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A relativity concept in mesenchymal stromal cell manufacturing

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Abstract

Mesenchymal stromal cells (MSCs) are being experimentally tested in several biological systems and clinical settings with the aim of verifying possible therapeutic effects for a variety of indications. MSCs are also known to be heterogeneous populations, with phenotypic and functional features that depend heavily on the individual donor, the harvest site, and the culture conditions. In the context of this multidimensional complexity, a recurrent question is whether it is feasible to produce MSC batches as "standard" therapeutics, possibly within scalable manufacturing systems. Here, we provide a short overview of the literature on different culture methods for MSCs, including those employing innovative technologies, and of some typically assessed functional features (e.g., growth, senescence, genomic stability, clonogenicity, etc.). We then offer our perspective of a roadmap on how to identify and refine manufacturing systems for MSCs intended for specific clinical indications. We submit that the vision of producing MSCs according to a unique standard, although commercially attractive, cannot yet be scientifically substantiated. Instead, efforts should be concentrated on standardizing methods for characterization of MSCs generated by different groups, possibly covering a vast gamut of functionalities. Such assessments, combined with hypotheses on the therapeutic mode of action and associated clinical data, should ultimately allow definition of in-process controls and measurable release criteria for MSC manufacturing. These will have to be validated as predictive of potency in suitable pre-clinical models and of therapeutic efficacy in patients.

Key Words: *cellular therapy, clinical trial, manufacturing, MSCs*

Introduction

The clinical use of mesenchymal stromal cells (MSCs) for tissue regeneration, immune modulation or graft enhancement has reached dimensions deserving serious consideration and critical discussion by the scientific and clinical communities [1]. Indeed, even high-profile journals have provided the forum for controversial debates, either challenging the soundness of putative therapeutic modes by MSCs or advocating their legitimate clinical experimentation despite the absence of demonstrated biological mechanisms [2]. Beyond the extreme positions taken by opposite fringes, it is becoming apparent that the field requires well-designed randomized, prospective, controlled trials that deliver quantitative outcome measures, on the basis of which it will be possible to verify or reject specific hypotheses.

To enable such trials, manufacture of MSC batches becomes of primary importance. It is necessary at this stage to make a distinction between those trials in which a "small-scale" production of MSCs is sufficient, for example, in an autologous use setting, from those which require the manufacture of 10 000 or more doses, typically for allogeneic transplants, in which large-scale production models are likely key to economic sustainability. Obviously, the latter can only be based on extensive expansion of MSCs, which in turn opens several questions about preservation of function and onset of senescence.

MSC preparations are considered advanced therapy medicinal products by European regulation (European Commission [EC] 1394/2007). In the United States, they are considered a more-than-minimal-manipulated cellular and gene therapy product regulated under section 351 of the Public Health Service Act (42 U.S.C. 262). In Europe, MSCs as advanced therapy medicinal products require an authorization of national regulatory authorities from the countries involved in a clinical trial. In the United States, for conducting clinical trials using MSCs, it is mandatory to have an approved Investigational New Drug Application from the Food & Drug Administration. Although some differences exist between

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Europe and the United States, in both cases, MSCs should be produced according to Good Manufacturing Practice (GMP) rules, associated with the requirement to define suitable release criteria and/or potency assays.

In this context, the purpose of the present article is to offer a concise perspective on the parameters to be considered in the production of large MSC batches and how these can be modulated by specific culture systems and operating conditions. A strategy is then proposed to define manufacturing processes integrating scientific developments and clinical perspectives with prospected industrial exploitation, in compliance with regulatory pathways.

Onset of senescence during MSC expansion

When MSCs are transferred from their native niche to a polystyrene culture dish, they change from small, mostly quiescent cells, to spindle-shaped, actively proliferating cells (Figure 1). At early passages, the cells divide about once a day, which contributes to their popularity for therapeutic purposes. It should be realized, however, that expansion comes at a price. As their time in culture progresses, their proliferation rate declines, and depending on the donor and culture conditions, MSCs enter a state of replicative senescence after 20–30 cell divisions [3–5]. During this process, MSC morphology changes from relatively small spindle-shaped cells to larger and flattened cells, with typically more pronounced actin cytoskeleton fibers. Concomitant with the process of senescence, MSCs tend to progressively lose their multi-potency. Especially well-documented effects are the loss of *in vitro* differentiation into the osteogenic, chondrogenic and adipogenic lineages after culture expansion, which occurs at an earlier population doubling than senescence itself, and does not occur at the same time for all lineages [6,7].

Loss of multi-potency and onset of senescence indicate that MSC properties change during culture expansion. Surprisingly, the conventional panel of cluster of differentiation (CD) markers used to characterize MSCs is only marginally affected. Thus, although the typical CD "signature" can be used to confirm the mesenchymal stromal nature of MSCs (i.e., positivity for CD73, CD90 and CD105) [8], surface markers correlating to MSC function and multipotency are largely lacking [9]. The expression of several factors such as alkaline phosphatase and STRO-1 does decline after culture expansion, but is not reliably predictive of differentiation capacity or other immunomodulatory properties [10].

MSCs are telomerase-negative cells and culture expansion is associated with a decrease in telomere length, which begins at the start of culture [11]. A number of immortalized MSC lines have been reported [12–15], exhibiting maintained multi-potency and thus suggesting a causal relationship between the two. Culture expansion has also been implicated in issues related to genomic stability. MSCs accumulate DNA damage during expansion, and an increase in DNA adducts such as 8-oxo guanine have been reported [6,16]. Moreover, after about 15 population doublings,

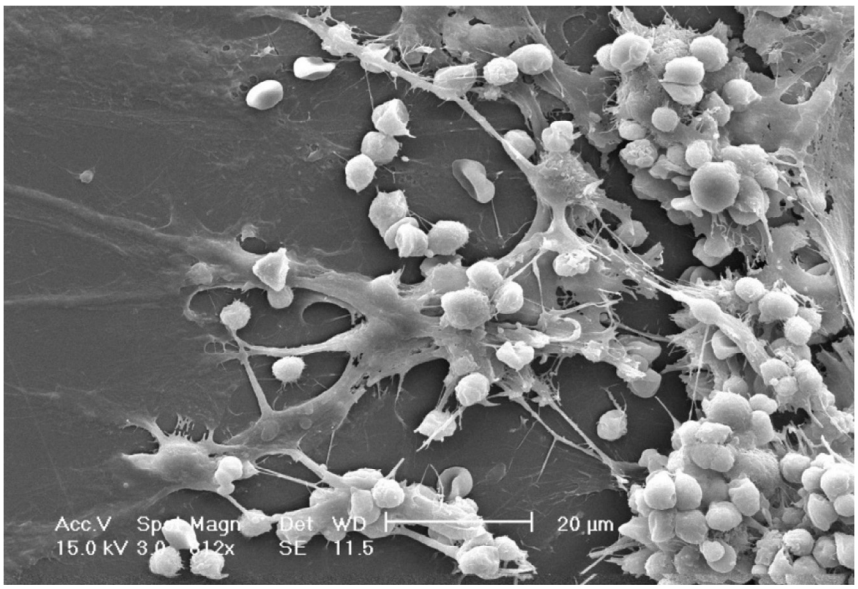

Figure 1. Changes in MSC morphology. Scanning electron microscopic image of a human bone marrow biopsy, 24 h after seeding onto tissue culture polystyrene. The image depicts the different shapes of cells, from small and rounded representing the native shape in the bone marrow, to cells that recently attached to the surface and large flat cells, which have spread and will start proliferating. Some non-adherent, round cells are likely of hematopoietic origin, including red blood cells displaying a concave morphology.

MSCs activate a DNA damage response, which occurs at around the same time as loss of multi-potency and before cells enter quiescence. It is well known that DNA damage can be mutagenic, and telomere loss is associated with genomic instability. This raises the concern that MSC expansion is associated with an increase in genomic mutations, which may lead to tumorigenesis upon implantation. However, spontaneous immortalization is almost never seen in human cells, in contrast to mouse cells, and the authors of this article have never observed spontaneous immortalization of MSCs or tumorigenicity upon implantation *in vivo*. This is supported by extensive cytogenetic analysis of human MSCs during culture expansion, which revealed their large genomic stability [17–19]. Indeed, when spontaneous immortalization of MSCs was reported, it was later attributed to the contamination of the culture with tumor cell lines [20]. However, other reports do describe genomic instability [3] correlated to oxidative damage.

Although senescence seems inevitable during extensive expansion, the biological properties of MSCs can be strongly influenced by the composition of the culture medium [21,22]. One important source of chemical heterogeneity is fetal bovine serum (FBS), which is still used for most MSC expansion protocols. MSCs grown in different serum batches display large differences in proliferation rate and differentiation, such that many labs devote considerable time to testing different batches of serum. FBS is no longer considered as an option for clinical applications, where autologous serum is preferred [23], with the associated disadvantages of donor variability and limited standardization [24]. Alternatively, platelet lysate may be used as substitute for serum [25]. Although donor specific differences in platelet lysate activity on bone marrow derived MSCs were also reported [26], platelet lysate has the advantage of containing non-xenogeneic products and to be a potent source of growth factors that is safe, reliable, practical and affordable, at least for small-scale academic production runs. Many xenogeneic serum-free and chemically defined medium formulations have also been reported in the literature. These media, although highly promising, are not yet homogeneous in formulation and contain diverse sets of factors, therefore leading to controversial results and additional variability/complexity in the field of MSC culture expansion [27,28]. Moving forward with chemically defined medium to standardize culture conditions requires groups to state the formulation used, despite issues with intellectual property. This would also have the advantage of de-risking medium supply during clinical development by not having to rely on a single supplier.

One of the most potent enhancers of MSC growth that is typically used in serum-free medium formulations and also as an additional supplement to reduce batch-to-batch serum variability is FGF2 [29]. The example of FGF2 is paradigmatic for the potential benefits and risks of expanding MSCs in the presence of growth factors. In fact, FGF2 stimulates MSC proliferation and maintains a less differentiated phenotype as well as a superior clonogenicity and multilineage differentiation capacity, possibly by selecting earlier progenitor populations with longer telomeres [29]. However, FGF2-expanded MSCs have a reduced capacity to support hematopoiesis and consistently express HLA-DR, with yet unpredictable consequences in the immunomodulatory and immunoprivileged properties [30].

The level of oxygen at which MSCs are expanded is known to influence their properties. In general, cell culture under normoxia poses high oxidative stress on the cells, and senescence is delayed under low oxygen levels. Expansion under hypoxia may positively affect multi-potency [31,32] but may also skew the differentiation capacity of MSCs [33]. In this regard, it is important to remark that culture conditions typically report the levels of oxygen in the air as v/v% and in many cases using only the ill-defined terms "hypoxia" or "normoxia." Instead, the relevant culture variable is the percentage of dissolved oxygen in the culture medium, which can greatly vary in culture dishes despite the nominal level being settled in the incubator air. Standardization of the parameter is crucial and can be best achieved when cell culture is performed in monitored/controlled bioreactor systems, as discussed in the next section.

Alternative MSC culture systems

Studies on different cellular systems have recognized that maintenance of "early progenitor" properties may be favored by the establishment of a tissue-specific microenvironment or niche. This principle has fostered the search for culture conditions that are possibly more "physiological" for MSCs than a traditional petri dish made of a rigid polystyrene material. Considering that the extracellular matrix (ECM) comprises important components of the niche, different groups have tested the possibility to grow MSCs on petri dishes coated with ECM molecules. In particular, it was demonstrated that denatured collagen coating improves the osteogenic and adipogenic differentiation capacity of human bone marrow–derived MSCs [34]. To mimic the stromal environment where MSCs are found *in vivo*, the use of devitalized ECM laid down by MSCs as substrate for their growth was also investigated. Results convincingly indicated a more efficient preservation of SSEA-4+, clonogenic MSCs, which retained their ability to form bone tissue *in vivo* after extensive passaging [35].

Chemical [36], stiffness [37] and geometric [38] modifications to materials have been implicated in the lineage specification and differentiation of MSCs. Recently, it was shown that defined nano-topographical patterns embossed in culture substrates can be used to maintain undifferentiated MSCs, possibly by regulating intracellular tension [39]. These studies highlight the complexity of interactions among several substrate parameters and call for the development of high-throughput technologies to identify improved material surfaces [40].

Various attempts have been reported to expand MSCs in three-dimensional (3D) environments, for example, with suspension culture in the presence of dynamic flow [41] or using microcarrier beads [42]. On the basis of the analogy with other industries, these systems would have a large potential to drive down production costs, at the same time allowing for monitoring and control of relevant parameters, which are important during process standardization. Despite the promising results obtained, however, these studies achieved a rather limited number of cell doublings during the 3D culture. Moreover, the approaches require an initial phase of MSC growth on plastic, which is intrinsically associated with selection of the adherent cellular fractions and the loss of most hematopoietic lineage cells. Indeed, non-mesenchymal or non-adherent bone marrow cells were proposed to be involved in regulating MSC function and have been demonstrated to enhance growth of MSCs with clonogenic properties [43,44].

An alternative strategy for MSC expansion relies on directly loading and culturing freshly harvested bone marrow cells into the pores of 3D scaffolds, thereby entirely bypassing the initial phase of selection on Petri dishes [45,46]. The system, which requires the use of a perfusion-based bioreactor system to improve the efficiency of MSC seeding and nutrition, supported the scalable expansion of clonogenic MSCs, with a transcriptomic profile closer to stem cell signatures [47]. Although no formal proof has been provided, the effect could be mediated by the generation of an *ex vivo* "stromal niche" [48], leading to the preservation in the culture system of subpopulations of earlier MSC progenitors less efficiently adhering [44] and/or of hematopoietic cells. The same paradigm was successfully validated for the growth of adipose tissue–derived MSCs, in which a population of functional endothelial lineage cells, otherwise lost in serial passaging, was preserved [49] and was instrumental in achieving accelerated engraftment of the corresponding engineered tissues [50]. It should, however, be pointed out that strategies based on MSC co-culture with supporting populations, although promising in their capacity to reproduce their native environment, require a downstream sorting system to eliminate the non-mesenchymal accessory cells and may introduce the challenge to harvest cells from the porous scaffolds without detriment to the functionality of the final product.

A roadmap toward suitable MSC manufacturing protocols

This short overview highlights the broad spectrum of MSC phenotypes and functions that can be reached by different culture protocols, obviously challenging the possibility to define a "standard" for MSCs or for a MSC manufacturing system. Recognizing that the conceptual framework around the use of MSCs is strictly dependent on the intended therapeutic application, we propose here a possible roadmap toward the identification of matching MSC culture conditions (Figure 2). In our view, the starting point of the process is the definition of a precise clinical indication, according to which a specific hypothesis on the mechanism of action of the delivered cells will have to be formulated. Such postulated therapeutic mode should in turn guide the definition of possible release criteria for the batches of MSCs to be grafted. For example, an expected direct contribution of MSCs to tissue regeneration may imply the need to maintain a differentiation capacity toward a certain lineage or possibly even multi-potency. Instead, the putative "hit-and-run" effect potentiating the repair mechanisms of endogenous cells may require assessing the secretion of molecules (e.g., interleukin-4, interleukin-1RA, prostaglandin 2) capable of establishing a "regenerative niche" (e.g., by inducing monocyte polarization toward M2) [25]. In this same perspective, recent papers have outlined a gamut of possible assays to quantitatively capture the immune modulatory properties of MSCs, toward the establishment of release criteria for the treatment of specific autoimmune or inflammatory diseases [51–53]. For example, functionality tests for MSCs to be used in clinical indications targeting immune disorders have been proposed to include changes in response to interferon-γ licensing, based on cytofluorimetric, mRNA gene expression and proteomic levels [54]. The same approach should be initiated for MSCs used in other clinical fields. Despite the ongoing controversies and challenges, it is becoming increasingly clear that currently used surface markers are not likely to capture specific MSC functionalities and that "defining robust and predictive markers and assays of potency remains the Gordian knot in the field" [53]. The complexity may be further increased by the fact that the criteria of "purity" typical for drugs and single cell populations need to be extended to multipotent cellular products such as MSCs, where interaction of diverse cell subpopulations may drive their effectiveness.

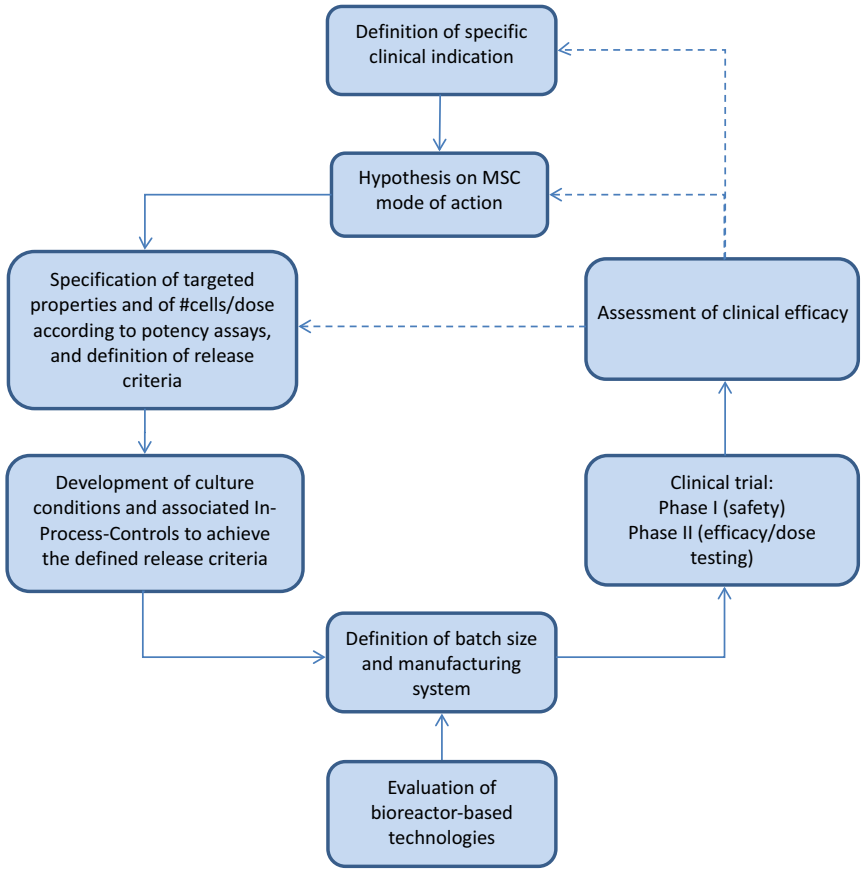

Figure 2. Proposed roadmap for MSC manufacturing. The definition of a specific clinical indication targeted by the delivery of MSCs should be based on a mode of action, postulated even arbitrarily on the basis of pre-clinical models. This should lead to the definition of targeted properties for MSCs, including the development of release criteria for vitality, identity, purity and potency, hypothesized to be predictive for the clinical outcome, and in turn guiding the selection of culture parameters monitored and controlled by in-process controls (IPC). At this stage, culture conditions should be identified that satisfy the selected IPC and lead to a product meeting the specific release criteria (RC). The parallel assessment of novel bioreactor-based technologies supporting scalable, standardized and possibly automated processes should lead to the definition of a manufacturing system to be used for cell production for a clinical trial. The clinical efficacy observed in such a trial should then be assessed in view of a possible correlation with the selected IPC and/or RC and the defined number of cells per dose. This could lead to a validation/refinement of the targeted properties and/or RC, or to the need to correct the intended clinical indications and/or underlying hypothesis in an iterative process (dashed lines). This roadmap could obviously be considered for manufacturing other cell types in cellular therapy.

With all the arbitrary assumptions that are necessary at early stages in development of new strategies, it is, in the authors' view, imperative that a set of targeted properties and their respective release criteria be defined for each intended clinical indication and that these drive the definition of culture conditions allowing maximizing the maintenance/acquisition of such properties. It is unlikely that the same batch of MSCs, even if well standardized and quality controlled, will be effective for the diversity of clinical indications that are currently being targeted using MSC preparations. Moreover, to be able to retrospectively revise the definition of the MSC features most directly predictive of clinical outcome, alternative parameters should be measured beyond those required for the initially selected release criteria. In a different area of cellular therapy, namely limbal stem cells for corneal regeneration, this strategy based on post hoc analyses has led to the identification of p63 expression as a parameter quantitatively associated with successful transplantation [55]. An additional element of complexity is related to the characterization of the cells before or after banking, because freshly thawed MSCs (i.e., in the typical state at the time of delivery) may display distinct biological properties from those typically examined in pre-clinical studies (i.e., in the log phase of growth) [56].

At this stage, MSC culture that is in compliance with GMP would be ready for clinical testing. As previously mentioned, the trial should follow design criteria that allow, as much as possible, to test a specific hypothesis that could inform on the proposed mechanism of action or possibly lead to the formulation of a different one. Results will ultimately determine whether

the target indication, along with the specific inclusion/exclusion criteria, need to be refined or more drastically considered inappropriate. Importantly, in the context of manufacturing, analysis of the number of cells prescribed for a graft dose will have to be combined with the extent of expansion allowing the defined release criteria to be maintained. Together, these data will offer an indication on the number of possible doses per MSC batch and therefore on the scalability of the process.

To industrialize manufacturing, a scaled-up or -out process, respectively, for allogeneic or autologous preparations should consider implementation in bioreactor systems, within a controlled physicochemical culture environment (e.g., in terms of dissolved oxygen or pH). Indeed, the introduction of monitoring features should simplify traceability and regulatory as well as safety compliance. Process automation within closed systems (e.g., for automated medium exchange during culture or streamlined storage operation) is expected to lead to minimized operator handling, improved reproducibility, possibility of predominant operation is GMP environments of Class C (as opposed to the more costly ones in Class A) and, as a consequence of all this, to superior cost-effectiveness. The introduction of bioreactor systems as early as possible in the phase of process definition, although requiring large upfront investments, has been critical for industrial exploitation in other biotech sectors (e.g., production of drugs, antibodies or vaccines). In the specific context of MSC expansion, bioreactor systems could go beyond replicating with robotic systems the sometimes-ineffective manual protocols and instead lead to streamlined, more effective manufacturing processes addressing the most critical scientific, regulatory and commercial challenges [57].

Conclusions

The extent of MSC expansion and the specific culture conditions critically regulate the phenotype, function and onset of senescence of MSCs. To date, in the absence of clinical data supporting the correlation of specific MSC properties with unambiguous positive outcome, no MSC preparation can be considered "standard" even if deriving from certified manufacturers. Although targeting standardized MSC preparations is likely to restrict scientific inquiry and innovation, standardization of protocols and of functional measurements is pivotal to bring continuity of data interpretation to the field [58]. To assess manufacturing comparability during process scale-up or between manufacturing sites, it was proposed that an MSC reference or calibration standard be established [59]. This proposition would not be conceptually sound if it was intended to generate a cell gold standard (i.e., MSCs representing a reference "optimal" biologic activity) because a scientific basis for that is not yet available [60]. Instead, the purpose of the cell ruler would be to "serve as a common calibration tool and provide a central data point, against which variation in any dimension could be reported" [61]. Even within this conceptual framework, however, several operational challenges related to sources, protocols, costs and distribution for a cell ruler remain. In a simplified framework, what then needs to be introduced are standard methods and guidelines to extensively characterize MSCs in parallel with their *in vivo* implantation.

We thus submit that following the proposed logic will maximize the chances of developing safe and effective uses for MSCs that are compliant with the sometimes-discrepant regulatory rules among different countries, as part of advanced toolkits for routine clinical practice. The process will also be relevant, thanks to the investigations on the underlying modes of action, to potentiate the biological functionality of MSC products and/or specialized delivery systems (e.g., by manufacturing MSCs genetically modified to express/overexpress trophic factors) [62]. This might ultimately lessen the large doses that are currently necessary, thereby reducing the burden on GMP.

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