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**REVIEW**

- 281 Dysfunctional stem and progenitor cells impair fracture healing with age  
*Wagner DR, Karnik S, Gunderson ZJ, Nielsen JJ, Fennimore A, Promer HJ, Lowery JW, Loghmani MT, Low PS, McKinley TO, Kacena MA, Clauss M, Li J*
- 297 Physical energies to the rescue of damaged tissues  
*Facchin F, Canaider S, Tassinari R, Zannini C, Bianconi E, Taglioli V, Olivi E, Cavallini C, Tausel M, Ventura C*
- 322 Effects of various antimicrobial agents on multi-directional differentiation potential of bone marrow-derived mesenchymal stem cells  
*Li H, Yue B*

**MINIREVIEWS**

- 337 Effect of aging on behaviour of mesenchymal stem cells  
*Fafián-Labora JA, Morente-López M, Arufe MC*

**ORIGINAL ARTICLE****Basic Study**

- 347 Similarities and differences between mesenchymal stem/progenitor cells derived from various human tissues  
*Kozłowska U, Krawczyński A, Futoma K, Jurek T, Rorat M, Patrzalek D, Klimczak A*

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Editorial Board Member of *World Journal of Stem Cells*, Anton Bonartsev, PhD, Assistant Lecturer, Senior Researcher, Senior Scientist, Department of Bioengineering, M.V. Lomonosov State University, Faculty of Biologly, Moscow 119234, Russia

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## Dysfunctional stem and progenitor cells impair fracture healing with age

Diane R Wagner, Sonali Karnik, Zachary J Gunderson, Jeffery J Nielsen, Alanna Fennimore, Hunter J Promer, Jonathan W Lowery, M Terry Loghmani, Philip S Low, Todd O McKinley, Melissa A Kacena, Matthias Clauss, Jiliang Li

**ORCID number:** Diane R Wagner (0000-0001-8013-0777); Sonali Karnik (0000-0002-1905-2885); Zachary Gunderson (0000-0003-0469-3644); Jeffery Nielsen (0000-0002-2329-1299); Alanna Fennimore (0000-0002-3285-3310); Hunter Promer (0000-0003-0047-9456); Jonathan Lowery (0000-0002-9942-2945); M Terry Loghmani (0000-0003-2286-0733); Philip S Low (0000-0001-9042-5528); Todd McKinley (0000-0001-6354-7685); Melissa A Kacena (0000-0001-7293-0088); Matthias Clauss (0000-0001-5180-3899); Jiliang Li (0000-0002-8180-3205).

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**Diane R Wagner, Sonali Karnik**, Department of Mechanical and Energy Engineering, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202, United States

**Zachary J Gunderson, Todd O McKinley, Melissa A Kacena**, Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Jeffery J Nielsen**, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, United States

**Alanna Fennimore, M Terry Loghmani**, Department of Physical Therapy, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202, United States

**Hunter J Promer, Jonathan W Lowery**, Division of Biomedical Science, Marian University College of Osteopathic Medicine, Indianapolis, IN 46222, United States

**Philip S Low**, Department of Chemistry, Purdue University, West Lafayette, IN 47907 United States

**Melissa A Kacena**, Richard L. Roudebush VA Medical Center, Indianapolis, IN 46202, United States

**Matthias Clauss**, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, United States

**Jiliang Li**, Department of Biology, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202, United States

**Corresponding author:** Diane R Wagner, PhD, Associate Professor, Department of Mechanical and Energy Engineering, Indiana University-Purdue University Indianapolis, 723 W. Michigan St. SL 260, Indianapolis, IN 46220, United States. [wagnerdi@iupui.edu](mailto:wagnerdi@iupui.edu)

**Telephone:** +1-317-2748958

**Fax:** +1-317-2744567

### Abstract

Successful fracture healing requires the simultaneous regeneration of both the bone and vasculature; mesenchymal stem cells (MSCs) are directed to replace the bone tissue, while endothelial progenitor cells (EPCs) form the new vasculature that supplies blood to the fracture site. In the elderly, the healing process is slowed, partly due to decreased regenerative function of these stem and progenitor cells. MSCs from older individuals are impaired with regard to cell

Government.

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number, proliferative capacity, ability to migrate, and osteochondrogenic differentiation potential. The proliferation, migration and function of EPCs are also compromised with advanced age. Although the reasons for cellular dysfunction with age are complex and multidimensional, reduced expression of growth factors, accumulation of oxidative damage from reactive oxygen species, and altered signaling of the Sirtuin-1 pathway are contributing factors to aging at the cellular level of both MSCs and EPCs. Because of these geriatric-specific issues, effective treatment for fracture repair may require new therapeutic techniques to restore cellular function. Some suggested directions for potential treatments include cellular therapies, pharmacological agents, treatments targeting age-related molecular mechanisms, and physical therapeutics. Advanced age is the primary risk factor for a fracture, due to the low bone mass and inferior bone quality associated with aging; a better understanding of the dysfunctional behavior of the aging cell will provide a foundation for new treatments to decrease healing time and reduce the development of complications during the extended recovery from fracture healing in the elderly.

**Key words:** Fracture healing; Aging; Bone; Angiogenesis; Mesenchymal stem cells; Endothelial progenitor cells

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**Core tip:** Bone fractures in the elderly are a significant issue, due to the prevalence of the problem, the difficulty of treatment, and the severe consequences of the extended healing period. The delay in fracture healing with advanced age has been attributed to both the decreased number and function of mesenchymal stem cells that regenerate the bone and the inferior performance of endothelial progenitor cells that direct angiogenesis. Some suggested avenues for potential treatments include cellular therapies, pharmacological agents, treatments targeting age-related molecular mechanisms, and physical therapeutics.

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## INTRODUCTION

Aging is the dominant risk factor for fractures, primarily due to low bone mass and poor bone quality in the elderly<sup>[1]</sup>. While persons 65 years or older currently account for 13% of the United States population<sup>[2]</sup>, they account for more than 50% of hospital admissions with a musculoskeletal injury which are primarily fractures<sup>[3]</sup>. Fractures in the elderly population are associated with a unique set of geriatric-specific management challenges. In addition to treatment for a fracture, elderly patients are more likely to be simultaneously treated for additional medical or surgical issues which affect healing and outcomes. In addition, low bone mass and poor bone quality impart technical difficulty in achieving stable internal fixation with plates, screws, nails and wires in surgically treated fractures<sup>[4-10]</sup>. For example, studies have demonstrated that arthroplasty is typically necessary to avoid predictable healing failure that results from loss of surgical fixation and fracture reduction in elderly fractures of the shoulder, elbow, and hip<sup>[4,5,11-13]</sup>. In addition, periprosthetic fractures that occur around hip and knee replacement prostheses are increasing exponentially and will continue to increase with the aging population<sup>[14-16]</sup>. These fractures are particularly challenging for orthopaedic surgeons and healing failure can result in amputation and complete lifelong immobility.

Successful fracture healing requires that both the mineralized tissue and vasculature regenerate simultaneously to repair the highly vascularized bone (Figure 1). In fact, the processes of bone tissue regeneration and angiogenesis have significant

interactions between them during fracture healing. In secondary fracture healing, *i.e.*, in the absence of rigid fixation, the healing process begins when a hematoma forms soon after the injury with subsequent acute inflammation at the fracture site. Inflammatory cytokines as well as growth factors are released to signal the recruitment of mesenchymal stem cells (MSCs) to the injury<sup>[17,18]</sup>. Resident and infiltrating macrophages also influence the homing and localization of MSCs<sup>[18]</sup>. The recruited MSCs are multipotent, mesodermally derived cells that are capable of proliferating and differentiating into various cell types including osteoblasts and chondrocytes<sup>[19]</sup>. Recent evidence supports that the MSCs that home to the fracture site for repair derive primarily from the local periosteum<sup>[20,21]</sup>. Once the MSCs have reached their target site, circulating growth factors such as bone morphogenetic proteins (BMPs) induce their differentiation into osteoblasts and chondrocytes to initiate the formation of a cartilaginous callus bridge between the bone fragments<sup>[21]</sup>. Subsequently, the chondrocytes become hypertrophic and undergo endochondral ossification. Both osteoblasts and hypertrophic chondrocytes express high levels of vascular endothelial growth factor (VEGF), a key mediator of angiogenesis and a requisite component of fracture healing<sup>[22,23]</sup>. VEGF modulates bone repair through the induction of endothelial progenitor cells (EPCs) to increase blood vessel density, providing access for nutrients and cells to the site. With an established vasculature, newly formed osteoblasts begin to replace the soft cartilaginous callus with a stronger osseous one, effectively uniting fragmented bones. Over time, the osseous callus is remodeled into vascularized lamellar bone with a central bone marrow cavity at the diaphysis.

Advanced age is a risk factor for impaired fracture healing<sup>[24,25]</sup> with increased morbidity and mortality<sup>[26-28]</sup> as well as increased costs. Increased age has been correlated to healing complications in the tibial shaft<sup>[29]</sup>, clavicle<sup>[30]</sup>, femoral neck<sup>[31]</sup>, and floating knee injuries<sup>[32]</sup>. Delayed fracture healing, evidenced by a longer time to regain the mechanical strength and mineral content in the bone, has been observed in rodents<sup>[33-35]</sup>. In general, delayed fracture healing in elderly patients is thought to result from a lower capacity for MSC differentiation and impaired angio-/vasculogenesis<sup>[25]</sup>. These phenomena were observed by Lu *et al.*<sup>[36]</sup>, who assessed the molecular, cellular and histological progression of tibia fractures in juvenile, middle-aged and elderly mice and reported delayed chondrocyte differentiation and maturation, vascular invasion, and bone formation in the older animals<sup>[36]</sup>. The extended healing time may play a role in the development of serious complications that emerge during prolonged immobilization and the consequent high mortality rate with fractures in the geriatric population<sup>[37,38]</sup>.

In this review, we describe the dysfunctional behavior of aging MSCs and EPCs that contribute to impaired fracture healing in the elderly (Figure 1). Although the causes of delayed fracture healing with advanced age are complex and multifactorial, we highlight the reduction in growth factor expression, effects of reactive oxygen species (ROS), and the role of the sirtuin-1 (SIRT1) signaling pathway as significant factors in aging at the cellular level in MSCs and EPCs. Finally, we discuss potential treatments to enhance bone fracture healing that may be beneficial for elderly patients.

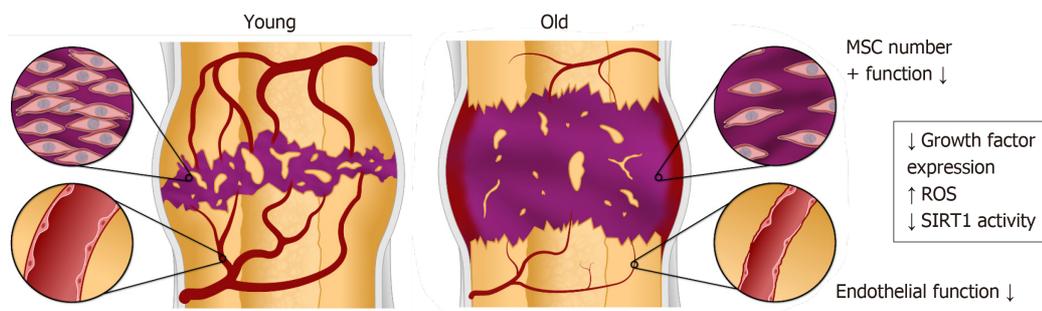
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## MSC IMPAIRMENT WITH AGE

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One of the factors for diminished fracture healing in the elderly is the altered behavior of MSCs with respect to number, proliferation, migration ability, and differentiation potential with age<sup>[20]</sup>. In bone marrow and adipose tissue from different species such as non-human primates<sup>[39]</sup>, humans<sup>[40-42]</sup>, mice<sup>[43-45]</sup>, and rats<sup>[46]</sup> there was a pronounced age-dependent difference in the number of MSCs based on the colony forming unit (CFU) assay; MSCs from younger individuals were more numerous as they formed up to 50% more CFUs than older individuals<sup>[40-44,46]</sup>. MSCs have also been characterized by their positive expression of surface markers such CD90, CD44, and CD73. In a study on human marrow-derived MSCs, Stolzing *et al.*<sup>[45]</sup> found that young cells expressed more CD90, CD105, and Stro-1 and old cells expressed more CD44. The effect of aging on cell surface markers was also observed by Yu *et al.*<sup>[39]</sup> in MSCs isolated from the bone marrow of rhesus macaques. The MSCs from young and middle-aged individuals had a higher percentage of CD90+ cells than the MSCs derived from older individuals, whereas, the MSCs from older individuals had a higher percentage of CD44+ cells.

The proliferative potential of MSCs also declines with age. The doubling times in MSCs isolated from human bone marrow was 0.9 and 1.7 days in cells from younger and older individuals, respectively<sup>[40]</sup>. This increase in cell doubling time with age was



**Figure 1** Fracture healing is impaired with advanced age, including delays in both bone and vascular regeneration due to dysfunction of mesenchymal stem cells and endothelial cells. MSC: Mesenchymal stem cell.

also observed in MSCs isolated from adipose tissue; cell doubling times increased from approximately 2.6 d in MSCs from younger individuals to 3.8 d in MSCs from older individuals<sup>[41,42]</sup>. The proliferation rate was also reduced in MSCs isolated from mouse bone marrow by 20% in older animals<sup>[44]</sup>.

The age of the patients not only affects the number and proliferative potential of MSCs but also their ability to migrate to the site of injury, which plays an important role in their regenerative function. It was observed that MSCs from older rats showed lower motility on uncoated filters than those from younger animals<sup>[47]</sup>. In a different study, twice as many bone marrow-derived MSCs from younger rats migrated towards the chemokine SDF-1 as those from older rats<sup>[48]</sup>. The decrease in the motility or migration potential of old MSCs may be due to their decreased expression of chemokine receptors<sup>[48,49]</sup>. In an interesting study on the effect of age on bone marrow microenvironment and migration of MSCs, Yang *et al.*<sup>[50]</sup> found that co-culture with bone marrow aspirate from old mice reduced the migration of an MSC cell line. The authors also found that the bone marrow aspirate from older mice expressed less SDF-1<sup>[50]</sup>. Together, these studies suggest that the reduced migratory potential of MSCs from older individuals may be due to reductions in both the MSC expression of chemotactic receptors and in chemotactic cytokines secreted by the older tissue. All of these factors together might contribute toward reduced migration of MSCs to the fracture site in elderly patients leading to poor fracture healing.

An important distinguishing feature of MSCs is their ability to differentiate to the osteogenic and chondrogenic lineages, among others. Various groups studying the age-related changes in differentiation potential of MSCs have concluded differently. Several groups have reported that the osteogenic differentiation potential of the MSCs isolated from either bone marrow or adipose tissue is reduced as age advances<sup>[41,42,45,51,52]</sup>. Zhang *et al.*<sup>[43]</sup> reported that osteogenic differentiation capacity of bone marrow-derived MSCs from mice increases in an age-dependent manner to 18 mo of age and decreases rapidly thereafter. In contrast to these studies, other groups found the MSCs maintained their differentiation potential even in aged donors<sup>[53,54]</sup>. There is also disagreement in the literature on whether age has an effect on the chondrogenic potential of MSCs. Some groups have reported an age-related reduction in their chondrogenic potential<sup>[41,42,52]</sup>. In other studies, the chondrogenic potential of MSCs was not affected with advanced age<sup>[45,51,55]</sup>. However, in all cases, the isolated MSCs were cultured and differentiated *in vitro* where they lack the microenvironment of the native tissue which might be different as the donors age. Conflicting findings in the literature with respect to differentiation potential of MSCs isolated from older individuals require further studies which take tissue microenvironments into consideration to understand any changes in differentiation.

A decline in the expression of growth factors that induce MSC chondrogenic and osteogenic differentiation have been proposed to contribute to impaired fracture healing with age. For example, expression of BMP-2 and Indian hedgehog were at significantly lower levels in the fracture calluses of older rats<sup>[56]</sup>. Additionally, the response of MSCs to growth factors like BMP-2 may be attenuated with age. As an example, markers of osteogenesis in canine MSCs increased in all animals when treated with BMP-2 in culture, but the increase was less robust in cells from older animals<sup>[57]</sup>. Similarly, pediatric human iliac crest MSCs were more responsive to exogenous BMP-2 than adult MSCs from the same anatomic location based on the *in vitro* expression of osteogenic markers<sup>[58]</sup>.

The accumulation of ROS is another factor that may affect MSC function in the aged population, resulting in oxidative damage to DNA, structural lipids and proteins as well as cellular senescence<sup>[46]</sup>. Oxidative stress has been shown to increase during fracture healing<sup>[59-61]</sup>, however the effect of ROS on MSCs during fracture repair in

aging is unclear. In a developmental model of bone formation, chondrogenesis was enhanced by ROS in the developing limb bud, where a cartilage template precedes long bone formation<sup>[62]</sup>. High levels of ROS have also been associated with hypertrophic chondrocytes that are undergoing endochondral ossification *in vitro*<sup>[63]</sup>. Furthermore, the addition of an antioxidant to cell culture media inhibited chondrocyte hypertrophy, while elevated ROS stimulated chondrocyte hypertrophy<sup>[63]</sup>. Osteogenesis through intramembranous ossification, on the other hand, is inhibited by elevated levels of ROS<sup>[64-66]</sup> and intracellular ROS levels have been observed to dramatically decrease upon osteogenic differentiation due to the upregulation of antioxidant enzymes superoxide dismutase 2 (SOD2) and catalase<sup>[66]</sup>.

Among the molecular regulators of aging, SIRT1, a NAD-dependent histone deacetylase, is of particular importance. SIRT1 expression and activation decrease with age, which modifies a wide range of cellular processes, including MSC proliferation and differentiation. For example, SIRT1 knockdown in human marrow- and adipose-derived MSCs resulted in reduced proliferation *in vitro*<sup>[67]</sup>. Additionally, MSCs isolated from Sirt1 knock-out mice showed reduced differentiation toward the osteogenic lineage<sup>[68]</sup>, while Sirt1<sup>+/-</sup> female mice had reduced bone mass and increased marrow adipogenesis<sup>[69]</sup>. Differentiation to the chondrogenic lineages were also inhibited in MSCs isolated from Sirt1 knockout mice<sup>[68]</sup> and with SIRT1 knockdown<sup>[70]</sup>.

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## IMPAIRED EPCS WITH AGING

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Blood supply is critical for fracture healing. Formation of sufficient vasculature at the fracture sites provides oxygen and nutrients for cell survival and proliferation. Aging has negative effects on angiogenesis which can lead to delayed healing or non-union of fractures<sup>[36,71]</sup>. Vascular changes such as the decline in endothelial function are reliable markers for aging<sup>[72-75]</sup>. Highly proliferative EPCs, also described as late outgrowth EPCs or endothelial colony forming cells (ECFCs), are believed to play an important role in maintenance of the viable endothelial layer in the vascular system<sup>[76-78]</sup>.

Aging decreases endothelial cell (EC) proliferation and migration, as well as the expression of EC growth factors and their cognate receptors<sup>[79-81]</sup>. Aging is also a major cause for endothelial dysfunction and microvascular hypermeability<sup>[82,83]</sup>. The mechanisms underlying age-related endothelial dysfunction likely involve increased oxidative stress and alterations in molecular pathways affecting common aging processes. Importantly, EPC dysfunction and senescence contribute to oxidative stress<sup>[84]</sup>.

Age related mitochondrial dysfunction is a likely candidate to explain this endothelial progenitor dysfunction. Mitochondria-derived production of ROS results in increased oxidative stress in ECs. Attenuation of mitochondrial oxidative stress in a genetically modified mouse model of overexpression of human catalase in mitochondria improved endothelial function<sup>[85]</sup>. Conversely, genetic deletion of the mitochondrial antioxidant proteins, mitochondrial SOD and glutathione peroxidase 1, exacerbated age-related vascular dysfunction<sup>[86,87]</sup>. Age-related oxidative stress may also be caused by increased activity of NADPH oxidase in ECs<sup>[88]</sup>. Increased oxidative stress in aged ECs inactivates nitric oxide (NO)<sup>[88,89]</sup>. Impaired bioavailability of NO negatively affects cell division and survival, mitochondrial function and cellular energy metabolism, and EPCs<sup>[90]</sup>.

SIRT1 is an important molecular regulator in ECs<sup>[91]</sup> in addition to its role in MSCs. SIRT1 expression and activity decreases with aging in the vasculature. Accordingly, pharmacological activation of SIRT1 significantly improves endothelial function in aged mice<sup>[92]</sup>. Similarly, cleavage of SIRT1 by cathepsin in EPCs mediates stress-induced premature senescence<sup>[93]</sup>.

Age is also a limiting factor for mobilization of EPCs including ECFCs<sup>[94-96]</sup>. Thus, it appears that the decrease in number and/or function of ECFCs, a homogenous population of EPCs, may be a major driver for failed fracture repair in elderly patients. Previous studies suggest age-related EPC dysfunction may be reversible by anti-aging intervention<sup>[97]</sup>. Preclinical studies also showed that the serum factors derived from young rats have beneficial effects on EPCs isolated from aged ones<sup>[98,99]</sup>.

In addition to their role in fracture healing, MSCs share properties with pericytes and are important for vascular network formation<sup>[99]</sup>. Pericytes have an important role in angiogenesis and could be a novel therapeutic target because of their involvement in regulation of capillary permeability, EC proliferation and extracellular matrix generation<sup>[100,101]</sup>. In fact, age-related loss of pericyte coverage of microvessels contributes to function and structural impairment of microcirculatory network<sup>[100]</sup>. Interestingly, when adipose derived mesenchymal and endothelial stem cells are

brought in close contact, a Wnt signaling specific mechanism favors osteogenic versus adipogenic differentiation<sup>[102,103]</sup>. It remains to be elucidated if treatment targeting pericytes could enhance bone healing in aging.

Dysfunction of aged ECs and EPCs lead to endothelial senescence and apoptosis and directly interfered with angiogenesis in aging<sup>[82,104-106]</sup>. Age-related changes in circulation factors might also contribute to impaired angiogenesis in aging. Pro-angiogenic endocrine factors, growth hormone, insulin like growth factor I, platelet derived growth factor (PDGF) and VEGF, which regulate multiple aspects of angiogenic processes, decline with aging<sup>[95,107-109]</sup>. This may be explained by reduced expression of, and responsiveness to, HIF-1 $\alpha$  during aging<sup>[110]</sup>. Impaired angiogenesis also results in age-related decline in vessel density, impaired adaptation to hypoxia, and ischemia<sup>[111]</sup>.

Impaired angiogenesis during fracture healing creates an ischemic environment at the fracture site and disrupts the interactions between the blood supply and MSCs that are required for bone healing. In a mouse model of fracture accompanied by vascular damage, ischemia significantly decreased the callus size, and the cartilage and bone formation, leading to delayed union<sup>[112]</sup>. Similar results have been seen in *in vitro* culture of MSCs in hypoxic environments. Hypoxia was found to be linked to reduced osteogenic potential of MSCs, evidenced by the down regulation of many osteogenic markers<sup>[113]</sup> and osteogenic pathways such as RUNX2<sup>[114]</sup>. Hypoxia has also been found to inhibit hypertrophic differentiation of chondrocytes and endochondral ossification<sup>[115]</sup>. Thus, a disruption to the angiogenesis process due to aging may have profound effects on MSC behavior at the fracture site, leading to delayed fracture healing.

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## POTENTIAL TREATMENT OPPORTUNITIES FOR IMPROVED FRACTURE HEALING IN AGING

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### ***Cell-based therapies***

Successful management of bone fractures in the elderly may require special measures not commonly indicated in younger individuals. As native MSCs and EPCs may be compromised with respect to number and/or function with advanced age, delivering these cells to the fracture site is one potential avenue to accelerate fracture repair.

Bone tissue engineering has been investigated intensively for three decades, but efforts to date have not yielded a cell-seeded implant which can be used clinically. Most tissue engineering approaches target intramembranous or direct bone formation, but this approach has had poor outcomes because the cells must initially survive in an avascular hypoxic environment before the invasion of vasculature. Without vasculature, nutrient delivery and waste removal are severely compromised in the center of the implant, causing cell necrosis and failure of cell-seeded implants<sup>[113,116]</sup>. A relatively new technique to address this issue exploits the tendency of MSCs to undergo a process resembling hypertrophy when cultured under standard chondrogenic differentiation conditions<sup>[117,118]</sup>. In this regenerative strategy, bone tissue is generated *via* the endochondral ossification pathway, where a cartilaginous template is first formed and later remodeled into mature bone. One advantage of endochondral bone tissue engineering is that the chondrogenic cells function much better than osteogenic cells in low-oxygen environments such as the avascular region of a bone defect<sup>[113,119]</sup>. Therefore, the chondrogenic cells are maintained in the implant site until the vasculature invades, at which time the hypertrophic cells induce bone formation, as in secondary native fracture healing. Because the cells undergo a process that resembles hypertrophy, they release an array of growth factors for vascular and bone formation that are spatially and temporally controlled. The feasibility of this technique has been demonstrated using embryonic stem cell<sup>[120]</sup>, marrow- and adipose-derived MSCs<sup>[121-129]</sup>, and the murine, chondrocytic cell line ATDC5<sup>[130]</sup>. Recently, fracture healing through endochondral ossification using hypertrophically primed MSCs in a collagen construct was demonstrated in a weight bearing femoral defect model in rats<sup>[131]</sup>. In fact, endochondral ossification has been shown to be a better alternative than intramembranous bone regeneration by Thompson *et al.*<sup>[132]</sup>, where the chondrogenically primed MSCs supported greater repair of a cranial critical-sized defect (CSD)<sup>[132]</sup>.

Another cell-therapy approach to improve bone healing is to enhance angiogenesis with ECFCs. Currently, implantation of ECFCs has been tested in animals and is currently being investigated in human clinical trials for other indications, such as myocardial infarction, ischemic stroke, liver cirrhosis, and diabetic foot<sup>[133]</sup>. Our group<sup>[134]</sup> and others<sup>[135-140]</sup> have recently shown the utility of using ECs for bone repair. ECFCs were selected based on their proliferative potential, expression of CD31 and

CD309, as well as their ability to take-up acetylated low-density lipoproteins<sup>[141]</sup>.

ECFCs can induce neovascularization at the bone defect site, and stimulate fracture repair and bone regeneration in young rats<sup>[134]</sup>. ECFCs ( $10^6$  cells) were seeded into a type I collagen sponge and transplanted into the bone defect during fracture surgery. The data showed that ECFCs induced more new blood vessels compared to the unseeded type I collagen controls<sup>[134]</sup>. Furthermore, new bone was formed within the defect area when implanted with ECFCs, but no bone was observed in the controls<sup>[134]</sup>. Histological examination showed that osteocytes, osteoblasts, and osteoclasts were observed in newly formed bone tissues in ECFC treated animals at 6 weeks<sup>[134]</sup>. These data suggest that ECFCs can increase neovascularization and stimulate new bone formation in the damaged bone area with a CSD that normally fails to heal.

In another study by our group, hydroxyapatite and tri-calcium phosphate (HA/TCP) scaffolds loaded with ECFCs ( $10^6$  cells) were placed into the fibula defect. Histological examination showed significantly greater newly formed bone in HA/TCP scaffolds loaded with ECFCs than that observed in the HA/TCP scaffold only animals<sup>[134]</sup>, suggesting that ECFCs may migrate and further enhance bone regeneration inside the scaffold.

### **Pharmacological agents**

Because the endogenous concentrations of bone anabolic agents that facilitate fracture repair can be significantly reduced in the elderly, one obvious remedy hypothesized for enhancement of their healing process has been to supplement the patient's natural levels of bone anabolic agents. Recognizing that BMP-2 can potentially augment the rate of bone fracture repair, Medtronic Inc. has explored the local application of BMP-2 (Infuse) to a fracture surface to accelerate the healing process. While success has been documented for enhancement of recovery from open tibial shaft fractures<sup>[142,143]</sup>, dental and facial reconstruction surgeries<sup>[144,145]</sup>, and spinal fusion procedures<sup>[146,147]</sup>, the same methodology cannot be applied when fracture surfaces cannot be physically exposed. Thus, those fractures that do not require surgical intervention cannot be treated with BMP-2 dosing through local delivery. Moreover, repeated administration of a bone anabolic agent is not possible with this strategy, since the fracture surface is usually only accessible during the initial reconstruction/stabilization surgery.

A second method to augment endogenous levels of osteogenic agents was explored by Eli Lilly and Co. when they examined the use of parathyroid hormone (Forteo) to accelerate the repair rate of tibial<sup>[148-150]</sup> and hip fractures<sup>[151,152]</sup>. While measurable improvement in the healing process was documented in many patients, the phase 3 trial failed to reach its clinical endpoint due to concerns over the induced hypercalcemia that was observed as therapeutically effective concentrations of drug were approached. Although the potentially deleterious consequences of the hypercalcemia forced discontinuation of the clinical trial, the results suggested that a more targeted form of parathyroid hormone might succeed if it could concentrate that drug at the site of the fracture and reduce its concentration in healthy tissues.

Looking to the future, a large number of peptide and protein hormones that are commonly released at the site of a wound have been reported to exhibit bone anabolic activity. These include FGF2<sup>[153-156]</sup>, PTHrP<sup>[157-159]</sup>, PDGF<sup>[160-162]</sup>, Prostaglandins<sup>[163-165]</sup>, IGF<sup>[166]</sup>, VEGF<sup>[167,168]</sup> and others. Because virtually all of these stimulants are known to have multiple anabolic activities that can cause undesirable changes in healthy tissues, it is unlikely that any will prove useful as bone fracture repair drugs unless they can be applied locally to the fracture surface or targeted to the same fracture surface following systemic administration. Hopefully, with the design of new bone fracture homing ligands, such fracture targeted anabolic agents can be developed for less invasive therapies of fractures in the elderly.

### **Therapies targeting age-related molecular mechanisms**

Aging at the cellular level is associated with increased ROS production and decreased endogenous antioxidant levels, leading to accumulation of oxidative damage and cellular senescence. Therefore, antioxidants have been studied as a therapy to improve a variety of health outcomes, including fracture healing. Antioxidants vitamin E<sup>[169,170]</sup>, melatonin<sup>[171,172]</sup>, and N-acetylcysteine<sup>[173]</sup> have all been shown to promote fracture healing in animal models. Cellular senescence itself can induce chronic inflammatory disease in mice, and depletion of senescent cells by so called senolytic agents can reduce systemic inflammation and extend life span in small rodent by 37%<sup>[174-178]</sup>. Importantly, targeting cellular senescence prevents age-related bone loss in mice<sup>[179]</sup>. Therefore, it appears to be promising to identify ways to reduce the generation and maintenance of senescent progenitor cells. This widely overlooked aspect is of particular interest because a high number of senescent cells with detrimental functions are to be expected during aging and aging-associated inflammatory conditions.

Another potential target for improving fracture healing in aging is SIRT1<sup>[91]</sup>; as described above, it appears to be involved in the age-related decline in both MSC and EC function. Furthermore, crosstalk between SIRT1 activity and ROS production plays a crucial role in the aging process<sup>[180]</sup>. SIRT1 expression and activity decreases with aging. Accordingly, pharmacological activation of SIRT1 improves the survival of aged MSCs upon transplantation<sup>[21]</sup> and also significantly improves endothelial function in aged mice<sup>[92]</sup>. Similarly, cleavage of SIRT1 by cathepsin in EPCs mediates stress-induced premature senescence<sup>[93]</sup>. Most notably, pharmacological activation of SIRT1 increased bone mass in mouse models of osteoporosis<sup>[181]</sup>.

### Physical therapeutics

Physical therapeutics are non-invasive and non-pharmacological treatments that cause physiologic cascades in the body to affect measurable change in molecular and tissue function, leading to improved functional outcomes<sup>[182-184]</sup>. Movement therapies and therapeutic modalities are two approaches frequently used in the clinic that should be considered within the context of fracture healing and aging.

Movement approaches may include ambulation and therapeutic exercise, both of which mobilize physiological responses secondary to mechanical loading. While the effects of these treatments have not been fully explored in humans, it has been shown that mechanical loading of cells *in vitro* can impact gene expression and bone-derived mesenchymal cell (MSC) differentiation into three types of tissue: Fat, bone, and cartilage<sup>[185]</sup>. Even short durations of compression can cause an increase in differentiation and calcium mineralization in certain cultures<sup>[186]</sup>. Another benefit of mechanical loading during cyclical compression, which mimics gait, of human MSCs is an improvement in oxygenation of the fracture's hematoma that benefits cellular metabolism and the ability to heal<sup>[187]</sup>. One study investigated the effects of functional mechanical loading on large bone defect regeneration *in vivo*. Bone CSDs in rat femora were stabilized using either stiff or compliant fixation plates that allowed compressive loading during ambulation. Findings demonstrated that functional transfer of axial loads during segmental bone repair enhanced bone formation and regeneration<sup>[188]</sup>. Meanwhile, a retrospective cohort study observed that early ambulation/mobilization of elderly patients with fractures improve outcomes faster than those who delay mobilization<sup>[189]</sup>. In contrast, immobility was associated with higher mortality and lower function<sup>[190]</sup>.

Several therapeutic modalities are used clinically to facilitate fracture healing, including whole-body vibration (WBV) and pulsed ultrasound. Recent systematic reviews suggest WBV is a safe and effective treatment. Pre-clinical trials with ovariectomized rats have shown those with diminished estrogen respond better to WBV than those with normal levels<sup>[191-193]</sup>. Interestingly, a study observed an increase in osteogenic potential of bone marrow with WBV during a period of hindlimb unloading compared to those with no treatment; this increase was expounded upon later during re-ambulation and concurrent WBV<sup>[194]</sup>. Pulsed ultrasound may offer another option for fracture healing in the elderly. Research has shown low-intensity pulsed ultrasound to decrease osteoclastic gene expression<sup>[195]</sup> decrease MSC adipocyte differentiation<sup>[195]</sup>, and foster MSC's commitment to osteogenesis<sup>[196,197]</sup>. However, according to recent systematic reviews, there is a low level of evidence to support its use in the early phases of fracture healing in elderly humans<sup>[198]</sup> and in those undergoing distraction osteogenesis<sup>[199]</sup>.

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## CONCLUSION

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Bone fractures in the elderly are a significant issue, due to the prevalence of the problem, the difficulty of treatment, and the severe consequences of the extended healing period. The delay in fracture healing with advanced age has been attributed to the decreased number and function of MSCs that regenerate the bone and the inferior performance of EPCs that participate in angiogenesis. The causes of cellular aging and the concomitant decline in functionality are wide-ranging, but provide some intriguing indications of potential targets for speeding fracture healing in older individuals. In the future, cell therapies that supplement the inadequate native cellular response with MSCs or EPCs; bone anabolic pharmacological agents, particularly in combination with strategies to localize their delivery to the bone fracture; drugs that reduce oxidative stress, cellular senescence, or activate SIRT1; and/or physical therapeutics may prove effective in promoting fracture healing in the elderly.

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