# Do mesenchymal stem cell improve bone healing in non-union?

Anita Akpunku

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2016

# Dedication

This project is dedicated to beloved family who has encouraged me through this entire process. My late father Chief J Akpunku, who passed away July 2015, for all his hard work in making me who I am today. My sweet mother whose love and prayers have protected through the hardest days in this journey. My sisters Ruth and Ogechi, my dearest brothers Cassa and Jude, whose love and faith in me is immeasurable.

Acknowledgements.

I would like to extend a special gratitude to Dr. Nitin Puri for being there in every step of this paper, guiding me with direction and encouragement. I could not have asked for a better mentor and advisor. I would also like to extend my gratitude to Dr. Hogue, the PA program director for all her dedication and hard work in making the UTPA program one of the best in the country. I also want to give thanks to all the professors and staff of the Physician Assistant department who collectively and individually have made the entire program a success. I would also like to acknowledge my classmates and special friends who have made the program a formidable experience. My friends Chelsea, Sarah, Iphie, Ada, Ethel, Onyi and all my dearest friends for all their enduring friendship throughout my time in Toledo.

## Definitions

A few of the terms that will be used frequently in this project can be ambiguous and as such, some definitions of those terms are listed below.

1. **Nonunion.** The US Federal Drug Administration (FDA) defined nonunion as a fracture for which a minimum of nine months has passed since the injury and there has been no radiologic signs of healing for at three months.
2. **Mesenchymal stem cells (MSC)** have been defined in various ways (Cuomo, Virk, Petrigliano, Morgan, & Lieberman, 2009), defined MSC as multipotent cells that has the potential to proliferate and differentiate into a wide variety of tissue such as bone, cartilage, adipose and muscle. MSC has also been defined as non-hematopoietic stromal cells which contains the ability to different into multilineage cells and stimulate growth of bone, cartilage, adipose and muscle tissue.
3. **Stem cell:** A cell that has two essential characteristics; the ability to differentiate into a particular cell type and the ability of self-renewal (Emara, Diab, & Emara, 2015).
4. **Homing:** This is the process by which cells migrate to, and engraft. It involves a cascade of events that leads to interaction between flowing cells and blood vessels at the target tissue site (Kariminekoo et al., 2016).
5. **Gene therapy:** This is a process of transferring genetic material from a donor site to the cell genome of the target site. This can be achieving using viral or non-viral vectors and either by *in vivo* and *ex vivo* (Emara et al., 2015).
6. **Critical-sized defect:** This is the smallest size bone defect that will not completely heal over the natural life of the animal without intervention (Spicer et al., 2012).

7. **Interfragmentary compression**: Static compression applied to a fracture plane imparts a high degree of stability to the fragments and thus reduces micromotion and strain. Bone surface resorption does not then occur. There is no demonstrable proof that interfragmentary compression, per se, has any effect upon internal remodeling of the cortical bone (Matter et al. 1974).
8. **Osteoinduction:** This refers to the stimulation and development of the primitive, undifferentiated and pluripotent cells into the bone-forming cell lineage. It is the process by which osteogenesis is induced (Albrektsson & Johansson, 2001).
9. **Osteoconduction:** This term means that bone grows on a surface. An osteoconductive surface is one that permits bone growth on its surface or down into pores, channels or pipes. It can also be defined as the process by which bone is directed so as to conform to a material's surface (Albrektsson & Johansson, 2001).

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## Introduction

The incidence of fractures requiring hospital stay has been estimated to be about one million in the United States (Zigdon-Giladi, Rudich, Michaeli Geller, & Evron, 2015). Non-union or delayed union of fractures is seen in about 5-10% of all fracture patients, this imposes undue health risk and frequent hospitalizations to patients (Desai et al., 2015). A 2012 multinational survey of orthopedic surgeons reported that non-union is multifactorial. A few factors attributed to non-union includes; the mechanism of injury, the degree of soft-tissue damage in a traumatic injury and the extent of vascular compromise. The health status of the patients such as smoking history, advancing age, diabetes, osteoporosis, and congenital bone disorders have also been implicated in non-unions (Bhandari, Fong, Sprague, Williams, & Petrisor, 2012).

There is currently no universally accepted definition of non-union amongst orthopedic surgeons, however, the Food and Drug Administration has defined a diagnosis of non-union as an injury in which at least nine (9) months have passed, and there is no radiological sign of healing for at least the last three. Non-unions have also been classified into two categories; hypervascular (hypertrophic) non-union, in which fracture ends are vascularized and thus, capable of biological activity and avascular (atrophic) non-unions where there is poor blood supply of the fracture ends (Morshed, 2014).

Bone tissue possesses an intrinsic tendency to heal and regenerate as part of repair process in response to injury and during continuous remodeling which occurs throughout adult life. Bone tissue has a unique ability to regenerate without scar formation as such, bone healing can be considered a form of tissue regeneration and in most cases the process occurs naturally and without complications. Despite the innate regenerative ability of bone tissue, in some

instances, this biological process fails leading to inadequate healing and non-unions (Marsell & Einhorn, 2011). Bone tissue is composed primarily of two parts; the bone matrix and the bone cells. The matrix contains about 90% type I collage and the remainder comprises of various non-collagenous proteins. There are two main types of bone cells. The bone-forming osteoblast, and the bone resorbing osteoclasts. Both cell types in conjunction with their precursors form specialized units known as bone multicellular units (Frost, 2001). The main function of this unit is to promote bone remodeling which maintains the integrity of the skeletal system by repetitive actions of the osteoclast and osteoblast. The osteoclasts function to remove old high mineral density bone prone to micro fracture while the osteoblast form new stable bone (Blair, Robinson, & Zaidi, 2005). The osteoblast are recruited from the MSCs found in the bone marrow while the osteoclasts come from hematopoietic stem cells through committed osteoclast progenitor cells that forms multinucleated cells (Blair et al., 2005).

Following fracture, the diamond concept, has been used to illustrate four conditions necessary for successful healing of fractures. The concept includes; (1) an adequate mechanical environment achieved by stable fixation, (2) osteoconductivity which are the scaffolds on which the regenerating cells use to produce and distribute hydroxyapatite and tricalcium phosphate, (3) osteoinductivity which requires growth factors to stimulate osteoprogenitor cells and (4) osteogenesis as a result of the osteoblast stimulation by growth factors (Verdonk, Goubau, Almqvist, & Verdonk, 2015).

Fracture healing occurs via direct or indirect healing depending on the mechanical structure in place. The fixation technique of the surgeon defines the mechanical structure determines the healing process of the fracture. Direct or primary bone healing is seen after surgical fixation of a fracture with a rigid object. This type of healing depends on an impeccable

and correct anatomical reduction with very little or no motion at the fracture site. (Hak et al., 2014). Primary bone healing occurs via direct contact healing or through gap healing. The cortical bone tries to re-establish mechanical continuity without the formation of a callus, if the gap between the bone fragments is less than 0.1mm after interfragmentary compression, direct contact healing is most likely to occur (Marsell & Einhorn, 2011).

Indirect or secondary healing is the most common form and is characterized by spontaneous healing without rigid fixation at the fracture site. In this case, callus forms within a mechanical environment that allows micro-motion and minor weight-bearing at the fracture site. Indirect healing may occur after a surgical fixation or nonsurgical management with a brace or cast (Hak et al., 2014). Indirect method consist of acute inflammatory response, recruitment of MSCs, formation of callus and revascularization at the fracture site. This a more complex process than the direct healing.

When trauma or any damage to the skeletal system occurs, it causes a disruption in the bone vasculature which could result in acute necrosis and hypoxia of the surrounding tissue (Glowacki, 1998). This disruption in blood vessels leads to an activation of clotting factors which activates the coagulation cascade and results in the formation of a hematoma. The hematoma consists of blood cells from bone marrow periphery and forms a template for formation of callus, a fibrin-rich granulation tissue which stabilizes the less stable structure of the fracture (Kanczler & Oreffo, 2008). Within the callus tissue, an endochondral formation occurs in between the fracture ends and external periosteal site. Injury and tissue disruption causes an acute inflammatory response, leading to the activation of endothelial cells, macrophages, cytokines and growth factors that recruit osteoprogenitor and mesenchymal cells to the injured site. Specific mesenchymal stem cells (MSC) must be recruited to proliferate and

differentiate into osteogenic cells in other to achieve bone adequate regeneration (Marsell & Einhorn, 2011). Bone morphogenic proteins (BMPs), possess osteoinductive properties and promotes bone healing by exerting their effects on undifferentiated cell by promoting their proliferation and differentiation into an osteoprogenitor pathway (Giannoudis, Jones, & Einhorn, 2011). Fracture healing also requires a blood supply thus, the ability to undergo revascularization is essential in achieving bone regeneration. Vascularization is process is regulated by an angiopoietin pathway and a vascular endothelial growth factor (VEFG) dependent pathway. The VEGF pathway is considered the key regulator in revascularization, it promotes the invasion of blood vessels and transform avascular cartilage into a vascularized osseous tissue. VEFG promotes both angiogenesis and vasculogenesis (Marsell & Einhorn, 2011). Tumor necrosis factor-a (TNF-a), interleukin-1 (IL-1), and IL-6 are released to promote vascular proliferation, stimulate angiogenesis, vascular endothelial growth factors (VEGF) production as well as the differentiation of osteoblast and osteoclast (Hak et al., 2014).

Non-union or delayed union can result if there is a disturbance in any of the processes required for bone regeneration. The cause of non-union is multifactorial and factors such as inadequate blood supply, instability at the fracture site, extensive soft tissue damage at site, advance age, smoking, malnutrition, infection, osteoporosis and chronic illnesses such as diabetes and cachexia has been implicated. Medication such as steroids and non-steroidal anti-inflammatory drugs (NSAIDs) can also be potential causes of nonunion (Zigdon-Giladi et al., 2015). Despite improvement in surgical orthopedic procedures, delayed, inadequate or incomplete bone healing still remains a challenging concept in clinical medicine. Non-unions are associated with high rate of healthcare utilization, higher cost and increased doses of opioid medications. The inability of fractured bone to achieve complete union after therapy may led to

morbidities, loss of dependence and even death in some cases (Tseng, Lee, & Reddi, 2008). A 2015 study concluded that patients diagnosed with non-union have a very low health-related quality of life. According to the study, the utility score of patient with non-union was 0.68, this score was considerably lower than in patients only diagnosed with stroke (0.81), diabetes mellitus (0.88) and Acquired immuno-deficiency syndrome (AIDS) (0.71). This study however did not take into consideration, other comorbidities that non-union patients may possess and how these comorbidities may contribute to a lower utility score (Schottel, O'Connor, & Brinker, 2015). There is also the issue of financial burden to a patient with non-union. The average direct cost to non-union have been estimated to be about $11,333. However, indirect cost such as lost wages and reduce productivity accounts for 67-79% of the total cost of treatment on non-unions (Hak et al., 2014). It is therefore important to study and implement diagnostic and treatment strategies that aims to improve healing and reduce bone-healing time.

Following treatment of a fracture, a routine part of orthopedic care is to determine when the fracture is adequately healed and when the patient can resume weight-bearing. It is important that the orthopedic physician makes the right decision for patients on appropriate time for removal of hardware, diagnosis and treatment of non-unions. When a patient is suspected of nonunion, assessment should include a clinical history, physical examination, imaging and lab studies (Hak et al., 2014). Clinical history should include the present and degree of pain with weight bearing and physical examination should inspect for signs of infection such as redness, deformity, drainage and problems at wound sites. In a 2008 review of fracture healing, absence of pain and tenderness to palpation at the site and the ability to bear-weight were the most commonly used criteria to determine bone healing (Morshed, Corrales, Genant, & Miclau, 2008). Radiological evaluation involves the use of plain radiographs, radiographic union scores,

computed tomography (CT), ultrasound and positron emission tomography (PET). Findings on plain radiographs includes presence and size of bridged callus, continuity of cortical and the disappearance of fracture lines (Bhandari et al., 2002). In spite of their development of more advance technology, plain radiographs remains the most commonly used tools due to increased physician familiarity, low cost, lower radiation exposure and widespread availability (Morshed, 2014). However, according to a study by Davis et al. the reliability of radiographs in detecting and staging of union are inconclusive and do not define union with adequate accuracy to enable its use as end-point in diagnosis of non-unions (Davis et al., 2004). Computed tomography has been described to be superior to plain radiographs in early detection of fracture, assessing union and visually extent of fracture. In a 2010 study conducted by Bhattachryya et al. CT has 100% sensitivity for detecting non-unions and only a 62% specificity (Bhattacharyya et al., 2006). However, high cost and increased radiation exposure limits the use of CT. Although there is no standardized acceptable tool for assessing non-union, Shmidhammer et al. concluded that micro CT with 3D reconstruction is the optimal method for diagnosing healing in non-unions. (Schmidhammer et al., 2006). PET is used to assess nonunion based on its capability to monitor metabolic activity. Ultrasound cannot detect cortical bone, but it has been used because of its ability to detect the formation of callus earlier than plain radiography (Craig, Jacobson, & Moed, 1999). Serological markers like C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), are becoming increasing popular as possibly early detectors of non-unions, they are commonly used to evaluate non-unions when infection is suspected as etiology of the non-union. However, they are not very specific and more research is needed to identify more sensitive and specific markers such as bone resorption markers, osteoclast regulatory proteins and bone-formation markers (Cox, Einhorn, Tzioupis, & Giannoudis, 2010). Despite all the advancement

in the assessment of fracture, physical exam remains the mainstay in assessing fracture union (Morshed, 2014).

In current practice, there are a number of acceptable protocols in the treatment of non-unions. These protocols include autologous bone graft, allografts, and bone stimulation. These methods of bone repair all have considerable drawbacks and the need for superior techniques to bone repair is warranted in the advancement of medicine. The use of stem cells for bone repair is a concept that is constantly being researched and explored in various areas of regenerative medicine. Due to their ability to grow into various tissue, stem cells have been applied successfully to other areas of medicine. Stem cells have been applied successfully to cardiovascular medicine for the regeneration of cardiac myocytes following an ischemic heart disease or heart failure. They have also been successfully in reconstruction of heart valves (Valina et al., 2007). In liver disease, studies have shown that stem cells have the capability to differentiate into hepatocytes when transplanted into an injured liver in mouse models (Seo, Suh, Bae, & Jung, 2005). Stem cells have also shown potential to differentiate *in vitro* into pancreatic cell lineage capable of expressing pancreatic cell markers and hormones such as insulin, somatostatin and glucagon (Hefei et al., 2015). The use of stem cells in brain and spinal cord repair is also been researched and could improve the next generation approach to clinical practices (Steinbeck & Studer, 2015). The application of stem cells to medicine is rapidly advancing and its potential is limitless. The aim of this literature review is to examine current and future studies of the application of stem cells in bone regeneration and to determine its effectiveness in future applications.

## Methodology

This project was carried out from January of 2015 through October of 2016. I reviewed published articles on nonunion, fracture healing and stem cell therapy. EndNote was used to organize my references and in-text citations.

**Search terms:** Mesenchymal stem cells, multipotent stromal cells, nonunion, stem cells, embryonic stem cell, bone healing, and fracture.

**Databases**: PubMed, Google Scholar, FDA, Access medicine, Elsevier, Clinical key and DynaMed.

**Inclusion criteria**: Articles from clinical review, clinical trials and peer review journals

**Exclusion criteria:** This review will exclude articles older that 15years, articles on chronic bone disease and articles not published in English language

## Literature review

Non-union of fractures still remains a clinically important challenge in orthopedic medicine. The skeletal system of the human body requires bones and muscles to maintain the structural integrity in other to achieve stability and balance in an upright man. Bones act as the hardware which together with the muscular system, propels various range of motion. Bone also possess some metabolic functions such storage of calcium, phosphorus and fat. Bone contains the bone marrow which has a major function of producing red blood cells. Bone- marrow is an organized tissue located within the bone cavities. It is highly innervated and vascularized but lacks and lymphatic structures. It is made up of two types of cells, the stromal cell and the parenchymal cells (Gulati, Ashton, & Hyun, 1988). As mentioned earlier, unlike other tissues, bone tissue has a unique ability to regenerate without scar formation and in most cases, the process occurs naturally and without complications and the newly formed bone eventually become in indistinguishable with the uninjured bone (Dimitriou, Jones, McGonagle, & Giannoudis, 2011). However, there are cases in which the normal bone regeneration is impaired and bone fails to heal. Some factors contributing to non-union can be attributed to age, infection, avascular necrosis, co-morbidity, trauma and the extent of displacement in a fractured bone (Egol et al., 2012).

### C**urrent therapy for non-union fractures.**

1. The current gold standard protocol for treating non-union includes surgical stabilization and autologous bone graft from the iliac crest of donor and implanted at recipient site (Qu, Guo, Fang, Cui, & Liu, 2014). Bone grafting is the safest and most effective procedure since it contains a person's own tissue and is therefore both compatible and non-immunogenic, thus reduces the chance of immune reactions. The autologous bone

graft usually combines BMPs and growth factors for osteoinduction, osteoprogenitor cells for osteogenesis and scaffolds used to provide a framework for osteoconduction (Bauer & Muschler, 2000). Bone grafting has also been shown to produce excellent results in achieving union, however, the supply of autologous bone is limited in the body and harvesting of grafts has been associated with a high morbidity and risk of infection (Griffin, Iqbal, & Bayat, 2011).

2. An alternative to autologous graft is the use of allografts tissue. Allografts are bone tissue coming from human cadavers from fresh-frozen banks which avoids problems with harvesting. The solution usually does not contain living cells and some components are destroyed during the freeze-drying or irradiation process and thus only possess osteoconductive properties (Finkemeier, 2002). Allografts however, also has the limitations of immunogenic reactions, rejections and the high possibility to introducing infections from a donor to a recipient (Giannoudis, Dinopoulos, & Tsiridis, 2005). The use of biosynthetic materials as substitutes for scaffold has also been explored. A wide change of these biomaterials such as calcium-phosphate ceramics, hydroxyapatite and tricalcium phosphate have been used as granules. These biomaterials are said to promote adhesion, proliferation and differentiation of osteoblast and produce collagen that would eventually undergo mineralization. Although most synthetic materials possess some osteoconductive and osteoinductive characteristics, they do not have the osteogenic properties (Gomez-Barrena et al., 2015). Scaffold and other synthetic bone substitutes have also been explored to improve repair of non-unions. Scaffold are primarily osteoconductors, and although they lack osteoinductive properties, synthetic bone substitutes are widely used for their osteoconductive property (Dimitriou et al., 2011).

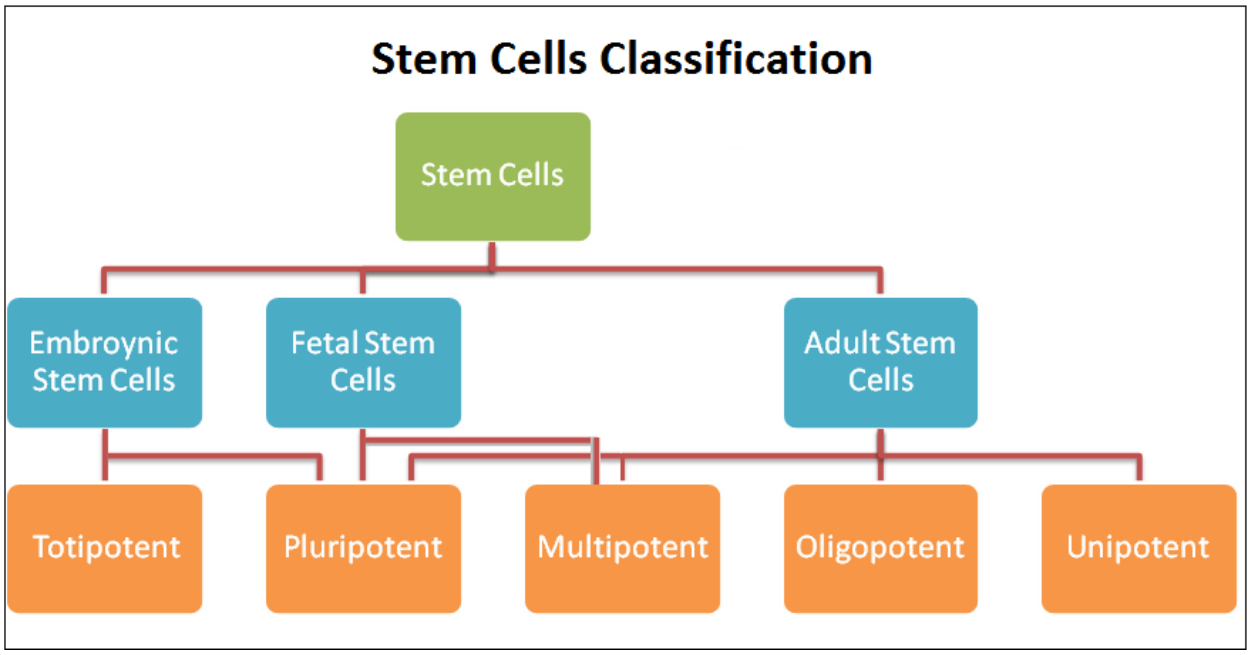
3. Tissue engineering is also a promising approach in the field of bone regeneration. It aims to generate new, cell-driven functional tissues, as opposed to the current implantation of non-living scaffold. This approach combines progenitor cells such as MSCs or in some cases mature cells, for their osteogenic property, with appropriate growth factors for osteoinduction and imbed them into biocompatible scaffold in other to generate and maintain bone (Dimitriou et al., 2011).
4. Bone growth stimulator is also being used as a therapy to improve bone healing in both acute fractures and non-unions. Some of the technologies used for bone growth stimulation includes direct current (DC), Inductive coupling, and Low-intensity pulsed ultrasound (LIPUS) (Behrens, Deren, & Monchik, 2013). Direct current stimulators are the oldest stimulators used. DC consisted of implanting wire leads at the fracture site and then removed 6-8 months later. DC stimulated provided constant uniform current at the site, however, battery life and the fact that it needed two different procedures were disadvantages to the DC stimulators (Aaron, Ciombor, & Simon, 2004). The most currently used stimulator is the low-intensity pulse ultrasound stimulator. A cohort study revealed that the overall success rate of LIPUS for non-union was between 55%-100%, in this study however it was unknown if the participants were healed spontaneously because no were no control groups (Watanabe, Matsushita, Bhandari, Zdero, & Schemitsch, 2010). It has also been shown that LIPUS can help decrease the economic burden that non-union fracture places on patient because it is less expensive and less invasive compared to a repeat surgery (Mehta, Long, DeKoven, Smith, & Steen, 2015).

**Mesenchymal stem cell therapy for non-unions.**

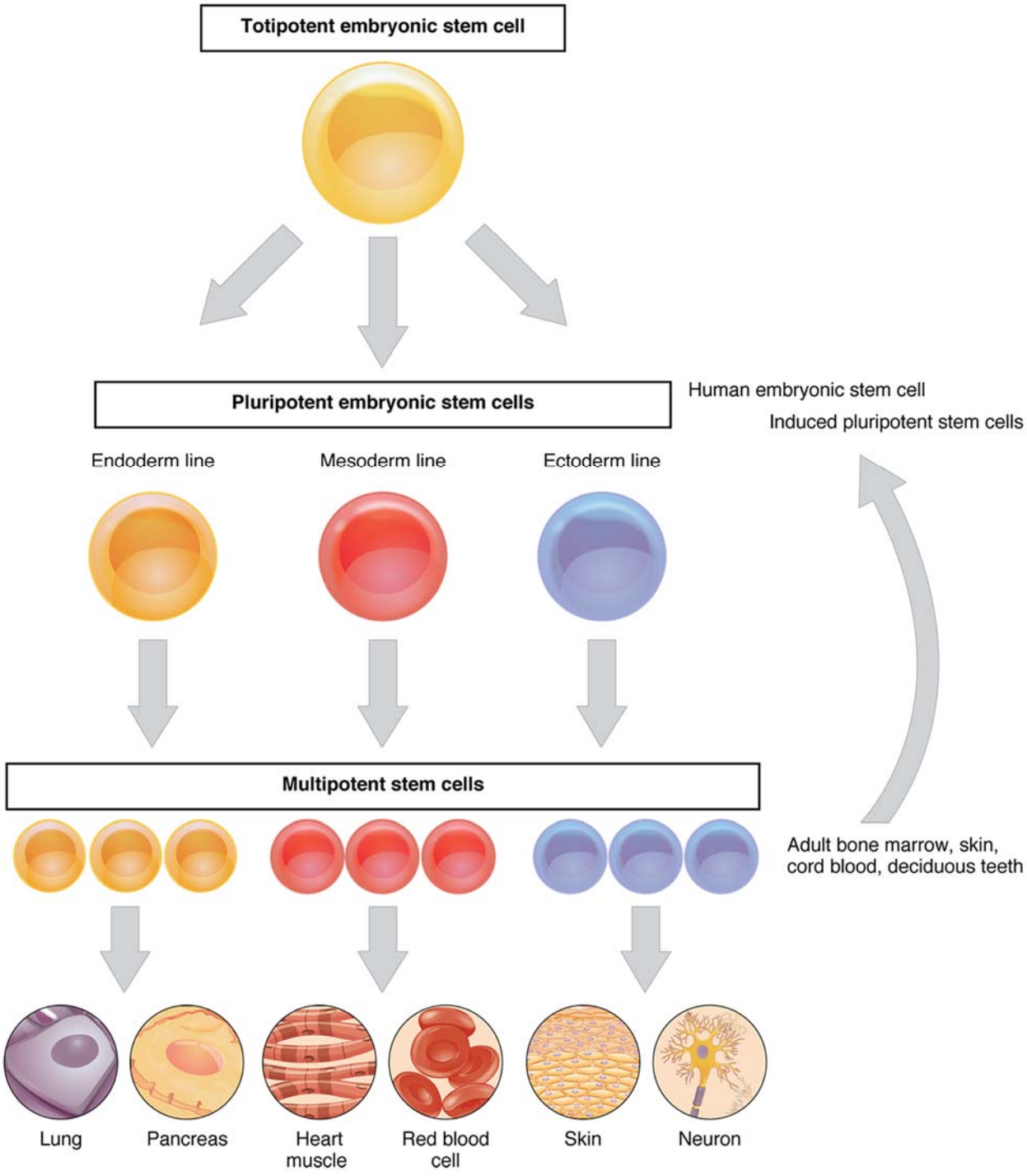
As the need for more effective therapy to achieve union increases, extensive research and clinical trials have been done to ascertain whether the use of mesenchymal stem cells can improve bone-healing time. The biologic function of a stem cell is its ability to regenerate into mesodermal tissues (Gamradt & Lieberman, 2004). Cells are considered the building blocks of life, and in most scenarios, cells are viewed as organized and specialized to form organs or tissues. Stem cells are a special category of cells that have two main characteristics that separate them from all other cell types. First, stem cells are unspecialized cells that have not differentiated into a cell with a specific specialized function but have the potential to give rise to any specialized cell type (Nam, Lee, Nam, & Joo, 2015). The second characteristic is that stem cells are able to undergo continual division and renewing in which they can self-regenerate and give rise to more stem cells or regenerate more specialized cells (Nam et al., 2015). Stem cells and all of their unique properties are at the center of some career long research and full understanding of them takes vigorous research and they are still not understood to their fullest potential.

Stem cells are undifferentiated and have two main characteristics; the capability of self-renewal and the ability to differentiate into distinct lineage of mature cells (Tseng et al., 2008). The stem cells can be grouped into, embryonic stem cell (ESC), fetal stem cell (FSC) and adult stem cell, based on where they are found. Stem cell can also be grouped into totipotent, pluripotent and multipotent based on their potency. Potency in this case describes their degree of differentiation (Cho, Fernandez, & Kwon, 2014). A totipotent cell has the ability to differentiate into every type of cell in the adult body and cells of extra-embryonic tissue necessary for fetal development. Totipotent stem cells are derived from fertilized egg and are thus the only true totipotent cells (Tseng et al., 2008). Pluripotent cells derived from further differentiation of the

cells. These cells can give rise to any specialized cells including the main tissue types namely, endoderm, mesoderm, and ectoderm. Pluripotent cells are however incapable of differentiating into cells required for fetal development. Of all three types, the ESC are the most pluripotent with a high degree of plasticity, they have the ability to differentiate into any type of cell found in the embryo and also possess a high capability to self-renew (Xu et al., 2002). ESC are isolated from the inner cell mass of the blastocyst (Zaidi & Nixon, 2007).




http://stemcelloverview.weebly.com/classes-of-stem-cells.html

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The third type of stem cells are the multipotent stem cells which are more specialized than the previous two. Adult stem cells are multipotent and have been the most studied out of the

group. They are have a lower level of plasticity when compared to ESC and totipotent but also have the potential to self-renew and differentiate (Polak & Bishop, 2006). There have been suggestions that adult stem cells may have more plasticity than previously proposed. A more pluripotent adult stem cell known as the multipotent progenitor stem cell has been isolated from more marrow of rats and are capable of differentiating into the three different germ layers, a characteristic of the pluripotent ESCs (Jiang et al., 2002). Adult stem cells can be further divided into mesenchymal stem cells and hematopoietic stem cells. Hematopoietic stem cells are capable of differentiating into cells that predominate in the circulatory system and their clinical use in treatment of leukemia, lymphoma, multiple myeloma and anemias are currently explored in clinical practices. Mesenchymal cells on the other hand, are non-hematopoietic and are found in numerous sources throughout the human body (Tseng et al., 2008). Adult human bone marrow, periosteum and fat tissue contain all contain mesenchymal multipotent progenitor cell in abundance. Few MSCs with osteoblastic potential can also be seen in other tissue such as muscle, umbilical cord, placenta, dermis, cartilage and synovial fluid (Fayaz et al., 2011). The bases of using stems cell for therapeutic clinical uses is brought about by their normal role in tissue repair, their ability to tolerate *in vitro* expansion and the fact that they are found in every tissue in the body. The belief is that MSC can migrate to sites of injury and engraft within, a process known as homing (Kariminekoo et al., 2016).

The proposed theory of osteoblast proliferation, is based on the thought that is if the pluripotent MSCs are provided the right environment will exhibit an osteoblastic phenotype (Cuomo et al., 2009). Bone marrow are currently the most appropriate cells used for bone regeneration due to their strong osteogenic properties and are easily obtained by culturing bone marrow aspirates from iliac crest. Recent studies have shown that MSC derived from small bone-

marrow aspirate are multi-potent and are capable of differentiating into various lineage. They have also been shown to be readily isolated and expanded (Zigdon-Giladi et al., 2015). A 2011 study compared the use of ESC derived MSC and adult bone marrow derived MSC in their use for repair of non-union fracture. The study concluded that BM derived MSC indicted a higher potential to induce fracture healing in non-union compare to ESC derived MSC, although it was also shown that the ESC-derived MSC showed a slightly higher healing potential compared to the control group with no MSC induction (Undale et al., 2011). The current approach is to administer a large number of osteoprogenitor MSC in combination with some growth factors, directly to injured site. This is a minimally invasive procedure and seem to produce satisfactory results in bone healing (Pountos & Giannoudis, 2005).

MSCs are minimally immunogenic, which means they do not express major histocompatibility II and stimulatory molecules, therefore, allogenic transplantation should not require that the host be immunosuppressed. All MSCs, can be induced *in vitro* and i*n vivo* to differentiate into various tissues such as bone, cartilage, muscle and tendon. MSCs could be used to facilitate fracture healing by being loaded in scaffold of predefined shape to fit in the defected bone (Pountos & Giannoudis, 2005).

**Isolation and expansion of mesenchymal stem cells**

MSCs are typically harvested from the iliac crest and usually requires and efficient method for its isolation. In most cases this is achieved by the use of density gradient centrifugation with Ficoll or Percoll. The marrow samples are washed with phosphate-buffered saline and then centrifuged. After the cells are centrifuged, layers of red blood cells, fat and other cells are separated according to their densities. The MSCs which are contained in the middle layer is then aspirated and purified (Griffin et al., 2011). The nucleated cells which are contained

in the interface layer are cultured in a medium with Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum (FBS) (Hao et al., 2016). Some media do not contain any animal product but instead is made of human platelet lysates for optimal safety in clinical applications (Rosset, Deschaseaux, & Layrolle, 2014).

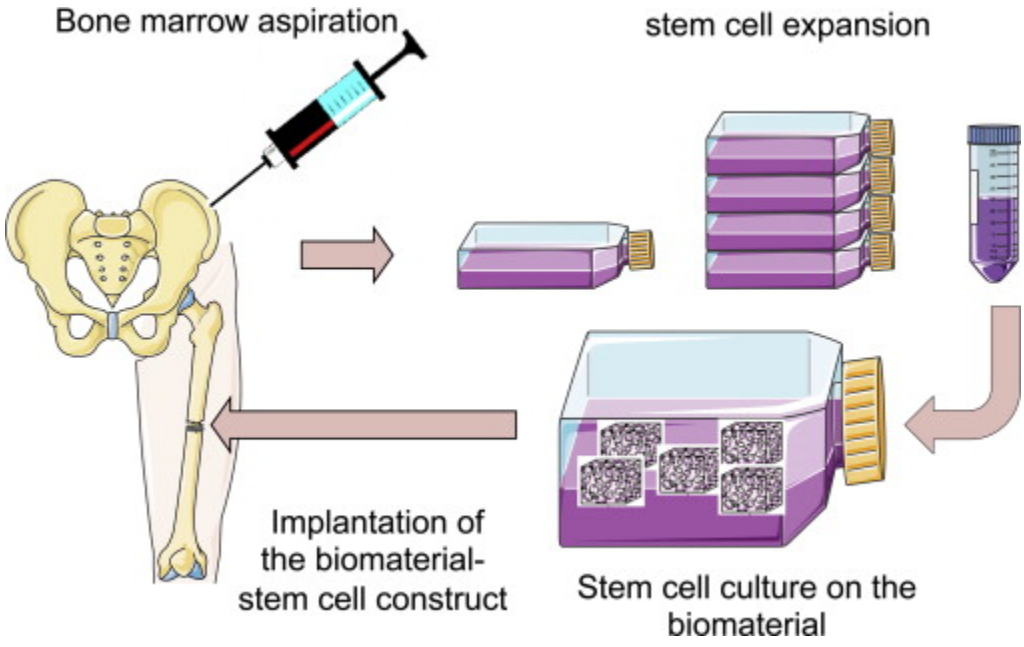


Figure1: Bioreactor principle allowing culturing of mesenchymal stem cells on a biomaterial prior to implantation (Rosset et al., 2014).

The cells are further expanded in a three-dimensional bioreactor which mimics the *in vivo* environment (Abdallah & Kassem, 2008). MSCs are capable of both *in vitro* and *in vivo* differentiation into various cell types. *In vitro,* the induction of differentiation is achieved a mixture of morphogens and chemical vital for the particular cell type. In osteoblast differentiation for example, combination of important players such as dexamethasone, calcitriol, ascorbic acid and β-gycerophosphate is added to the medium. Differentiation is then verified by presence of specific gene and protein expressions (Post, Abdallah, Bentzon, & Kassem, 2008). MSC can also identified *in vitro* based on their ability to adhere to the plastic culture plates and

their ability to generate colony forming units after culture in a standard medium (Rosset et al., 2014).

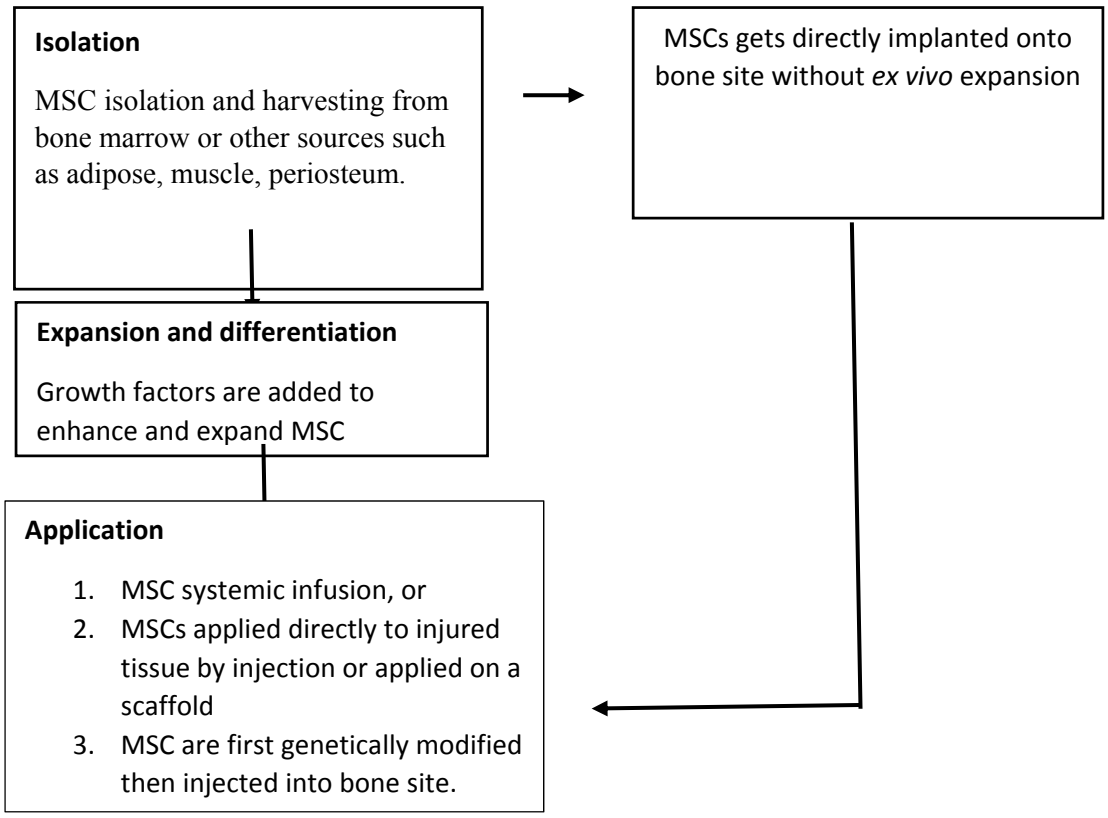


Fig 2. Diagram showing the process of applying mesenchymal stem cells for bone repair. (Griffin et al., 2011).

The cells are also concentrated in three to six fold to increase the number of mononuclear cells, and hence the MSC. It has also been shown that the healing rate is proportional to the concentration of MSCs that were administered.

In the treatment of tibial non-unions in sixty patients via percutaneous injection of concentrates from iliac crest bone-marrow aspirate, there was evidence of a positive correlation between the volumes of callus formed and the concentration of stem cell aspirate used. In seven patients who failed to achieve union after treatment, it was shown that both the concentration and the number of cells that were injected into these patients, was significantly lower than in patients who achieved union (Hernigou, Poignard, Beaujean, & Rouard, 2005). After the MSC are

expanded *ex vivo,* they are then introduced by systemic fusion, direct application to the site or on a scaffold and then applied directly to the site of the affected bone, in some cases, they are first genetically modified before their application into the bone lesion (Griffin et al., 2011).

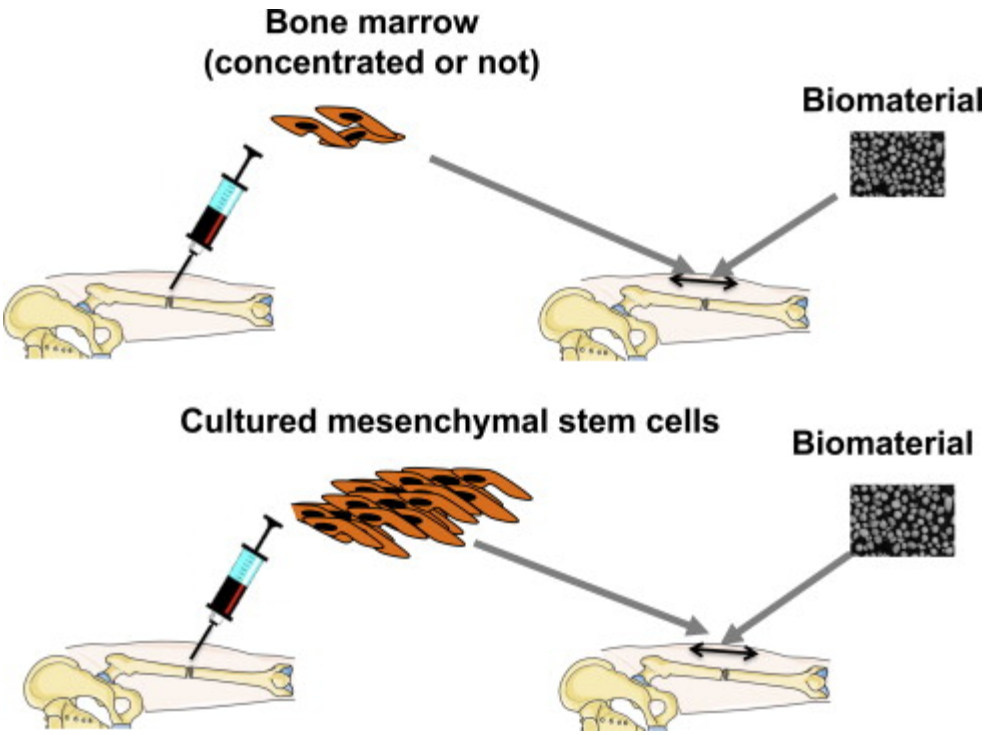


Figure 3: Use of bone marrow or cultured mesenchymal stem cells (Rosset et al., 2014).

**Expanded MSCs introduced by systemic infusion.**

It has be shown that MSC can differentiate into different cell types required for various tissue engineering (Zaidi & Nixon, 2007). Studies with animal model have also demonstrated the ability of MSC to migrate to the bone marrow of the host after peripheral injection, these cells have the capacity to remain there for a long period of time (Devine et al., 2001). Also, a study by Liu et al. to determine the safety of intravenous (IV) infusion of MSCs, demonstrated a successful infusion of the *ex vivo* expanded MSC into human volunteers and rhesus. The study was able to demonstrate allogenic MScs were capable of homing in the bone marrow of recipient patient. The results also concluded that there were no obvious side effects after IV infusion of

MSC into host and thus, the study called for further research in systemic infusion (Liu et al., 2006). The systemic infusion approach have also been used in treating osteogenesis imperfecta (OI) in children. According to the study a total of six children with OI, were infused systemically with MSCs obtained from sibling donors, of the six children, five showed MSC homing into various parts of the body, including bone skin, skin and marrow. These five children also showed an increase in bone growth between 60% and 94% increase compared to the negligible growth these patients showed 6months before the trial (Horwitz et al., 2002). A more recent study published in June 2016, evaluated the safety of IV infusion of adipose-derived MSC in patients with refractory rheumatoid arthritis (RA). In this experiment, fifty-three patients were infused with allogenic adipose-derived MSC. The study concluded that the infusion was generally well tolerated and no evidence of dose- related toxicity existed. They study also recommended further experiments into systemic infusion of MSC to treat RA (Alvaro-Gracia et al., 2016).

Despite this success with systemic infusion, the technique has not be successful in the repair of fracture. A study by Shrepfer et al. done by IV infusion of MSC in rats showed that most of the MSCs, where concentrated in the lungs of the rats with only a negligible amount reaching the target site of injury (Schrepfer et al., 2007). Perhaps, this is a potential limitation on the use of this method for fracture healing. Research into systemic infusion is still on going.

**Application of MSCs directly on the fracture or grown on scaffold.**

Direct application of the MSC at the fracture site is deemed more practical than the infusion method. Scaffold act as a building block on which the concentrated MSCs can be implanted. In other to get the desired effect, scaffolds need to mirror the natural environment of bone matrix (Frenkel et al., 2005). There is also a benefit of using growth factors, which acts as osteoinductors, to increase the formation and vascularization of bone. A wide variety of scaffold

exists for implantation however, studies have shown that hydroxyapatite (HA) is the most favorable, due to its exceptional osteoconductive properties (Tanaka et al., 2011). It has also been shown that HA possess a good strength compared to other products such as beta-tricalcium phosphate (β-TCP), however, unlike β-TCP, it is non-resorbable. In most cases, a combination of HA and β-TCP are used for optimal results. Despite its osteoconductive properties, HA also possess poor mechanical properties and thus, using HA alone may not be feasible in maintaining the mechanical loading required for bone remodeling (Lin et al., 2007). Mechanical stability of the fracture bone has been recognized as an essential factor in fracture repair. A study by Lin et al. shows that three-dimensional polymer scaffolds has excellent stability. HA has also been combined with other biodegradable polymer/bio ceramic, to allow for better control when shaping the composites required for bone regeneration (Lin et al., 2007). Another study done by Quarto et al. used autologous bone-marrow derived MSCs from three patients which large bone defects and of varying ages (41, 16, and 22). The autologous cells where expanded *ex vivo* and were imbedded on microporous hydroxyapatite ceramic scaffolds. The grafts were sized and shaped to meet the varying bone defects in each patient and mechanically stabilized via external fixation. Although no complications was noted or reported during the long term follow up of these patients after successful surgery, it was noted that the graft was not resorbed and remained the same after 7 years. This was attributed to the high density of the mineral and its low permeability. Angiographic studies of these bones after 7 years revealed adequate vascularization of the grafted zone. In addition, in all three patients, x-rays and (computed tomography) CT scans showed an increase in callus formation at the site of the implants and good communications with the host bone just two months after surgery (Quarto et al., 2001). Marcacci et al. in a 2007 study, the isolated bone marrow aspirate from patients were expanded *in vitro* up

to 12-14 folds and placed on porous HA scaffold designed to match the shape and size of the defect. Five to seven months post-surgery, it radiographs and CT scans showed complete fusion between the host and the grafted implant. Good vascularization and integration was also reported seven years post-surgery (Marcacci et al., 2007).

Another promising technique is the differentiation of MSCs into specialized osteoblasts before injecting it into the host. In the treatment of bone tumors, Morishita et al. added trypsin to MSCs to prepare a cell suspension. HA ceramic scaffolds were soaked in the suspension and further cultured in an osteogenic medium augmented with beta-glycerophospate, vitamin C and dexamethasone. After 2 weeks, osteogenic differentiation of the MSCs on the HA ceramics was achieved. In the cultured plates, the MSCs high alkaline phosphatase activity, indicating osteogenic ability. After harvesting and curettage of the tumor, the HA was used to fill the patient's bone cavity. The implants revealed a new bone formation in pre regions of the ceramics after ectopic implantation, immediate healing potential was evident on serial plain radiographs and CT images.

An alternative method is the combination of bone-marrow aspirate concentrate (BMAC) with osteoinduction agents such as demineralized bone matrix (DBM) and /or recombinant human bone morphogenic protein-2 (rhBMP-2). The randomized cohort study included 49 patients with atrophic tibial non-union and excluded patients with any, previous open grafting and stress fractures where the gap could not be measure. After concentration of the BM aspirates, it was mixed with DMB and/or rhBMP and injected into the patients fracture site. Prior to the injection, the fracture site biopsied and cultured to rule out any infection or pathology. A space was then created using a curettage, the mixture of BMAC, DMP and/or rhBMP-2 was slowly injected into space. The skin is then sutured to avoid leakage of the BMAC. The primary

outcome was the radiologic healing of fracture. Over all, the average radiographic healing rate was 79.6% at an average of 4.7 months. Although not statistically significant, the DMP group showed a healing rate of 86.4% compared to the rhBMP-2 group of 70.8%. (Desai et al., 2015). The low rate and longer duration to achieve union in this study was attributed to the fact that the patients all had more refractory non-union for longer periods and also in all cases, the patients have had some form of internal fixation and there was no possibility of a union without further intervention (Desai et al., 2015). One limitation of this study is that the sample size was relatively small and there was no control group that received n BMAC injections without osteoinductive materials. Huang et al. also reported that BMP-2 have also been shown to increase bone formation *in vivo* and *in vitro.* The study showed that addition of BMP-2 greatly increased the expression of osteocalcin, and thus concluded that addition of BMP-2 and other growth factors such as VEGF appeared to be an important factor in the enhancement of osteogenesis (Huang, Ren, Ma, Smith, & Goodman, 2010).

A recent study has also been carried out to evaluate the effectiveness of BMAC and fibrin glue to treat atrophic non-union in animal. Fibrin glue has been shown to have an excellent biocompatibility and thus will act as a sufficient carrier to administer the BMAC (Babhulkar, Pande, & Babhulkar, 2005). In this study, thirty-six male Lewis rat, were divided into three-groups; (A) a control group, (B) atrophic non-union group and (C) an experimental group. Non-union of groups A and B was achieved by cauterization of the femoral periosteum. After bone marrow aspiration the osteogenic activity of the bone marrow stem cells (BMSC), was examined by alkaline phosphatase (ALP) and Alizarin Red stain. The BMSC were stained strongly by both ALP and Alizarin red stain. The BMSC were then trypsinized and concentrated via centrifugation. The fibrin glue was prepared using fibrinogen, fibrin-stabilizing factor and

thrombin. The BSMC suspension was added with the fibrin and fibrinogen stabilizing-factor and then later mixed with thrombin in a sterilized syringe. The fibrin-glue alone was injected into femur gap of rats in group B, while the BMSC fibrin glue was injected into the femur of rats in group C, and then sutured. Prior to injection all rats were stabilized using a customized external fixator, which was attached to the femur. Histologic and radiographic analysis of the procedure was carried out eight weeks post operatively. The results from the radiography showed bridging of the osteotomy gap with formation of new bone tissue in group A. Group B, the atrophic non-union, injected with just fibrin gap demonstrated the presence of fibrous connective tissue in the osteotomy gap, also in group B, and the osteotomy gap appeared larger in 10 rats. In group C, the experimental group, the femurs achieved complete bony bridging of osteotomy gap with formation of a large number of woven bone tissue. Thus, Hao et al. concluded that repair of atrophic non-union can be achieved with the use of BMSCs and fibrin glue (Hao et al., 2016).

A different process is the use of the patient as his/her own bioreactor. In a 2004 experiment done by Warnke et al. achieved a successful reconstruction of the 7-cm mandibular defect caused by mouth cancer on a 56-year-old man. They use custom fit titanium mesh as external scaffold. The mesh was then loaded with hydroxyapatite (HA), recombinant human Bone Morphogenic Protein-7 (rhBMP-7) and BM derived MSC. The scaffold was then implanted at the patient's latissimus dorsi muscle to allow for growth. The mandible replacement was then harvested 7 weeks later and transplanted to repair the severe mandibular defect on the patient. One advantage of this procedure is that transplantation rejection is very unlikely as the patient's own cells is used as a substrate for the grated tissue. The study showed success as the patient regained full masticatory function (Warnke et al., 2006). This procedure is also suitable for patients with large bone defects who are contraindicated to the other previous approaches.

There have been cases in which MSCs have been used for bone regeneration without expansion. This is majorly due to cost and time influences. In a 1995 study, Connolly, showed in a series of 100 bone-healing problems, such as non-union fractures, arthrodesis and other bone defects, that MSC were also effective in bone regeneration when used ex vivo without expansion. This study however, failed to report the required amount of MSC needed to achieve optimal healing in bone defects (Connolly, 1995).

**Genetically modified mesenchymal stem cells.**

Despite the success in combination of growth factors and scaffold to MSCs for tissue regeneration, limitations exists in the long-term release of growth factors for proliferation and maintenance of MSCs (Griffin et al., 2011). Hence, genetic modification of MSCs to produce growth factors and express transgenes of osteogenic proteins have been explored. The rational is that this will decrease the number of MSCs needed for implantation and could inadvertently eliminate in vitro culture and expansion (Tseng et al., 2008). Genetic modification of the cell can be done sing viral or non-viral vectors and by either *in vivo* or *ex vivo* process. In *in vivo*, processes involves the direct transfer of genetic materials into the genome of the target MSC using a viral vector. Viral vectors show a better expression of the desired protein and they have a higher efficiency in transfection. Thus viral vectors are optimal for gene modification of MSCs (Gamradt & Lieberman, 2004). The indirect *ex vivo* method requires the transduction of target cells with a vector encoding the desired genetic expression. This method involves a series of stems which includes; collection of cells by tissue harvest, expansion of the cells in culture, then genetically modifying the cells *in vitro,* before transferring back into the host. Although this process is more demanding, it has been considered safer due to the opportunity to test the cells for the presence of any abnormality and the selection of cells with the highest gene expression

before injecting into the host (Dimitriou et al., 2011). An advantage of the *in vivo* method is that it does not require and expansion and the direct injection of the viral vector is a simple process and can result in the expression of transgenes at the injection site. Studies have shown that using the *in vivo,* direct injection of BMP-2 producing adenovirus in animal models can induce bone formation and union in critically sized mouse radial defects (Gamradt et al., 2006).

**Safety and regulatory requirements.**

Currently, only autologous MSCs are used for stem cell therapy in bone repair. Intra-operative BM concentration in the operating room using small centrifuges and CE-marked kits does not require authorization and is performed under the responsibility of the surgeon (Gomez-Barrena et al., 2015). In a study conducted by Hernigou et al using this procedure, in 1873 patients, no complications were recorded (Hernigou, Poignard, Zilber, & Rouard, 2009). Clinical trials that assessed the safety of MSC transplantation for treatment of disease, did not show any significant adverse disease, however, some studies have found that MSC have the ability to interact with tumor cells. Thus MSCs can support tumor angiogenesis and proliferation by providing matrix required for tumor scaffolding, therefore, caution should be used in treatment of neoplastic patients (Zigdon-Giladi et al., 2015).

**The future.**

The biology and application of stem cell therapy is on the rise in various field of medicine. Despite this, it is important to note that delayed union, non-union and bone defects will be the major indications of stem cell therapy in routine clinical practice. The development of hydrogel-based solutions for supplying cells and biomaterials percutaneously can be expected in the future. Furthermore, the immunosuppressive properties of MSCs may allow the transplantation of allogeneic MSCs in various orthopedic conditions, with the establishment of

cell banks for regenerative medicine (Rosset et al., 2014). Trials evaluating allogeneic MSCs (Mesoblast) in delayed union are under way in Australia.

The use of allogenic MSC have also been proposed as an alternative approach. According to Arinzeh et al. the use of allogenic MSC will be beneficent in providing cells for a much larger population. The article proposed that MSC isolated from donors could be expanded and cryopreserved for future use as an "off-the-self" therapy. Donors and recipient need not be related or immunogenic ally matched. It is believed that some features of MSC may enable it to avoid being rejected by host immune system since the MSCs do not express Class II molecules (Arinzeh, 2005). A recent study investigate the use of allogeneic MSCs to promote osteoblastogenesis and prevents glucocorticoid-induced osteoporosis (GIOP). The study explores the systemic infusion of MSCs without genetic manipulation. Allogenic MSC were harvested and systemically infused into mice with excess dexamethasone. They concluded the systemic infusion of allogenic MSC prevented reduction of bone mass and strength in GlOP. No anticatabolic effects or systemic immunomodulatory effects of the allogenic MSCs were detected in the mice (Sui et al., 2016).

We can also begin to consider medico-economic evaluations of the benefits of bone repair cell therapy. Advances in tissue engineering techniques for the treatment of large bone defects are expected in the future as majority of the current therapies are more efficient in small critical-sized defect. A multidisciplinary approach will be required to improve implanted cell survival and to ensure prompt vessel ingrowth into the biomaterial via careful selection of structure and shape, together with addition of cytokines and growth factors (Rosset et al., 2014).

## Discussion and Conclusion

Non-union fracture pose an increasing problem in orthopedic medicine and the patients. For most patients, the inability to achieve complete union of fractures leads to constant chronic pain, loss of independence, financial burden and in some cases psychological issues. The issues surrounding treatment of non-unions has been a long and involved and ongoing expedition. The current modalities employed in clinical setting for treatment of non-unions includes, physical therapy, pain management, autologous bone graft and administration of specific growth factors, all resulting in different outcomes with regards to benefit to risk ratio (Hao et al., 2016). The future of orthopedic medicine relies on research and advancement studies to keep up with the therapeutic demand. As the comprehension of bone biology and physiology continues to expand, many new strategies in treatment of non-union fractures are being developed and studied. Many researchers agree that stem cell is the future of medicine. There are numerous ongoing research in the use of stem cells in to advance different areas in medicine. Researchers have thus, explored the use of human mesenchymal stem cells in regeneration of bone. Although there seem to be promise in use of MSC for future therapies orthopedic medicine, there are still questions to be answered in the field of MSC. There is insufficient information about the cellular basis for the use of MSC-mediated fracture repair and bone regeneration *in vivo* in animals. There are concerns regarding the efficacy of transplanted cells and their mechanism of engraftment, homing and differentiation in an *in vivo* environment. Further understanding into this mechanism could be integral for future therapeutic advancements. There is also a safety concern in transplantation of adult stem cells. Some studies have demonstrated the potential of MSCs to interact with tumor cells, however, the research in this area is sparse and the need for further investigation into this mechanism of interaction is warranted.

The studies in this literature review where limited in the number of participants in the study. Findings from various studies concluded that bone union after treatment is proportional to the number of MSCs available, thus, a higher amount of MSCs, are required for optimum results. The downside of this is that this study is difficult to perform on a large scale due to the availability of MSCs, the variability in host tissue, the surgical technique and other unique factors in a clinical study .One study also acknowledged its failure to evaluate patients' subgroups (age, smoking, body mass index etc.) in their experiment and thus may have led to different outcomes. Another study did not employ a standardized protocol in the treatment and each surgeon was allowed to treat the patients using their own different perspective and approach (Egol et al., 2012). Another limitation of these studies is the lack of an agreement in the modalities used to access bone healing. This makes it difficult to determine the accurate healing time-frame. Further advances in the assessment of healing should be explored and a single modality should be put to place for accurate measurement of bone healing. There need to be criteria that determines when the MSCs have been fully integrated and immunologically compactible with the host tissue. There also need to be consensus on when function has been restored by the MSCs. The long-term stability of bones repaired using MSCs are difficult to access as most of the studies are still very recent. Follow up on patients who achieved repair will be needed for accurate documentation of long-term effect (Tseng et al., 2008).

Questions of ethical issues in harvesting of stem cells are also been raised as the stem cell rise is being researched. Although, most of the research use adult-stem cells, there have been a few who utilize umbilical cord blood or embryonic stem cells due to their increased plasticity. The harvesting of these cells for clinical research is cornering and some questions surrounding their ethical and moral basis needs to be addressed before using them in them in clinical

practices. Concerns about the risk of genetic enhanced cells are also being addressed. The rational for genetic is that it has the potential to minimize the risk of tumorigenicity and immune rejection. This also potentially reduce the need number of stem cells needed for implantation. However, most methods of genetic delivery involves viral vectors and there are concerns of the risk of immune reactions. Further research into the gene expansion and delivery with safer vectors to minimize adverse reactions.

Finally, as the advancement in the use of stem cell therapy in different clinical settings continues to expand. The demand to increase the harvest and culture of stem cells for future purposes are on the rise and thus, there will be a need for research into large-scale culture, storage and distribution of stem cells.

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## Abstract

**Objective:** The aim of this literature review is to examine the efficacy of mesenchymal stem cells in the treatment of non-union fracture. To date, a wide variety of modalities have been explored to achieve unions and stability of fractured bones. Researchers and orthopedic surgeons aim to explore the versatility of mesenchymal stem cells and to understand the importance of their role in bone healing. **Methods:** A number of literature and published articles were gathered from PubMed, Elsevier, Clinical key, and Google Scholar. Search terms included, "mesenchymal stem cells", "pluripotent", "embryonic stem cell", "non-union", "fracture healing", "multipotent", "osteogenesis", "bone marrow" "long bone fracture". **Results:** Almost all the studies used in this literature review observed improvement in bone healing time with the use of stem cells. There was no standardized technique employed in the delivery of stem cells into the host. The technique and mode of delivery heavily depended on the orthopedic surgeons and their level of familiarity with the method used. **Conclusion:** All the articles agree that there are measurable benefits in the use of stem cells to improve bone healing, however, there are inconsistency in the methods of observations. Majority of the studies were also performed on a small scale and as such makes it less to be statistically significant. There is wide scale agreement that further research is needed in the department to orthopedic surgery to consistently generate results on a larger scale with a high degree of accuracy and precision.