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# Mesenchymal Stromal Cells and Their Uses in Bio-Regenerative Therapies for Bone and Cartilage: A Review

**Nathan Smernoff**

Texas College of Osteopathic Medicine, University of North Texas Health Science Center, Fort Wort, Texas, USA
Email: nathansmernoff@my.unthsc.edu

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## Abstract

Mesenchymal stromal cells (MSCs) are a top candidate for new clinical treatments in the repair of bone and cartilage. In several clinical trials, they have shown reliable, effective, and safe management of inflammation, pain, and the regenerative capabilities of resident tissues. MSCs are likely derived from pericytes. They modulate the environment they are placed in by secreting immunomodulatory and signaling molecules to reduce inflammation and direct resident cells to create new tissues. They are easily isolated from several different adult tissues, and inexpensive to grow in a lab. However, a mistake made in the initial classification of MSCs as stem cells has created deeply engrained misconceptions that are still evident today. MSCs are not stem cells, despite a large fraction of research and therapies using the name "mesenchymal stem cells". This mistake creates false narratives attributing the observed positive outcomes of MSC treatments to stem cell characteristics, which has led to distrust in MSC research. Despite inconsistencies in their classification, MSCs demonstrate consistent positive effects in numerous animal studies and human clinical trials for non-unions and osteoarthritis. With an aging population, regenerative techniques are very promising for novel therapies. To produce trusted and safe new treatments using MSCs, it is essential for the International Society for Cellular Therapies to re-establish common ground in the identity, mechanism of action, and isolation techniques of these cells.

## Keywords

Mesenchymal Stromal Cells, Osteoarthritis, Non-Unions

## 1. Introduction

Non-unions occur when a fractured bone fails to reanneal. The Food and Drug

Administration (FDA) officially classifies non-unions as fractures that have not healed after nine months and have shown no healing for three months. Doctors functionally diagnose a non-union when they believe the bone will not heal without intervention. Around 5% to 10% of fractures fail to heal correctly, and this number is increasing as the developed nation's elderly population increases [1]. Non-unions occur from various pathways. Infections, old age, and even diabetes can reduce the body's ability to heal broken bones. Improper alignment of a fracture will also cause non-unions. Treatment of non-unions is done on a case-by-case basis. Non-surgical methods include immobilization to stabilize the fracture or electrical stimulation to generate beneficial proteins like bone-morphogenic proteins. Most severe non-unions respond well to surgical intervention, where bone fragments cleaned of infection, fixated together using metal aids, or implanted with bone grafts. However, surgery is costly to the patient and creates several opportunities for mistakes and further infection. Furthermore, they do not solve the problem that a significant majority of non-unions face; old age decreases the body's ability to heal. The price, complications, and limitations associated with surgery to treat non-unions generate a healthy curiosity towards new avenues for treatment and cure.

Osteoarthritis is characterized as the progressive loss of articular cartilage, resulting in joint pain, stiffness, and loss of functional mobility. Osteoarthritis is the primary factor contributing to disability in the elderly and is also expected to increase in number with increases in the elderly population worldwide [2]. The causes of osteoarthritis range from genetic predispositions to age, sex, body mass index, and previous trauma. Modern treatment is severely limited and primarily consists of non-steroidal anti-inflammatory drugs, hyaluronic acid injections, and as a last resort, joint replacement. Joint replacement is not only expensive and dangerous, but joints such as the knee do not typically respond well to the implantations. All treatments for osteoarthritis are limited in their ability to reverse the effects of the disease. Cartilage is slow to heal because it is avascular, and chondrocytes are segregated in lacunae, making them hard to reach when damage occurs. The best treatments available can only slow the progressive course or hide it. Regenerative therapies are a top candidate in the hopes of a treatment that can treat the pain and inflammation and rebuild and repair damaged joints.

With an increasingly older population, pathology affecting bone and cartilage are rising in number and severity. Bioregenerative techniques to reinstitute healthy cartilage and heal broken bones can offer permanent and less invasive solutions to increasingly common afflictions. The musculoskeletal system comprises several tissues, including muscle, bone, tendon, ligament, cartilage, and blood vessels. Each type of tissue within the skeletal system depends on stem cells and progenitor cells for growth, development, repair, and maintenance. Stem cells are self-renewing and differentiate into new cell lineages. Progenitor cells also contain the ability to differentiate; however, they are more specific towards selecting a cell fate and limited in their ability to replicate. Cells with this

replicative potential can be isolated based on their structural and functional characteristics, grown, and used for regenerative purposes to induce bone healing or produce new cartilage. Mesenchymal stromal cells (MSCs) are a top candidate in the pursuit of regenerative cells for several orthopedic applications. They are easily extracted from several tissues, cheap to grow, and have shown several therapeutic benefits in orthopedic applications.

While research using mesenchymal stromal cells shows promising results, inconsistencies in defining and isolating MSCs have created doubt in the scientific community. Despite numerous clinical trials, it is still up to debate where MSCs come from, how they contribute towards healing, how to isolate them properly, and if they are, in fact, stem cells. To develop new treatments using MSCs, or other replicative cells, consensus in basic scientific protocol needs to be met. There needs to be an updated standardized method of isolating and growing MSCs. More research should be done to tease out the specific actions MSCs undergo to contribute towards healing before treatments are made available to the public. Animal studies show promising results for bone healing and cartilage regeneration. Clinical trials corroborate these findings. However, if MSCs are going to replace current therapies, the scientific community needs to agree on common facts.

## 2. What Are Mesenchymal Stromal Cells?

Mesenchymal stromal cells are plastic adhering cells that differentiate into several mesenchyme tissues when grown *in vitro*. The mesenchyme is a connective tissue responsible for the development of the skeletal system during embryonic growth. Tissues within the mesenchyme include bone, cartilage, and muscle, making MSCs an interest in orthopedic regenerative medicine. Arnold Caplan first coined the term "mesenchymal stem cells" to describe self-renewing, multipotent stromal cells that gave rise to both bone and cartilage when grown on plastic plates [3]. This discovery created a boom in research for several reasons. First, MSCs are isolated and cultured from adult tissue and thus avoid the ethical controversy that embryonic stem cells generate. Second, researchers could study the formation of specific tissues in a lab setting for the first time. Third, and most importantly, if MSCs can produce bone and cartilage when grown on a plastic plate, then maybe they can be isolated, grown, and re-implanted in patients to induce bone and cartilage growth.

The initial classification of MSC as stem cells was incorrect, and in 2006 the International Society for Cellular Therapies renamed these cells "mesenchymal stromal cells" (MSC) and established minimum criteria for their isolation. Modern MSCs are cells that adhere to plastic *in vitro* and express CD73, CD90, and CD105, and lack expression for CD14, CD34, CD45, CD11b, CD79a, CD19, or HLA-DR surface molecules. These cells must also demonstrate tri-lineage potential *in vitro* by differentiating into osteoblasts, chondroblasts, and adipocytes [4]. These requirements established common ground to both define and isolate MSCs for research.

Mesenchymal stromal cells originate from pericytes. Perivascular cells isolated from human adipose tissue, bone marrow, and skeletal muscle all display the officially recognized cell surface markers of MSCs [5]. Under the correct circumstances, pericytes grown in a plastic dish could be stimulated to differentiate into chondrocytes, adipocytes, and osteocytes, in a mechanism similar to MSCs [5]. Isolated pericytes are remarkably similar to MSCs. Pericytes derived from adipose tissue demonstrated the ability to differentiate *in vitro* through MSC pathways, expressed nearly identical cell surface markers to MSCs, and genetic evidence revealed nearly identical transcriptomes for both cells [6]. The origin of MSCs may explain why they can be isolated from various tissues. Pericytes wrap around the endothelium of blood vessels throughout the body. Each tissue that MSCs are isolated from has one thing in common: a blood supply. Pericytes from surrounding vasculature may respond to injury by progressing into MSCs. More research should be done to understand the signals that induce pericytes to become MSCs and how they contribute towards healing.

Mesenchymal stromal cells are not stem cells *in vivo*. Stem cells are defined by their self-regenerative ability and their capacity to differentiate into new cells *in vivo*. While MSCs have been observed to do both *in vitro*, there is no evidence of *in vivo* stem cell character for either criterion. The origin of MSCs further discredits their classification as stem cells. While pericytes isolated and grown *in vitro* could differentiate into several distinct MSC lineages, pericytes labeled with GFP *in vivo* did not demonstrate the same plasticity [7]. Thus, it is assumed that the artificial environment MSCs are grown in likely induces differentiation and self-renewal.

Both researchers and unapproved products are falsely using the "mesenchymal stem cell" name for clout despite calls from several agencies to stop. A significant amount of current research still refers to MSC as "mesenchymal stem cells" despite a call from the developer of the term MSCs to use alternative labeling. Caplan, the founder of MSCs, has proposed changing the name to "medicinal signaling cells" to clarify these cells' origin and function *in vivo*. Caplan claims he was wrong in his original assessment. With current research, the scientific community should conclusively agree that MSCs originate from perivascular cells, likely aid in regeneration by the secretion of immunomodulatory molecules and are not stem cells *in vivo* [8]. For the remainder of this paper, "MSC" (singular) or "MSCs" (plural) will be used as a broad abbreviation for either 'Medicinal Signaling Cell/s,' 'Mesenchymal Stem Cell/s,' or 'Mesenchymal Stromal Cell/s,' regardless of what the paper being discussed classifies them as. There is no unified name used in the literature besides the abbreviation of "MSC."

While a fundamental benchmark for classifying MSCs for research exists, high cell heterogeneity creates controversy regarding their designation. Bone marrow was the original source of MSCs. However, MSCs can be isolated from adipose tissue, the umbilical cord, synovial fluid, and nearly any compartment in the body, even outside of the mesenchyme. MSCs derived from bone marrow, mus-

cle, periosteum, and perinatal cord tissue displayed distinct differentiation capacities when grown under identical conditions and transplanted *in vivo* to similar locations in mice kidneys. Gene-expression analysis of these isolated MSCs revealed a significant distinction in their expression of genes, suggesting no uniform identity for MSC [9]. The MSCs isolated from various locations in the body may contain mixtures of other cells that change therapeutic outcomes. With inconsistencies in the designation of MSCs and evidence for heterogeneous cell populations, the term 'MSC' has become a broad umbrella phrase and likely encompasses various cells grouped together. There have been several proposals to abandon the term mesenchymal stromal cells because it describes heterogeneous mixtures of cells exhibiting stem cell features, not a homogenous sample of cells. A more precise name for these cells would encompass their replicative potential and denote their tissue source [10] [11]. The term "multipotent stromal cells" has frequently circulated as a possible replacement that clarifies the likelihood of multiple cell types from various tissues and clarifies that origin doesn't necessarily have to be the mesenchyme.

Growing cells according to their plastic adherence could be contributing to high cell heterogeneity in MSC cultures. The standard method of isolation via MSC's adherence to plastic may alter cell surface marker expression over time. When grown under the same conditions that MSCs are cultured, endothelial cells derived from adipose tissue lost expression of their identifying cell surface markers and developed the same cell surface markers as MSCs [12]. Thus, the practice of isolating cells and growing them according to their plastic adherence changes their expression profiles to be identical with MSCs. High cell heterogeneity claims are a valid concern for research because multiple cell types in one solution could lead to differential healing. Multiple cell types disguised as MSCs and at varying concentrations within the solution could affect that treatment outcome. One treatment may do better than another, and researchers could never know why.

Skeletal stem cells (SSCs) designate a more precise vocabulary for isolated stem cells because they are homogenous and have clearly defined origins. SSCs are self-regenerating cells that differentiate into chondrocytes, osteoblasts, and osteocytes *in vivo*, but not adipose cells. SSCs exhibit
PDPN+CD146-CD73+CD164+ cell surface markers. This is a similar marker to MSCs, but more specific towards skeletal stem cells. Skeletal stem cells are separated using Fluorescence-Activated Cell Sorting (FACS), a superior isolation method compared to culture adhesion to plastic plates, as commonly seen with MSCs. FACS assures cells are separated based on the fluorescent labeling of cell surface molecules, leading to higher yields of homogenous cell colonies. Compared to plastic adherence isolation methods that select a broad phenotype, the PDPN+CD146-CD73+CD164+ phenotype is limited to only SSCs. Human SSC lineage can be traced down to a single cell called the bone cartilage stromal progenitor cell (BCSP) [13]. The cell hierarchy goes down from SSC to pre-BCSP to BCSP to various progenitors, including osteocytes, chondrocytes, and even

MSCs. This result might explain why there is an overlap in functionality between MSCs and SSCs, both of which produce bone and cartilage.

## 3. Bone Repair

Studying fracture healing in animal studies has led to an increased understanding of the role MSCs play in healing. Systemic injections of MSCs in mice lead to localized homing of MSCs to the injured limb and increased healing in a dose-dependent manner [14]. MSCs were extracted by clearing bone marrow from femurs and tibias of mice. These cells were isolated using magnetic cell sorting techniques to generate a 90% homogenous sample of MSC expressing the CD11b-, CD38-, and CD45- surface markers. Closed tibia fractures were induced using steel pins with a bending device that applied uniform force. Assays and bioluminescent techniques revealed that the MSCs localized to the endosteal niche upon fracture and likely contributed to bone healing by producing bone morphogenic protein 2 (BMP-2) and reducing inflammatory cytokines and interleukins [14]. Fractured tibias were removed and analyzed using distraction-to-failure tests, $\mu$-CT scanning, and histology techniques. The calluses of the mice treated with MSCs displayed more toughness, ultimate force, and volume when compared to untreated mice. The systemic MSC treatment led to the cells localizing to the fracture site's endosteal niche, production of BMP-2, reduced inflammation, and significantly increased bone/callus strength and size. The production of BMP-2 and the reduced inflammation may explain the fracture's healing, but there are more immunomodulatory effects to tease out before wide clinical applications can begin.

Genetically engineered MSCs are another option and may gain advantages towards differentiation, mobilization, and fracture healing. When MSCs were driven to overexpress Sox11, a transcription factor, they demonstrated almost twice the levels of tri-lineage differentiation and mobilization *in vitro* [15]. They also demonstrated nearly twice the bone formation *in vivo* leading to accelerated fracture healing in rat models. A limiting factor in regenerative therapy is the capacity for MSCs to migrate towards and differentiate into bone-producing cells when injected. It is imperative that injected cells, whether systemic or local, can find the injury to aid in recovery. MSCs modified to overexpress SOX11 traveled across an 8 μm pore-sized membrane faster than wild-type MSCs [15]. Once localized to the injury, the second most crucial factor is the secretion of immunomodulatory molecules by the MSCs. MSCs modified to overexpress SOX11 exhibited higher yields of osteogenesis, adipogenesis, and chondrogenesis when directed towards selective differentiation *in vitro* [15]. It is unverified if MSCs differentiate *in vivo*. However, their manipulated differentiation ex vivo could still be helpful for both understanding the effects of SOX11 and potential engraftment techniques of pre-differentiated MSCs. The modification of MSCs to overexpress SOX11 led to significantly increased bone formation for *in vivo* bone grafts and accelerated healing time in rat models with induced femur fractures [15]. It is still unknown what the full effects of SOX11 are. However, MSCs

can be modified to gain advantages from their overexpression, indicating that Sox11 is an important factor in regulating MSC's differentiation and migration.

MSCs directed to differentiate into chondrogenic cells may aid in the repair process of non-unions better than undifferentiated MSCs. Differentiating bone marrow derived MSCs towards chondrogenic cells produced significantly faster bone mineralization and increased volumes of newly regenerated bone than non-specific MSC injection [16]. Chondrocytes produce cartilage and likely stimulate the beginning stages of fracture healing by forming a callus that bridges the gap between bones. This callus serves as the template for future bone mineralization. Injecting chondrocytes to repair non-unions offers a more direct approach to MSC therapy. However, the study's cells were not isolated adequately to the uniform standards needed for consensus, and sample sizes were too small. MSCs were isolated from human bone marrow and used to observe fracture repair in rats. Not only were these stem cells taken from humans and used in rats, but these cells were isolated based on their adherence to plastic only, and cell surface markers were not accounted for [16]. With a sample size of three, a lack of cell homogeneity might explain why the second rat in the trial group receiving chondrocyte differentiated MSC did not display any significant difference in bone remodeling when compared to the undifferentiated MSC groups. Without more trials, it is hard to conclude that differentiated MSCs are more therapeutically active. A lack of proper cell dosing was noted as a possible explanation for the second trial rat's difference. However, there were no remarks about the non-uniform cell isolation methods or lack of cell purity proof. Without uniform standards of isolation for these MSCs, it is hard to say this experiment could be repeated with similar results or that the MSCs were the sole beneficiary. Understanding the limitations of this research can guide future goals of understanding differentiated MSCs and their therapeutic potential.

Biomaterials such as biphasic calcium phosphate are a promising tool to be used in conjunction with MSCs to provide a matrix for osteoconduction. Fluorescently labeled MSCs showed that upon mixing a matrix with MSCs, most cells attached to the calcium phosphate pores and remained alive, confirming that the biomaterial provides a high surface area template for MSCs to engraft in ways similar to their plastic adherence. Twenty-six out of twenty-eight patients receiving the treatment healed their non-union fractures [17]. A comparative double-blind clinical trial should be used in the future to examine differences between non-treatment or even treatments used today. The direct effects biomaterials have on MSCs should be investigated further, but they may provide a medium that induces MSCs to behave more similarly to their *in vitro* counterparts. If this is the case, *in vitro* differentiation could be manipulated to occur *in vivo*.

Mesenchymal stromal cells can also be modified to play a role in fracture healing for patient subpopulations. MSCs have been shown to improve fracture healing and angiogenesis in patients with diabetes mellitus. Diabetes is associated with poor fracture healing due to a lack of *in vivo* angiogenesis. Culturing MSCs in hypoxic conditions directed MSCs towards angiogenesis and mitigated

this effect [18]. Diabetes is associated with the decreased healing potential of surrounding blood supply and decreased bone production, leading to an increased prevalence of non-unions. Because vasculature is vital for bone formation, a conditioned medium of MSCs was used to direct cells towards angiogenesis. MSCs were isolated from human volunteers according to their plastic adherence, cell surface markers, and differentiation capabilities *in vitro*. The conditioned media featured MSCs grown in alpha-minimal essential medium in a hypoxic environment. Gelatin grafts of MSCs grown with or without the conditioned medium were transplanted in either diabetic or healthy rats. Immunohistochemical imaging revealed decreased competencies for diabetic rats to produce capillary endothelial cells and mesenchymal tissues and that treatment with conditioned medium MSCs could rescue these effects to near normal. Fibular fractures induced in diabetic and non-diabetic rats allowed researchers to investigate the effects of conditioned MSCs on fracture healing *in vivo*. There were no unions of the fibula observed in the diabetic rats after eight weeks; fracture healing is severely reduced in rats with diabetes. This is likely due to the reduced angiogenesis observed. Around 40% of non-diabetic rats displayed healed unions to the fibula after eight weeks. Diabetic rats treated with the conditioned MSCs displayed rescued healing outcomes comparable to rats without diabetes, about 40%. Diabetic rats also displayed reduced capillary endothelial cell counts compared to normal rats; however, treatment with hypoxic grown MSCs rescued this effect to near normal [18]. The adverse effects diabetes has on fracture healing and non-unions can be alleviated by treatment with conditioned cultured MSCs.

Skeletal stem cells expand locally in response to fractures and correlate to age-related declines in cartilage and bone health in adults. Human bone xenografts implanted in mice showed higher yields of SSCs and BCSP when fractures were induced [13]. A local increase in SSCs correlated with fractures indicates their use in repair mechanisms of injury healing. FACS isolated significantly fewer SSC in older mice than young ones [19]. These cells also demonstrated less *in vitro* clonal capacity when compared to the cells from younger mice. Not only do SSC contribute to fracture healing by localizing to the injury site, but they also decrease in number and capability with age, possibly explaining the age-related decline in bone and cartilage health. This result does not clarify *in vivo* clonal capacity; therefore, this is not a complete understanding of the effects of SSC and aging. However, preliminary knowledge of age-related stem cell decline is necessary to understand the disease's mechanism before any new therapies or cures are introduced. Degenerative afflictions, like osteoarthritis, non-unions, and osteoporosis, are likely caused by a reduction in SSC count and regenerative capacity with age. SSCs are involved in healing and age-related disorders, and future therapies aim to target these healing mechanisms.

## Osteoarthritis

Intra-articular injection of adipose-derived MSCs shows promising results for

the pain management and functional regeneration of articular cartilage in osteoarthritic joints. Numerous clinical trials are outlining the safety and efficacy of intra-articular injection of MSCs to treat osteoarthritis. Intra-articular injection of $1.0 \times 10^8$ adipose-derived MSCs improved the knee joint function and pain associated with osteoarthritis while demonstrating no adverse effects [20]. MSCs were cultured from adipose tissue resected from patients and sorted based on their common surface molecules. Eighteen patients were divided into three groups: low, medium, and high dose. Before and after MSC treatment, patients were examined using objective measures like arthroscopy, MRI, histological imaging, and subjective pain indexes. After six months post-treatment, patients in the high dose group demonstrated significant improvement in each benchmark due to the new hyaline-like articular cartilage production [20]. The result was cartilage production and reduced pain. Other studies have demonstrated similar results and further attributed the effects of MSCs to paracrine signaling and reduced inflammation.

Other studies have corroborated beneficial findings with adipose-derived MSC intra-articular injections. Intra-articular injection of adipose-derived MSC shows promising osteoarthritis treatment results in clinical trials [21]. Twenty-four patients with measurable osteoarthritis in their knees were enrolled in a double-blinded, randomized clinical trial. Twelve patients received the MSC injection into the knee, and twelve patients received a placebo saline injection. The MSCs collected were autologous, meaning each patient received MSCs from their own adipose tissue. The treatment group cells were cleaned, pelleted, and confirmed to be homogenous by the presence and absence of the cell surface markers [4]. All patients were assessed at 3- and 6-months post-injection for various parameters, including pain, stiffness, function, and size of the cartilage defect measured by magnetic resonance imaging (MRI). The group that received the MSC injection demonstrated statistically significant progress regarding pain, functional mobility, and reduced cartilage loss compared to the group that received the saline placebo group [21]. Larger sample sizes should be used in future research, but clinical trials continue to expand and generate better findings.

Long-term clinical trials also show promising results for basic injection of MSCs to treat osteoarthritis. Five years following the intra-articular injection of bone marrow derived MSCs, patients displayed no adverse effects while demonstrating improved baseline scores in functional mobility [22]. Functional mobility scores started to drop after six months of improvement; however, they never returned to baseline or dipped below. While there were only three patients in the trial, this is a preliminary indication of the benefits just one injection of MSC can have long term. Most approved intra-articular injections for arthritis, for example, hyaluronic acid or cortisone, are usually injected multiple times over months or years. While there is evidence that a single injection of MSCs can have long-term positive results, future research aims to understand the effects of multiple injections.

Repeated injection of allogeneic mesenchymal stromal cells demonstrates su-

perior beneficial effects for osteoarthritic joints. While autologous MSCs are derived from the patient and re-injected, allogeneic MSCs are derived from different individuals than the one receiving treatment. Intra-articular injection allogeneic umbilical cord derived MSCs provided significantly more pain relief and functional mobility when given in multiple doses in a clinical trial [23]. Twenty-eight patients were assigned to one of three groups. The first group received one dose of MSCs, the second group received repeated doses of MSCs, and the third group received a hyaluronic acid injection. Umbilical cord derived MSCs were isolated and cultured using the International Society for Cellular Therapies guidelines. Baseline scores in pain, arthritic index, and MRI structural analysis were compared to scores 6- and 12-months post-treatment. Patients receiving repeated doses of MSCs demonstrated reduced pain by 86% and reduced disability by 89%. The hyaluronic acid group had a 38% reduction in pain and a 50% reduction in disability. MSC treatment may be a more efficient than what is currently available for osteoarthritis. However, there were no significant differences observed in the structural analysis of the knee joint using [23]. More data is needed to conclude the reasons why MSCs provide better relief in osteoarthritis. Intra-articular injection of MSCs does not generate new cartilage but may halt degeneration.

Allogenic MSC treatment would be advantageous in a clinical setting because it would allow for pre-packaged immediate treatment. This would remove the need for two procedures to first isolate cells from the patient before they are later re-injected. However, there have been some concerns regarding allogeneic MSC treatment and the immune response to repeated injections. Repeated injection of allogenic MSCs in horses led to antibody production for the major histocompatibility complex in the MSCs [24]. Repeated injection could lead to immune repression of MSC activity. However, more research needs to be done to verify these effects. More animal studies should be used before clinical trials continue.

MSC injection could be the next most promising treatment for pain management associated with osteoarthritis. The degenerative nature of osteoarthritis wears down joints and leads to increasing pain and loss of functional mobility. So far, intra-articular injection of MSCs has not been able to generate new articular cartilage. However, they may not need to be considered the next new safe and effective treatment. Intra-articular injection of MSCs led to significant increases in anti-inflammatory molecules while also decreasing proteins associated with pain and chondrolytic enzymes [25]. Osteoarthritis was induced in rat shoulders using monoiodoacetate (MIA). Immunofluorescence was used to assess the expression levels of anti-calcitonin gene-related peptide (CGRP), tumor necrosis factor-alpha stimulated gene/protein 6 (TSG-6), and A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5).

The expression of CGRP in the C5 dorsal horn was used to quantify the sensitization of pain in the shoulder. The rats with induced osteoarthritis displayed significantly elevated levels of CGRP (pain) than the non-arthritic control rats used for comparison. The rats with induced osteoarthritis and injected MSCs

displayed similar levels of CGRP (pain) to the non-arthritic controls. This result suggests the MSC treatment reduced pain associated with osteoarthritis to near-normal levels. TSG-6 is an anti-inflammatory molecule that may protect cartilage. The MSC group had significantly elevated levels of TSF-6 (less inflammation), while the controls and the induced osteoarthritis groups had similar levels. This suggests that MSCs likely aid osteoarthritis by reducing inflammation to protect cartilage. ADAMTS5 is a cartilage damage indicator. All groups in the study demonstrated low levels of ADAMTS5 (cartilage damage) except for the induced osteoarthritis group [25]. This result suggests that MSC treatment rescues the effects of osteoarthritis by reducing cartilage damage. Together, these results support the notion that intra-articular injection of MSCs reduces inflammation, pain, and cartilage loss via precise mechanisms. While injections may not re-create lost cartilage, they could prevent a large proportion of people from having to replace their joints when their articular cartilage begins to wear away. For those who have little to no articular cartilage left, treatment using the injection of MSCs may require aid.

Co-implantation of MSCs with allogeneic cartilage can potentially regenerate new cartilage and advance healing in osteoarthritis. High tibial osteotomy is a surgical procedure that realigns knee joints to shift weight towards the lateral condyle of the knee. For patients with asymmetric deformities in the knee, a high tibial osteotomy can reduce cartilage degeneration and subsequent osteoarthritis. However, it has only been shown to regenerate cartilage in younger patients. High tibial osteotomy procedures followed by the implantation of MSCs with allogeneic cartilage resulted in higher levels of cartilage regeneration when compared to high tibial osteotomy with MSCs alone [26]. Eighty patients with knee osteoarthritis that elected to undergo a high tibial osteotomy were selected for the clinical trial. Adipose-derived MSCs were isolated from patients before surgery and cultured according to the previous standards set forth by the International Society of Cellular Therapies. The allogeneic cartilage was taken from donor cadavers. All patients received a high tibial osteotomy. Following surgery, half of the patients received the allogeneic cartilage with MSCs, and half the patients received MSCs only.

After approximately 12 months, both groups displayed similar Knee Injury and Osteoarthritis Outcome Scores (KOOS). However, after 24 months, the allogenic cartilage MSC group displayed significantly higher overall improvement levels than the MSCs alone. Co-implantation may demonstrate its advantages in the long term. Using arthroscopic procedures, the cartilage regeneration was ranked according to the Kanamiya grading scale. While around 40% of the MSC group displayed partial or total cartilage regeneration, around 55% of the allogenic cartilage MSC group displayed the same partial or total regeneration. Implantation of cartilage directly helped the regeneration of cartilage. The Kanamiya grade and KOOS were correlated so that as the regeneration of cartilage increased, so did the clinical outcome for the patient [26]. While this study might be investigating a specific surgical technique, allogenic cartilage may pro-

vide cartilage regeneration for several different applications. Further studies should be made to investigate allogeneic cartilage and MSC implant alone to see if this could be a viable scaffold for engraftment.

Scaffolds such as poly-lactic-co-glycolic acid (PLGA) can be used in conjunction with MSC injection to enhance their effects on damaged joints. MSC delivery with a PLGA scaffold could localize MSCs and direct their anti-inflammatory and anti-immune effects to joint tissues [27]. Furthermore, the number of cells required to reach a therapeutic effect was reduced to 5% - 20% because MSCs were localized using a 3-dimensional matrix. Arthritic scores of wild-type rats were compared to rats with collagen-induced arthritis. The arthritic rats were treated with either nothing (arthritis control), intra-articular injection of MSCs, intra-peritoneal injection of MSCs, or the PLGA nano-scaffold with MSCs.

Arthritic scores were highest for the arthritis induced rats and the intra-peritoneal MSC group. Both the arthritis free rats and the MSC with the PLGA scaffold displayed similarly the lowest arthritis scores. The intra-articular injection group displayed reduced arthritis, but about half the reduction as seen with the MSC scaffold group. These results indicate that the injection of MSCs with a scaffold significantly increases the therapeutic benefits. Using GFP-labeled MSCs, immunohistochemical staining revealed that MSCs were found only in the scaffold MSC group and only in the ankle following treatment. The increased therapeutic effects observed with scaffold are likely due to increased localization and implantation following injection. It was also observed that scaffold MSC treatment reduced the weight of lymph nodes and mRNA levels of various inflammatory molecules. Treatment of MSCs with a scaffold decreases systemic inflammation. Serum concentrations of anti-CII IgG were collected two and three weeks after treatment to quantify the immune response to collagen-induced arthritis. An ELISA revealed that all treatments using MSCs displayed a reduction in antibody levels. However, the scaffold MSC group contained the most significant reduction of antibodies than the intra-articular or intra-peritoneal groups compared to induced arthritis alone [27]. This further indicates that the effects of MSCs have on healing is likely due to their immunomodulatory response.

An MSC sheet encapsulated in a PLGA/MSC scaffold produced more cartilage, and that cartilage was better integrated into the host [28]. Not only are there alternative methods of MSC injection, but even advancing the delivery of MSCs with scaffolds can affect the incorporation of new tissues. Several different techniques are being explored that offer new and improving methods of tissue regeneration. Using synthetic scaffolds increases the surface area that MSCs can attach to, therefore increasing their immunomodulatory response *in vivo*. Synthetic matrixes may also allow MSCs to perform similar to how they act *in vitro*. However, this claim needs more research. If it is true, scaffolds could make MSCs into stem cells *in vivo*.

Biomaterials increase the paracrine effects of MSCs by increasing cell-cell in-

teractions [28]. Biomaterials with macro-pores (120 μm) demonstrated significantly advanced cytokine secretion profiles when compared to nano-pores (5 nm) and 2-dimensional plastic adhered cells [28]. Cytokine array analysis showed that macro-pore scaffolds provided an environment where MSCs could produce various cytokines in higher variety and concentrations than micro-pores or plastic. Immunological staining revealed that MSCs were spread out among the scaffold and connected to neighboring cells within the macro-pore environment. In contrast, in the micro-pore environment, MSCs were spread out, circular, and not connected to other cells [29]. Together these findings indicate that the synthetic environments MSCs are placed in can promote cell-to-cell interactions that may induce higher levels of paracrine signaling.

Increasing cell-cell interactions increases beneficial effects while also reducing the number of cells needed. Optimal pore size and concentration of MSCs are to produce beneficial effects in osteoarthritic mice. Gelatin mycrocryogels were prepared at various gelatin concentrations (4%, 6%, and 8%) [30]. Umbilical cord derived MSCs were loaded on each gelatin concentration. After seven days, MSCs were analyzed by live and dead staining techniques. Cells grew more densely in 6% gelatin mycrocryogels (MSC-GM). Histological staining and various cartilage index scores assessed the differences $3 \times 10^4$ MSCs, MSC-GM with $3 \times 10^4$ cells, and MSC-GM with $3 \times 10^5$ cells. The reduction in osteoarthritis progression was 34.1%, 63.4%, and 62.2%, respectively. MSCs injected with the gelatin performed better than MSCs alone. Also, both MSC groups with gelatin were comparable despite there being more cells in one group. The maximum threshold for MSCs appears to be around $3 \times 10^4$, and using a gelatin matrix around 6% reduced arthritis almost twice as much as MSC treatment alone. Luciferase tagged MSCs also demonstrated that the gelatin scaffold increased retention of MSCs by almost twice as much after 14 days. Increases in cell-cell interactions and cell density can optimize the benefits seen in MSC therapy by increasing the paracrine output and retention of the implanted cells.

Modified micro-fracture (MF) surgeries show promising results for rebuilding articular cartilage. An MF procedure involves surgeons drilling small holes in the chondral epiphysis of a long bone. These holes go down to the bone marrow, where they are thought to release residing stem cells, leading to fibrous cartilage formation. While this fibrocartilage relieves short-term joint pain and stiffness by reducing bone on bone movement, it is not as mechanically effective as the native articular cartilage in the long term [31]. MFs may improve mobility and pain, but they do not reintroduce the desired tissue, leading to shortcomings over time. Murphey et al. (2020) found that SSCs are locally released to the site of interest in response to MFs. Not only were SSC released to the fracture callus, but they also exhibited transcriptomes similar to the SSC in younger mice and humans. SSC's response to MF is to localize to the damaged tissue and express genes essential for self-renewal and cartilage generation. By co-transplanting bone morphogenic protein 2 (BMP2) and an antagonist for vascular endothelial growth factor (VEGF-) along with MF surgery, researchers were able to direct

mobilized SSC to produce articular cartilage in mice [19]. MF following injection of BMP2 and VEGF, has promising results in generating new articular cartilage for osteoarthritis.

Micro-fracture surgery followed by the injection of MSCs may be another valuable treatment for articular cartilage defects. MF surgery followed by the insertion of autologous bone marrow derived MSCs resulted in significant increases in cartilage healing and quality of life when compared to MF alone [32]. This randomized clinical trial divided fourteen patients with articular cartilage lesions randomly into either the MF alone or MF with MSCs group. Quantitative assessments using MRIs were used to observe cartilage tissue 6-, 24-, and 48-weeks post-surgery. The mean score given for cartilage tissue was almost 30 points higher for the treatment group, indicating that the use of MSCs aids articular cartilage repair following MF surgery. Qualitative tests such as the Knee Injury and Osteoarthritis Outcome Score (KOOS) were used to assess pain improvement after surgery. Forty-eight weeks post-surgery, the KOOS quality of life score was higher for the treatment group; however, higher sample sizes should be used in the future [32]. Because cartilage healing is minimal, the idea of minimally invasive surgeries to induce natural healing mechanisms is extremely valuable. MSC injection can increase the quality of cartilage repair and thus provided improvements to functional and subjective outcomes.

## 4. Conclusions

When MSCs were first classified, they were falsely named as stem cells, their isolation was based solely on plastic adherence *in vitro*, and their actions *in vivo* were not understood. MSCs have had a tough go at correcting these issues while maintaining their reputation for a promising regenerative cell line. Outdated research has claimed that "mesenchymal stem cells" contribute towards healing by differentiating into progenitor cells *in vivo*. However, there is no proof for these claims. These false attributions and inconsistent isolation methods have led to controversy over the validity of MSC research.

Research into possible bone and cartilage regeneration techniques has allowed for a more accurate understanding of effects MSCs have on healing *in vivo*. There is strong evidence suggesting that MSCs are paracrine signaling cells originating from pericytes. These cells are likely localized to vascular tissue and respond to injury by moving towards the damaged site and secreting various molecules to reduce inflammation and direct resident cells to contribute towards healing. Depending on their environment, MSCs have been observed to display differential effects; they likely have many different capabilities of injury response. Understanding the individualized response of MSCs *in vivo* is vital for new treatments for specified ailments. It's not enough to understand that MSC treatment has positive outcomes. Future research should aim to clarify the effects MSCs have on healing.

There are several unique avenues for new orthopedic treatments using mesenchymal stromal cells, ranging from generic injections to the specified treat-

ment of non-unions in people with diabetes. Repeatable results have been observed in several clinical trials. The most promising of these is the intra-articular injection of MSCs into the knee to treat osteoarthritis. There is definitive proof that this treatment relieves pain, reduces inflammation, and carries positive results in the long term. While this is not necessarily a technique to regenerate lost cartilage, it does show promise to delay or even halt the progressive nature of cartilage loss. Future studies will need to conduct double-blind comparative clinical trials with large sample sizes to add more evidence to this claim.

MSCs can be manipulated to enhance and specify their effects using bio-material scaffolds, gene techniques, and growing conditions. While MSCs can differentiate *in vitro*, they do not display this same capability *in vivo*. However, the plastic conditions that stimulate *in vitro* differentiation may be replicated *in vivo* using bio-material scaffolds. Understanding the subtypes of MSCs and how to direct them towards the desired effect requires ample understanding of what MSCs are doing *in vivo*. In order to investigate this, researchers need a unified and undebatable way to isolate pure colonies of MSCs to conduct *in vivo* research. The International Society for Cellular Therapy should clarify better methods of isolation to unite researchers. Before releasing new therapies, a more solid foundation in the identity and mechanism of action MSCs play is needed.

To generate accepted conclusions from MSC research, cells should be isolated by FACS or some other type of immediate cell sorting procedure before being grown on plastic plates. Isolating cells, growing them on plastic, and then confirming their identity with cell surface markers may not establish homogenous samples. Therefore, the International Society for Cellular Therapies needs to re-clarify what defines an MSC and how they should be isolated and grown. The boundary between cells is hard to define because all cells have the same genome. Any distinction between cells is a difference in gene expression and is entirely relative. The choice for a concrete border between one cell and the other is arbitrary, and researchers do not always agree. The Human Cell Atlas Project aims to use modern genetic techniques to create a database of all human cell types. This project would be an extensive reference map for researchers, a lot like the human genome project is. Having a unified database for distinguishing cell types would advance MSC research tremendously. Much of the research discussed in this paper exhibit positive and repeatable results while using techniques used to isolate or classify MSCs that are still in question. Having a unified base will create a more solid foundation to provide consensus for the researcher's conclusions.

The confusion surrounding MSCs complicates the promising potential they have in therapeutic applications because the mechanism of action is not understood or clarified. It is unknown to what degree cell populations are beneficial because they vary from study to study. A lack of homogenous isolation discredits any inference that the cell population will carry repeatable therapeutic effects. However, the experimental results surrounding the blanket term 'MSC' should not be ignored based on a lack of proper terminology, description, or isolation.

Despite the lack of precise classification, these reports contain valuable data, and many MSC treatments produce beneficial and repeatable effects. A systematic review of MSCs and their therapeutic applications can identify sound results and inconsistencies when the practice of isolating and defining cells is considered. If there are questionable isolation techniques, repeated results from various researchers can help clarify conclusions. Despite controversy over the identity of MSCs, studies of MSCs are among the most promising avenues for regenerative therapies that could replace expensive and high-risk orthopedic surgeries.

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## Conflicts of Interest

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