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# Bone Marrow Mesenchymal Stem Cells for Future Orthopaedics

[1]Baby Joseph, [1]Biby T. Edwin, [2]Boby T. Edwin, [1]S. Justin Raj and [1]P. Sankarganesh
[1]Interdisciplinary Research Centre, Malankara Catholic College, Mariagiri, Kaliyakkavilai-629153, Tamilnadu, India
[2]Department of Botany, T.K.M. College of Arts and Science, Kollam-691005, Kerala, India

*Corresponding Author: Biby T. Edwin and Baby Joseph, Interdisciplinary Research Centre, Malankara Catholic College, Mariagiri, Kaliyakkavilai-629153, Tamilnadu, India*

## ABSTRACT

Bone Marrow Mesenchymal Stem Cells (BMMSCs) are a group of non-hematopoietic cells residing within the bone marrow. These are adhesive cells which can differentiate into osteogenic, adipogenic and chondrogenic lineages under appropriate conditions, results in maintaining the homeostasis mechanism. Though extracellular matrix is meant only for MSCs cell adhesion, it has some role in the lineage change. Apart from the extracellular matrix, growth factors play a pivotal role in the morphogenesis. In this review, we discuss the characteristics of BMMSCs, their differentiation potential toward different skeletal tissues (cartilage and bone) and bone tissue engineering and its need.

**Key words:** Bone marrow mesenchymal stem cells, bone, bone tissue engineering, extra cellular matrix

## INTRODUCTION

The mechanical performance of bone is of paramount importance for the quality of life we experience. The structural integrity of bone, its hierarchical structure, organization and its physicochemical constitution, all influence its ability to withstand loads, such as those seen occasionally in everyday life loading scenarios, which are either above the norm, prolonged, or repetitive (Gupta and Zioupos, 2008). The bone tissue is a mineralised connective tissue. Bone-forming cells called osteoblasts deposit a matrix of collagen, but they also release calcium, magnesium and phosphate ions, which chemically combine and harden within the matrix into the mineral hydroxyapatite (Martini, 2004).

## BONE AND ITS EXTRACELLULAR MATRIX

Bone is a complex, highly organised and specialized connective tissue. It has relatively high compressive strength but poor tensile strength. While bone is essentially brittle, it does have a degree of significant elasticity contributed by its organic components (chiefly collagen). Bone can be either compact or cancellous (spongy). The basic functional unit of mature compact bone is the osteon or Haversian system. In an osteon, the osteocytes are arranged in concentric layers around a central canal, or Haversian canal. This canal contains one or more blood vessels that carry blood to and from the osteon. Central canals generally run parallel to the surface of the bone. The lamellae of each osteon form a series of nested cylinders around the central canal (Martini, 2004).

In cancellous or spongy bone, lamellae are not arranged in osteons. Cancellous bone is trabecular (has an open, meshwork or honeycomb-like structure). It has a relatively high surface area, but forms a smaller portion of the skeleton (Maribe, 2004).

Bone provides internal support and confers marked rigidity, strength and elasticity through the secretion of a well-organized mineralized Extracellular Matrix (ECM) (Ge *et al.*, 2008), which is approximately two third inorganic. This inorganic mineral phase consists primarily of calcium and phosphate ions, with traces of magnesium, carbonate, hydroxyl, chloride, fluoride and citrate ions (Ratner *et al.*, 2004). The majority of bone consists of extracellular matrix proteins and the mineral hydroxyapatite (HA) (Xu *et al.*, 2009). Natural ECM is a condensed matrix mainly composed of locally secreted proteins and polysaccharides, arranged as a molecular network formed by an intricate agglomerate of weaves, struts and gels interconnecting cells with matrix proteins. Amounts and organizations of these molecules are variable with tissue site and type and during tissue development; in terms of resistance to tensile and compressive forces and transport properties. In physiological conditions, ECM composition derives from homeostasis, a fine dynamic balance of regeneration, differentiation and programmed cellular death (apoptosis), which continuously remodels ECM through protein breakdown and synthesis (Meredith *et al.*, 1993). Cells synthesize assembly and degrade ECM components responding to specific signals and, on the other hand, ECM controls and guides specific cell functions. This continuous cross-talk between cells and ECM is essential for tissue and organ development and repair. Components of ECM are Collagens, Proteoglycans, Hyaluronic acid, Laminins, Fibronectin (Ungaro *et al.*, 2006). Dynamic properties of ECM are controlled by proteoglycans and a number of signaling molecules, such as Growth Factors (GFs), which mediate cell-ECM and cell-cell interactions. Cell adhesion to the ECM is mediated by integrins, a class of heterodimeric cell surface receptors that form a direct physical linkage between the ECM and the cytoskeleton and also activate biochemical signaling networks by nucleating signaling proteins on the cytoplasmic side of the plasma membrane (Hynes, 2002).

**Mesenchymal stem cells:** MSCs are a rare, heterogeneous conglomerate of cells, which can be isolated from most connective tissues in the body and typically reside in perivascular areas (Caplan, 2008) and can proliferate *in vitro* as plastic adherent cells (Iyer *et al.*, 2010). MSCs were initially isolated from bone marrow but are now shown to reside in almost every type of connective tissue (Da Silva Meirelles *et al.*, 2006). They have the capacity of self renewal (Caplan, 2005) and it gives rise to multiple mesodermal tissue types (Bruder *et al.*, 1997). During embryologic development, precursor cells of the mesodermal layer give rise to multiple mesenchymal tissue types including bone, cartilage, tendon, muscle, fat and marrow stroma (Blair *et al.*, 2002). These are endowed with potent immune regulatory and neuroprotective properties, have recently emerged as promising cellular vehicles for the treatment of multiple sclerosis. Preclinical evaluation in experimental models of multiple sclerosis have shown that MSCs are efficacious in suppressing clinical disease. Mechanisms that may underlie these effects predominantly involve the secretion of immunomodulatory and neurotrophic growth factors, which collectively act to limit CNS inflammation, stimulate neurogenesis, protect axons and promote remyelination (Christopher *et al.*, 2010). Qualitative and quantitative analysis of mesenchymal stem cells from fat of rabbits were analysed by Mazzetti *et al.* (2010) and they proved that lipectomy of adipose panicule is a very satisfactory method to extract stem cells from fat. MSCs are distinguished from hematopoietic cells by being negative for the cell surface markers CD11b, CD14, CD34, CD45 and Human Leukocyte

Antigen (HLA)-DR but expressing CD73, CD90 and CD105 (Ghannam *et al*., 2010). Moallem and Jahangiri (2007) proved that mature mesothelial cells could transform into fibroblast-like cells *in vivo* and invade the underlying subserosal connective tissue, quite unusual for mature epithelial cells.

**Bone marrow mesenchymal stem cells:** Bone marrow is a complex tissue composed of hematopoietic and Mesenchymal Stem Cells (MSCs). MSCs are present in many human tissues and can be directly derived from marrow (Guo *et al*., 2009). The presence of non-haematopoietic stem cells in bone marrow was first suggested by the observations of the German pathologist Clonheim 130 years ago. His work raised the possibility that bone marrow may be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair (Prockop, 1997). MSC are thought to have at least five primary roles *in vivo*: as progenitor cells for bone formation during bone remodelling or repair (Blair *et al*., 2002), cartilage formation (Pittenger *et al*., 1999), vascular support (Hegner *et al*., 2005), haematopoietic support (Jang *et al*., 2006) and as progenitors for adipocytes (Pittenger *et al*., 1999). These precursor cells, also present in the postnatal organism, are referred to as mesenchymal stem cells (Caplan, 1991). These cells have also been shown to retain their developmental potential following extensive subcultivation *in vitro* (Bruder *et al*., 1997), thus supporting their characterization as stem cells.

**Characteristics of bone marrow mesenchymal stem cells:** Recently, techniques for the isolation and extensive subcultivation of human marrow-derived MSCs have been developed, along with a series of monoclonal antibody probes which react with the surface of these cells both *in vitro* and *in situ* (Haynesworth *et al*., 1992). MSCs are easily isolated from a small aspirate of bone marrow and expanded with high efficiency (Siniscalco *et al*., 2008). Mesenchymal stem cells can be identified by their capacity to form adhesive cell colonies *in vitro*. This feature described some 40 years ago (Friedenstein *et al*., 1968) is still the gold standard for the isolation of human MSCs from bone marrow (Alhadlaq and Mao, 2004). The most desirable source to obtain autologous mesenchymal stem cells is bone marrow which avoids the need for an open surgery, possible donor-site complications of pain, infection and damage to nerves and blood vessels (Nair *et al*., 2009).

**Effect of age on mesenchymal stem cells:** Though MSCs have considerable regenerative capacity, it also undergo aging process and senescence. In *in vitro* expansion, senescence is associated with telomere shortening. The rate of telomere shortening was 100 bp in every two passage (Bonab *et al*., 2006). These deficiencies may contribute to the aging process and age-related diseases including osteoarthritis and osteoporosis. It is characterized by excessive loss of bone and deterioration of bone tissue due to an overall imbalance between osteoblast-mediated bone formation and osteoclast mediated bone resorption. However MSC deficiencies, either in terms of number or cellular function, seem to be involved in musculoskeletal diseases (Bonyadi *et al*., 2003) and also a number of diseases remote from the musculoskeletal environment including atherosclerosis (Hegner *et al*., 2005) and diabetes (Kume *et al*., 2005). The number of multipotent MSCs of bone marrow cells rapidly decreases with donor age and thereby causes only limited tissue repair in adults (Nakashima and Reddi, 2003; Jadlowiec *et al*., 2003). So, it is important to investigate markers of cellular aging that might underly the above-mentioned changes in MSC number and differentiation potential. These include p53 and p21, Reactive Oxygen Species (ROS), Nitric Oxide (NO), Advanced Glycation End (AGE) products and Receptor for AGEs (RAGE) and

lipofuscin all of which have been found in elevated levels in aged cells and tissues and are associated with age-related degeneration (Medrano and Scrable, 2005; Rosso *et al.*, 2006). Therefore, considering the clinical application for injury/trauma, disease and congenital defects (Nakashima and Reddi, 2003; Alsberg *et al.*, 2001) to hard tissue and soft tissues, it is necessary to efficiently proliferate the MSCs without damaging their differentiation potentials (John *et al.*, 2002).

## MESENCHYMAL STEM CELLS DIFFERENTIATION

One of the characteristic features of MSCs are their multidifferentiation potential under culture conditions, comprising lineage specific regulators. Here, we will discuss the chondrogenic and osteogenic differentiation of MSCs. Chondrogenic differentiation of MSCs *in vitro* is often achieved by culturing them in a three dimensional (3D) condition in the presence of TGF-β superfamily growth factors (Varghese *et al.*, 2008). Differentiation of MSC into cartilage is characterized by upregulation of cartilage specific genes, collagen type II, IX, aggrecan and biosynthesis of collagen and proteoglycans. This is a multistep process that involves mesenchymal cell recruitment, migration, proliferation and *in vivo* condensation (Goldring *et al.*, 2006). When TGF-β1, IGF-1 and insulin were added to the culture medium, the human mesenchymal stem cells rapidly lost their fibroblastic morphology (Ikeda *et al.*, 2007). This process is controlled by cellular interactions with the surrounding matrix, growth and differentiation factors and other environmental factors that initiate or suppress cellular signaling pathways and transcription of specific genes in a temporal-spatial manner. Additionally, a number of morphogenetic factors including hedgehog proteins (Hhgs), Wnt proteins, Notch ligands, members of TGF-β superfamily of growth factors, IGFs and FGFs have been implicated to play important roles in controlling cartilaginous tissue development (Pogue and Lyons, 2006). Morphogenetic signals from chondrocytes also play an important role in regulating skeletal differentiation of MSCs (Hwang *et al.*, 2007; Grassel and Ahmed, 2007).

MSC differentiation for osteogenesis is controlled by a complex network of hormones and transcription factors, of which Cbfa1 (Ducy *et al.*, 1997) and osterix (Nakashima *et al.*, 2002) are pivotal. By binding to gene promoters Cbfa1 increase expression of bone-specific genes such as osterix, collagen type IA, osteocalcin and bone sialoprotein II (Kern *et al.*, 2001). Alkaline phosphatase (ALP), collagen type IA and bone sialoprotein II, transforming growth factor b1, osteonectin and bone morphogenetic protein-2 are expressed early to intermediate in the differentiation process (Young, 2003) and osteocalcin and osteopontin are expressed later (Zhu *et al.*, 2001). Basic fibroblast growth factor (bFGF) stimulates osteoblast proliferation *in vitro*, alkaline phosphatase (APase) activity, collagen synthesis and osteocalcin synthesis is inhibited (Hurley *et al.*, 1993). Inhibition of $\alpha_5$ or $\beta_1$ integrin subunits using isotype-specific antibodies has been shown to reduce mineralization by ~20 and 45%, respectively, while antibodies to $\alpha_v\beta_3$ and $\alpha_2\beta_1$ reduced mineralization by 65 and 95%, respectively (Schneider *et al.*, 2001). These results suggest that preferential adhesion and signaling via $\alpha_v\beta_3$ and $\alpha_2\beta_1$ may be required for osteoblastic differentiation. However, one contradictory report revealed that overexpression of $\alpha_v\beta_3$ decreases matrix mineralization, reduces alkaline phosphatase (ALP) activity and reduces expression of osteocalcin, type-I collagen and bone sialoprotein (Cheng *et al.*, 2001).

**Osteoblast:** Osteoblasts are sophisticated fibroblast, mononucleate cells that are responsible for bone formation. Osteoblasts (OB) produce osteoid, which is composed mainly of Type I collagen. Osteoblasts are also responsible for mineralization of the osteoid matrix. Dynamic shape of bone is

due to the activity of osteoblast cells. Cells of the osteoblastic lineage are derived from precursors originating in the mesodermal layer of the trilaminar embryo and are referred to as Mesenchymal Stem Cells (MSCs). Possible osteoblast precursor cells may be the Bone Marrow Stromal Cells (BMSC) found in the marrow space adjacent to the trabeculae. The osteogenic potential of these cells has been well established both *in vivo* and *in vitro* (Friedenstein, 1990) and the osteoblastic differentiation of BMSC has been (Rickard *et al.*, 1995). As increased bone formation *in vivo* is characterized by an increase in the number of mature OB and not by an increase in bone apposition rate (Marie, 1995), these *in vitro* results would tend to suggest that mature OB are not the major target cell for bone anabolic substances and that perhaps OB precursor cells may be better candidates. The osteoblasts can be identified by immunochemical detections of osteocalcin and osteopontin (Nakamura *et al.*, 2009).

**Osteoinductive growth factors:** During bone formation, multiple growth factors are expressed, such as Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factor (FGF), insulin-like growth factor-1 (IGF- 1), platelet-derived growth factor-BB (PDGF-BB), tissue growth factor β-1 (TGFβ-1) and Vascular Endothelial Growth Factors (VEGF), each of which plays different roles that may overlap (Wildemann *et al.*, 2007). Key transcriptional regulators of bone formation are Runx2 (runt-related transcription factor 2) and Osterix that orchestrate the differentiation of MSCs into functional bone forming cells known as osteoblasts (OB) (Zaidi, 2007). Bone Morphogenic Proteins (BMP1-12) are low-molecular-weight noncollagenous glycoproteins that belong to an expanding TGF-β super family of growth and differentiation factors (Urist *et al.*, 1984) .The BMPs have a myriad of functions ranging from embryonic organogenesis to bone regeneration (Wozney, 1992). The BMP family includes a large number of factors implicated in osteoinduction and consists of three subclasses: (1) BMP-2, BMP-4, etc., (2) BMP- 5, BMP-6, BMP-7 (OP-1), BMP-8 (OP-2) and (3) BMP- 3, which is the least related subclass (Wozney, 2002). In the process of bone repair and regeneration, cellular proliferation and differentiation are tightly regulated by an ever-changing ECM and growth factor synthesis. Fracture healing is viewed by Gerstenfeld *et al.* (2003) as an example of specialized postnatal bone regeneration. Although, the regeneration process is not exactly homologous with that which takes place during embryogenesis, fracture healing recapitulates a number of crucial processes that control bone generation during embryonic skeletal development. During fracture repair, there are three key groups of soluble factors: pro-inflammatory cytokines, the TGF-β superfamily and angiogenic factors are involved. TGF-β and other growth factors are released by platelets found in bone matrix and secreted by osteoprogenitor cells. These proteins are thought to stimulate cellular proliferation as well as differentiation of osteoblasts, resulting in direct bone matrix formation. Pro-inflammatory cytokines which initiate the repair process are considered to arise from marrow or bone matrix within the initial injury site and includes the Macrophage Colony Stimulating Factor (M-CSF), receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), tumor necrosis factor-alpha/beta (TNF-α/β), etc. The TGF-β superfamily involves BMPs (1-8), Growth differentiation factors (1, 5, 8 and 10), TGFb1-3, etc., which facilitate intramembranous and endochondral bone formation. BMPs are related through amino acid homology to the supergene family of transforming growth factor-betas (TGF-B) (Centrella *et al.*, 1991). While, TGF-B itself appears to have some synergistic effects with BMPs, TGF-B1 is unable to initiate the entire osteoinduction cascade by itself and form ectopic bone, a property uniquely exhibited by the BMPs (Ludwig *et al.*, 2000). The angiogenic group consists of VEGFa-d, angiopoietin-1 (Ang1), pleiotrophin (PTN), which induce formation of new

blood vessels that satisfy the demand for increased blood flow during fracture repair (Gerstenfeld *et al*., 2003). Many other growth factors are likely involved in the bone graft healing process. Fibroblast Growth Factor (FGF) acts as an angiogenic factor promoting graft bed neovascularization (Scutt and Bertram, 1999). Platelet Derived Growth Factor (PDGF) can act as a local tissue growth regulator in addition to Insulin Growth Factors (IGF). Platelet-Rich Plasma (PRP) has been in use in much clinical and experimental bone reconstructive surgery (Kitoh *et al*., 2004). The activated platelets are a source of growth factors, such as platelet derived growth factor, transforming growth factor beta, fibroblast growth factor, vascular endothelial growth factor and insulin-like growth factor (Harrison and Cramer, 1993). Currently, single BMPs are available through recombinant gene technology and mixtures of BMP extracted from human or bovine bone are available for basic science research and clinical trials (Hahn *et al*., 1992).

## BONE TISSUE ENGINEERING AND ITS NEED

Stem cell research leading to prospective therapies in reparative medicine has the potential to affect the lives of millions of people around the world and there is a good reason to be optimistic (Mirzapour *et al*., 2011). In large bone defects, healing process is non-spontaneous. But it is important to restore the structural integrity and its function within the shortest possible period. A common approach is to isolate specific cells through a small biopsy from a patient and then permit them to grow on a scaffold under controlled conditions. This limits the need for the migration and differentiation of indigenous cells within defect sites and in turn accelerates tissue regeneration (Temenoff *et al*., 2000). But this method is time consuming. The shortcomings of clinical strategies like autografts and allografts, both of which have inherent limitations, such as limited supply, increased morbidity and disease transmission potential (Martina *et al*., 2005), have inspired the development of tissue engineering (Zhao *et al*., 2002). In the past decade, the science of Tissue Engineering (TE) has been gaining attention as an excellent option for bone graft systems by applying the principles of engineering and life sciences for the development of biological substitutes that restore, maintain, or improve tissue functions (Langer and Vacanti, 1993). Since, these cells would be of autogenous origin, there would be no risk of immune rejection and pathogen transfer (Ishaug *et al*., 1997). Genetic disorder, osteogenesis imperfecta characterized by defective type I collagen is the first disease for which stem cell-based therapy was proposed (Saeed and Mesaik, 2005). In the same way bone tissue engineering involves mimicking and creating a complex biomechanical environment for cell-cell and cell-matrix interactions. Such an environment should promote maturation of the cell-scaffold construct *in vitro*. One of the most important aspects of bone tissue engineering is the introduction of bioactive bone cells into the three-dimensionally porous scaffold (Livingston *et al*., 2002). The three-dimensional scaffold used can support the cell growth, proliferation and finally lead to the implantation to the contextual site (Joseph *et al*., 2010) and should enable the modulation of key mechano transduction pathways in a dynamic loading environment *in vivo* within the patient (Yang and El-Haj, 2005). The optimization of such a process relies on the interplay between two interdependent elements other than the bone substitute: (1) progenitor cells for osteogenesis and (2) growth factors for osteoinduction (Bruder *et al*., 1998).

Animal and clinical studies have shown that MSCs have the ability to differentiate to several cell lineages (adipocytes, chondrocytes and osteoblasts) and promote bone-specific protein synthesis and mineralization in an osteoconductive environment (Zangi *et al*., 2006) and also capable of repairing damaged skeletal tissue or large bone segmental defects (Derubeis and Cancedda, 2004). Moreover, MSCs do not appear to be rejected by the immune system, allowing for large-scale

production and appropriate characterization and the subsequent ready availability of allogeneic tissue repair enhancing cellular therapeutics (Martin and Pittenger, 2006). As MSCs present more advantages than other cells, they have already been widely used in bone tissue engineering (Gomillion and Burg, 2006). However, there are few MSCs present in human bone marrow. It is estimated that there is only one MSCs out of $10^4$-$10^5$ mononuclear cells (Pittenger *et al.*, 1999). Therefore, separation, culture and expansion of adult human bone marrow-derived mesenchymal stem cells *in vitro* are prerequisite for clinical use and tissue engineering.

## CONCLUSION

Unlike any other organ system of the body, bone has the unique ability to regenerate and repair itself without scar following injury. But in several indications like nonhealing fractures, spine fusion, or for filling large bone defects, bone grafts and bone substitutes are required. With recent advances in the knowledge regarding biomaterials and the availability of osteoinductive proteins, we have entered into a new era of biologic manipulation of bone formation. The factors focused on this review are likely to play critical roles in cell-scaffold interaction, cell-cell interaction and osteogenesis. The better understanding of the fascinating property of MSC and the formulation of a new scaffold having all properties of extracellular matrix might realize non-problematic bone tissue engineering.

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