain and replace terminally differentiated cells within their own specific tissue. Several subpopulations of adult stem cells were identified, such as bone marrow-derived stem cells (BMSCs), adipose-derived stem cells (ASCs), mesenchymal stem cells (MSCs), blood- and muscle-derived CD133+ cells, and neural stem cells. In the latest 20 years, stem cell therapy has taken on a fundamental role in the treatment of chronic diseases, which are still incurable by pharmacological or conventional approaches: Duchenne and Becker muscular dystrophies (DMD, BMD) [26, 37], Miyoshi Myopathy (MM) [38] and limb-girdle muscular dystrophies (LGMD-2B) [38], amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), Parkinson's disease (PD), multiple sclerosis (MS), and Alzheimer's disease (AD) [39].

Satellite Cells

Satellite cells are progenitor cells located between the basement membrane and the sarcolemma of the muscle fibers. In physiological muscle conditions, satellite cells are quiescent, they don't differentiate, and they don't undergo cell division. Under specific stimuli originated from the environment, they start to differentiate, to proliferate, and to activate myogenic differentiation in order to form new muscle fibers. In 2005, Montarras et al. purified a predominantly quiescent population of satellite cells expressing Pax3, CD34, and Pax7 from the diaphragm muscle of Pax3-GFP knock-in mice [40]. These cells, once transplanted into irradiated dystrophic mice, are able to form a pool of satellite cells expressing both Pax7 and Pax3 markers [40]. Otherwise, satellite cells were poorly considered in a clinical perspective, because they are difficult to isolate and reluctant to proliferate; many expansion cycles are necessary to obtain a significant number of cells for transplantation experiments. Moreover, the in vitro growth of freshly isolated satellite cells significantly reduced their in vivo myogenic potential (Fig. 14.1).

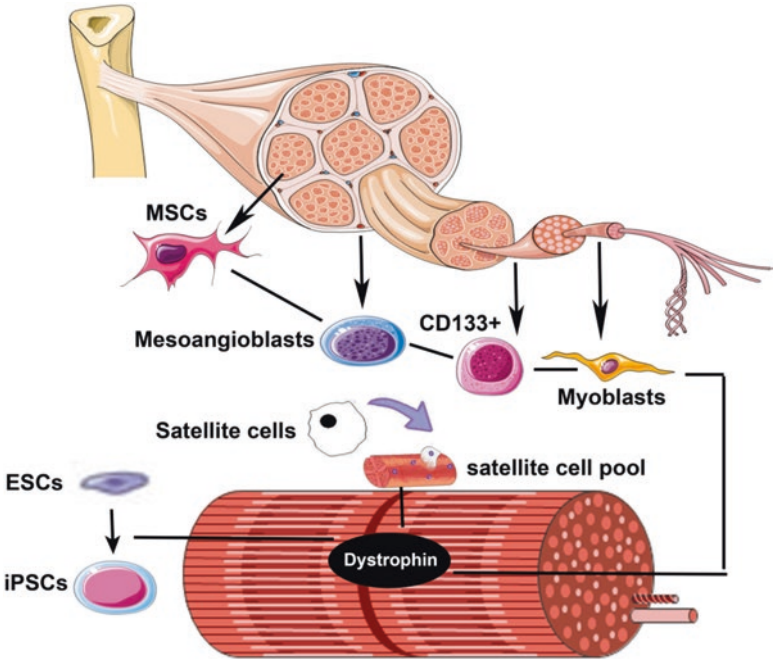

Fig. 14.1 Schematic representation of cell therapy approach in muscular dystrophies

Mesoangioblasts

Mesoangioblasts are multipotent progenitors derived from mesodermal tissues; they are physically associated with the embryonic dorsal aorta both in avian and mammalian species and are able to differentiate in various mesodermal phenotypes. In order to increase the efficiency of muscle reconstitution driven by mesoangioblasts, their capacity of migration into skeletal muscle and their unspecific trapping in the capillary filters of the body were deeply studied (Fig. 14.1). Galvez and colleagues have exposed mesoangioblasts to several cytokines, to stromal-derived factor (SDF)-1 or tumor necrosis factor (TNF)-α, and to α4 integrins or L-selectin to enhance the mesoangioblast migration into dystrophic muscles. In fact, following injection of treated mesoangioblasts into α-sarcoglycan KO mice, they demonstrated a significant reconstitution of muscular fibers [41]. Notably, mesoangioblasts obtained from patients' cardiac muscle biopsies not only differentiate into myocytes but also participate into formation of capillaries [42]. In 2015, Cossu et al. for the first time reported the results of the first-in-human, exploratory, nonrandomized open-label phase I–IIa clinical trial of intra-arterial HLA-matched donor cell transplantation in five DMD patients. Four consecutive escalation doses of donor-derived mesoangioblasts were infused into the limb arteries of the patients under immunosuppressive therapy (tacrolimus). Infusions were performed at 2-month intervals, and clinical and laboratory analysis were conducted. Two months after the

last infusion, a muscle biopsy was performed. The primary endpoint of the study was the safety: only one treated patient showed a thalamic stroke. Moreover, all the included patients were analyzed by MRI in order to follow the progression of the disease. No functional improvements were observed. Unfortunately, low levels of dystrophin were detected. Future implementation of the protocol, together with a younger age of patients, will be needed to improve the efficacy of the approach [43].

CD133+ Stem Cells

CD133 antigen was found to be expressed by hematopoietic-endothelial progenitors, so it was considered as a cell surface marker of adult stem cells [44, 45]. CD133 expressed on endothelial progenitor cells (EPCs) exerted a fundamental function in angiogenesis and neovasculogenesis during both tumor growth and wound healing [46]. In 2004, Torrente et al. demonstrated that CD133+ cells isolated from peripheral blood of normal and dystrophic patients possessed stem cell behavior, as they give rise to small adherent colonies and showed robust telomerase activity. When cultured in myogenic medium, CD133+ stem cells express several myogenic markers suggesting a muscular commitment [47]. Limsuwan et al. obtained clones from CD133+ stem cells and showed that they were able to differentiate into blast cells, macrophages, and CD31+/VE-cadherin+ endothelial cells [48]. A myogenic population expressing CD133 antigen together with CD34, CD45, and KDR from human normal and DMD biopsies was identified. They represented 2% of the total dystrophic muscle-derived nucleated cells. The expression of Pax7, Myf-5, MyoD, m-cadherin, MRF-4, and myogenin markers after 24 days of culture and their ability to differentiate into multinucleated myotubes expressing MyHCs suggested a myogenic commitment. In a clinical setting, Torrente et al. investigated the safety of muscle-derived CD133+ cells after transplantation in DMD muscles [49]. Intramuscular transplantation of muscle-derived CD133+ cells in DMD patients results as a safe procedure and feasible [49]. Following the injection of CD133+ stem cells, the patients showed an increased number of capillaries per muscle fiber and expressed a change in the ratio of slow-to-fast myosin myofibers.

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) arise from the stromal compartment of bone marrow; their main role is to provide a stromal support system for hematopoietic stem cells into the bone marrow [50]. MSCs can be isolated from several tissues as adipose tissue, umbilical cord blood [51], placenta [52], thymus [53], dental pulp [54], muscle tissue biopsies [55] (Fig. 14.1), and/or surgical waste tissues [56]. MSCs are characterized for a high capacity of in vitro expansion and for the expression of CD73, CD90, and CD105 surface markers. MSCs are able to differentiate into adipocytes, chondroblasts, and osteoblasts [57]. Therefore, they differentiate not only into osteogenic, chondrogenic, and adipogenic lineages but also in mesodermal (myocyte, osteocyte, endothelium, adipocyte, cardiomyocyte), ectodermal (neuronal), and

endodermal (hepatic, pancreatic, respiratory epithelium) lineages. The immunomodulatory properties of MSCs were deeply studied in clinical perspectives. MSCs have roles in controlling inflammation by the expression of receptors for SDF-1, lysophosphatidic acid, and CCL2 [58]. Probably the MSC immunosuppressive function could be enhanced through stimulation by Toll-like receptor (TLR) agonists and IFN-γ [59]. The transplantation of MSCs obtained from human liposuctioned fat eliminates the requirement of immunosuppressive drugs [60]. Recent works have demonstrated that human MSC-like cells could be isolated from adult skeletal muscle. The muscle tissue used to harvest the cells was obtained from healthy muscle tissue biopsies [55], surgical waste tissue from orthopedic reconstructions [56], or surgically debrided muscle tissue following orthopedic trauma [56]. These evidences support the idea that skeletal muscle may be an important clinical source of MSCs for use in therapeutic applications [61]. Non-induced MSCs transplanted into injured tissue showed specific differentiation in post-myocardial infarct models [62, 63], in stroke [64], kidney damage [65], pulmonary fibrosis [66], and bone fractures [67]. The expression of cell adhesion molecules, integrins, and chemokine receptors supports the idea that MSCs, in case of injured or inflamed tissues, can home migration across endothelial cell layers where they can enhance wound healing, support tissue regeneration, and restore the BM microenvironment. The mechanism beyond this is not yet fully understood, but it is likely that injured tissue expresses specific receptors or ligands to facilitate trafficking, adhesion, and infiltration. For these reasons, MSCs are used as therapeutic delivery agents to repair damaged tissues [68]. Various in vitro and in vivo studies have evaluated the safety, feasibility, and efficacy of transplanting MSCs for clinical trials [69]. Cellular transplantation into animal models has demonstrated that MSCs can engraft into organs like the liver, bone, lung, and kidney after infusion. Several groups have used these cells to repair infarcted myocardium [70], some of them injected isolated murine MSCs directly into healthy adult myocardium and noted neoangiogenesis near the injection site within 1 week after transplantation [71]. Transplantation of MSCs together with erythropoietin treatment in rat models of acute myocardial infarction has proved to favor capillary density and reduction of infarct size and fibrotic areas, in comparison to groups that received only MSCs [72]. MSCs were shown to enhance the survival of existing myocytes in mice through paracrine mechanisms [73]. Interestingly, Hofstetter and colleagues injected rat MSCs into the spinal cords of paraplegic rats 1 week after injury. They showed that MSCs form bundles that bridge the epicenter of the injury, guiding regeneration through the spinal cord lesion, thus promoting recovery [74]. These data open the hypothesis that the beneficial effect of MSCs in sites of injury could not necessarily involve their differentiation into the regenerating tissue type but rather the local production of growth or other factors, as well as physical attributes like forming guiding strands in the injured spinal cord. Thus, the ability of MSCs to differentiate into various injured tissue, including muscle, as well as ability to complement dystrophin deficiency [75], makes them an attractive therapeutic candidate for DMD. The use of mesenchymal stem cells as inhibitors of inflammation is conceptually appealing. With focus on the bone marrow, it has been speculated that one of their main functions is the protection of hematopoietic precursor from inflammatory damage [76]. This potent activity of MSC is best exemplified in an experiment where these cells

were capable of inhibiting one of the most potent inflammatory processes, septic shock. The investigators demonstrated that administration of bone marrow-derived MSCs was capable of increasing survival in the lethal cecal puncture ligation murine model through modulation of macrophage activity [77]. Moreover, inhibition of chronic inflammatory processes such as models of autoimmune arthritis and diabetes [78], multiple sclerosis [79], and lupus was well documented by syngeneic, or in some cases allogeneic, MSCs. Mechanistically, MSCs play multifactorial roles in controlling inflammation. De Bari et al. published that a small population of the implanted MSCs isolated from human synovial membrane persisted as functional satellite cells in muscle tissues for over 6 months [80]. Human mononuclear cells recovered from first recipient mice displayed in vitro phenotypic and functional properties of primary myoblasts and retained their myogenic capacity into second recipient mice [80]. In addition, transplanted hSM-MSC restored the dystrophin expression at the sarcolemma of muscle fibers and rescued, at least in part, the capacity of mdx mouse muscle cells to produce MGF, a critical factor controlling local muscle maintenance and repair [81] not detectable in dystrophic mdx muscles [81]. Starting from previous work demonstrating their capacity to differentiate into osteoblasts and adipocytes [Erices, 2000 #82;Goodwin, 2001 #83;Romanov, 2003 #84], Gang and collaborators showed that MSCs isolated from human umbilical cord blood (UCB-MSCs) were capable to develop into skeletal muscle [82]. They demonstrated that these cells—during myogenic induction by myogenic medium—can express two fundamental muscle-specific transcription factors, such as MyoD and myogenin. Moreover, they observed weak myosin expression in 3-week cultures after myogenic induction and significantly enhanced expression at 6 weeks, when more than half of the cells were myosin positive [82].

Fibro-/Adipogenic Progenitors (FAPs)

In 2010, Joe et al. described a murine lin$^-$α7$^-$Sca-1$^+$ population positive for the expression of the surface PDGFRα and which represent up to the 85% of muscle-resident PDGFRα-expressing cells in undamaged muscle and up to 98% in damaged muscle. They refer to this muscle-derived subpopulation as fibro/adipogenic progenitors (FAPs) [83]. They subcutaneously or intramuscularly transplanted a purified population of FAPs in a murine model of fatty infiltration (muscle injected with glycerol) and in healthy mice, and they demonstrated that they are able to generate ectopic white fat only into the fatty infiltration model, not in healthy one. These evidences demonstrated that this subpopulation is quiescent in intact muscle but proliferates efficiently in response to damage. FAPs do not generate myofibers alone but can increase the differentiation potential of primary myogenic progenitors in co-culture experiments. FAPs could provide a source of pro-differentiation signals for proliferating myogenic progenitors but only after muscle damage [83]. Within muscle, myofibroblasts express α-smooth muscle actin (α-SMA) and play a fundamental role in tissues affected by fibrosis. Particularly, FAPs express PDGFR-α and the transcription factor Tcf4; it seems that these characteristics determine the role of FAPs in connective tissue synthesis and in the origin of myofibroblasts. Contreras et al. showed

that cells positive for Tcf4 and PDGFR-α are expressed in normal skeletal muscle and are increased in damaged tissues as dystrophic muscle (muscles of mdx mice), in muscle after sciatic denervation, and in muscles subjected to chronic damage [84].Up to now, no evidences of expression of FAPs in human muscles were showed yet.

Induced Pluripotent Stem Cells

Embryonic stem cells (ESC) are pluripotent cells derived from the early embryo. They are able to proliferate over prolonged periods of culture remaining undifferentiated and maintaining a stable karyotype [85–87]. They differentiate into all embryonic germ layers and share self-renewal, immortality, and pluripotency abilities. According to these findings, ESCs are feasible tools for therapeutic interventions in neuromuscular and neurodegenerative diseases (Fig. 14.1). However, the major limitation in this approach is due to the teratoma formation after ESC injection. The genetic stability of hESC lines over longer time periods is not well understood so that they can proliferate without control accumulating aberrant modifications. This way, all undifferentiated cells need to be removed from a graft. Furthermore, blastocyst destruction and oocyte donation that are needed to obtain ESCs implicate fundamental moral and ethical problems. Recently, induced pluripotent stem cells (iPSCs) have been derived from murine tissues and human cells [36, 88, 89] introducing transcription factors, expressed in undifferentiated ES cells, in differentiated cells like fibroblasts apt to reprogram adult cells. These cells show the ability to differentiate into all adult cell types. However, the overexpression of certain oncogenic genes such as c-myc caused the transformation of iPSCs into tumorigenic cells [36]. For this reason, Nakagawa et al. have generated pluripotent stem cells without c-myc overexpression, but, unfortunately, the efficiency was low [88]. The generation of patient-specific iPSCs overcomes the ethical concerns associated with the use of embryonic or fetal material, avoiding the risk of immune rejection [90]. One of the principal problems linked to the use of iPSCs for clinical application is that parts of the viral genome that is used to reprogram the cells are oncogenes. To overcome this issue, new protocols were standardized in order to generate iPSCs without viral integration [90, 91]. Large production protocols for safe proliferation of iPSCs and technological improvements for their differentiation are needed to render these cells commercially feasible [92]. Nowadays, no unique cell type maintained, amplified in vitro, and injected to the patients without immune rejection has been described. The most promising tool in terms of myogenic potential seems to be the human iPSCs. The creation of hiPSCs suggests the possibility to create personalized medical treatment involving the generation of these cells for each patient. Costs for treating diseases with devastating evolution as DMD represent a significant burden for national health systems, so patient-tailored approaches could be variably accessible depending on the country and national healthcare organization. One solution may be the development of haplobanks matching the largest majority of the population, as suggested by Wilmut et al. in 2015. Obviously, these haplobanks have to respect genetic differences between populations: ideally, it will be necessary to have specific haplobanks for Caucasians or Japanese or African population.

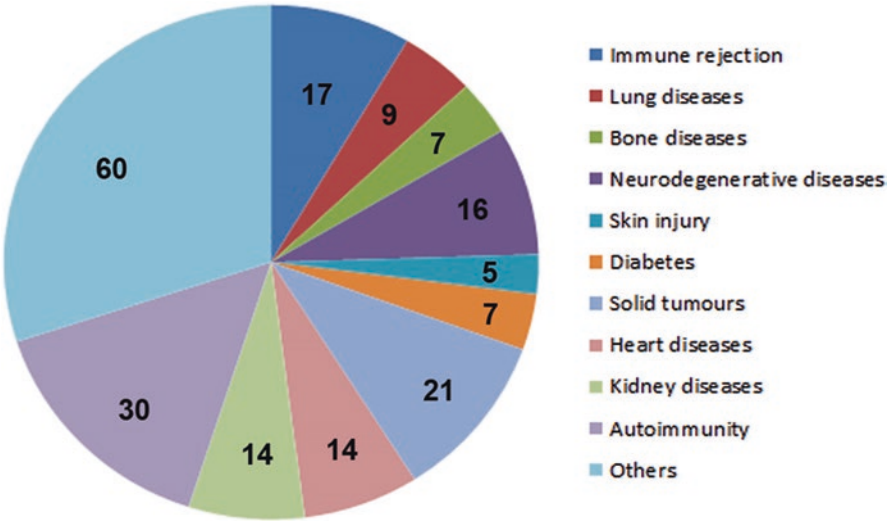

Fig. 14.2 Diseases being addressed using mesenchymal stem cells (MSC) for clinical trials ($n = 200$)

Conclusions

The most important requisite of the cell-based therapeutic approach is to replace damaged cells with stem cells to improve patient's pathology. Skeletal muscle is one of the most plentiful tissues in the body, accounting for approximately one third of body weight in a healthy individual [93]. The capacity of muscle to autorepair after injury suggests that it serves as a reservoir for cells that participate to tissue regeneration processes [94]. Different muscle-derived stem cell populations were isolated and characterized for their ability to differentiate into multiple cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts, and endothelial cells. They could be obtained via minimally invasive muscle biopsy from healthy and dystrophic muscle. In particular, MSCs can be also isolated from muscle tissue following traumatic injury and could provide surgeons with clinically versatile populations of stem cells.

In this chapter, we have described several stem cell populations derived from human skeletal muscle able to regenerate a variety of tissue types, many of which are nearing clinical translation. These findings show the great potential of the cell therapy approach to treat muscular dystrophy and at the same time underline the critical importance of research efforts directed at elucidating and harnessing the regenerative properties of skeletal muscle-derived stem and progenitor cells. Nowadays, MSCs are one of the most suitable subpopulations for clinical use. More than 5000 clinical trials using MSCs were described, in Fig. 14.2 we showed the last 200 trials shared on diseases typology (Table 14.1).

Table 14.1 Ongoing and completed studies related to the injection of stem cells in patients affected by DMD

Study	Pathology	Stem cells	Phase	Sponsor	Identifier
Allogeneic transplantation of human umbilical cord mesenchymal stem cells (UC-MSC) for a single male patient with Duchenne muscular dystrophy (DMD)	DMD	UC-MSC	Safety/efficacy	Allergy and Asthma Consultants, Wichita, Kansas	NCT02235844
Phase I/II study of stem cell therapy in patients with Duchenne muscular dystrophy	DMD	UC-MSC	Safety/efficacy study, phase I/II	Shenzhen Beike Biotechnology Co., Ltd., China	NCT01610440
Efficacy of allogenic mesenchymal stem cell therapy in ambulatory and non-ambulatory children with Duchenne muscular dystrophy	DMD	UC-MSC	Safety/efficacy study, phase I-II	University of Gaziantep, Turkey	NCT02484560
Transplantation of myoblasts to Duchenne muscular dystrophy (DMD) patients	DMD	Myoblasts	Safety/efficacy study, phase I-II	Centre Hospitalier Universitaire de Québec, CHU de Québec, Canada	NCT02196467
Efficacy of umbilical cord mesenchymal stem cells in Duchenne muscular dystrophy	DMD	UC-MSC	Safety/efficacy study, phase I-II	Acibadem University, Istanbul, Turkey	NCT02285673

(continued)

Table 14.1 (continued)

Study	Pathology	Stem cells	Phase	Sponsor	Identifier
The role of autologous bone marrow mononuclear cell therapy in Duchenne muscular dystrophy	DMD	UC-MSC	Safety/efficacy study, phase I	Neurogen Brain and Spine Institute, Mumbai, India	NCT02241434

Mesoangioblasts [95] and blood-derived CD133+ [96] have the ability to migrate through the vasculature, and most do not. Potential future methods to increase the migratory ability of stem cell population include the identification of cell surface markers like adhesion molecules [97] and appropriate growth factors [97]. A Phase I/II clinical trial using mesoangioblasts in DMD patients did not show a sufficient expression of dystrophin, and no functional improvement was observed [43]. Expanded satellite cells or myoblasts were isolated from wild-type mice and intramuscularly injected in dystrophic mdx mice [98, 99]; unfortunately it was demonstrated that myoblast transplantation is an ineffective technique because of the low efficiency of the dystrophin production in muscle fibers of DMD patients and no functional or clinical improvement in treated children [35]. In a possible future scenario, adult stem cells purified from patients suffering for neuromuscular disorders could be ex vivo engineered and reinjected in the initial donor intra-arterially. The intra-arterial injections of the patient's own stem cells after transduction allow the distribution of the cells to the whole-body musculature so that it could be possible to take care of severe-affected patients that have reduced mass body, as in DMD and BDM pathology (Brignier and Gewirtz). The European Medicines Agency (EMA) issued the guideline to replace the Points to Consider on the Manufacture and Quality Control of Human Somatic Cell Therapy Medicinal Products (CPMP/BWP/41450/98). In general, when a cell-based medicinal product (CBMP) enters the clinical development phase, the same requirements as for other medicinal products are applied. The clinical development plan should include pharmacodynamic studies, pharmacokinetic studies, mechanism of action studies, dose finding studies, and randomized clinical trials in accordance to the Directive 2001/20/EC and to the existing general and specific guidances for the evaluated condition. A risk analysis approach can be used by the applicants to justify the development and evaluation plans and can be a basis for the preparation of a risk management plan. Special problems might be associated with the clinical development of human cell-based medicinal products. Guidance is therefore provided on the conduct of pharmacodynamic/pharmacokinetic studies, dose finding studies, and clinical efficacy and safety studies. The guidelines describe the special consideration that should be given to pharmacovigilance aspects and the risk management plan for these products. The relevant genotypic and phenotypic characteristics of

the primary cell cultures, of the established cell lines, and of the derived cell clones should be defined and their stability with respect to culture longevity determined. Consistency/repeatability of the cell culture process should be demonstrated, and the culture conditions including the media and the duration should be optimized with respect to the intended clinical function of the cells.

Cell therapy should offer the chance to distribute the corrected stem cells to the whole-body musculature providing new corrected myofibers and the rescue of dystrophin expression in DMD patients. In comparison with drugs, cells are able to survive and integrate into host tissue; self-renewing residual potency of donor cells is sufficient to maintain tissue homeostasis for a long period, and cells can control inflammation and consequent sclerosis, either stimulating or inhibiting angiogenesis, thanks to the secretion of many factors.

Many trials have already been designed and carried out to find a clinical treatment to DMD: both stem cell-based studies, specifically using mesoangioblasts ("Optimization of stem cell therapy for degenerative epithelial and muscle diseases"—OPTISTEM; Grant Agreement number: 223098 (HEALTH-2007-1.4-6)), and gene therapy approaches, using molecules able to rescue genetic mutations, are still ongoing. Nevertheless, all of them have shown safety but limited efficacy because of incurring impasses, mainly due to modest dystrophin production in the case of cell and gene therapy and the need of repeated administrations in the case of oligonucleotide-mediated exon skipping strategies, with associated long-term possible toxicity. On March 15, 2011, a phase I/II clinical trial in DMD pediatric patients, using donor mesoangioblasts as a treatment, and supported by the 7th European Framework (Call FP7-Health-2007B-Project No. 223098), started out at the San Raffaele Hospital in Milan; infusions were completed on December 21, 2011, in three patients (DMD0301, DMD0302, DMD0303) and in November 2013 in two more (DMD0305 and DMD0306) out of a cohort of $n = 5$. This trial was based upon intra-arterial transplantation of donor stem cells from an HLA-identical donor under a regimen of immune suppression. While results are still being analyzed, they may conclude that the trial proved to be safe but of limited efficacy. They observed a transient functional stabilization (16 months in patient 02 and 16 months—still lasting—in patient 05) in two out of the three ambulant patients, limited engraftment (microchimerism from 0.1 to 0.7% in 4/5 patients) and dystrophin expression in 3/5 patients, though only in one we can conclude that this was donor derived because of a large deletion in this patient (the other two patients have a point mutation and molecular analysis is ongoing). Limited efficacy was mainly due to: (1) the age of patients (8–14 years at the onset of transplantation) who were already compromised at the onset of the trial, (2) the lower dose administered and the different functional outcome of targeting only limbs in bipeds versus quadrupeds, and (3) the gene dosage effect since the very few nuclei engrafted could not possibly produce enough dystrophin to compensate for the large majority of resident, genetically defective nuclei. In addition, T cells reactive toward donor cells were found in some patients, although clinical control by immune suppression with tacrolimus [43].

It has to be taken into account the involvement of inflammation in muscular dystrophies (MDs). For a successful transplantation, the possible link between inflammation and the immune system has to be investigated, because the mechanisms underlying its activation are not completely understood. Inflammation and innate immune response activation are firstly a consequence of skeletal muscle physiological function, but their chronic activation is determined by continuous cycles of muscle fiber degeneration/regeneration. The number of transplanted cells is another important factor to be considered. Stem cells have to be selected on the basis of their biological characteristics: (1) optimal number of stem cells after isolation, (2) optimal proliferation rate, and (3) optimal migration and myogenic potential. The efficacy dose is a crucial parameter to consider and, consequently, the proliferation capacities of the selected cells. For example, satellite cells do not proliferate enough to use them in case of systemical treatment of dystrophic patients. Moreover, other obstacles were found in the relevant costs related to GMP-cell preparation and validation.

Stem cell therapy is still under the process of research, and there are several question marks that need to be addressed before stem cell approaches might enter widespread clinical practices.

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