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Mesenchymal progenitor cells for the osteogenic lineage

Noriaki Ono, DDS, PhD[1] and **Henry M. Kronenberg, MD**[2]

Noriaki Ono: noriono@umich.edu; Henry M. Kronenberg: hkronenberg@mgh.harvard.edu

[1]Department of Orthodontics and Pediatric Dentistry, University of Michigan School of Dentistry, Ann Arbor, MI, 48109, USA, Phone: (734)647-8450

[2]Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA, 02114, USA, Phone: (617)726-3974, Fax: (617)726-7543

Abstract

Mesenchymal progenitors of the osteogenic lineage provide the flexibility for bone to grow, maintain its function and homeostasis. Traditionally, colony-forming-unit fibroblasts (CFU-Fs) have been regarded as surrogates for mesenchymal progenitors; however, this definition cannot address the function of these progenitors in their native setting. Transgenic murine models including lineage-tracing technologies based on the *cre-lox* system have proven to be useful in delineating mesenchymal progenitors in their native environment. Although heterogeneity of cell populations of interest marked by a promoter-based approach complicates overall interpretation, an emerging complexity of mesenchymal progenitors has been revealed. Current literatures suggest two distinct types of bone progenitor cells; growth-associated mesenchymal progenitors contribute to explosive growth of bone in early life, whereas bone marrow mesenchymal progenitors contribute to the much slower remodeling process and response to injury that occurs mainly in adulthood. More detailed relationships of these progenitors need to be studied through further experimentation.

Introduction

The lifecycle of an organism is marked by a number of biologically important events, including morphogenesis and development, growth and functional maturation, homeostasis and maintenance of proper architecture and function, and response to injury. The significance of stem and progenitor cells playing major roles in these processes has been emphasized. Stem cells are characterized by two important functions; self-renewal, which is the ability of replicate themselves while maintaining their properties, and multipotency, which is the ability to give rise to multiple types of differentiated cell types. Progenitor cells are their downstream offspring with similar but potentially more limited capabilities. Stem and progenitor cells exist in a tissue-specific manner in each organ and tissue during

Conflict of Interest

Noriaki Ono and Henry M. Kronenberg declare that they have no conflict of interest.

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

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development and are also, present in some organs in adult life. In mammals, stem and progenitor cells are well documented not only in tissues that rapidly turn over, such as blood (hematopoietic stem cells) and skin (epithelial stem cells), but also in tissues with much slower turnover such as skeletal muscle (satellite cells) (1) (reviewed in (2)) and the central nervous system (neural stem cells) (reviewed in (3)). The primary function of 'connective' tissues is to support other important tissues or organs; while exhibiting remarkable regenerative capacities in response to injury, their turnover is generally slow. Mesenchymal progenitors (sometimes called as 'mesenchymal stem cells') are found virtually in all connective tissues, such as bone, tendon, ligament, dermis and dental pulp.

The most intensively studied mesenchymal progenitors are those in bone. The primary functions of bones are to provide protection for vital organs and to act as levers whereby muscle contraction leads to movement of the organism. Bone cells also support hematopoiesis in the adjacent marrow (4–6) and secrete hormones that regulate carbohydrate and mineral ion metabolism, as well as male fertility (7) and brain function(8). Bone growth is substantial in fetal life and early postnatal life. Even after bone growth slows or stops, bone cells turn over continually throughout life and provide substantial capability for repair from injury. In this short review, we will discuss recent advances on the biology of the mesenchymal progenitors of the osteogenic lineage that provide the flexibility for bone to grow, maintain its homeostasis, and conduct repair when necessary. We will focus primarily on mammalian models of bone.

Mesenchymal progenitor cells for the osteogenic lineage in adult life

Colony forming-unit fibroblasts (CFU-Fs): Perivascular stromal cell populations

The bulk of knowledge on mesenchymal progenitors in adult life has been accumulated based on experiments using human and rodent bone marrow cells, by combining *in vitro/ex vivo* cell culture approaches and heterotopic transplantation of cultured cells into immunodeficient mice. These mesenchymal progenitors are found in a perivascular location, assuming the morphology resembling adventitial reticular cell on bone marrow sinusoids or pericytes on arterioles(9). The discovery that bone marrow may include mesenchymal progenitors capable of making bone was almost serendipitously discovered in 1960s; when bulk human bone marrow cells were subcutaneously transplanted into immunodeficient mice, they formed ossicles that included blood cells inside(10). Later, colony-forming unit fibroblasts (CFU-Fs), which are defined as cells capable of adhering to a plastic culture dish and establishing colonies, were found to be responsible for ectopic ossicle formation after subcutaneous transplantation(11). These ossicles contain osteoblasts and stromal cells of the donor origin with blood cells of the recipient origin; this finding was strongly indicative of the fact that human adult bone marrow includes mesenchymal stromal progenitors capable of reconstituting bone marrow in a new environment; analogous experiments in rodents yielded similar results. However, these criteria for identifying mesenchymal progenitors did not clarify the location and the properties of these cells *in situ*, because these cells can be identified expansion in cell culture. Therefore, the newer approaches using cell surface markers and cell sorting have been pursued to identify their native locations and to prospectively isolate these cells. Initially, the STRO-1 antibody was used to identify and

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enrich clonal human bone marrow mesenchymal cells(12). Further studies (13) showed that all CFU-Fs were recovered in the CD146$^+$ fraction of human bone marrow cells. In addition, these cells were serially transplantable subcutaneously and could generate CD146$^+$ cells upon secondary transplantation (indicating self-renewal), thus meeting the criteria of "skeletal stem cells". Sacchetti et al also found that adventitial reticular cells lining bone marrow sinusoids *in vivo* were positive for CD146. Later, another group further demonstrated that CD51(αV integrin)$^+$PDGFRα$^+$ cells represent a small subset of CD146$^+$ cells in human bone marrow with even more enriched colony-forming activity (14). Therefore, these findings have established the idea that human CFU-Fs are found within perivascular stromal cells and can generate bone and stroma upon ectopic transplantation. Whether these cells represent precursors of osteoblasts formed during normal development or during bone remodeling, or perhaps represent cells that respond to injury, is an important question to answer in the future.

In vivo insights into mesenchymal progenitor cells: Use of transgenic markers and cell surface markers

The use of flow cytometry and of mice engineered to express easily assayable proteins such as green fluorescent protein (GFP) or *E. coli* β-galactosidase has allowed identification of distinct mesenchymal cell types in their native environment. One study showed that PDGFRα$^+$Sca1$^+$ non-hematopoietic cells ("PαS cells") reside in the perivascular space *in vivo* and are highly enriched for CFU-Fs(15). Further, more importantly, co-transplantation of uncultured GFP$^+$ PαS cells with hematopoietic stem cells (HSCs) into irradiated donor resulted in engraftment of GFP$^+$ cells in bone marrow: they became osteoblasts, adipocytes, stromal cells and PαS cells themselves, suggesting their self-renewal *in vivo*. Interestingly, cultured PαS cells did not have such capability. Further insights into mesenchymal progenitors in adult bone marrow have been accrued as byproducts of studies to investigate the HSC 'niche' in adult mouse bone marrow. Cxcl12 (chemokine (C-X-C motif) ligand 12) is a stromal cell-derived factor and a critical regulator of HSCs. One group generated a *Cxcl12*-GFP knock-in allele and noted that perivascular reticular cells adjacent to sinusoids or in the endosteum were particularly positive for GFP expression; they named these cells CAR (Cxcl12-abundant reticular) cells(16). They further demonstrated that individual CAR cells express high level of the osteogenic transcription factors, *Runx2* and *Osx*, and adipogenic transcription factor *PPARγ*. In addition, ablation of CAR cells significantly impaired the formation of osteoblasts and adipocytes *in vitro*(16). The theory that mesenchymal and hematopoietic stem cells form a unique bone marrow niche was proposed in 2010(17). These investigators discovered that *Nestin*-GFP was expressed in perivascular stromal cells and was especially bright in pericytes of bone marrow arterioles (Nestin is an intermediate filament protein and a marker for neural stem cells). These *Nes*-GFP$^+$ stromal cells colocalized with HSCs, included all CFU-F activities and formed self-renewable 'mesenspheres' that could pass serial heterotopic transplantations. Their group subsequently showed that CD51$^+$PDGFRα$^+$ cells constituted a population of *Nes*-GFP$^+$ cells enriched for supporting hematopoiesis and exhibiting properties of skeletal progenitors in murine and human cell preparations (14). Alpha-smooth muscle actin (αSMA) is a marker of pericytes. One group generated *αSMA*-GFP/Cherry transgenic mice and showed that transgene-expressing cells were associated with microvasculature in bone marrow and periosteum and

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exhibited trilineage differentiation potential *in vitro*(18). Further, another group showed that peri-trabecular stromal cells expressed *Osterix (Osx)*-Cherry (Osx is an important transcription factor in osteoblast differentiation) (19) and *Connective Tissue Growth Factor (CTGF)*-GFP(20), and the latter cells appear to behave as mesenchymal progenitors at least *in vitro* and upon transplantation. Therefore, use of transgenic markers and sophisticated cell surface markers in combination with more traditional *in vitro*, CFU-F and transplantation assays has proven to be useful in delineating mesenchymal progenitors in adult bone marrow, and revealed an emerging complexity of mesenchymal stromal populations, with subpopulations expressing different genes and residing at different locations of bone marrow.

Further insights into mesenchymal progenitor cells: Murine lineage-tracing system

Much of the work on stem cells in mesenchymal tissues has been derived by the ideas of regenerative medicine. Thus, *in vitro* expansion and transplantation assays have revealed important stem cell-like properties of cells isolated from tissues. These studies cannot, however, address the function of stem cells in their native setting. To explore such functions of progenitor cells, lineage-tracing technologies have been exploited. Typically, such approaches use the *cre-lox* technology to allow promoter-specified expression of *cre* recombinase. *Cre* then acts on reporter genes driven by ubiquitously expressed promoters that are blocked in protein expression by the presence of sequences that stop continued transcription and translation. These “STOP” sequences are flanked by *lox* sites; thus, the reporter protein is synthesized and expressed only after the action of *cre* recombinase. Typically, the *cre* recombinase is covalently bound to the ligand-binding domain of the estrogen receptor (creERt) that has been mutated so that tamoxifen but not estradiol can bind. After tamoxifen administration, the recombinase is active only for about 24 hours that tamoxifen is present (21), though, after recombinase action, the reporter gene is continually expressed in that cell and its descendants. This approach has been increasingly used to establish progenitor-descendant relationships in an unperturbed environment. The major challenges in using this approach result from identifying promoters with expression in a narrow array of desirable cell types, and particularly, promoters without expression in putative descendant cells.

Mendez-Ferrer et al(17) were the first to use such a system to mark and trace the fate of bone marrow mesenchymal progenitors using this system; the authors used a *Nestin-creERt* transgenic line for this purpose. When they induced recombination in adult mice, no descendant was detectable after a month of chase. However, after 8 months of chase, some descendants of cells marked after *cre* recombinase action became osteoblasts, osteocytes and chondrocytes(17), although more detailed kinetics of marked cells was not shown in this study. The same group further demonstrated that pericytes of bone marrow arterioles were marked by a *NG2-creERt* line(22); however, the authors did not analyze the fate of these cells thereafter. Grcevic et al(18) generated *aSMA-creERt* line and analyzed the fate of these cells. Cells in the primary spongiosa and the periosteum were marked, and their descendants increased over a few weeks during normal bone turnover (they did not examine a longer time of chase in this study), and participated in fracture repair. Whether the cells that participated in fracture repair came from perivascular stromal cells or periosteum was not

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evident from their data. One group used another inducible promoter, the interferon-inducible *Mx1* promoter, to drive expression of *cre* in response to administration of polyI-polyC; *Mx1-cre* marked bone marrow mesenchymal progenitor cells(23). *Mx1-cre* marked both mesenchymal stromal and hematopoietic cells in bone marrow; the authors carefully eliminated the possibility of the latter's contribution by removing them with irradiation and bone marrow transplantation. They found that *Mx1-cre* marked stromal cells included cells that continually, for the life of the mouse, became osteoblasts in normal bone and fracture repair, and were intravenously transplantable. These cells, when cultured *in vitro,* were capable of becoming adipocytes, chondrocytes and osteoblasts. In contrast, their differentiation was limited to 'osteolineage' cells (osteoblasts and stromal cells) in normal bone marrow, highlighting the dichotomy between their multi-cellular fates *in vitro* and their fates *in vivo*. Because of the extended mode of induction that the authors used in their study (pIpC injection on every other day for 10 days) and the expression of *Mx1-cre* in both osteoblasts and their precursors, they could not ascertain the location and the precise identity of the cells responsible for continually generating osteoblasts in the native environment.

A number of studies using genetic models in recent years have uncovered more detailed biology of mesenchymal progenitors of the osteolineage; however, heterogeneity of cell populations of interest marked by a promoter-based approach complicate overall interpretation of presented data. This is particularly true in studies using a 'constitutively active' version of *cre* recombinase. The fundamental difference of 'constitutively active' *cre* from 'inducible' *creERt* should not be overlooked; unlike the latter, the former induces recombination whenever the promoter becomes active (no temporal 'time' factor). Therefore, if the promoter is active during early development, the *cre* line inherently marks greater numbers of descendants, rendering overall interpretation more complicated. If that promoter becomes active in other cell types later in development, then the possible relationships between the differing cell types marked by a reporter cannot be delineated. For example, one group showed that, among the 'PαS' fraction, CFU-Fs were exclusively found within the *Prx1-cre* targeted fraction. The authors concluded that *Prx1-cre* marks early mesenchymal progenitors in adult bone marrow(24). While this might be the case, because *Prx1-cre* marks essentially all limb mesenchymal cells and their descendants, the promoter-reporter combination cannot be used to identify mesenchymal precursors by histological techniques. More recently, one group reported that perisinusoidal mesenchymal stromal cells expressing leptin receptor (LepR) was the main source of bone-forming cells in adult mice. They showed that, using a *LepR-cre* knock-in allele, the descendants of *LepR-cre* expressing cells contributed to a large fraction of CFU-Fs, and adult osteoblasts and adipocytes; in addition, these cells were intrafemorally transplantable after sublethal irradiation(25). Interestingly, $LepR^{+}$ cells differentiated into osteoblasts on the bone surfaces only after two months of age; before that, they were in the primary spongiosa and in stromal regions. Because the expression of *LepR-cre* could not be limited in time, however, the authors were limited in their ability to identify *in vivo* the specific precursors of adult osteoblasts and adipocytes.

In summary, we now have substantial evidences that several populations of bone marrow stromal cells behave as 'mesenchymal stem cells' when taken out of their native

environment. However, the identities of cells that are the precursors of adult stromal, osteoblasts, and adipocytes during postnatal growth and homeostasis in adults need further clarification.

Mesenchymal progenitor cells of the osteogenic lineage in development

During early limb development, cells in the lateral plate mesoderm express Prx1 and serve as precursors of all limb mesenchymal cells. In fact, *Prx1-cre* marks essentially all mesenchymal cells in bone, including osteoblasts, chondrocytes and stromal cells, but not cells in skeletal muscles(26). Subsequently, mesenchymal condensations within the limb bud determine the domain for future bone, and these cells express Sox9. As *Sox9-cre* marks essentially all mesenchymal cells in bone, early cells expressing Sox9 also serve as precursors for osteoblasts and chondrocytes(27). These fate-mapping studies indicate that developmental mesenchymal progenitors arise locally within the bone anlage. Subsequently, cells within condensations express type II collagen (Col2) and differentiate into chondrocytes, and further into hypertrophic chondrocytes expressing type X collagen (Col10). Around the same time, cells in the surrounding region, the perichondrium, express Osterix (Osx), a critical transcription factor in osteoblast differentiation. Use of an *Osx-creERt* line demonstrated that Osx^{+} cells in the perichondrium invaded into the cartilage template together with blood vessels and subsequently, became osteoblasts and stromal cells inside the ossification center(28). Cells in the perichondrium also expressed *Nes*-GFP, and these cells were derived from cells within the cartilage template expressing *Col2-cre*(29). Further studies showed that the Osx^{+} cells in the fetal perichondrium only transiently contributed to bone marrow stroma; in contrast, early postnatal Osx^{+} cells contributed to bone marrow stroma persistently for a very long time(30,31). Adult Osx^{+} cells did not have such capability(30). These findings indicate that Osx^{+} cells at a specific time of development are recruited to a perivascular location in bone marrow and become stromal cells, some of which may later behave as mesenchymal progenitors. The underlying reason for differential expression of Osx in a specific subtype of progenitors at a specific time is unknown, and requires further studies. In line with these findings, *Osx-cre* also marks not only osteoblasts and osteocytes, but also stromal cells and adipocytes(32,33), complementing the notion that Osx is expressed in these mesenchymal progenitors sometime during their lineage progression.

Recent lines of evidence suggest that cells marked by the expression of genes expressed in mesenchymal condensations represent the major source for chondrocytes, osteoblasts and stromal cells populations in the limb. Cells expressing type II collagen (Col2) have been implicated to include osteochondroprogenitors, as conditional gene deletion using *Col2-cre* affects both bone and cartilage(34–36). One recent study showed that, using an inducible genetic labeling system with multiple transgenes expressed in mesenchymal condensations and early growth cartilage, *Col2-creERt*, *Sox9-creERt* and *Acan-creERt* marked cells at P3 continued to provide osteoblasts and stromal cells for more than a year(31). In fact, a majority of osteoblasts, *Cxcl12*-GFP^{+} stromal cells (‘CAR cells’) and bone marrow mesenchymal progenitors (CFU-Fs and PαS cells) were derivative of cells marked by *Col2-cre*. Further, another group showed that, using *Col10-cre* and *Acan-creERt*, chondrocytes were the source of osteoblasts in endochondral bones during development and fracture

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healing(37). Yet another group showed that, using *Col10-cre* and *Col10-creERt*, hypertrophic chondrocytes could become osteoblasts(38). Therefore, these recent studies suggest that chondrocytes themselves may also be a source of osteoblasts and stromal cells during endochondral bone ossification. The idea that chondrocytes or cells closely related to them in the growth plate cartilage or its surrounding areas represent a novel population of developmental mesenchymal progenitors of the osteogenic lineage emphasizes that, during the fetal and early postnatal period of comparatively explosive increase in the number of osteolineage cells, multiple cell types are enlisted to form bone. More recently, one group showed that ‘mouse skeletal stem cells (mSSCs)’, which could generate bone, cartilage and stroma upon transplantation, could be identified by a combination of cell surface markers and found in proximity to the growth plate(39). Another group showed that, using *Grem1-creER* (Gremlin 1 is an antagonist for bone morphogenic protein, BMP), osteochondroreticular (OCR) stem cells were concentrated within the metaphysis of long bones, but not in the perisinusoidal space(40); in addition, they showed that these ‘OCR stem cells’ were more clonogenic than perisinusoidal ‘MSCs’. This pair of studies, more strikingly, took advantage of multicolor ‘*Brainbow*’ or ‘*Confetti*’ reporter alleles and elegantly demonstrated the existence of clonogenic precursors in the growth plate region. While *Grem1-creERt* marked early cells that become chondrocytes and osteoblasts *in vivo*, the transgene did not mark adipocytes, illustrating further the variable fates of cells capable of generating multiple mesenchymal lineages.

Conclusion

In this short review, we have discussed recent advances in our knowledge on mesenchymal progenitor cells of osteogenic cells both in adult life and development. It is increasingly evident that there are two distinct types of bone progenitor cells. Growth-associated mesenchymal progenitors are found in proximity to the growth plate cartilage in the metaphysis and epiphysis of endochondral bones, and contribute to explosive growth of bone in early life. In contrast, heterogeneous bone marrow mesenchymal progenitors are found in perisinusoidal locations, and contribute to the much slower remodeling process and response to injury that occurs mainly in adulthood. Furthermore, growth-associated mesenchymal progenitors may be eventually recruited to perivascular locations, and thus serve as the source of bone marrow mesenchymal progenitors in adult life. More detailed mechanisms on this transition need to be studied through further experimentation.

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