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Origin of Reparative Stem Cells in Fracture Healing

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Abstract

Purpose of Review The identity and functional roles of stem cell population(s) that contribute to fracture repair remains unclear. This review provides a brief history of mesenchymal stem cell (MSCs) and provides an updated view of the many stem/progenitor cell populations contributing to fracture repair.

Recent Findings Functional studies show MSCs are not the multipotential stem cell population that form cartilage and bone during fracture repair. Rather, multiple studies have confirmed the periosteum is the primary source of stem/progenitor cells for fracture repair. Newer work is also identifying other stem/progenitor cells that may also contribute to healing.

Summary Although the heterogenous periosteal cells migrate to the fracture site and contribute directly to callus formation, other cell populations are involved. Pericytes and bone marrow stromal cells are now thought of as key secretory centers that mostly coordinate the repair process. Other populations of stem/progenitor cells from the muscle and transdifferentiated chondroctyes may also contribute to repair, and their functional role is an area of active research.

Keywords Skeletal stem cell · Fracture · Mesenchymal stem cell · Periosteum · Pericyte

Defining Stem Cells

Stem cells are defined as having the ability to differentiate to specialized cell types and being capable of self-renewal, such that some fraction of the progeny remains un-differentiated. Based on the differentiation potential, or "potency" of the stem cell, they can be classified as totipotent, pluripotent, multipotent, or unipotent. Totipotent describes stem cells that are able to generate all of the cell fates of the animal including the extraembryonic tissues, such as the placenta. These are the zygote and early blastomeres. Pluripotent cells, also called embryonic stem cells (ESCs), are able to generate the embryonic tissues from all three primary germ layers and are isolated from the inner cell mass of the blastocyst. In addition to these native pluripotent stem cells, it is also possible to experimentally derive a pluripotent stem cell population from adult somatic cell. These "induced" pluripotent stem cells (iPCS) [1, 2] are generated by genetically reprograming cells: the technology was named the Nature Method of the Year in 2009 and Yamanaka/Gurdon received the Nobel Prize 2012 for their discovery.

Once stem cells lose the ability to form all cell types, they are considered multipotent. Multipotent cells generate a limited number of cell fates of an animal in closely related families of cells, for example, all the tissues within a specific germ layer or organ system. Tissue-specific stem cells are known as "adult" or "somatic" stem cells, and are somewhat specialized to generate multiple, organ-specific cell types. Adult stem cells are located in specialized niches that maintain the stemness through interaction with the local microenvironment. These stem cells contribute to tissue homeostasis and injury response.

The musculoskeletal system supports a number of multipotent adult stem cells. The best characterized adult stem cell population is the hematopoietic stem cell (HSC), which is the source for all blood cells. HSCs are localized and

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maintained within the bone marrow. Also located in this niche are the bone marrow stromal cells (BMSCs), also known as mesenchymal stem cells (MSCs) or skeletal stem cell (SSC). As discussed later in the review, there remains disagreement as to the definition and biological role of these cells in the adult animal.

Lastly is the unipotent stem cell, which is able to generate only a single cell fate. There is some debate within the field on whether unipotent stem cells should be considered "stem cells" or if "stem cells" must have the capacity to differentiate into multiple cell types. Within the musckuloskeletal system, satellite cells are unipotent progenitors that have self-renewal capacity, but only give rise to muscle cells.

While differentiation capacity is the defining characteristic of a stem cell, to be considered a bonafide stem cell, it must also have self-renewing properties. Experimentally, it is much more difficult to demonstrate self-renewing properties. The gold standard for experimentally demonstrating self-renewal is in vivo implantation and serial transplantation [3]. This involves isolating the discrete cell population of interest followed by implantation and following the formation of ectopic tissue. This provides the initial in vivo evidence that the cell population of interest can give rise to de novo tissues. Next, self-renewal capacity must be shown through re-isolation of the cell population from this de novo tissue, followed by a second implantation demonstrating subsequent de novo tissue formation. In the case where self-renewal has not been experimentally demonstrated, it is more accurate to use the term "progenitor" cell to describe the cell population. Progenitor cells are an intermediate between the stem cell and specialized cell, have a high proliferative capacity, and are non-self-renewing.

This review will focus on the endogenous stem and progenitor populations that contribute to fracture repair. Bone is unique within the musculoskeletal system in that under normal conditions a broken bone can truly regenerate, producing a de novo tissue that is indistinguishable from the original, in form and function. We aim to present a current perspective on both the individual cell types involved in bone regeneration and how cross talk between cell populations coordinates healing. Importantly, this review aims to highlight the many unanswered questions and areas of ongoing debate that relate to the type and location of these different stem and progenitor cell populations.

The "MSC"

The History and Debate

Some of the earliest studies aimed at bone regeneration are by Urist in 1965 where he was able to induce heterotopic ossification (HO) or de novo bone formation in the musculature of animals by implanting demineralized bone [4]. Later studies by Urist first identified Bone Morphogenetic Proteins (BMPs) as the key protein driving HO development [5, 6]. However, it was Tavassoli and Crosby that originated the concept that a population of adult "stem cells" respond and give rise to the de novo bone formation also in the 1960s. Their experiments showed that boneless fragments isolated from the bone marrow could be transplanted into multiple heterotopic sites and produce HO. The size of the HO appeared to depend upon the amount of isolated tissue implanted [4, 7]. It was concluded that the bone marrow must consist of an entity that had ostegenic potential. This work was followed by Friedenstein et al. who continued this work from the late 1960s to 1990. During this time, he isolated the bone marrow-derived "stem cell" and demonstrated osteogenic capacity. The osteogenic potential of these cells were non-hematopeoietic, tissue culture plastic adherent cells, and were clonogenic in culture at low density. Further, transplantation of a single clonogenic cell had multipotent potential and could generate a variety of tissues in addition to bone, including cartilage, adipocytes, and fibroblasts [8–14].

In 1990, Arnold Caplan coined the term "mesenchymal stem cell", or MSCs, to describe these multipotent progenitor cells with the capacity to form adipose, cartilage, and bone tissue or the "ABCs" [15, 16]. The mesenchymal stem cell theory originated and developed from the idea that during embryogenesis the mesoderm consists of multipotent progenitors that will give rise to bone, cartilage, muscle, and other mesenchymal tissues. Similarly, cells from the bone marrow had osteogenic potential in vivo and were shown to differentiate into multiple lineages such as bone, cartilage, tendon, muscle, and fat in vitro.

Through Caplan's work, along with others, it was thought that these MSCs were perhaps more broadly distributed throughout the body rather than located solely within the bone marrow. Progenitor cells showing similar in vitro differentiation potential to the bone marrow-derived MSCs have subsequently been isolated from adipose tissue [17], periosteum [18, 19], the synovial lining [20, 21], and muscle [22, 23] tissue. Crisan et al. later demonstrated that MSCs expressed similar markers with pericytes (cells located on the abluminal surface of vessels) and that pericytes had equivalent multipotent properties in vitro [24, 25]. This suggested that MSCs were pericytes and perhaps explains how they can be isolated from many tissues. Caplan wrote in an article to accompany the Crisan paper that "…my suggestion is all MSCs are pericytes…" [26].

We would like to point out that not all pericytes are MSCs and that experimentally there are inherent differences between MSCs isolated from different tissues [26–28]. This may be explained by the fact that the multipotency of these cells has only been rigorously demonstrated in vitro. As such, it remains unclear, and actively debated, whether the

perivasculature is another MSC niche, if they are the same cell, or if pericytes represent some MSC precursor population. However, the previous studies from the 1960s and 1970s showing bone marrow stromal cells capable of forming ectopic bone in vivo suggests a presence of a stem cell or tissue-specific progenitor(s) that are more linage restricted than the 1990 Caplan postulated MSC [29–31]. Current research suggests that tissue-specific progenitor cell populations may actually contribute to the pericyte fate [27•], providing a possible link with pericytes.

Despite remaining uncertainty over the true multipotency of the MSC, recent work has suggested that in vivo MSCs function as secretory centers rather than a progenitor population [32, 33]. Rather, the current model postulates that injury activates MSCs/pericytes to migrate away from vessels and become activated to secrete factors that promote local tissue regeneration [32, 34–38]. A specific population of MSCs/pericytes did not contribute to tissue homeostasis over a 2-year period or contribute to the regeneration after injury (brain, heart, and muscle) [39]. A secretome of the MSCs has been demonstrated as both trophic and immunomodulatory in that it can stimulate tissue repair by activating local progenitor cells and secreting anti-inflammatory cytokines. In fact, in 2017, Caplan purposed to change the name of "Mesenchymal Stem Cell" to "Medicinal Signaling Cell" due to their main function as signaling centers during injury [40, 41].

MSC Identity

When using the term MSCs, details of the cell population one is referring to should be clearly defined, as history shows there is discrepancy in naming convention. Naming convention is challenged by a lack of consensus over the cell surface markers for a "MSC." Moreover, there appears to be species-specific differences, with possible differences in cell surface markers between human and mouse MSCs. In 2006, the International Society of Cellular Therapy published a proposed minimal criteria for multipotent MSCs; they suggested that cells must be adherent to plastic, show multipotent differentiation in vitro to osteoblasts, adipocytes, chondrocytes, and have specific surface antigen expression for CD105, CD73, and CD90 while negative for CD45, CD35, CD14, CD79alpha, and HLA-DR. (Table 1).

Stem Cell Contribution to Fracture Repair

Molecular and Cellular Overview

Bone has a high regenerative capacity, and the majority of fractures will heal with a tissue that is indistinguishable from the native tissue in form and function. One reason for the reparative nature of bone is the existence of many active local progenitor cells connected systemically through networks of vascularity. Fracture healing proceeds through four biologically distinct, but overlapping phases—inflammation, intramembranous ossification, endochondral ossification, bone remodeling—with stem cells differentially contributing to each stage of healing.

The cascade of bone regeneration begins when the bone breaks. This exposes the bone marrow and results in rapid formation of a hematoma to contain the bleeding and help debride the injury. In this first phase of repair, a pro-inflammatory response dominates healing through activation of neutrophils, macrophages, and other inflammatory cells. While a prolonged pro-inflammatory state can have a negative effect on bone repair, the early pro-inflammatory response influences differentiation and establishment of the initial fracture callus [42–47].

The progenitor cells that heal the fractured bone come locally from the endosteum and periosteum [29, 48–50]. These progenitor cells respond to the fracture by upregulating genes associated with proliferation, as well as numerous cytokines and chemokines that help enable osteogenic or chondrogenic differentiation [51]. Importantly, differentiation of these stem cells is mechanically sensitive and the relative stability of the microenvironment drives fate decisions [52–55]. Along the endosteal and periosteal surfaces, where the bone itself provides a high degree of stability, the progenitor cells differentiate directly into osteoblasts and contribute to fracture repair through intramembranous bone healing. In the fracture gap, where there is more mobility, periosteal progenitor cells differentiate into chondrocytes to form a soft cartilage callus to provide a temporary bridge between the broken bone ends.

The endochondral phase of healing describes the process by which this soft cartilage callus becomes bone. During this phase of healing, it is important that the pro-inflammatory state is resolved or healing can be delayed [44, 45, 47, 56]. Macrophage polarity is critical towards regulating the inflammatory microenvironment and resolution involves modulating from the pro-inflammatory "M1" macrophages towards an anti-inflammatory "M2" state.

As endochondral healing proceeds, the soft cartilage callus is replaced by bone through a combination of chondrocyte to osteoblast transformation and new bone formation. It was once believed that hypertrophic chondrocytes were fated for cell death [57, 58], but now there is a preponderance of new data using modern genetic tools demonstrating chondrocytes can give rise directly to osteoblasts during development, growth, and repair [59–65]. While data suggests that chondrocytes give rise to the majority of osteoblasts during fracture healing [59, 60], pericytes or invading osteochondral progenitors from the vasculature may also contribute to the new bone formation from the cartilage template [66].

The final stage of fracture healing involves conversion of the newly formed woven bone to a cortical bone structure. Bone resorption is tightly coupled to bone formation and

Table 1 MSC cell surface markers

Marker	Description
CD29	Marker of MSCs recruited by Substance P to wounded sites
CD31	Endothelial/angiogenic progenitor cell marker
CD34	Early hematopoietic/endothelial progenitor cell marker
CD44	Early MSC marker putatively associated with Nanog expression
CD45	General hematopoietic stem cell marker
CD73	Marker of MSCs that demonstrate osteogenic capacity in vitro
CD105	Marker of MSCs that demonstrate osteogenic capacity in vitro
CD146	Marker of cells with ability to regenerate the hematopoietic stem cell niche and have osteogenic properties
CD271	Marker of MSCs that demonstrate osteogenic capacity in vitro
STRO-1	Marker of MSCs that demonstrate osteogenic capacity in vitro
Sca-1	Marker of MSCs that demonstrate osteogenic capacity in vitro
C-Kit	Marker of early and supposedly self-renewing endothelial progenitor cell
CXCR4	Receptor to SDF-1a

required for functional healing. Osteoclasts are the multinucleated giant cells responsible for resorption [67]. Osteoclast-mediated degradation of the bone releases matrix-sequestered factors, such as TGFβ, as well as factors produced by the osteoclast itself, such as complement 3a, which are both hypothesized to be critical in the subsequent stimulation of osteogenesis [68, 69]. Osteoclast mediated bone resorption is concluded with the apoptotic death of the osteoclasts [70].

While the initial fracture callus is established by osteochondral progenitor cells from the periosteum and endosteum, multiple other progenitor populations influence their differentiation and coordinate their ultimate phenotype. In the sub-sections below, we aim to describe the contribution and cross talk between various progenitor populations and experimental tools for continuing to investigate their role in fracture repair (Fig. 1).

Periosteal Progenitor Cells

Covering the surfaces of bone is a highly vascularized bilayer membrane called the periosteum. In the outermost layer of the periosteum, collagens and elastin provide fibrous structure that assists in regulating the flow of molecules between bone and muscle [71, 72]. The innermost layer consists of the progenitor cells that contribute to bone homeostasis and fracture repair. The cellular response to fracture within the periosteum is observed as early as 24–48 h post-injury through rapid proliferation. While the disruption of the periosteum has been shown to delay fracture repair [73], the specific identification of the population(s) of progenitor cells that reside within the periosteum is still unclear. Functional studies suggest that periosteal progenitors are bipotent, with an osteochondral potential [48], and that they express some MSCs cell surface markes: CD73, CD90, and CD105 [52, 72, 74, 75].

Elegant studies by Celine Colnot used the combination of bone grafting and lineage tracing to show that periosteal and endosteal cells are functionally distinct [48]. Using reporter animals as donors for bone grafts, it was shown that the endosteum has only an osteogenic potential, while the periosteum has both osteogenic and chondrogenic potential. This characteristic appears intrinsic to the cell populations. This was experimentally supported by demonstrating a differential response of periosteal and endosteal cells to BMP, where the chondrogenic potential of endosteal cells is significantly reduced given exogenous application of BMP [76]. This differing response to BMP signaling in the periosteal cells is further supported by an interesting study by Morgan et al. where BMP signaling was inhibited using a soluble form of the BMP receptor type Ia. Only the periosteal response was blunted, cartilage maturation was delayed, and periosteal bone bridging was decreased, resulting in impaired strength and toughness. Although other cell populations showed an osteogenic response with increased osteogenic gene response and mineralized tissue [77].

Using reporter mice Prx1 has been identified as a periosteal marker, first identified by expression in mesenchymal cells during embryological development of the limb [29, 78–80]. Studies in the adult animal later showed Prx1 expression in the periosteum and suggested they represents a population of progenitor cells that contributes to the fracture callus [29, 49•]. Knockout of BMP2 in Prx1-Cre cells in the limb mesenchyme resulted in spontaneous fractures in adults and a lack of callus formation [81]. Within the periosteum, a subset of cells along the perivascular domain that were labeled by αSMA were also found to give rise to the fracture callus [50, 82•]. Characterization of these αSMA^{+} cells from the periosteum revealed a very heterogeneous population that expresses different markers of MSCs (Sca1 (9% of cells), PDGFRα (2%), Leptin receptor (2%), and

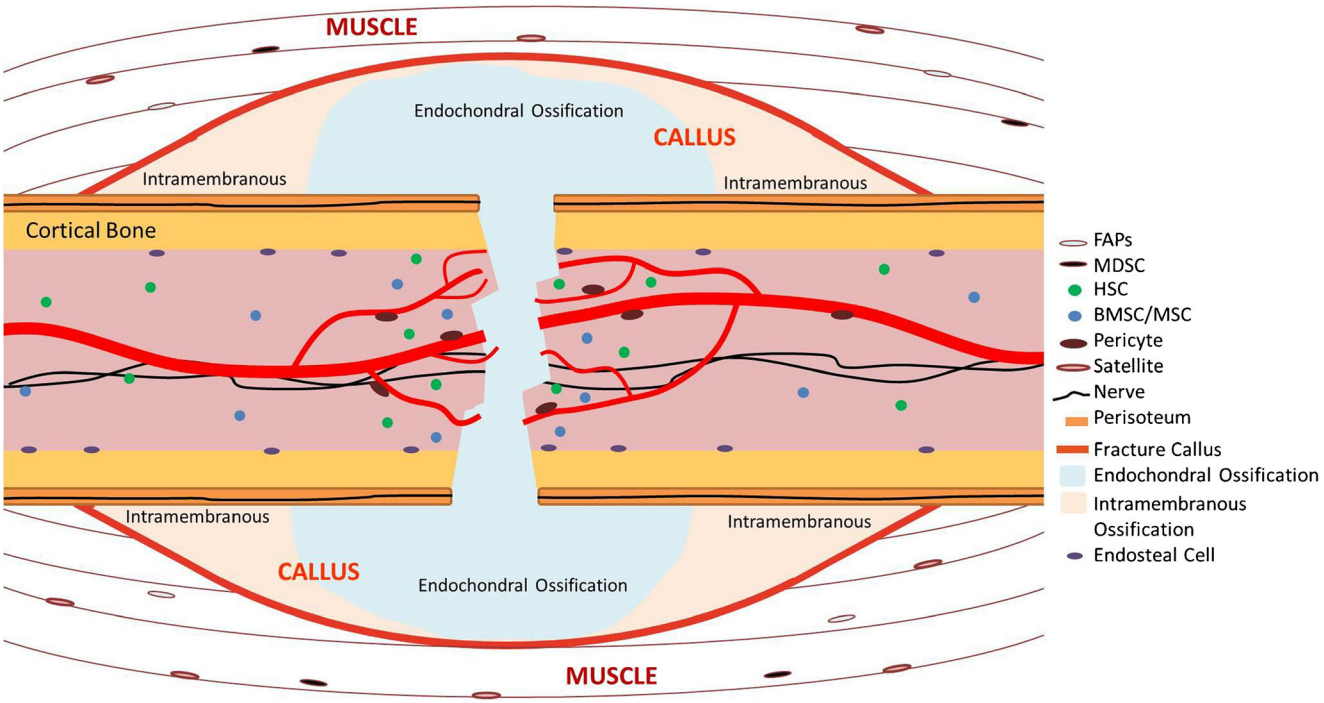

Fig. 1 A drawing depicting different cell populations that contribute to fracture repair. The fracture callus and surrounding muscle along with areas of intramembranous (light red area), endochondral ossification (blue area), blood vessels, and nerves are indicated

pericytes (PDGFRβ (2%)). Taken together, these studies indicate a heterogenous periosteum where the cell populations have a distinct function and potential from other progenitor cells.

MSCs

Because of the excitement generated over the discovery of the "MSC," and their multipotent capacity to form cartilage and bone in vitro, it is often falsely assumed that bone marrow-derived MSCs are the primary cells that heal bone. This misconception is fueled clinically by therapeutic application of BM-MSCs to promote bone regeneration. However, as discussed above, and rigorously demonstrated experimentally, periosteal progenitors are the primary source of cells that give rise directly to fracture callus. Importantly, it has been shown experimentally that bone marrow-derived cells have a very minimal direct contribution to fracture healing using bone marrow ablation/transplantation [44, 83].

While BM-MSCs may not play a direct role in forming the fracture callus that heals the bone, they nonetheless play an important role in supporting healing through paracrine secretion of trophic and immunomodulatory factors. The "trophic," or a stimulatory influence, of MSCs on other cells is another term that has been famously coined and promoted by Caplan [32]. Secretory molecules produced by MSCs that influence tissue resident progenitor cells include TGF-β, stem cell factor (SCF), insulin-like growth factor (IGF), epidermal growth factor (EGF), and granulocyte and macrophage colony stimulating factors (G/M-CSF) [32, 33]. Current evidence suggests that these paracrine trophic factors, rather than direct differentiation and engraftment of MSCs [32, 84–87], are responsible for observed repairs following MSC therapy in disease conditions such as stroke [88, 89], osteogenesis imperfecta [90], myocardial infarct [91], and fracture repair [92, 93].

In addition to this trophic role, MSCs are key regulators of local inflammation during fracture repair. As mentioned above, transition from a pro- to anti-inflammatory state is critical for stimulating both initial differentiation of the periosteal progenitor cells and transition from cartilage to bone during endochondral ossification [42–47]. Specifically, macrophages are a key cell population that contribute to polarizing the inflammatory state and MSCs have an immunomodulatory effect on macrophages. The mechanism(s) by which MSCs polarize macrophages remains an active area of research. While it is established that paracrine regulation of the macrophage polarity can be achieved by secretion of factors such as IL-10, IL-13, IL-4, and TGFβ, there may also be direct interaction of these cell types [32, 84, 94–96].

Tracking the in vivo fate and contribution of tissue resident MSCs and/or pericytes to fracture healing is complex and has been limited to studies that can identify novel cell populations by in vivo markers and subsequent functional studies. Within the bone marrow, "MSC-like" cells that contribute to bone regeneration with unipotent potential towards the osteogenic lineage were identified by expression of myxovirus resistance-1 (Mx-1) [30]. These Mx1-cells were shown to have clonogenic capacity, single cell-derived multilineage differentiation capability ex vivo, and in vivo osteogenic

differentiation and new bone formation after transplantation [30]. Work from Ivo Kalajzic's group has demonstrated a set of αSMA$^+$ cells located in the perivascular region of the periosteum, bone marrow, and muscle [50, 82]. In vitro αSMA$^+$ cells have multilineage potential and in vivo αSMA-CreERT::Ai9 contribute directly to the bone and cartilage callus formed during fracture healing [50]. Continued work in these reporter systems will help to clarify both the cellular contribution and functional role for these "MSC" populations during fracture healing and help to define the relationship of these cells to other progenitor populations (Table 2).

Cartilage to Bone Transition in the Fracture Callus

Formation of the cartilage callus by the periosteal cells is functionally important towards rapidly stabilizing the two bone ends. This provisional cartilage callus forms rapidly within the fracture gap, around days 5–7 in a mouse, and has been postulated to serve an evolutionary role in minimizing weight bearing until healing has been completed. Chondrocytes within this fracture callus proliferate and mature to hypertrophy through molecular signaling mechanisms presumed to parallel endochondral ossification in the growth plate [97].

Our understanding of the process by which bone forms from the cartilage callus is evolving. Previously, it was understood that hypertrophic chondrocytes underwent programmed cell death and that bone was formed by osteoprogenitors that invaded and remodeled the provisional cartilage matrix [58, 66, 98]. While multiple earlier studies had suggested that chondrocytes could become osteoblasts [99–104], it is only recently that modern murine genetics has enabled lineage tracing to demonstrate that chondrocytes can give rise directly to bone through cellular transformation or transdifferentiation [59–65].

The mechanisms by which hypertrophic chondrocytes become osteoblasts remains unclear. Chondrocytes have been suggested to "de-differentiate" when cultured in vitro as early as 1960 and these "de-differentiated" chondrocytes could reexpress specific collagen genes to show a differentiated phenotype again [105, 106]. Later, Song and Tuan [107] suggested this enabled transdifferentiation in vitro. More recently, a few in vivo studies found expression of the pluripotency transcription factors during fracture healing [51, 59, 60]. In particular, Hu et al. identified Sox2 at the osteochondral transition of the fracture callus using immunohistochemistry and genetic labeling in hypertrophic chondrocytes, and importantly, showed that conditional deletion of Sox2 impaired fracture healing by producing a significantly smaller callus with a decreased bone and increased cartilage fraction [60]. Outside of fracture repair, Sox2 has been identified as a marker of the adult stem cell niche and found to maintain multipotency and cell proliferation [108–112]. While no studies to date have rigorously tested if hypertrophic chondrocytes meet the technical requirement of a "stem cells," in the sense that they have multipotent potential, hypertrophic chondrocytes at the chondro-osseous boarder have been shown to re-enter the cell cycle prior to transdifferentiation as evident by BrdU incorporation [60, 113], and may be capable of asymmetric cell division [103, 114].

Irrespective of whether hypertrophic chondrocytes in the fracture callus take on some sort of "stem cell like state" to become more plastic, it is clear that these cells switch their genetic profile to become more osteoblastic [60, 115, 116]. The Wnt/β-catenin pathway appears to be particularly central to this genetic transformation. At a molecular level, chondrogenic (Sox9) versus osteogenic differentiation (Runx2, Wnt/β-catenin) is regulated by direct repression of the opposing pathway [117–120]. Functionally, two important studies have shown that genetic deletion of β-catenin from chondrocytes in the developing bone causes a severe decrease in trabecular bone formation and changes to osteoclastogenesis, while overexpression of β-catenin causes ectopic or accelerated bone formation [65, 121, 122]. In fracture repair, immunohistochemistry has shown β-catenin and Runx2 are activated in hypertrophic chondrocytes at the fracture callus. Further, promoting activation of β-catenin through PTH treatment during fracture repair increased not only chondrogenic proliferation and differentiation, but also Runx2 and Osterix expression. The resulting callus was larger with significantly increased total bone volume [123].

Vasculature and Pericytes

The vasculature contributes to postnatal skeletal repair through both indirect and direct mechanisms. At a fundamental level, the vasculature is necessary for providing a permissive repair environment. This includes bringing in oxygen and nutrients, carrying away waste, and bringing in the immune cells that initiate a healing response. Angiogenesis, or new blood vessel formation, is critical for proper healing and impaired blood flow is often linked with poor clinical outcomes [124–126]. These new blood vessels typically form at the regenerative front of well healing tissues like bone, and the vascular endothelial cells have been shown to secrete trophic factors that promote osteogenesis [59, 76, 127]. Secreted factors from the vascular endothelial cells may also play a role in stimulating stem cells within their niche to enter a regenerative state by promoting Sox2 expression [60].

In addition to this direct role of the vasculature in supporting repair, blood vessels also provide the structural basis for pericytes. Following injury, pericytes are thought to enter the injured tissue in an activated state, where they serve a trophic function of providing growth factors to drive tissue regeneration and also play an anti-inflammatory role [32, 36, 37]. Although the complete array of cytokines and growth factors that are secreted during injury is not known to date, it was shown that even cultured human pericytes (passages 1–10) secrete high levels of heparin-binding epidermal growth

Table 2 Genetic labeling of stem cell populations

Cell type	Cell marker	In vitro differentiation	In vivo role in fracture	Inducible mice for reporter/functional studies	References
Stem cells	Sox2	Pluripotent	Expressed by hypertrophic chondrocytes KO delays FX healing	Sox2-CreER; Jax 017593	60, 108–111
	Oct4 (Pou5f1)		Possibly expressed during FX healing	Oct-MerCreMer; Jax 016829	59, 60
	Nanog		Possibly expressed during FX healing	none to date	51,60
Pericytes	PDGFRβ	Bone, cartilage, fat, fibroblast, muscle, tendon (?)	Trophic (paracrine supply of growth factors)	PDGFRβ-CreER^{T2}; Jax 029684, 030201	24, 25, 129
	neuro-glial 2 proteoglycan (NG2)			NG2-CreERT	130
	alkaline phosphatase (AP)			Tg:TN-AP-CreER^{T2}	151
Periosteal stem cells	CD 73+, CD 90+, CD 105+	Bone, cartilage	Bi-potent osteochondral progenitor cells	Prx1-Cre; Jax 005584	29, 78–80
"MSCs"	Myxovirus resistance-1 (Mx1)	?	Unipotent progenitor in bone marrow that gives rise to osteoblasts during FX healing.	Mx1-Cre; Jax 003556	30
	smooth muscle α-actin	Bone, cartilage, fat	Forms cartilage and bone in FX callus	αSMA-CreER^{T2}	50, 51
Hypertrophic chondrocytes	Type X collagen	Hypertrophic chondrocyte	Precursor to osteoblast in FX	Col10a1-CreER^{T2}	61
	Osterix (Sp7)	Hypertrophic chondrocyte/ osteoblast	Preosteoblast in Fx	SP7-CreERt	60, 66
FAPs/muscle-derived stem cells	Sca1+, CD 34+, CD45-, PDGFRα	Bone, cartilage, fat, fibroblast, muscle	May contribute directly to FX callus	PDGFRα -CreER^{T2}; Jax 018280	163, 164
Satellite cells	Sca1+, CD 34+, CD 45-, Myf5, Vcam, Pax7, Pax3	Muscle	Trophic (paracrine supply of growth factors)	Pax7-CreER^{T2}; Jax 017763 Pax3-Cre; Jax 025663	146, 151–155 155

factor, basic fibroblast growth factor, platelet-derived growth factor-B chain, vascular endothelial growth factor, keratinocyte growth factor, and thrombopoietin which all have been related to repair [128]. And observations showed that BMP2 and VEGF protein increased with the regenerated bone due to the implantation of human perivascular cells at a critical calvarial bone defect [92]. During repair, pericytes also support angiogenesis by structurally facilitating tubule formation and vascular remodeling.

At least two pericytes markers have become fairly well established using in vivo lineage tracing studies in other fields, platelet-derived growth factor receptor-β (PDGFRβ) [24, 25, 129] and neuro-glial 2 proteoglycan (NG2) [130], and many others have been suggested [131]. To date these pericytes-reporters have not been used to study in vivo fate during fracture healing. However, these pericyte markers along with mesenchymal stem cell markers (CD146, CD44, CD73, CD90, CD105) [25] has enabled the purification of pericytes populations for mechanistic data on the functionality of pericytes during skeletal tissue repair. For example, it has been shown that cultured purified human pericytes isolated from various tissues (skeletal muscle, pancreas, adipose, and bone marrow) are capable to differentiate to multiple lineages including osteoblast/osteocyte, adipose, muscle, and chondrocytes in vitro and formed bony nodules in vivo [24]. While demonstrating in vivo rescue of a non-union fracture rat tibia model with the use of purified human pericytes derived from adipose tissue, the key mechanism was that of the pericyte trophic function, and the role direct ossification was not clear [93]. Another study implanted pericytes within muscle and showed ectopic bone formation [24, 92]. Incorporation of human purified perivascular cells have also been observed with a murine critical-sized calvarial bone defect [92].

Muscle

Fractures are typically traumatic injuries that involve damage to the nearby muscle tissue in addition to the bone. The importance of skeletal muscle to fracture repair has long been appreciated in a clinical setting where open fractures are classified according to the Gustilo-Anderson scale, which characterizes severity of the fracture almost solely upon the extent of the soft tissue (muscle) damage [132, 133]. Muscle is known to be highly permissive for bone formation. This can be capitalized upon therapeutically by applying muscle or fasciocutaneous flaps to improve surgical outcomes of open fractures [134]. Conversely, diseases such as fibrodysplasia ossificans progressive, polytraumatic injuries (burns or traumatic brain injury), and some surgeries can lead to unwanted bone formation in the muscle.

Despite the long standing clinical consideration of muscle in fracture healing, the underlying molecular and cellular mechanisms by which muscle contributes to fracture healing are not well understood. This is due in part to the complex and multidimensional cross talk between muscle and bone. For example, muscle influences fracture healing indirectly by providing a vascular bed in close proximity and by modulating load on the bone. The role of the vasculature was covered in detail previously, and it was described briefly how the periosteal progenitor cells appear to be mechanosenstive since fracture fixation and local stability contributes to differentiation patterns. The influence of load can also impact healing independent of orthopedic hardware as studies have shown fracture healing is delayed by muscle paralysis, disuse atrophy, or immobilization [135–138]. Dissecting the cellular mechanisms that transmit mechanical signals is an active area of research that requires significantly more work, but recent work suggests that the primary cilia is a sensory organelle on cells that can sense and transduce load [139, 140], and that both TGFβ [141] and connexin43 [142–145] are mechanosenitive pathways in the skeleton.

Muscle may also contribute to fracture healing more directly, by providing either cells or paracrine stimuli to promote healing. Multiple subpopulations of progenitor cells have been described in skeletal muscle. The satellite stem cell, originally described in 1961 by Mauro [146], is located between the basal lamina and sarcolemma of myofibers and reliably identified by expression of the paired box transcription factor Pax7 [146, 147]. Satellite cells are traditionally considered unipotent myogenic precursor cells that remain quiescent until triggered into a regenerative state by external stimuli to then generate myoblast that fuse to form new multinucleated myofibers. Importantly, stemness has been experimentally evaluated with transplantation and lineage tracing studies to show satellite cells are capable of replenishing the existing stem cell pool via self-renewal [148–151]. Genetic deletion of Pax7 satellite cells in adult mice entirely blocks regenerative myogenesis [152–154] and also severely impairs fracture healing [155]. In fracture healing, Pax7 ablation acts primarily to support the periosteum through production of key osteogenic growth factors (bone morphogenetic protein, insulin-like growth factor 1, and fibroblast growth factor 2) [155]. The muscle secretome has been expanded to identify over 200 proteins, many of which are key for fracture repair and modulating inflammation [156, 157]. Interestingly, transplanted muscle, and to a lesser extent transplanted Pax7 satellite cells, were also shown to contribute to the fracture callus as chondrocytes [155]. However, the Pax7 satellite cells do not contribute to HO formation [158], indicating a possible multipotency of the Pax7 cells under the correct conditions of injury.

In addition to the satellite cell, recent evidence supports non-satellite skeletal muscle resident mesenchymal progenitor cells exhibit multilineage potential. In 2008, Gharaibeh et al. experimentally isolated a muscle-derived stem cell (MDSC) population of slowly adhering cells by preplating digested muscle biopsies [22]. These cells have been shown to have a similar marker profile with bone marrow-derived MSCs and pericytes (CD73, CD90, CD105, CD44, CD56, CD146 high:

CD45 negative) and exhibit myogenic, osteogenic, chondrogenic, and adipogenic capacities in vitro [159]. MDSCs have been shown to be effective for bone regeneration when supplemented with exogenous BMP, but an endogenous role of MDSCs in bone formation has not been demonstrated [160–162].

Later, in 2010, two groups identified a skeletal muscle resident progenitor cell with fibro/adipogenic potential (FAPs) that cumulatively can be identified as PDGFRα+, Sca1+, CD34+, CD45−, CD31−, SM/C2.6− [163, 164]. While these cells were initially characterized for giving rise to fibroblasts and adipocytes in vivo, experiments in a Tie2-CreERT::GPF reporter revealed that GFP+, PDGFRα+, and Sca1+ cells give rise to cartilage and bone in an experimental model of heterotopic ossification [164]. In fracture healing, it has been shown that a muscle-derived cell contributes to fracture healing, but the identity of that cell is unclear [155]. Taken together, these data suggest both a paracrine and possibly direct cellular role for muscle during fracture healing.

Genetic Mechanism for Studying Stem Cells In Vivo

Modern genetics has made it possible to use murine models to study the role of stem cells in regeneration and injury. Based on the current data, there are a number of potential progenitor populations that should be considered for their role in fracture repair, but there is an evolving understanding of their functional role that justifies continued research efforts. Specifically, as more markers are discovered, the overlap between these cell types, both in their nomenclature and at a functional level, is unclear. Even more importantly, we need to think of the cells more cross-functionally, beyond individual contributions and take into consideration their interaction with other cell types. In Table 2 below, we compile many of the mice that have been used to study progenitor populations.

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Compliance with Ethical Standards

Conflict of Interest Bragdon reports grants from the NIH and NIAMS, during the conduct of the study and fees as a grant reviewer from Musculoskeletal Transplant Foundation.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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