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Mesenchymal Stem Cells, Osteogenic Lineage and Bone Tissue Engineering: A Review

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Abstract: Mesenchymal Stem Cells (MSC) are multipotent non-hematopoietic progenitor cells with an extensive proliferation potential and ability to differentiate into various cell types including osteoblasts, chondroblasts, myocytes, adipocytes and neurocytes. MSC is a cell of bone marrow population that plays a key role in bone marrow homeostasis and regulates the maturation of both hematopoietic and non-hematopoietic cells. Mesenchymal stem cells have been emerged in promising therapeutic modality for tissue regeneration and repair. Treatment of graft versus host disease, heart regeneration following infarction, cartilage and bone repair and treatment of osteogenesis imperfecta are only few therapeutic application of MSC. Tissue engineering in recent years opened a new frontier to replace the affected tissue by MSC assistance. Mesenchymal stem cell seems to be the future powerful tool in therapeutic application of regenerative medicine in repairing and replacing the impaired organs. The main aim of current review is to have a more precise look to MSC, its proliferation and differentiation potentials, therapeutic application and bone tissue engineering.

Key words: Mesenchymal stem cell, regenerative medicine, bone tissue engineering, osteoblasts, scaffold, Malaysia

INTRODUCTION

Stem cell: Stem cells are being defined as the undifferentiated cells with the capacity of both differentiating into diverse range of specialize cell in the body with respect to their potency and maintaining self-renewal property. Potency of stem cell is the competency of the stem cell to differentiate into the specialized type of tissue cells of the body. Respected to the definition, there are five class of potency defined for the stem cell which are totipotent, pluripotent, multipotent, oligopotent and unipotent.

Totipotent stem cells are the cells that have the capacity of differentiating into embryonic and extraembryonic cell types. Such cells can fabricate a complete, viable and organism (Hans, 2007). These stem cells are produced from the fusion of an ovum and sperm cell. Cells generated by the 1st few divisions of the fertilized egg are also totipotent (Hans, 2007). Pluripotent stem cells are the progenies of totipotent cells and can differentiate into almost all cells in the body such as cells of any of the three germ layers. Multipotent stem cells can differentiate into a number of cells but from a germ layer. Oligopotent stem cells can differentiate into only a few cells and unipotent cells can differentiate only one cell type, their own but have the property of self-renewal which distinguishes them from non-stem cells (Mitalipov and Wolf, 2009; Hans, 2007). There are two different class of stem cell including embryonic and adult stem cell. Embryonic stem cells are the cells that are derived from the inner cell mass of an early stage of the embryo known as a blastocyst. Human embryos reach the blastocyst stage 4-5 days post fertilization at which time they consist of 50-150 cells. Embryonic Stem (ES) cells are pluripotent stem cells. It means they are capable of differentiating into all of the three primary germ layers; ectoderm, endoderm and mesoderm (Mitalipov and Wolf, 2009). Adult stem cells are undifferentiated cells found throughout the body after embryonic development. They can be isolated from adult body. There are different adult stem cells through the body such as hematopoietic stem, epithelial stem, neural stem, muscle stem and mesenchymal stem cell (Ulloa-Montoya *et al*., 2005). Mesenchymal Stem Cells (MSC) are multipotent adult stem cell with ability of differentiating into various types of tissue. Bone marrow is the most abundant site of harvesting MSC other than adipose tissue blood stream and omentum.

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BONE MARROW MICROENVIRONMENT

Sited within large bones are hollow compartments that include the bone marrow microenvironment. The non-cellular constituent of this environment consist of growth regulatory molecules such as growth factors and cytokines and a supporting matrix composed of collagen fibers and extracellular matrix glycoproteins that encourage cell to cell and cell to extracellular matrix interactions (Alberts *et al*., 2002; Majumdar *et al*., 1998; Short *et al*., 2003; Simmons and Torok-Storb, 1991). Blood vessels richly supply the bone marrow called blood sinuses in to which newly formed blood cells are discharged (Alberts *et al*., 2002). Stromal tissue is consists of a heterogeneous cells include reticular cells, adipocytes, osteogenic cells, smooth muscle cells in vessel walls, macrophages and vascular endothelial cells (Dexter *et al*., 1997; Charbord *et al*., 1985; Strobel *et al*., 1986; Nuttelman, 2005). In addition, two types of stem cells are found in the stromal environment, Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs) (Majumdar *et al*., 1998; Short *et al*., 2003). Hematopoietic stem cells present in the bone marrow can undergo both self-renewal to keep their high population and differentiation and maturation to generate the distinct lineages that involve the hematopoietic system (i.e., T lymphocytes, B lymphocytes, dendritic cells, monocytes, neutrophils, eosinophils, basophils, megakaryocytes and erythrocytes) (Majumdar *et al*., 1998; Albert *et al*., 2002). Stromal tissue plays a significant role in the proliferation, differentiation and maturation of hematopoietic stem cells into different lineages of the hematopoietic system (Majumdar *et al*., 1998; Short *et al*., 2003). Mesenchymal stem cells are the other stem cells in the bone marrow and maintain a level of self-renewal. MSCs are able to give rise to the cells that can differentiate into the cells of various types of connective tissue such as bone, muscle, tendon, fat and cartilage (Nuttelman, 2005).

MESENCHYMAL STEM CELL

Found in bone marrow two completely different types of adult stem cells, hematopoietic stem cell and mesenchymal stem cell. Mesenchymal stem cells have various advantages for applications in regenerative medicine such as ease of aspiration and isolation (Ballas *et al*., 2002), highly proliferative capacity (Bruder *et al*., 1997a; Haynesworth *et al*., 1992a; Banfi *et al*., 2002) and ability to differentiate into many of cell types (bone, mussle, lipid) (Kaveh *et al*., 2008) with capability of regenerate different tissues. In human, a bone marrow aspiration yields 50 mL of whole bone marrow. Of the total of 1-2×10^6 cells mL^{-1} aspirated bone marrow, roughly one cell in every 1×10^4 cells is MSC; hence, it is not so hard to harvest between 5,000 and 10,000 stem cells from a single time aspiration (Nuttelman, 2005). In addition, these MSCs can undergo a 6-8 fold expansion in cell number once a week so after two passages, they undergo over a 200-fold increase in cell number (Nuttelman, 2005).

Fig. 1: Shows the mesenchymal stem cells 2 days after bone marrow aspiration at *in vitro* culture medium following hematopoietic stem cell washing

Under the suitable environment, the MSCs are competent of differentiating into the cells of bone, cartilage, tendon, muscle, fat and others (Caplan and Bruder, 1997; Caplan, 1991; Haynesworth *et al*., 1992b; Pittenger *et al*., 1999). MSCs can also be differentiated into non-mesenchymal cells such as neuronal cells (Kopen *et al*., 1999; Deng *et al*., 2001; Sanchez-Ramos *et al*., 2000; Sanchez-Ramos, 2002). MSCs are thought to put down their tissue niche of the bone marrow and circulate through the bloodstream, assisting in repair of tissues while required (Nuttelman, 2005). Besides, MSCs have been demonstrated to travel from bone marrow to skeletal muscle to help in the repairing of muscles (Ferrari *et al*., 1998). Moreover, MSCs have been shown to enhance regeneration (neovascularigenesis) and repair of blood vessels in infracted myocardium (Fuchs *et al*., 2001; Jackson *et al*., 2001; Kobayashi *et al*., 2000) and as well MSCs were detected to differentiate into cardiomyocytes, endothelial cells, pericytes and smooth muscle cells after direct injection into the adult heart (Gojo *et al*., 2003). Figure 1 shows the mesenchymal stem cell *in vitro*.

DISCOVERY OF MESENCHYMAL STEM CELL

Friedenstein *et al*. (1966) discovered the bone-forming progenitor cells in rat bone marrow. Pioneering studies in the field demonstrated *in vitro* growth of adherent colonies of cells morphologically similar to

fibroblasts derived from explants of bone marrow (Friedenstein *et al*., 1970; Castro-Malaspina *et al*., 1980; Owen, 1988). When bone marrow is plated onto tissue culture plastic dish or flask, a small fraction of these cells are adherent and when cultured for several weeks, they will give rise to small, clonogenic colonies derived from individual cells (Nuttelman, 2005). These initial adherent cells as Colony Forming Unit-Fibroblasts (CFU-F) (Friedenstein *et al*., 1970). CFU-Fs are defined as the rapidly adherent, non-phagocytic clonogenic cells equipped to extended proliferation *in vitro* and each CFU-F can give rise to a large number of colonial cells (Short *et al*., 2003).

Moreover, there is non-uniformity in colony growth of these CFU-Fs; some cells quickly give rise to colonies soon after adherence to tissue culture plastic whereas other cells do not yield colonies until after several days of culture (Bruder *et al*., 1997b).

From discovery of the CFU-F, this cell has been renamed to the variety of terms. Current and most usually used nomenclature includes the Marrow Stromal Fibroblast (MSF), Marrow Stromal Cell (MSC), Mesenchymal Progenitor Cell (MPC), Bone Marrow Stromal Stem Cell (BMSSC) and Mesenchymal Stem Cell (MSC). The various nomenclature used generally refers to the same cell which is the original CFU-F obtained when fresh bone marrow is plated onto tissue culture plastic (Minguell *et al*., 2001). Currently, the most commonly referred to name for this cell is the Mesenchymal Stem Cell (MSC).

Studies indicate that in cell cycle of CFU-Fs, a small fraction is actively occupied in proliferation (10% of all cells are in the S, G2 and M phases of the cell cycle). However, the great majority of cells are waiting at the G0/G1 phase of the cell cycle (Conget and Minguell, 1999) this high proportion of cells at the G0/G1 boundary suggests a high competence for them to differentiate into various type of tissues (Tamir *et al*., 2000).

ISOLATION OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells can be isolated likewise to the original isolation of the CFU-F in the pioneering studies conducted by Friedenstein *et al*. (1970) by plating whole fresh bone marrow onto tissue culture plastic. Whole bone marrow is to be aspirated from the iliac crest or other trabecullar bone. Marrow has also been isolated from the tibial and femoral marrow cavity (Murphy *et al*., 2002; Oreffo *et al*., 1998) also from the thoracic and lumbar spine (D'Ippolito1 *et al*., 1999).

Isolation of MSCs relies on the adhesion of the stromal progenitor cell populations to tissue culture flask and their subsequent rapid proliferation

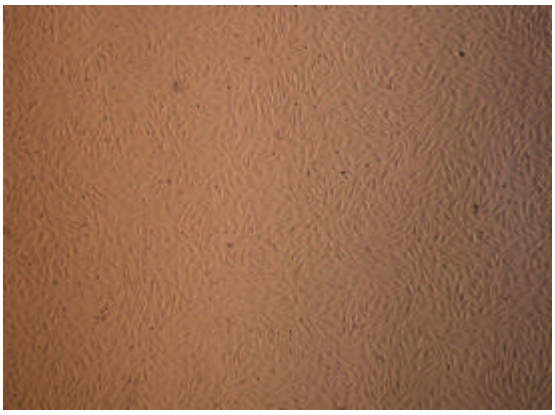

Fig. 2: Shows differentiated osteoblast from MSC 4 weeks post bone marrow culture and 4 days following differentiation

(Friedenstein *et al*., 1987; Kuznetsov *et al*., 1997). Once bone marrow obtained from the donor, cells are plated at the density ranging from 1×10^{4}-4×10^{5} cell cm^{-2} at the presence of suitable medium. In this condition, MSCs proliferate vary rapidly. These primary cultures are then maintained 2-3 days after which the non-adherent hematopoietic cell fraction is depleted. The plastic-adherent population of cells is referred to as MSCs (Minguell *et al*., 2001). The concentration of MSCs in whole human bone marrow is very small: estimates include 1 cell per $1\text{-}20\times10^{5}$ mononuclear cells (Gronthos and Simmons, 1996) or 1 in $1\text{-}10\times10^{5}$ nucleated cells (Pittenger *et al*., 1999) and most certainly varies from individual to individual. In addition, there is a negative correlation of CFU-F with age. Despite the low population of MSCs, their rapid expansion allows to get therapeutic numbers of cells from autologous whole bone marrow (Nuttelman, 2005). Figure 2 shows osteoblasts in differentiation culture medium.

CHARACTERIZAION, PURIFICATION AND DIFFERENTIATION OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells are in general characterized by their ability to proliferate in culture with an attached, well-spread morphology, the presence of a reliable set of marker proteins on their surfaces and their consistent differentiation ability to multiple lineages cells under controlled *in vitro* conditions (Pittenger *et al*., 1999). Previously, it was believed that MSCs represent a homogeneous population (single multipotent stem cell) however, recent studies supported the hypothesis that the population of MSCs derived from the adherent division when whole bone marrow is plated onto tissue

culture plastic (flask or plate) is a mixture of combination of committed progenitor cells in which, each progenitor cell with varied, restricted potential to differentiate to tissues of mesenchymal origin, such as fat, muscle, tendon, fat, cartilage and bone. On the other word, the CFU-F fraction in bone marrow represents a mixture of multi-, bi and uni-potential progenitors at different stages of differentiation (Pittenger *et al*., 1999; Owen, 1988; Kuznetsov *et al*., 1997).

In one of the pioneering studies intended on typifying the population of plastic-adherent MSCs, Pittenger *et al*. (1999) tested expanded MSCs for a variety of markers. Significantly, they did not identify markers pinpointing of the hematopoietic lineage, such as CD14, CD35 and CD45. Furthermore, hematopoietic cells were never identified in expanded cultures since they can easily be washed out. These investigators also expanded MSCs from 50 different donors and all cultures contained cells that differentiated successfully to the cells of bone, cartilage and fat. Besides, the cells did not differentiate spontaneously during culture expansion. They also demonstrated that mature fibroblasts did not differentiate in the three lineage assays. Moreover, the researchers isolated 6 colonies that had been derived from individual single cells thus they represented true clonogenic colonies (Pittenger *et al*., 1999). In close agreement in another research, it was found that 30% of CFU-Fs exhibited a tri-lineage potential (osteogenic/adipogenic/ chondrogenic) and the rest were constrained to a bi-lineage potential (osteogenic/ chondrogenic) or purely osteogenic potential. Clones restricted to only the chondrogenic/adipogenic or osteogenic/adipogenic bi-lineages were not detected (Muraglia *et al*., 2000). Interestingly, a clone with properties of a quadripotential mesenchymal progenitor cell, termed BMC9 clone was isolated.

As well as the osteogenic/chondrogenic/ adipogenic differentiation potential this clone could be differentiated to a hematopoietic-supporting stroma phenotype (Muraglia *et al*., 2000). Considering these discoveries, there is a mixed potential for MSCs to differentiate to at least four various lineages which are osteogenic, chondrogenic, adipogenic and hematopoietic-supporting stroma linage (Nuttelman, 2005).

Pioneering investigations focused on characterizing surface markers of CFU-Fs. The subsequent monoclonal antibodies were found as surface markers which are to be reactive towards CFU-Fs; STRO-1, SB-10, SH-2, SH-3 and SH-4. The SB-10 antibody reacted with an antigen found on undifferentiated MSCs which disappeared as soon as the cells initiate to osteogenic differentiation and began to produce alkaline phosphatase on their cell surface (Bruder *et al*., 1997a, b; Nuttelman, 2005). The SB-10 antigen was later identified as CD166 (activated leukocyte-cell adhesion molecule, ALCAM) (Bruder *et al*., 1998; Nuttelman, 2005). The SH-2 antibody reacts with an epitope present on the TGF-β receptor endoglin that also known as CD105 (Barry *et al*., 1999). The SH-3 and SH-4 antibodies were found to identify distinct epitopes on the membrane-bound ecto-5'-nucleotidases which is also known as CD73 (Barry *et al*., 2001). Other researchers showed that MSCs were positive for these markers; SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a and CD124 (Pittenger *et al*., 1999). This is important point that all of these antibodies with the exception of STRO-1 are expressed on a variety of other cell and do not grant the specificity required to extend these studies to *in vivo* evaluation (Barry *et al*., 2001).

Hence, researches were then focused on characterizing the STRO-1 antibody which reacted with non-hematopoietic cells found in whole bone marrow (Simmons and Torok-Storb, 1991). STRO-1 received its name, since it reacts with bone marrow stromal elements *in vitro* and *in vivo*. In closer study, STRO-1 was found to react with uncharacterized cell surface marker expressed by a small and heterogeneous population of adult bone marrow mononuclear cells. It was found that STRO-1-positive (STRO-1+) cells are able to differentiate into multiple stromal cell types in cluding adipocytes, smooth muscle cells, fibroblastic elements, osteoblasts and chondrocytes (Dennis *et al*., 2002; Gronthos *et al*., 1994). Additionally, STRO-1+ cells can amplify the generation of clonogenic cells and mature hematopoietic cells, imitating the native bone marrow environment (Nuttelman, 2005). Included within the STRO-1+ fraction are essentially all detectable clonogenic CFU-Fs and it is found that every CFU-F reacts positively with the STRO-1 antibody; therefore, the STRO-1 antibody can be used to select for all CFU-Fs (Simmons *et al*., 1991; Nuttelman, 2005).

Since the CFU-F division contains multi-, bi- and uni-potential MSCs in vestigations were looking for better ways to detect the truly multipotent MSC. In addition, the STRO-1 antibody is not sufficient enough to obtain the purity of MSCs, since there are contaminating populations of glycophorin-A-positive nucleated red cells and a small subset of B-lymphocytes (Gronthos *et al*., 2003). Gronthos *et al*. (2003) developed a technique to enrich the CFU-F fraction for truly multipotent MSCs by utilizing the STRO-1 antibody in combination with an antibody directed to vascular cell adhesion molecule-1 (VCAM-1/CD106). Each single cell of this STRO-1+/VCAM-1+population showed osteogenic, adipogenic and chondrogenic differentiation potential (Gronthos *et al*., 2003) representing a unique method to

detect true multipotent stem cells. Consequently, bone marrow contains hematopoietic and nonhematopoietic cells in cluding reticular cells, adipocytes, osteogenic cells, smooth muscle cells in vessel walls, macrophages and vascular endothelial cells. Hematopoietic stem cells giving rise to the lineages of the hematopoietic system (T lymphocytes, B lymphocytes, dendritic cells, monocytes, neutrophils, eosinophils, basophils, megakaryocytes and erythrocytes) and the multipotent mesenchymal stem cells were found within the CFU-F fraction. It is now generally accepted that the CFU-F fraction is a small subset of all bone marrow cells (Nuttelman, 2005). As the CFU-F population contains multi, bi and uni-potential progenitor cells, only a fraction of these progenitor cells are true multipotent MSCs. These truly multipotent MSCs can be detected by using the STRO-1 antibody in combination with the VCAM-1/CD106 antibody, yielding a highly enriched population of MSCs with clonogenic potential (Gronthos *et al*., 2003).

INTRACELLULAR SIGNALING PATHWAYS IMPLICATEDINOSTEOGENICDIFFERENTIATION OF MSC

The Mitogen-Activated Protein (MAP) kinase pathway is known to control and regulate the proliferation and differentiation of MSCs (Jaiswal *et al*., 2000). Growth factors and hormones such as estrogen, parathyroid hormone and dexamethasone, stimulate and activate two members of the MAP kinase family: extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) (Migliaccio *et al*., 1996). The two other members of the MAP kinase family, c-Jun N-terminal Kinase (JNK) and p38-reactivating kinase, are also activated by cytokines, environmental stress and other stimuli (Cano and Mahadevan, 1995). Sustained activation of ERK2 has been associated with up-regulation of osteopontin expression, matrix deposition and initiation of mineralization by MSCs (Jaiswal *et al*., 2000). ERKs are activated by dual phosphorylation on tyrosine and threonine residues separated by a glutamate residue by the upstream kinase MEK (MAP kinase or ERK kinase) (Jaiswal *et al*., 2000). In addition inhibition of ERK using the MEK inhibitor PD98059 led to the reduction of ALP activity and calcium deposition in MSC cultures; introduction of a constitutively active form of MEK led to an increase in both ALP activity and calcium deposition by MSCs (Jaiswal *et al*., 2000). Bone Morphogenic Proteins (BMP) influence on MSCs through heteromeric type I and type II receptor complexes which will causes the activation of intracellular Smad proteins as well as MAP kinase (Lee *et al*., 2002, 2000). As soon as BMP binding to the type I/type II receptor, an intracellular signal is produced. The signal is transmitted into the nucleus to adjust the gene expression of smad proteins (Marrony *et al*., 2003). These heteromeric smad complexes subsequently affect regulation of specific gene transcription in MSCs by binding to the Cbfa1 transcription factor (Hanai *et al*., 1999). Figure 2 shows osteoblast *in vitro*.

OSTEOGENIC DIFFERENTIATION MEDIA

Mesenchymal stem cells can be induced to differentiate into osteoblasts using glucocorticoids (Barling *et al*., 1989; Kamalia *et al*., 1992; Leboy *et al*., 1991; Simmons *et al*., 1991; Maniatopoulos *et al*., 1988; Benayahu *et al*., 1989), osteogenin (Vukicevic *et al*., 1989), Bone Morphogenetic Proteins (BMPs) (Thies *et al*., 1992) and basic Fibroblast Growth Factor (bFGF). Typical osteogenic differentiation media contains dexamethasone, ascorbic acid or an ascorbic acid analog and β-glycerophosphate (β-GP). Each constituent is found to support in osteogenic differentiation of mesenchymal stem cells *in vitro* (Nuttelman, 2005).

Role of dexamethason: Dexamethasone is a synthetic corticosteroid. While it is not found naturally in the body, it imitates the actions of various glucocorticoids located naturally in the body such as cortisol, estradiol, testosterone, vitamin D3, thyroxine and retinoic acid (Alberts *et al*., 2002). Glucocorticoids influence the expression of many genes that are known as stress-related genes. Glucocorticoid-activated genes work through gene activator proteins. Activator proteins are bound to the regulatory regions of DNA but only by themselves, they are not sufficient to activate transcription of genes. In presence of glucocorticoids, they would bind to glucocorticoid receptors in the nucleus of the cell and the activated glucocorticoid receptors will then bind to the regulatory region of each gene regulated in this style. In the absence of a glucocorticoid, activator proteins are kept in the cytosol and can not bind DNA. By this fashion, glucocorticoids can affect a number of genes simultaneously; each different gene has a different activator protein that launches transcription as soon as glucocorticoid binds (Nuttelman, 2005). The effect of the glucocorticoid on cell function eventually depends on cell type, the gene regulatory proteins enclosed within the cell and the regulatory region of the gene (Alberts *et al*., 2002). Each cell has a different set of regulatory proteins able of gene activation in this way, thus every cell type undergoes a different response to glucocorticoids and

this response is at last reliant on the specific activator proteins contained within the cell. Dexamethasone supports osteogenic differentiation (Liu *et al*., 2002; Bellows *et al*., 1990; Atmani *et al*., 2002; Peter *et al*., 1998; Cheng *et al*., 1994) by binding to some special regulatory proteins in the cell and then activating transcription of osteoblast-specific genes. *In vitro*, constant treatment with dexamethasone would increases alkaline phosphatase activity which is required for matrix mineralization and transformation of the morphology of MSCs from spindle-shaped to cuboidal (Cheng *et al*., 1994). There are also evidences that dexamethasone functions at multiple points in the differentiation process to stimulate osteoblastic maturation (Porter *et al*., 2003). If dexamethasone is removed from *in vitro* culture, a major amount of cells may regress towards a more undifferentiated condition or differentiate toward alternative pathways such as the adipogenic pathway. Therefore, a constant dexamethasone presence in culture medium is required to achieve maximal osteoblastic differentiation of MSC cultures (Porter *et al*., 2003; Nuttelman, 2005) however, very long presence of dexamethasone in differentiation media would have toxic effect on osteoblasts and cause lysis hence, they have to be harvested and utilized soon after they are completely differentiated.

Many researches have manifested that while dexamethasone can induce terminal differentiation of MSCs into osteoblast in culture, the presence of dexamethasone is not an absolute and necessary requirement for *in vitro* osteogenesis in rodent MSCs however it can help the differentiation (Kamalia *et al*., 1992; Benayahu *et al*., 1989; Bellows *et al*., 1990; Falla *et al*., 1993). This is in contrast with human MSCs in which dexamethasone is found to be required for the differentiation of hMSCs *in vitro* (Cheng *et al*., 1994; Nuttelman, 2005).

Long-term intake of glucocorticoids would result in osteoporosis *in vivo* (Baylink, 1983; Sambrook *et al*., 1990; LoCascio *et al*., 1990) and decreas osteoblastic activity and finally can lead to Cushing's syndrome. In prolonged exposure to glucocorticoids, it can direct to obesity, changes in skin complexion and tone, hypertension, myopathy in sulin resistance and osteopenia (Miyachi, 2000). The contradictory effects that dexamethasone enhances osteogenic activity and differentiation of MSCs *in vitro* (i.e., stimulates bone formation) leads to *in vivo* osteoporosis and osteopenia (i.e., bone degeneration and loss) could be explained by the fact that while supporting the MSC differentiation into osteoblasts, dexamethasone inhibits MSCs proliferation (Jaiswal *et al*., 1997). Therefore, regimens of dexamethasone may limit and curtail the amount of osteoprogenitor sources, such as MSCs in the body and lead to diminished numbers and bone loss due to the reduction in the available number of bone-forming cells (Nuttelman, 2005).

Role of ascorbic acid: Ascorbic acid was shown to be essential for the survival of human osteoblasts *in vitro* (Koshihara *et al*., 1987). Ascorbic acid also assists in facilitation of osteoblast proliferation and maintaining the cells in an osteoblastic phenotype by increasing the total protein, collagen synthesis and alkaline phosphatase activity (Graves *et al*., 1991; Hitomi *et al*., 1992). In some researches, it was found that alkaline phosphatase expression by hMSCs cultured in presence of ascorbic acid was no different than the same cells cultured in the absence of ascorbic acid (Gronthos *et al*., 1994). Ascorbic acid could be substituted with the more stable analog such as ascorbic acid phosphate as the half-life of ascorbic acid in culture is only seven hours whereas ascorbic acid phosphate has a half-life of seven days (Gronthos *et al*., 1994). In general, there are controversies on utilizing ascorbic acid as an osteogenic inducing agent in the culture.

Role of β-glycerophosphate: Organic phosphates aids osteogenesis by starting mineralization in cell cultures and is thought to modulate osteoblastic activities by promoting a bone-like mineral phase (Tenenbaum *et al*., 1992; Chung *et al*., 1992). Robison and Soames (1924) 1st noticed that organic phosphate could be an origin of inorganic phosphate. Despite many studies so far, the exact mechanism by which β-glycerophosphate can induce mineralization is still unclear but it is believed its support is closely related to the ability of alkaline phosphatase to hydrolyze organic phosphate and release inorganic phosphate (Fortuna *et al*., 1980; Nuttelman, 2005). This free inorganic phosphate can provide the chemical potential for promoting mineral deposition on the surface of tissue culture plastic and other materials.

Mesenchymal stem cells are shown to undergo osteogenic differentiation when they are grown on mineralized surfaces (Darimont *et al*., 2002; Gilbert *et al*., 2003). This osteogenic differentiation might be owing to the presence of osteopontin which strongly adsorbs to the charged mineral phases created by presence of β-glycerophosphate and other organic phosphates. Adhesion of mesenchymal stem cells to bone or mineralized surfaces, mostly through the cell adhesion protein; osteopontin is one of the major turning points in osteogenic differentiation (Yabe *et al*., 1997). Therefore,

an exogenously-added organic phosphate group within the osteogenic differentiation media contributes indirectly to the osteogenic differentiation of MSCs (Nuttelman, 2005). Besides, free phosphates are competent of directly influencing the differentiation of MSCs to osteoblasts by inducing the mRNA and protein expression of osteogenic markers like osteopontin. Furthermore, free phosphates have numerous effects on the construction of the key osteogenesis regulatory transcription factor and core binding factor α1 (Cbfa1) (Beck Jr. *et al*., 2000; Fujita *et al*., 2001).

The function of exogenously-added free phosphate groups is not the only factors affecting mineralization but also cell density as well plays an important role in mineralization. Jaiswal *et al.* (1997) found that higher MSC seeding densities would lead to significantly more mineral deposition, demonstrating that a certain threshold cell density should to be reached prior to mineralization. Furthermore, cultures that were allowed to concentrate their soluble products in the media produce more mineralized matrix in dicating an autocrine or paracrine role of factors synthesized by MSCs which are undergoing osteogenic differentiation (Jaiswal *et al*., 1997). This is in close agreement with other studies that have found that differentiation of MSCs is triggered by increasing cell density (Caplan *et al*., 1983; Nuttelman, 2005).

CHARACTERISTICS OF OSTEOGENIC DIFFERENTIATION

There are three distinct phases of MSC differentiation and bone formation: Proliferation, Extracellular matrix maturation and Matrix mineralization (Lian and Stein, 1992). In the proliferative level, MSCs are extremely mitotic in this phase, they exhibit high expression of H4 histone and c-fos (Pockwinse *et al*., 1992). Also in this phase, peak levels of genes associated with extracellular matrix production are present such as type I collagen, fibronectin and transforming growth factor β (Aronow *et al*., 1990).

Mesenchymal stem cells are able of differentiating into osteoblasts under the suitable environmental circumstances or stimuli. There is no single, definite marker of cells of the osteogenic lineage (osteoblast); however, there are several individual, unique and distinguishing characteristics (Gronthos *et al*., 1994). During osteogenic differentiation of MSCs, several changes in gene expression take place. Most remarkable, alkaline phosphatase, osteopontin, osteocalcin, collagen type I and core binding factor α1 are up-regulated. Up-regulation of these genes in MSCs is indicative of osteogenic differentiation. As osteoprogenitor cells undergo the procedure of new bone formation, alkaline phosphatase activity is primarily low but would increases during differentiation and matrix maturation. Finally, alkaline phosphatase activity decreases and while osteoblats turn into osteocytes this activity would be absent. Alkaline phosphatase activity generally appears before osteocalcin, implying that alkaline phosphatase is an early marker of the osteoblast lineage while osteocalcin is thought to be pinpointing of a more mature osteoblastic phenotype which is present in the osteocyte (Owen *et al*., 1990; Aronow *et al*., 1990; Bronckers *et al*., 1987; Malaval *et al*., 1994). As the mater of fact in newly formed bone, there is little osteocalcin staining rather osteocalcin deposition is a late event in new bone formation (Groot *et al*., 1986; Vermeulen *et al*., 1989). The situation *in vitro* is similar to *in vivo* (Nuttelman, 2005) when proliferation decreases, the expression of alkaline phosphatase increases and osteocalcin expression occurs at the latter phases of osteogenic differentiation (Turksen and Aubin, 1991). In general, alkaline phosphatase activity and osteocalcin have been used as identifying markers for osteoblasts.

Another protein marker of osteoblasts and osteogenic differentiating mesenchymal stem cells is osteopontin. Osteopontin is expressed in developing bone cells during early periods of osteogenesis prior to mineralization or osteocalcin expression (Mark *et al*., 1987a, b). Hence, expression of osteopontin is an early indicator of osteogenic differentiation used to identify osteoprogenitor cells.

THERAPEUTIC APPLICATION OF MESENCHYMAL STEM CELL AND OSTEOBLAST

Attributed to the wide plasticity of mesenchymal stem cells and the fact that they can differentiate into the cells of many different mesenchymal and non-mesenchymal tissues, scientists have begun to look at their potential in the clinical and therapeutic setting. There is accumulating evidences of the hypoimmunogenic nature of MSCs and there is little evidence of host immune rejection or Graft-Vs.-Host Disease (GVHD) even when there is a mismatch of allogeneic donor cells (Nuttelman, 2005).

The 1st clinical trials with MSCs was focused on the systemic infusion of *ex vivo* expanded autologous MSCs to figure out whether it was safe in the short term (Koc *et al*., 2000a; Lazarus *et al*., 1995). Mesenchymal stem cells are currently being investigated in a variety of clinical situations, such as treatment of myocardial infarction using autologous cells (Al-Khaldi *et al*., 2003), osteogenesis imperfecta using allogeneic cells (Horwitz *et al*., 1999), large or critical-sized segmental bone defects using autologous cells and a scaffold

(Quarto *et al*., 2001), Metachromic Leukodystrophy (MLD) and Hurler syndrome using allogeneic cells (Koc *et al*., 2000b) and severe idiopathic anemia using allogeneic MSCs (Fouillard *et al*., 2003). Very soon, MSCs might be capable of helping in the regeneration of diseased or chemotherapy-associated damaged bone marrow stroma (Galotto *et al*., 1999) as well as therapies to treat osteoarthritis, osteoporotic fractures, menisectomy and muscular dystrophy (Nuttelman, 2005).

Tissue engineering a young field of research and is an interdisciplinary field that applies the principles of engineering and life science toward development of biological substitutes that restore, maintain and improve tissue function (Langer and Vacanti, 1993). Tissue engineering is based on the profound understanding of tissue formation and regeneration and aims in growing new functional tissues rather than building new spare parts (Kneser *et al*., 2006). Tissue engineering is therefore tightly associated with the field of regenerative medicine and opens new windows for scientists to discover the new approaches of tissue healing following excessive tissue damages in the body. Tissue engineering of bone as a branch of tissue engineering is concerned to find the most suitable cell-scaffold combination to rally round the affected bone recover as soon as possible with the least possible complications.

As more and more is known and discovered about mesenchymal stem cells, there undoubtedly will be many more therapeutic applications using this highly plastic and convenient cell source to repair and regenerate a variety of diseased or damaged tissues.

BONE TISSUE ENGINEERING

Bone Tissue Engineering (BTE) is classically referred as the implantation of a scaffold seeded with appropriate population of seed cells and/or growth factor at the bone defect site. As apparent in the definition, there are two key elements involving in BTE that are the seed cells as well as scaffold. To overcome the drawbacks of the current bone graft and graft substitute materials, bone tissue engineering utilizing bone marrow mesenchymal stem cells is a promising technique to reconstruct bone defects since, MSCs have easy harvesting technique and are capable of proliferating differentiating into bone forming linage *in vitro*. Indeed, many studies have been performed and still on going to investigate the better materials for bone tissue engineering. Scaffold is the other key element in bone tissue engineering. Function of scaffold is to allow cells to attach and migrate onto or within the scaffold (Vats *et al*., 2003). An ideal scaffold should be: three dimentional and highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste, biocompatible and bioresorbable with a controllable degradation or resorption rate to match cell/tissue growth *in vitro* and *in vivo*, suitable surface chemistry for cell attachment proliferation and differentiation and finally it should be capable of osteogenesis, osteoconduction and/or osteoinduction in tissue engineering bone (Zhi and Zu-Bing, 2005).

Tissue engineering techniques involve imitating the natural milieu by placing the cells and/or growth factors in natural or synthetic scaffolds. However, there are numerous variations of this approach depending on:

- The source of the osteogenic cells: autologous, allogenic or xenograft
- Source of scaffold can be synthetic such as poly lactic acid and poly glycolic acid blends, bioactive glass particles, hydroxyapatite/chitosan-gelatin networks (Nuttelman, 2005), ceramic or glasses and bioactive glass (Vats *et al*., 2003) or natural such as autograft or allograft (Zhi and Zu-Bing, 2005; Kaveh *et al*., 2009), collagen, glycosamin glycans (Vats *et al*., 2003)
- Presence of absence of scaffold: direct injection of cells and/or signaling molecule into the defect site may be appropriate for damaged tissue confined to a small region. Larger regions however will perhaps need the scaffold as a structural cue
- Whether the scaffolds seeded with cells are cultured before surgery or the cells are seeded into the matrix and immediately implanted at the time of surgery (Hannjorg, 2009)

However, it is obvious that there is no single approach or dosage of cells and growth factors which satisfies all clinical needs. The best clue will depend upon the particular application and the relative health status of the patient (Hannjorg, 2009).

To engineer, the ideal bone graft material factors that are capable of triggering osteogenesis must be included. Osteoinductive growth factors or progenitor cells should be employed or recruited. When the scaffold is seeded with a population of cells is termed as cell-based bone tissue engineering while growth factor-based bone tissue engineering is the technique of seeding the growth factor on the scaffold (Meijer *et al*., 2007).

Since, Friedenstein *et al*. (1987) publications in the 1980s, we have known that Mesenchymal Stem Cells (MSCs) can be used to engineer mesenchymal tissues such as bone and cartilage. Therefore, researchers worldwide are seeking to provide the right carrier or

scaffold and the appropriate set of cells that once transplanted will ensure bone repair. Bone marrow has been found to be the richest source of MSCs which have a high proliferative ability and great capacity for differentiation into osteogenic linage (Haynesworth *et al*., 1992a, b; Caplan, 1993). Also from another point of view, bone marrow is an easily accessible source of osteogenic cells, since it can be collected using a relatively simple aspiration method. As such this method is less invasive than collecting osteogenic cells by taking biopsies from periosteum (Miura and O'Driscoll, 1998) or trabecular bone (Robey and Termine, 1985). For a successful BTE outcome, four fundamental prerequisites are required:

- Sufficient population of cells with osteogenic capacity
- An appropriate scaffold to seed the cells
- Growth factors to stimulate osteogenic differentiation *in vivo*
- Sufficient vascular supply (Caplan, 1991)

All these factors apart from number four could be fulfilled *in vivo* by creating suitable environment for osteogenic cell to growth and proliferate. Blood supply is the only factor depended on the patient health status. Texture of scaffold also affects the blood supply to some extent. Consequently now-a-days, researchers are employing all equipments to find the faster and easier avenues for bone healing by creating more amount of new bone regeneration at the defect site.

SCAFFOLDS AND OSTEOGENIC LINAGE

Scaffold is referred as a stand which can feel the osseous defects. Regarding the function, scaffold must allow cell attachment, migration onto or within and cell proliferation and differentiation. Scaffold must provide an environment that the cells maintain their phenotype and synthesize the requirement to augment the healing. An ideal scaffold should be three dimensional and highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste; biocompatible, biodegredible and bioresorbable with a controllable degradation or resorption rate to match cell/tissue growth *in vitro* and *in vivo* to facilitate remodeling; suitable surface chemistry for cell attachment proliferation and differentiation and capable of osteogenic osteoinduction and osteoconduction provide structural support does not initiate or boost the inflammatory process low toxicity (Kaveh *et al*., 2009; Zhi and Zu-Bing, 2005). There are two different types of scaffold: natural material such as collage, auto, allo or xenografts and synthetic scaffold such as different polymers, ceramic, glasses and cements and other different types of synthetic bone grafts. Each type of scaffold have different advantageous and drawbacks; the choice of choosing the scaffold depends on the reason of implanting the scaffold.

Either types of scaffold are capable of carrying the osteogenic linage cells (osteoblasts) into the defect site. The notable issue is the scaffold to be as close as possible to the ideal scaffold. Bone graft substitute have numbers of disadvantageous from which the most important ones are lack of osteogenic and/or osteoinduction properties. Allografts also suffer from many drawbacks, the significant one is disease transmission such as AIDS or hepatitis. Autograft despite carrying many advantageous have a few weaknesses. Donor site morbidity has become an over emphasized weak point of the autogenous graft. Although, many researches pointed the donor site morbidity of autograft this graft material is still the most common graft material used in human as well as veterinary surgery either alone or seeded with osteogenic linage (Kaveh *et al*., 2010). Still many investigations should be conducted to detec the best promising scaffold or to carry the osteogenic linage into the defect site and encourage the bone healing in the safest and fastest fashion as possible.

CONCLUSION

Mesenchymal stem cells display remarkable regulatory and therapeutic versatility. MSC clearly illustrates the potentials pitfall inherent in the extrapolation of inefficacy of medical treatments not utilizing the stem cell. Mesenchymal stem cells due to profuse number, ease of aspiration, fast proliferation and differentiation potential is one of the most common stem cells type used in medicine.

Stem cell application in regenerative medicine is one of the horizons and therefore a basic understanding of stem cell biology is important for clinicians and especially surgeons. Although, there are numeral existing studies suggesting the exercise of adult stem cell in tissue lineage in disputable *in vivo* data is lacking (Lisa, 2005).

RECOMMENDATIONS

There is still much to do if the full promise of stem cell and tissue engineering is to be realized. The understanding form stem cell especially, adult stem cell biology continues to increase however, we must be able to not only control but also optimize the differentiation of stem cell into different cell lineages. This includes determination of signals and specific genes that trigger

differentiation as well as role of understanding of physical and environmental factors with the latter being particularly unexplored (Polak and Bishop, 2006). Recently, bone tissue engineering; combination of scaffold-seed cell have been heralded as the strategy to regenerate bone since it can provide adequate amount of new bone volume and satisfactory bone regeneration potential; however, there are still so many controversies regarding the most suitable scaffold to carry osteogenic cells into the defect site. Researches still conducting investigations to hit upon the most suitable scaffold from different views of material, fabrication method, pore size, surface wax and costs to help the osteogenic cells migrate into and proliferate within to encourage the affected site to recover. In closing, finding the most effective way of using stem cells from embryonic, fetal and adult sources and triggering their differentiation in controlled manner would provide cell banks for *in vitro* growth of tissues for *in vivo* transplantation cell replacement therapy. Developing these concepts from experimental trials to hospitals and clinics will be crucial in meeting healthcare requirements in the new century.

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