

Circulating Progenitor Stem Cells Are Important Biomarkers of Chondrogenesis and Osteogenesis: Employment in Diagnosis and Treatment Follow Up

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Abstract

Adult multipotent mesenchymal stem cells (MSCs) arouse great scientific interest because of their many possible clinical applications. Besides the therapeutic potential for tissue regeneration and repair, they also offer new opportunities as diagnostic tools. For diagnostic purposes peripheral blood represents an easily accessible source of circulating MSCs which show similar characteristics compared to bone marrow MSCs. Dysfunctions in chondrogenic and osteogenic differentiation of MSCs are involved in the genesis of age-related degenerative disorders as osteoarthritis and osteoporosis. Altered expression profiles of specific transcription factors may be monitored in the *ex vivo* analysis of patients' peripheral blood MSCs; they represent important biomarkers for diagnosis. Furthermore, changes in MSCs expression profiles induced by pharmacological treatments are useful biomarkers for therapy follow-up. MicroRNAs also play a crucial role in chondrogenic and osteogenic differentiation of progenitor cells; differentially expressed microRNAs have been associated with osteoarthritis and osteoporosis, respectively. MicroRNAs can be recovered from blood, urine, and synovial fluid and represent useful supplemental tools for the diagnosis of these degenerative disorders.

In conclusion, circulating MSCs can be obtained by a non-invasive approach and provide an "*ex vivo*" source for monitoring various differentiation pathways in normal and pathological conditions; thus they represent a remarkable resource for various clinical applications.

Keywords: Circulating MSCs; Osteogenesis; Chondrogenesis; microRNAs; Biomarkers; Gene expression analysis

Abbreviations

bALP: Bone Alkaline Phosphatase; BM-MSC: Bone Marrow Mesenchymal Stem Cell; BMP: Bone Morphogenetic Protein; CMP: Cartilage Matrix Protein; COMP: Cartilage Oligomeric Protein; ECM: Extra Cellular Matrix; ESC: Embryonic Stem Cells; FGF: Fibroblast Growth Factor; GH: Growth Hormone; GR: Glucocorticoid Receptor; HSC: Hematopoietic Stem Cell; IGF1: Insuline-Like Growth Factor 1; miRNA: microRNA, MNC: Mononuclear Cell; OA: Osteoarthritis; OC: Osteocalcin; OP: Osteoporosis; OPN: Osteopontin; OSX: Osterix (SP7 transcription factor); PB-MSC: Peripheral Blood Mesenchymal Stem Cell; PTH: Parathormone; RUNX2: Runt-Related Transcription Factor 2; SOX#: SRY-box#; SPARC: Osteonectin; TGF- β : Transforming Growth Factor- β ; TNF- α : Tumor Necrosis Factor- α ; WNT#: Wingless-Type MMTV Integration Site Family Member#1; ZA: Zoledronic Acid

Background

Stem cells biology, circulating stem cells

The great interest surrounding stem cells biology is prompted by their remarkable capacities of multipotency and self-renewal. Embryonic Stem Cells (ESCs) are pluripotent, since they can differentiate into the three germ layers and are capable of unlimited undifferentiated proliferation *in vitro*. Adult multipotent stem cells, primarily isolated from bone marrow, can be distinguished in two different types: Hematopoietic Stem Cells (HSCs) committed to differentiate into blood cells, and stromal stem cells which are named Bone Marrow Mesenchymal Stem Cells (BM-MSCs), capable of differentiating into several lineages of mesodermal and non-mesodermal origin. Other sources of MSCs have been identified through the years, including placenta, adipose tissue, dental pulp, synovium, endometrium, breast

milk [1]. Circulating MSCs can be recovered from umbilical cord blood as well as from adult peripheral blood [2]. Peripheral blood MSCs (PB-MSCs) have shown similar characteristics in terms of multipotency and proliferation, compared to BM-MSCs. Unfortunately their number is very low in physiological conditions: approximately $1:10^8$ mononuclear cells (MNCs). Appropriate enrichment methods must be devised for their selection and utilization. Potential clinical applications of such cells, obtainable by a non-invasive approach, are very interesting; the possibility to make them proliferate and differentiate *in vitro* discloses also therapeutic applications for tissue regeneration.

Mesenchymal stem cells in cartilage and bone

BM-MSCs are the precursors of cartilage cells (chondrocytes), bone cells (osteoblasts), as well as fat cells (adipocytes). Thus chondrogenesis and osteogenesis arise from the same progenitor cells and are subject to complex regulation by interplaying agents such as systemic hormones, e.g. growth hormone (GH), insulin-like growth factor 1 (IGF-1), parathormone (PTH), estrogens et al., and local factors such as fibroblast growth factors (FGFs), transforming growth factor beta (TGF-beta),

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bone morphogenetic proteins (BMPs), WNT signaling pathways. Common chondrogenic/osteogenic precursors express the master transcription regulator Runt-related transcription factor-2 (RUNX2). SRY-box9 (SOX9), the chondrogenic differentiation marker, warrants chondrogenesis versus osteogenesis by negatively regulating RUNX2 [3]. Two other SOX family members (SOX5 and SOX6) are regulated by and co-expressed with SOX9; their combined activity is required for transformation of progenitor cells into early chondroblasts. In the endochondral ossification process, which characterizes long bones development, chondrocytes provide a template through proliferation, terminal differentiation to the hypertrophic state and finally apoptosis, whereby calcified cartilage is replaced by osteoblast-produced osteoid. In the articular cartilage, a resilient tissue which covers the ends of long bones at the joints, chondrocytes remain quiescent; they are responsible for the production and homeostatic maintenance of extracellular matrix (ECM) components. Structural ECM components, which determine articular cartilage mechanical and frictional properties, are: collagens (mainly type II collagen), proteoglycans (including aggrecan, decorin, biglycan, and fibromodulin), non-collagenous proteins, such as cartilage oligomeric protein (COMP), cartilage matrix protein (CMP), fibronectin. The primary matrix degrading enzymes involved in cartilage turnover are metalloproteinases and cathepsins. In healthy articular cartilage, balanced degradation and synthesis by chondrocytes ensure tissue homeostasis. The osteogenic commitment of pluripotent BM-MSCs is controlled by RUNX2, which in turn activates the osteogenic master regulator OSTERIX (OSX) [4]. RUNX2 expression, however, must be suppressed for the accomplishment of osteoblastic maturation, as demonstrated in animal models and in human pathological conditions [5,6]. The differentiation process implies the expression of downstream genes coding for specific osteoblastic proteins such as collagen type I, osteocalcin (OC), osteonectin (SPARC), osteopontin (OPN), bone sialoprotein (BSP), which are the organic constituents of bone ECM. Bone alkaline phosphatase (bALP) is also produced by osteoblasts and is involved in the mineralization of bone matrix (Figure 1) [7].

Epigenetic factors, such as microRNAs (miRNAs) are also involved in BM-MSCs commitment, differentiation and survival. These small noncoding RNAs act as negative modulators of gene expression. They interfere with translation by binding to partially complementary sequences in the 3'UTR sites of target mRNAs. Circulating miRNAs can be found in different biological fluids such as serum/plasma, urine, and synovial fluid. MiRNAs can be secreted by MSCs and transported by extracellular vesicles, e.g. exosomes, which in turn will undergo endocytotic internalization to recipient cells. Thus exosomes play important roles in cellular communications which occur in bone modeling and remodeling [8]. MiRNA profiling in exosomes isolated from BM-MSCs at various stages of osteogenic differentiation allowed the identification of differentially expressed miRNAs at each stage [9]. When administered to uncommitted BM-MSCs, in the absence of any other osteogenic supplement, exosomes deriving from each stage were able to induce osteogenic differentiation. However, only exosomes released by cells at the late stages of differentiation could induce the mineralization process. The regulatory effects on osteogenesis exerted by miRNAs are certainly entitled to further investigation.

Dysfunctions of MSCs in osteoarthritis and osteoporosis

Osteoarthritis (OA), a degenerative musculoskeletal disease of the joints, is characterized by cartilage alteration, bone remodeling at subchondral level, osteophytosis and pain. Chondrocytes, which are surrounded by the cartilage ECM, have a limited ability in intrinsic

regeneration [10]. In joints, subchondral bone supports the articular cartilage and, when microfractures occur, BM-MSCs are released in order to repair damaged cartilage. In addition to subchondral bone, also synovium contains MSCs. However, MSCs harvested from OA joints are not able to proliferate and differentiate properly [11] probably because of the altered inflammatory environment [12,13]. It has been reported that a subpopulation of progenitor cells, found in OA patients' cartilage, exhibits an early senescent phenotype [14]. This subpopulation, expressing CD271 antigen, also lacks proliferative capacity; senescence of progenitors and loss of proliferation potential may contribute to OA pathophysiology [15]. Importantly, macrophages residing in the synovia, which produce pro-inflammatory cytokines, act as negative regulators of chondrogenesis [16,17]. Decreased expression levels of SOX9, the master gene of chondrogenesis, have been observed in MSCs of OA patients. In addition, dysfunctions in proteasome and autophagic machinery have been reported in chondrocytes of OA patients [18]. Epigenetic factors may as well influence chondrogenesis. Altered expression patterns of specific miRNAs [19], as well as altered chromatin remodeling [20] can impair the commitment and differentiation of MSCs in OA patients' joints.

Osteoporosis (OP) is a common degenerative skeletal disease affecting most postmenopausal women and aged people in general. Alterations in BM-MSCs differentiation are involved in its genesis. OP is the consequence of an imbalance between bone formation and bone resorption. It has been reported that alterations in osteogenic differentiation of progenitor cells prevent bone formation and impair the physiological process of bone remodeling. MSCs' commitment is regulated by a network involving various transcription factors and signaling molecules [21]. Among them, several WNT family members (e.g. WNT1, WNT6, WNT10A, and WNT10B) play an important role for the osteogenic commitment of MSCs. Epigenetic inhibition of WNT pathway in OP animal models suppresses osteogenic differentiation [22]. Tumor Necrosis Factor-alpha (TNF- α) associated with estrogen deficiency (which is considered the most important cause of OP), impairs osteogenic differentiation by reducing antioxidant defenses [23]. The pro-osteogenic action of transcription factor RUNX2 is often affected by factors inducing osteopenia conditions. An elevated production of glucocorticoids impairs bone microarchitecture causing bone loss and skeletal deterioration. It has been reported that glucocorticoids antagonize RUNX2 during osteogenic differentiation. This effect is actually due to a physical interaction between RUNX2 and activated glucocorticoid receptor (GR) [24]. As for epigenetic factors, miRNA 204 and miRNA 211 are endogenous repressors of RUNX2 [19]; MiRNA-29a, on the contrary, can reduce glucocorticoid-induced bone loss by rescuing RUNX2 activity [24].

Gene expression profiles of circulating MSCs as markers of bone /cartilage homeostasis in health and disease

Several years ago, we developed a method for isolating MSCs from peripheral blood (Figure 2) [25]. In brief, cMSCs are obtained from 50ml of peripheral blood using an antibodies cocktail to deplete other blood cells. The protocol entails two subsequent density gradient centrifugations. In the first, mononuclear cells from heparinized peripheral blood are obtained. Then, mononuclear cells are mixed with 200 μ l of an antibodies cocktail containing bi-specific antibody complexes against red blood cells (glycophorin A) and CD3, CD14, CD19, CD38, CD66b positive cells (hematopoietic cells). Hematopoietic cells crosslinked with red blood cells are then discarded by an additional gradient centrifugation; the phenotype of isolated cells is thereafter determined. Evaluation of surface markers (cluster

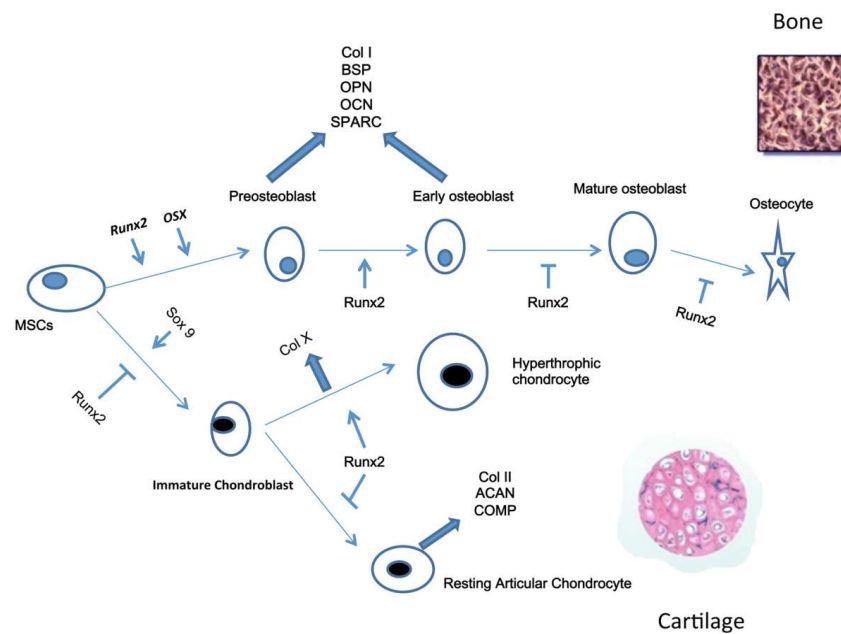


Figure 1: Regulators of osteoblast and chondrocyte differentiation steps. In the osteogenic differentiation of bipotent BM-MSC, RUNX2 and, sequentially OSX, play a crucial role, promoting the expression of bone ECM proteins. For further osteoblast maturation, RUNX2 must be downregulated. SOX9 expression drives the chondrogenic differentiation pathway; in the endochondral ossification process RUNX2 is necessary for chondrocyte maturation to hypertrophic chondrocyte, inducing COLX and other ECM components production.

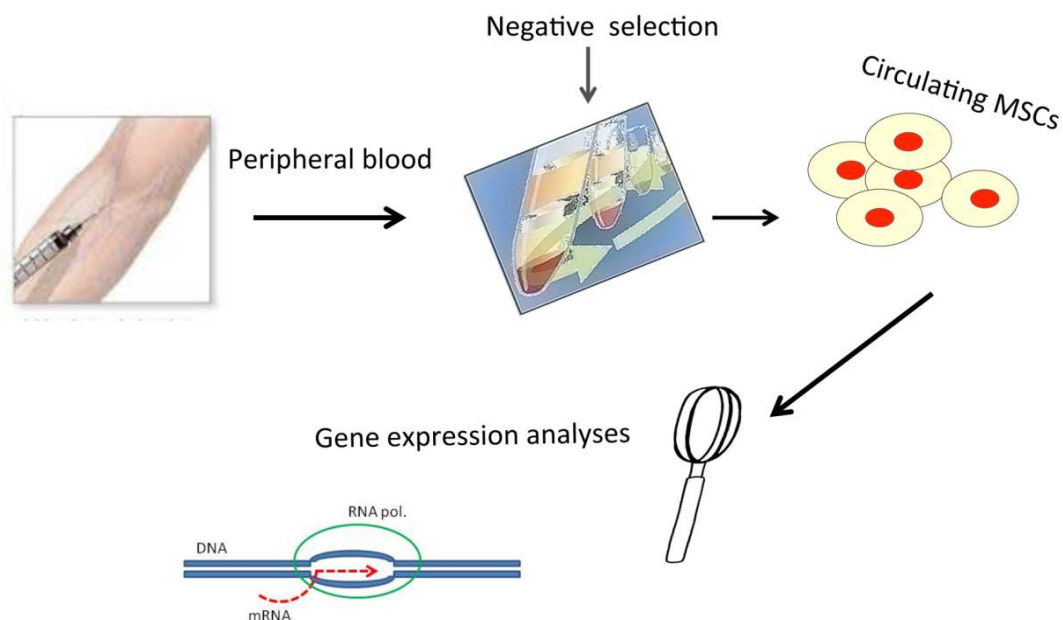


Figure 2: Recovery of circulating MSCs. Circulating MSCs can be obtained from peripheral blood by a two-step method including an enrichment of mononuclear cells followed by depletion of unwanted cells (Ref.#25). Thereafter cMSCs can be used to perform gene expression analyses.

differentiation) of PB-MSCs is important for diagnostic purpose. Usually, isolated PB-MSCs with a percentage of positive markers (such as CD105 and CD73) > 60% and with a low % of hematopoietic negative markers (such as CD3, CD19, CD14, CD45) are considered suitable for diagnostic analyses [6]. By this method we were able to define the

PB-MSCs profiles during commitment and differentiation in normal subjects. Thereafter we have identified up- or down-regulated genes in disorders affecting bone [6,26-28], as well as cartilage [29]. In PB-MSCs from OP patients, we observed a reduced expression of RUNX2 as well as an enhanced expression of the adipogenic transcription factor

PPARG2 [26]. Interestingly, data obtained by analyzing PB-MSCs confirmed the positive correlation between the atherogenic lipid profile and osteopenia features in OP post-menopausal patients [30] and suggested PB-MSC as important biomarkers for early diagnosis. An ex vivo analysis of zoledronic acid (ZA) therapy in OP patients was made by evaluating gene expression profiles in PB-MSCs [31]. The analysis highlighted the anabolic activity of ZA and also suggested the use of these biomarkers for therapy follow-up. Recently, we found a reduced expression of SOX9, the chondrogenesis master gene, in PB-MSCs obtained from patients affected by OA [29]. This observation suggests SOX9 expression as a useful PB-MSC biomarker for the identification of cartilage dysfunction. In addition, we showed an enhanced expression of SOX9 in patients PB-MSCs, upon treatment with clodronate correlating with the reduction of clinical symptoms. Gene expression profiling is a useful tool also for OA monitoring.

Expression profiles of circulating miRNAs as disease biomarkers

The expression profiles of specific miRNAs can also be associated with OA and OP features, respectively. They may represent useful supplemental tools for the diagnosis of these degenerative disorders. MiR-181-5p, which targets COL2A1 in chondrocytes and is associated with cartilage degeneration, can be recovered from peripheral blood [32]; it may therefore represent a marker for screening OA patients [33]. Members of the miR-183 cluster (miR-183/-182/-96), that can inhibit the osteogenic differentiation of BM-MSCs, may as well be used as diagnostic biomarkers for OP. The presence of these miRNAs actually increases in exosomes released by aged bone [34]. Similarly, miR-31-5p, involved in different steps of bone differentiation, can be found in exosomes; it has been suggested as a biomarker for osteoporosis [35]. Interestingly, a study describing the human miRNome in OP and OA patients highlighted different circulating miRNAs as biomarkers for the screening of these complex diseases [36].

Are PB-MSCs useful diagnostic tools?

Circulating blood cells may somehow be regarded as signallers of alterations occurring in the macro- or micro-environment of organs and tissues. This observation suggests the use of circulating cells as “surrogate tissue” for diagnostic analyses [37]. In particular, PB-MSCs may be useful tools directing towards the identification of early pathological conditions related to skeletal diseases as well as towards a correct diagnosis. Besides the traditional cytofluorimetric analyses of PB-MSCs, new technologies are requested to investigate these cells at different molecular levels (i.e. epigenome, transcriptome, methylome). We may overcome their paucity, a factual drawback, thanks to technological advancements. Gene expression analyses performed by array approaches or by Next Generation Sequencing (NGS) are effective, despite some limitations due to the background noise [38]. Certainly, data obtained by PB-MSCs analyses have to be confirmed and supported by additional clinical and biochemical observations.

Potential therapeutic applications of PB-MSCs

Besides the diagnostic applications discussed above, therapeutic applications for PB-MSCs (e.g. autologous/heterologous transplantation) appear very promising, provided that efficient protocols for isolation and in vitro culture expansion are devised. There are several examples of MSC-based therapy attempts for bone and cartilage defects, both in animal models [39,40] and in humans [41,42]. Most studies have employed BM-MSCs. Rare reports of studies in animal models employing PB-MSCs have been published to our

knowledge [43]. The laborious steps necessary for their isolation and self-renewal in vitro may have discouraged their employment so far. However, there is an increasing expectation on cell-based therapies employing MSCs for the treatment of degenerative and inflammatory diseases. Exploring all accessible sources of such precious cells is of paramount importance.

Conclusion

Circulating mesenchymal stem cells show characteristics of proliferation and multipotency similar to those of BM-MSCs. They are normally present in minimal concentrations; it is known that their number increases after mobilization from BM in response to injury [44,45].

PB-MSCs can be obtained by a non-invasive approach and provide an ex vivo source for monitoring various differentiation pathways in normal and pathological conditions. They therefore represent a remarkable resource for various clinical applications as well as for pre-clinical research in order to indicate how and where molecular pathways get disrupted in degenerative diseases. Altered expression profiles of commitment-specific mRNAs and miRNAs in PB-MSCs may be used as diagnostic and prognostic indicators in degenerative disorders such as OA and OP. Moreover, changes in relevant expression profiles can be monitored in patients under pharmacological treatment, and provide useful information for therapy follow-up. Circulating miRNAs can be recovered from various body fluids: their altered expression patterns in OA and OP are further biomarkers for patients' screening and treatment monitoring. Finally, the potential therapeutic use of PB-MSCs in regenerative medicine deserves proper consideration and further investigation.

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Author Contribution

MT Valenti contributed to the conception, design, and data acquisition; drafted and critically revised the manuscript; M Mottes contributed to the conception, design, and data acquisition; drafted and critically revised the manuscript; L Dalle Carbonare contributed to the conception, design, and data acquisition and critically revised the manuscript. All authors gave final approval for publication.

Competing Interests

The authors declare that they have no competing interests.

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