CHAPTER 2

Review of literature

Introduction

Regenerative medicine is a promising and rapidly growing field of research. Discovery of stem cells with the ability to rejuvenate or replace the defective organs or tissues of the body have given new dimensions for the search for ‘the fountain of youth’ by the humankind. Understanding the mechanisms of tissue regeneration, the cells involved, the in vivo regulatory microenvironment are mandatory for translating the enigma of regeneration for therapeutic applications.

In this chapter first, we discuss about the scope and different approaches of regenerative medicine, the types of stem cells, and its role in tissue regeneration. This is followed by an overview of mesenchymal stem cells, their osteoblast differentiation and the regulatory role of the extracellular matrix. Finally, we explore the progress in the field of cell based bone tissue engineering emphasising the biomimetic approach of bone regeneration. This chapter will cover the review of literature in the following order:

2.1 Regenerative medicine

2.2 Stem cells in regenerative medicine

2.3 Mesenchymal stem cells

2.4 Osteoblast differentiation

2.5 Extracellular matrix
2.6 Extracellular matrix, mesenchymal stem cells and osteoblast differentiation

2.7 Bone tissue engineering

2.8 Biomimetic approach of bone tissue engineering

2.1 Regenerative medicine

It is man’s centuries old dream to remain young forever. His quest for preventing aging and death goes back to time immemorial. The fantasy to regenerate tissue or organs existed since the observations of Prometheus. The amphibians like salamanders’ ability to regenerate limb, heart, tail, brain, eye tissues, kidney, brain and spinal cord throughout life also inspired and kindled the imagination of men. This has led to the emergence of a new branch of medicine –regenerative medicine. The regenerative medicine is defined as a technique to repair or replaces diseased or damaged cells, tissue and organs. According to Stocum (2002), regenerative biology seeks to understand the cellular and molecular differences between regenerating and non-regenerating tissues. Regenerative medicine seeks to apply this understanding to restore tissue structure and function in damaged, non-regenerating tissues.

There is selective distribution of regenerative capacity in the animal kingdom and that follows the hierarchy. The invertebrates like planaria and hydra can regenerate the whole animal from tiny body pieces or cell aggregates. Humans and other higher vertebrates have a very limited regenerative capacity.

Potency specifies the potential of the stem cell to differentiate into different cell types (Scholer, 2007). Life begins from a stem cell, the zygote, which will divide and differentiate to form the whole organism. The early cells that have the potential to develop into the whole organism are termed as totipotent. During the fetal development,
the inner cell mass of the blastocyst are capable of differentiating into all types of cells seen in an adult organism and are termed as pluripotent stem cells. With continued fetal development, further specialization of pluripotent stem cells results in multipotent stem cells with limited differentiation potential. Multipotent stem cells have the ability to give rise to multiple cell types like muscle, nerve or blood cells. Oligopotent stem cells can differentiate into only a few cells, such as lymphoid or myeloid stem cells. Unipotent cells can produce only one cell type, their own, but have the property of self-renewal, which distinguishes them from non-stem cells (e.g., muscle stem cells).

**Regenerative medicine: The different strategies**

Different strategies of regenerative medicine are aimed at either stimulating tissue regeneration or by producing the tissue ex vivo. These strategies are formulated and improved based on the cues from natural tissue regeneration. Stem cells, growth factors and biomaterial scaffolds are the three basic cogs that devise the various strategies of regenerative medicine. These different approaches of regenerative medicine are given in Figure 2.1.

**2.2 Stem cells in regenerative medicine**

Discovery of cells capable of self-renewal and differentiation has revolutionized the development in regenerative medicine. Those cells the ‘stem cells’ have fascinated the scientific world for more than a century. In the current scenario, stem cells are generally defined as clonogenic cells capable of both self-renewal and multilineage differentiation (Weissman, 2000). A stem cell is capable of dividing indefinitely (self-renewal) and having the potential to develop into of other cell types (differentiation). They achieve this either by undergoing symmetrical division giving rise to identical
daughter cells (self-renewal) or by undergoing asymmetrical division giving rise to specific cells of somatic tissue (differentiation) (Avery et al., 2006).

Figure 2.1 The different strategies of regenerative medicine.

Stem cells have been established from embryos and identified in the fetal tissues as well as in specific niches in many adult mammalian tissues and organs such as bone marrow, brain, skin, eyes, heart, kidneys, lungs, gastrointestinal tract, pancreas, liver, breast, ovaries, and prostate. Their self-renewal property, which enables prolonged in vitro propagation and their multi lineage differentiation potential, makes them the heart and hope of regenerative medicine. Hodgkinson et al (2006) described certain basic
criteria for the stem cells to be used in cell based therapies: (i) they should be found in abundance; (ii) they could be collected and harvested by a minimally invasive procedure; (iii) they could be differentiated along multiple cell lineage pathways in a reproducible manner; and (iv) they could be safely and effectively transplanted to either an autologous or allogenic host.

Stem cells may be broadly classified into four categories based on their origin and differentiation potential:

(i) Embryonic stem cells
(ii) Induced pluripotent stem cells
(iii) Fetal stem cells
(iv) Adult stem cells

**Embryonic stem cells**

Human embryonic stem cells (hESCs) are derived from the inner cell mass of a developing embryo (blastocyst) (Stojkovic et al., 2004) and are pluripotent (Mayhall et al., 2004). Thomson et al (1998) proposed that the essential characteristics of primate ES cells should include: (i) derivation from the pre-implantation or peri-implantation embryo; (ii) prolonged undifferentiated proliferation; and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. They demonstrated that the hESCs possessed the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). Human ESC lines are characterized by the stem cell characteristic features like undifferentiated
proliferation, high nucleus to cytoplasmic ratio, stable karyotype, expression of cell surface markers like SSEA3, SSEA4, OCT4, TRA-1-60, and TRA-1-81, and pluripotency genes Oct-4 and nanog (Sidhu and Tuch, 2006).

**Induced pluripotent stem cells**

Induced pluripotent stem cells (iPSC) are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state expressing pluripotent genes and maintaining ESC characteristic features. These cells had an embryonic stem cell-like morphology, including a round shape, large nucleoli and scant cytoplasm. Takahashi et al in 2007 (Takahashi et al., 2007) demonstrated for the first time that, iPSCs can be generated from adult human fibroblasts using the factors Oct3/4, Sox2, Klf4, and c-Myc which was again confirmed by Park et al (2008) in a separate study. Taura et al (2009) later demonstrated the differentiation of human iPSCs into osteogenic and adipogenic lineage. IPSC technology could generate patient specific stem cells for therapy that evades the ethical issue concern of ESCs. However, iPSCs have the same drawbacks of ESCs such as the *in vivo* teratoma formation, lack of genome integrity, variable propensity for lineage specific differentiation.

**Fetal stem cells**

The discovery and isolation of fetal stem cells, which have better proliferation rate and plasticity than adult stem cells are the novel addition to the pool of stem cells, which constitutes the promising candidates for tissue regeneration. Fetal stem cells are primitive cell types of fetal origin, which finally develop into the various organs of the body. Fetal blood, placenta and umbilical cord are rich sources of fetal hematopoietic stem cells. Amniotic fluid stem cells, amniotic membrane stem cells, umbilical cord blood stem
cells, Wharton’s jelly stem cells and placental stem cells are the five major sources of fetal MSCs (Pappa and Anagnou, 2009).

**Amniotic fluid stem cells:** The stem cell derived from amniotic membrane epithelium that is present and isolated from amniotic fluid is termed as the amniotic fluid stem cells. Prusa *et al* isolated these stem cells from the amniotic fluid for the first time in 2003. De Coppi *et al* (2007) demonstrated the differentiation of amniotic fluid derived MSCs (AFMSCs) to osteogenic lineage cells forming tissue-engineered bone.

**Amniotic membrane stem cells:** Amniotic membranes are rich source of stem cells which possess some characteristic features of pluripotent ESCs and multipotent MSCs. Amniotic mesenchymal or amniotic membrane mesenchymal stromal cells (AM-MSCs) possessed stem cell properties and shown multi lineage differentiation (Marcus *et al.*, 2008).

**Umbilical cord blood stem cells:** Umbilical cord blood (UCB) is rich in stem cells as it contains large amount of hematopoietic stem cells (HSCs) as well as a small portion of MSCs (Zhao *et al.*, 2006). Approximately 1% of the mononuclear cells present in cord blood are CD34⁺ HSCs (Pappa and Anagnou, 2009). UCB-MSCs possess the property of fetal stem cells, as they closely resemble ESCs (Tondreau *et al.*, 2005). Studies on the differentiation potential of UCB-MSCs show their superior osteogenic potential and inferior adipogenic potential over bone marrow derived MSCs (Chang *et al.*, 2006).

**Wharton’s jelly stem cells:** Wharton's Jelly is the embryonic mucous connective tissue of the umbilical cord, first named by Thomas Wharton in 1656. Apart from the hMSC markers, they also expressed hESC markers like oct-4, nanog, rex-1 and sox-2 and genes associated with the three principal germinal layers (ectoderm, mesoderm and endoderm) (Weiss *et al.*, 2006). Ease of isolation, abundance, no ethical issues and high *in vitro*
proliferative rate and multilineage differentiation potential make WJ-MSCs an ideal candidate for regenerative medicine.

**Placental stem cells:** Recently placenta, the feto-maternal organ with cells of fetal and maternal origin, has been identified as a rich source of stem cells. These cells with stem cell properties and comparative characteristic features of hMSCs are generally termed as placenta-derived stem cells (PDSC) or placenta derived mesenchymal stem cells (P-MSCs) (Li et al., 2007). Battula et al (2007) showed that P-MSCs derived from the chorionic placenta expressed the ESC makers like Oct-4 and nanog.

**Adult stem cells**

Adult stem cells are undifferentiated cells that occur in the differentiated tissue, such as bone marrow, liver, pancreas, skin, skeletal muscle, dental pulp, limbal tissue, adipose tissue, or the brain, in the adult body. They replenish themselves throughout the lifetime of the organism, or differentiate to yield the cell types of the tissue of origin. Adult stem cells have been isolated from bone marrow, blood, eye, adipose tissue, brain, skeletal muscle, dental pulp, liver, skin, the lining of the gastrointestinal tract, heart, hair follicle and pancreas (Hodgkinson et al., 2009; Pessina and Gribaldo, 2006; Barrilleaux et al., 2006). Adult stem cells are found in specific niches *in vivo* and are responsible for maintenance and repair of the tissue in which they reside. Based on the origin and lineage specificity, adult stem cells are broadly classified into hematopoietic stem cells and mesenchymal stem cells.

**Hematopoietic stem cells:** Hematopoietic stem cells (HSCs) are multipotent stem cells that give rise to all the cells of hematopoietic lineage that include blood cell types from the myeloid and lymphoid lineages. They are mainly present in the bone marrow and have been isolated from bone marrow, peripheral blood and umbilical cord blood. They
are characterized by a set of surface markers (cluster of differentiation or CD). They are positive for CD34, CD133 and Bcrp-1; and negative for CD38 and Lin (Hodgkinson et al., 2009). HSC transplantations are widely used for treating both malignant and non-malignant disorders (Copelan, 2006).

2.3 Mesenchymal stem cells

The existence of a different population of stem cells apart from hematopoietic stem cells were first reported by Friedenstein in 1974 (Friedenstein et al., 1974). It was the discovery of mesenchymal stem cells or mesenchymal stromal cells (MSCs) which revolutionized the history of regenerative medicine. MSCs are multipotent stem cells present in various fetal and adult tissues. They are generally characterized in vitro by their plastic adherence, fibroblast like morphology, expansion potential and tri-lineage (Osteoblasts, adipocytes and chondrocytes) differentiation potential. The Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed a set of standards to define human MSC (hMSC) for both laboratory-based scientific investigations and for pre-clinical studies. They proposed the following three criteria to define MSC (Dominici et al., 2006): (i) adherence to plastic, (ii) specific surface antigen expression, and (iii) multipotent differentiation potential.

MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. More than 95% of the MSC population should express CD105, CD73 and CD90 as measured by flow cytometry. In addition, these cells must lack expression (≤ 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Mafi et al (2011) in their review article describes and summarises the cell surface markers (CD markers) used for the characterization of MSCs in various studies. The cells
must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard
in vitro differentiating conditions (Pittenger et al., 1999).

Sources of hMSCs

As mentioned above, hMSCs can be isolated from a variety of tissues, but only the hMSCs from sources like Wharton’s jelly, term placenta, bone marrow, adipose tissue and dental pulp are well characterized and widely explored for their potential application in regenerative medicine. Human MSCs from Wharton’s Jelly and placenta have been already discussed under fetal MSCs in section 2.2. So here, we will be discussing only the hMSCs from the adult tissues.

Bone marrow derived hMSCs: Bone marrow is one of the richest sources of hMSCs. In the bone marrow compartment, MSCs represent a very small population – 0.001 to 0.01% of the total nucleated cells (Pittenger et al., 1999). Bone marrow derived hMSCs (BM-hMSCs) are the most extensively studied and widely used stem cells in tissue engineering and regenerative medicine. MSCs are isolated from an aspirate of bone marrow harvested from the superior iliac crest of the pelvis in humans (Pal et al., 2009a; Pittenger et al., 1999). MSCs are obtained from the mononuclear fraction of the bone marrow by density gradient centrifugation and the primary cultures are usually maintained for 12–16 days. By this time, the non-adherent haematopoietic cell fraction is depleted and plates will have a confluent adherent population of MSCs. The cells are then characterized by immunophenotyping and tri-lineage (osteogenic, adipogenic and chondrogenic lineages) differentiation potential. Pal et al (2009a) showed that hMSCs could be expanded for long passages (up to 25 passages) without loss of functionality or stemness indicating their potential application in large-scale production of cells for autologous/allogenic transplantation in appropriate clinical conditions.
Adipose tissue derived hMSCs: The adipose tissue has been recently identified as another rich source of hMSCs and is easily obtained through liposuction, a cheaper and less invasive procedure than bone marrow puncture (Bunnell et al., 2008; Helder et al., 2007). MSCs are isolated from the stromal vascular fraction (SVF) of the adipose tissue obtained by collagenase treatment and subsequent centrifugation. Adipose tissue derived MSCs are generally termed as adipose derived stem cells or ADSCs. The abundant source, easy isolation; rapid expansion in culture and multilineage potential makes ADSC an attractive cell source for tissue engineering and regenerative medicine (Hodgkinson et al., 2009; Yarak and Okamoto, 2010).

Dental pulp derived hMSCs: Gronthos et al in 2000 (Gronthos et al., 2000) identified a population of adult human stem cells in the dental pulp which were termed as dental pulp stem cells (DPSCs). The isolated DPSCs showed self-renewal and differentiation to odontoblasts and osteoblasts. DPSCs are primarily derived from pulp tissues of primary incisors, exfoliated deciduous and permanent third molar teeth (Kerkis et al., 2011).

Advantages of hMSCs in regenerative medicine

MSC are considered great candidates for tissue engineering and regenerative medicine due to the following criteria (Yarak and Okamoto, 2010):

(i) They can be easily isolated and harvested
(ii) They can be harvested from the patient himself / from a donor enabling both autologous or allogenic transplantation
(iii) It is possible of harvest an adequate number of cells for transplantation, due to the high cellular proliferation in vitro
(iv) Multipotent capacity of cell differentiation
(v) Easy laboratory handling
They have little immunogenicity or they are immunosuppressive.

They have the ability to integrate into the host tissue and interact with the surrounding tissue.

**Differentiation potential of hMSCs**

The tri-lineage differentiation potential of hMSCs into osteoblast, adipocytes and chondrocytes are well established and well documented. Various studies show that hMSCs have the ability to differentiate into cells beyond the mesenchymal lineage (Jackson *et al.*, 2007). Differentiation of hMSCs to cardiomyocytes, hepatocytes, endothelial, and neuronal cells under appropriate *in vitro* culture conditions are also been reported (Wang *et al*., 2011).

**Human MSC niche**

The concept of a stem cell ‘niche’ was first introduced by Schofield in 1978 (Schofield, 1978). A stem cell niche can be defined as the microenvironment surrounding the stem cells in its native tissue, which includes the non-stem cells, the extra cellular matrix (ECM) and soluble factors (cytokines and growth factors). The function of the niche is to replenish and maintain the stem cells in its undifferentiated states to ensure tissue homeostasis.

**2.4 Osteoblast differentiation**

Human MSCs present in the bone marrow are the major source of osteoprogenitor cells, which can differentiate into osteoblasts under appropriate stimuli (Friedenstein *et al*., 1987; Pittenger *et al*., 1999). Human MSCs from Wharton’s jelly, adipose tissue (Gimble and Guilak, 2003) and dental pulp were also reported to have good osteogenic potential. The differentiation pathway of osteoblasts from hMSCs is depicted in figure
2.1. *In vitro* osteoblast differentiation from hMSCs is generally induced with osteogenic induction media containing ascorbic acid, β-glycerophosphate and dexamethasone.

The ‘master switch’ responsible for the initiation of osteoblast differentiation from MSCs is RUNX2 (runt-related transcription factor 2), which is also known as osteoblast specific factor (Osf2) or core binding factor α (Cbfa1) (Komori, 2001; Lian *et al*., 2004). The subsequent stages of differentiation can be divided into three phases: (i) the proliferative phase; (ii) the extracellular matrix synthesis and maturation phase; and (iii) the mineralization phase (Lian and Stein, 1992; 2003). Figure 2.2 depicts the different stages of differentiation and the associated markers and regulatory factors specific for each phase.

**The proliferative phase:** The proliferative phase is constituted by the lineage committed osteoprogenitor cells that have significant proliferative capacity. This phase is marked by rapid cell proliferation. The osteoprogenitor cells express the cell-growth associated genes H4 histone and cfos (Pockwinse *et al*., 1992) and thus undergo frequent mitosis resulting in high cell proliferation. Genes associated with ECM synthesis like collagen type Iα1 (COL1A1), fibronectin and TGF-β are also expressed in the proliferative phase. Osteopontin (OPN) shows the first peek in expression during this phase.

**The extracellular matrix synthesis and maturation phase:** The matrix maturation phase is marked by the increase in the expression of alkaline phosphatase liver/bone/kidney (ALPL), integrin binding sialoprotein (IBSP) and COL1A1. ALPL plays an important role in preparing the extracellular matrix for mineralization. This phase is constituted predominantly by pre-osteoblasts. There is minimal cell proliferation in this phase. During this phase, the pre-osteoblasts mature into osteoblasts and there is active
synthesis of ECM. Early stage is marked by the presence of COL1, COL3, COL5, fibronectin followed by ALPL.

The mineralization phase: The final mineralization phase is distinguished by a second peak in OPN expression and the expression of osteocalcin (OCN), IBSP and parathyroid hormone (PTH) receptor by mature osteoblasts. There is no cell proliferation in this phase and mature osteoblasts are the predominant cells. The alkaline phosphatase (ALP) activity also declines once the mineralization begins. In this phase, the nodule cells begin to mineralize the collagen-based extracellular matrix. Ninety percentage of the organic matrix is constituted by collagen type1 and the remaining 10% is non-collaginous proteins (NCP) like OCN, matrix gla-protein (MGP), osteonectin (ON) and fibronectin. Most of these proteins exhibit calcium-binding properties. The mineral matrix of bone consists of calcium phosphate in the form of hydroxyapatite (3Ca$_3$(PO$_4$)$_2$(OH)$_2$).

Figure 2.2 Different phases of osteoblast differentiation (Adapted from Lian et al., 2003). (PGE2, prostaglandin E2).

2.5 Extracellular matrix

Extracellular matrix (ECM) is the acellular components surrounding the cells. ECM is an important factor of cellular microenvironment, which along with various
growth factors plays an important role in cell adhesion, proliferation differentiation and development (Adams and Watt, 1993). ECM is mainly made up of various proteins and glycosaminoglycans. The main functions of ECM include: (i) provides mechanical support to cell and tissue; (ii) provides structural stability to the tissue; (iii) signal transduction; (iv) regulates cell adhesion, proliferation, migration, differentiation and development (v) serve as the reservoir for growth factors, cytokines and other soluble signalling molecules.

**Structure**

The two main components of ECM are proteins and proteoglycans (PGs). The major ECM proteins include collagen, elastin, laminin, fibronectin and vitronectin. They constitute the structural elements of the ECM and provide the structural integrity to the tissue. PGs are composed of glycosaminoglycan chains covalently linked to a specific core protein (Schaefer and Schaefer, 2010). PGs fill the majority of the extracellular interstitial space within the tissue. ECM functions as adhesive substrate, provides structure, presents growth factor to their receptor, sequesters and stores growth factors; and plays a role in signal transduction (Rozario and DeSimone, 2010).

**ECM proteins**

**Collagen:** Collagens are the most abundant fibrous protein and the major structural element of the ECM. Collagen type I, III and V are the major collagens present in the bone tissue (Gelse et al., 2003). Fibrillar collagens provide most of the biomechanical properties essential for the functioning of the organ systems like bone, tendon, fascia and articular cartilage. Collagens maintain structural integrity of tissues and organs. Collagen type I is the major constituent of bone matrix and play a significant role in bone mineralization.
**Elastin:** Tropoelastin is a key component of the elastic fibres. Mature cross-linked tropoelastin gives rise to elastin, which confers resilience and elasticity on a diverse range of tissues (Wise and Weiss, 2009). Elastin fibres provide recoil to tissues that undergo repeated stretch. This function is crucial for arteries, lung, skin and other dynamic connective tissues that undergo cycles of extension and recoil.

**Fibronectin:** Fibronectin (FN) is another major ECM protein and plays a major role in directing the organization of the interstitial ECM. In addition, FN has a crucial role in mediating cell attachment and function (Pereira et al., 2007). It has a heparin-binding N-terminus, a collagen-binding domain and a cell binding Arg-Gly-Asp (RGD) domain. Fibronectin has been shown to control adhesion dependent cell growth (Sottile et al., 2000).

**Vitronectin:** Vitronectin (VN) is a multifunctional adhesive protein found in ECM of different tissues and in serum. Like fibronectin, vitronectin also play key roles in the attachment of cells to their surrounding matrix and may participate in the regulation of cell differentiation, proliferation, and morphogenesis (Underwood and Bennett, 1989).

**Laminin:** Laminins (LNs) are cross-shaped proteins mainly present in the basal lamina. LN binds to membrane receptors (integrins) of the overlying cells and attaches the cells to the basal lamina. The laminins can self-assemble, bind to other matrix macromolecules, and have unique and shared cell interactions. Through these interactions, laminins critically contribute to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival (Colognato and Yurchenco, 2000).
**Glycosaminoglycans**

GAGs are unbranched polysaccharide, composed of repeating disaccharide units. GAGs are highly negative charged and are extremely hydrophilic which is responsible for their hydrogel like structure. The major glycosaminoglycans found in ECM include hyaluronic acid, heparan sulphate, heparin, dermatan sulphate, keratin sulphate and chondroitin sulphate. GAGs differ in the type of sugars in the disaccharide units. Except hyaluronic acid, all other GAGs are sulphated.

**Hyaluronic acid/ hyaluronan:** Hyaluronic acid is the only GAG that is non-sulphated and the only GAG that is not linked to a protein core. It is a massive polysaccharide consisting of a linear chain of repeating disaccharides of glucuronic acid and N-acetyl glucosamine. Its high negative charge makes HA extremely hygroscopic and gives it a hydrogel like nature. Through receptors such as CD44 and RHAMM (receptor for HA-mediated motility expressed protein) HA can mediate signal transduction, triggering various signalling pathways like MAPK (mitogen activated protein kinases), c-Src, Ras and P13kinase / Akt (Necas et al., 2008). HA is known to regulate cell growth, differentiation, adhesion and migration (Zhu et al., 2006).

**Heparan sulphate:** Heparan sulphate (HS) has alternating uronic acid and D-glucosamine units (Esko and Lindahl, 2001). The structural heterogeneity of heparan sulphate allows it to interact with a wide range of functionally diverse proteins, such as growth factors, cytokines, chemokines, proteases, lipases and cell-adhesion molecules (Parish, 2006; Kirn-Safran et al., 2009).

**Heparin:** Heparin is closely related to heparan sulphate but is highly sulphated than HS and has the highest negative charge density among all the known biological molecules. It consists of many repeating disaccharide units. Heparin is secreted mainly by mast cells
and it act as an anti-coagulant preventing blood clotting (Riley et al., 1955). Heparin is also widely used in tissue engineering to improve the biocompatibility and hemocompatibility of scaffold materials (Nillesen et al., 2007). Heparin has the ability to form and degrade collagen and thus plays a significant role in bone formation and resorption, (Shibata et al., 1992).

**Keratan sulphate:** Keratan sulphate-GAG (KS-GAG) is a linear carbohydrate chain consisting of repeating disaccharides composed of galactose (Gal) and N-acetyl glucosamine (GlcNAc) with sulphation at the 6-O positions on the saccharides. Keratan sulphate (KS) is the predominant GAG present in the cornea of the eye.

**Dermatan sulphate:** Dermatan sulphate (DS), which was earlier known as chondroitin sulphate B (CS-B), is composed of linear polysaccharides having repeating disaccharide units of N-acetyl galactosamine or glucuronic acid joined by B 1,4 or 1,3 linkages respectively. DS is linked to core proteins like decorin, biglycan, thrombomodulin, endocan, epiphycan and versican to form DS proteoglycans (DS-PGs). These DS-PGs are present in the ECM of a variety of tissues like skin, cornea, cartilage, bone and endothelia (Trowbridge and Gallo, 2002). DS could bind to growth factors like FGF-2 and can enhance cell proliferation. DS is the major PG present in the wound tissue and is considered as the multifunctional facilitator of the wound repair process (Trowbridge and Gallo, 2002).

**Chondroitin sulphate:** Chondroitin sulphate (CS) is a homopolymeric GAG containing only one type of repeating disaccharide unit of glucuronic acid and N-acetyl galactosamine. Chondroitin sulphates are classified as CS-O, A, B, C, D, E, F and K based on the pattern of their sulphation. Differences in sulphation pattern are known to deliver diverse, properties and functions to various CSs. It is the major component of
cartilage ECM and it provides the structural integrity to the tissues like cartilage, bone, skin, blood vessel, ligament and tendons. Loss of chondroitin sulphate from the cartilage is a major cause of osteoarthritis (Lee et al., 2010).

**Integrins**

Integrins are cell surface receptors that mediate cell adhesion and ECM induced signal transduction. Integrins can activate intracellular signalling pathways and thus play a crucial role in regulating cell behaviour and functions. Table 2.1 gives the integrins associated with major ECM proteins (Humphries et al., 2006; Giancotti, 2000).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Integrin</th>
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<tbody>
<tr>
<td>Laminin</td>
<td>$\alpha_1\beta_1$, $\alpha_{10}\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_4$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_3\beta_1$ and $\alpha_2\beta_2$</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>$\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_V\beta_6$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_D\beta_2$, $\alpha_4\beta_1$, $\alpha_4\beta_7$ and $\alpha_V\beta_8$,</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>$\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_8\beta_1$ and $\alpha_{IIb}\beta_3$</td>
</tr>
<tr>
<td>Collagens</td>
<td>$\alpha_1\beta_1$, $\alpha_2\beta_2$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$ and $\alpha_D\beta_2$</td>
</tr>
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Table 2.1: Major integrins associated with adhesive ECM proteins

The most studied integrin mediated signalling pathways includes the mitogen activated protein kinase (MAPK) pathway (Yee et al., 2008), extracellular-regulated kinase (ERK) pathway and focal adhesion kinase (FAK) (Meng et al., 2009) pathway. The MAPK cascade, leading to extracellular-regulated kinase (ERK) activation, is a key regulator of cell growth and proliferation.

2.6 **Extracellular matrix, mesenchymal stem cells and osteoblast differentiation**

**Extracellular matrix and mesenchymal stem cells**

The cells are surrounded by a highly dynamic and complex extracellular microenvironment that carries a great amount of information in the form of biophysical
and biochemical signals. Thus, the molecular interaction of the cell with the ECM effectors significantly influences and regulates cell properties like differentiation, proliferation, migration, apoptosis (Lutolf and Hubbell, 2005; Ode et al., 2010). According to Reilly et al (2010), stem cells are exquisitely sensitive to the intrinsic properties of their ECM and the mechanical interaction between the cell and the ECM is one of the key mediators of differentiation. The intrinsic properties of the ECM such as, matrix structure, elasticity, and composition have a major impact on the differentiation potential of stem cells (Hanein and Horwitz, 2011). These biophysical cues obtained from the ECM can trigger signal transduction that would commit the stem cells to a specific lineage. The major ECM associated signalling pathway is the MAPK (mitogen-activated protein kinase) pathway and the focal adhesion kinase pathway (Salasznyk et al., 2007; Rubinfeld and Seger, 2005).

ECM proteins like laminin, fibronectin have shown to increase hMSC adhesion and proliferation in vitro. Hashimoto et al (2006) proposed that laminin-5 supported the self-renewal of MSCs by enhancing their proliferation and suppressing their differentiation. Enhanced adhesion and spreading were observed when hMSCs were cultured on fibronectin-coated plates (Ogura et al., 2004). Lin et al (2010) showed that fibronectin and laminin promoted the differentiation of hMSCs into insulin producing cells through activating Akt and ERK signalling pathways.

Proteoglycans and glycosaminoglycans are also shown to influence the growth and differentiation of hMSCs. ECM-GAGs like heparin and heparan sulphate can interact with fibronectin and can mediate many cellular functions like adhesion and differentiation (Gallagher et al., 1986).
Extracellular matrix and osteogenic differentiation

In bone, there are two juxtaposed ECMs, the inorganic matrix and the organic matrix. The inorganic matrix is made up of calcium phosphate in the form of hydroxyapatite. The organic matrix is a complex network of proteins and proteoglycans. Collagen type I is the major constituent of the organic bone matrix and it also contain other proteins like type III, IV, V, and VI collagens; non-collagenous proteins like fibronectin, osteocalcin and bone sialoprotein; and proteoglycans like biglycan, decorin, syndecan and perlecan (Chen, 2010).

Osteoblasts and osteoprogenitor cells express integrins like α1β1, α2β1, α4β1, α5β1, α6β1, α8β1 and αvβ3. These integrins can bind to a number of ECM components and these integrin-mediated interactions with extracellular proteins are the key regulators of osteoblast functions (Garcia and Reyes, 2005; Kundu et al., 2009). Lai et al (2001) demonstrated that ERKs are essential for the growth and differentiation of osteoblasts. ERKs also influenced osteoblast adhesion, spreading, migration, and integrin expression.

ECM induced MAPK signalling pathways also have a regulatory role in osteoblast differentiation of hMSCs (Datta et al., 2010). MAPK signalling is associated with the expression of osterix in hMSC. Osterix is a transcription factor that is essential for the development of a mature osteogenic phenotype.

ECM plays an important role in hMSC proliferation and osteoblast differentiation (Marie, 2009; Decaris et al., 2012). Sun et al (2011) demonstrated the functional recovery of aged MSCs when exposed to younger ECM. There was improvement in cell proliferation and osteoblast differentiation potential. When MSCs were exposed to ECM left by osteoblasts there was higher expression of alkaline phosphatase and enhanced calcium deposition (Dumas et al., 2010).
Many studies demonstrated the crucial role of fibronectin in osteoblast differentiation, mineralization and survival (Globus et al., 1998; Martino et al., 2007). Schleicher et al (2005) demonstrated that vitronectin enhanced the attachment and migration of osteoblasts in a 3-D culture.

Glycosaminoglycans and proteoglycans also play important role in osteogenesis (Nakamura et al., 2006; 2007). Hwang et al (2011) reported that an ECM based hydrogel containing hyaluronic acid significantly enhanced osteoblast differentiation. Slater et al (1996) demonstrated the role of chondroitin sulphate in initiating and promoting osteoblast differentiation and mineralization.

2.7 Bone tissue engineering

“Tissue Engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ” (Langer and Vacanti, 1993). Bone tissue engineering (BTE) applies the principles of tissue engineering for the development of viable substitutes that restore and maintain the function of human bone tissue.

Bone is a dynamic tissue, which is highly vascularised. It is a unique tissue capable of regeneration and remodelling throughout the lifetime. However, clinical situations like extensive injury, non-union or delayed union fractures, congenital malformations or diseases requires surgical intervention and tissue grafting. The cell-based bone tissue engineering (BTE) combines living osteogenic cells with biomaterial scaffolds with or without osteoinductive growth factors ex vivo to allow the development of a three-dimensional, functional bone tissue construct (Laurencin et al., 1999). The three
fundamental requirements for bone tissue engineering include: (i) scaffolds, (ii) osteogenic cells, and (iii) osteoinductive growth factors.

Figure 2.3 Diamond model of bone tissue engineering (Adapted from Giannoudis et al., 2007).

The standard tissue engineering approach to provide solutions for impaired fracture healing, bone restoration and regeneration includes the utilisation of growth factors, scaffolds and mesenchymal stem cells (triangular concept). Since, mechanical environment is also an important element in bone regeneration, Giannoudis et al (2007) introduced the diamond concept of fracture healing and BTE (Fig. 2.3).

Scaffolds

Scaffolds are considered as the in vitro replacement for the in vivo ECM. The basic properties of scaffolds include porosity, biocompatibility and biodegradability. Scaffolds serve as the three dimensional structural template for cell adhesion, proliferation, differentiation, ECM deposition and consequent bone in-growth (Laurencin et al., 1999).
According to Porter et al (2009), an ideal scaffold used for BTE should possess the following properties.

(i)  The scaffold should provide temporary mechanical support to the affected area.

(ii) It should act as a substrate for osteoid deposition.

(iii) It should contain a porous architecture to allow for vascularisation and bone in-growth.

(iv) The scaffold should encourage bone cell migration into it (osteoconduction).

(v) It should support and promote osteogenic differentiation in the non-osseous, synthetic scaffold (osteoinduction).

(vi) It should enhance cellular activity towards scaffold-host tissue integration (osteointegration).

(vii) It should degrade in a controlled manner to facilitate load transfer to developing bone.

(viii) On degradation, it should produce only non-toxic products.

(ix) It should not evoke any adverse immune response.

(x) It should be possible to sterilize the scaffold without the loss of bioactivity.

(xi) The scaffold should deliver bioactive molecules or drugs in a controlled manner to accelerate healing and prevent pathology.

Generally, scaffolds made of osteoconductive materials with large interconnected pores (which facilitate the cell infiltration and matrix deposition) and rough inner surfaces (which facilitate cell attachment), and with mechanical properties similar to native bone are the most suitable scaffolds for bone formation (Szpalski et al., 2011). A variety of scaffolds made from ceramics, metals, polymers (natural and synthetic) as well as composite scaffolds are used in bone regeneration and tissue engineering applications.
**Osteogenic cells**

BTE involves seeding cells with osteogenic capabilities onto scaffolds in the presence of osteogenic growth factors that mimic the natural environment of bone (Szpalski et al., 2012). The cells used for bone tissue engineering should have isolation and expansion efficiency, stability of osteoblastic phenotype, *in vivo* bone formation capacity and long-term safety for a successful clinical application (Colnot, 2011). Adult bone tissue and periosteum are good sources of primary osteogenic cells (Jonsson et al., 1999). Since hMSCs have the potential to differentiate into osteoblasts, they constitute an ideal cell source for the development of a bioengineered bone construct using various biomaterial scaffolds (Mauney et al 2005; Pittenger et al., 1999).

**Growth factors**

Growth factors like, bone morphogenic proteins (BMPs), the transforming growth factor-beta (TGF-b) super family, platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) are identified to play a significant role in osteogenesis and fracture healing (Tsiridis et al., 2007; Dimitriou et al., 2005). **BMPs:** BMPs are the most extensively studied osteoinductive growth factor. They induce the mitogenesis of MSCs and other osteoprogenitors, and there by enhance their differentiation to osteoblasts (Dimitriou et al., 2011). BMP has been reported to improve the performance of scaffolds by improving the osteoinduction (Yilgor et al., 2009). Incorporation of BMP-gene in bone tissue engineering applications is another emerging strategy to improve osteoinduction and bone regeneration (Tang et al., 2008).
FGFs: FGFs are known to promote growth and differentiation of a variety of cells such as fibroblasts, myocytes, osteoblasts, epithelial cells and chondrocytes. FGFs appear in the early stages of fracture healing and they act as a mitogen for mesenchymal cells (Dimitriou et al., 2005). Transfer of gene encoding basic FGF (bFGF) to MSCs increased their osteogenic potential with enhanced capillary regeneration, thus providing the vasculature necessary for new bone formation (Guo et al., 2006). Basic FGF has been used in various BTE applications to enhance osteogenesis (Qu et al. 2011).

TGF-β: TGF-β is a potent mitogen and chemotactic factor for bone forming cells like MSCs, osteoprogenitor cells, osteoblasts (Dimitriou et al., 2005). TGF-β is a potent chemotactic stimulator of MSCs and induces the production of bone extracellular proteins such as COL, PGs, OPN, ON, and ALP (Tsiridis et al., 2008). Although few BTE studies used TGF-β to improve osteogenesis (Keogh et al., 2010), its osteoinductive potential seems limited, and concern for unforeseen side effects has limited its use in BTE for enhancing bone regeneration (Lieberman et al., 2002).

VEGF: Poor vascularisation is the major cause of bone graft failure in higher animals and humans, where the fracture or wound size will be larger. Vascular endothelial growth factor (VEGF) is a growth factor with angiogenic properties that is widely for BTE applications to improve angiogenesis and osteogenesis (Salgado et al., 2004).

Bioreactors

It is difficult to sustain long-term culture in conventional static culture conditions. The alternative dynamic culture system provides continuous exchange of nutrients and metabolic products. This helps to sustain more viable and metabolically active cell populations for long time. Dynamic cultures are carried out in bioreactor systems that could provide an optimal supply of the oxygen and nutrients to the cells within the
scaffold. A bioreactor can be defined as a device that uses mechanical means to control and regulate the biological processes. Cells respond to mechanical stimulation and bioreactors can be used to apply mechanical stimulation to cells. The most commonly used bioreactors for bone tissue engineering are the spinner flask, perfusion bioreactor and the rotating wall vessel reactor (RWVR) (Yeatts and Fisher, 2011).

2.8 Biomimetic approach of bone tissue engineering

An ideal scaffold should mimic the beneficial properties of the native tissue ECM for tissue regeneration. According to Ma (2008), the natural ECM or a mirror image of it, need not be the ideal scaffold for tissue engineering applications because tissue engineering should be an accelerated regeneration process compared to the natural development program. Therefore, the biomimetic approach of bone tissue engineering can be simply defined as that mimicking certain advantageous features of the natural ECM to facilitate cell recruiting, adhesion, proliferation, differentiation and new bone tissue genesis (Ma, 2008). Three types of information from the ECM are relevant for biomimetic approach of tissue engineering: (i) physical properties like elasticity, stiffness, resilience of the cellular environment; (ii) specific chemical signals; and (iii) the nanoscale topography of the microenvironmental adhesive sites (von der Mark et al., 2010). Willie et al (2010) emphasises the need for a scaffold design that more closely mimics the natural healing cascade by providing an optimal mechanical, physical and chemical environment necessary for the desired cell behaviour.

Biomimetic scaffolds for bone tissue engineering

Bone tissue have mineralized matrix that can withstand significant compressive loads. Therefore, the scaffolds used for BTE should be able to provide a framework for mechanical stability along with interconnected large pores for osteointegration (Grayson
et al., 2009). An ideal scaffold should be osteoinductive, which can initiate the spontaneous induction of bone formation \textit{in vivo}. The biomimetic approach aims at the induction of bone formation by the implantation of smart, self-inducing biomimetic matrices endowed with shape memory geometries that per se initiate the ripple-like cascade of bone differentiation by induction even without the addition of any exogenous growth factors (Ripamonti and Roden, 2010). Developing bone ECM based scaffold is method of choice for mimicking the \textit{in vivo} microenvironment to improve osteogenesis. Construction of highly biomimetic scaffolds with specific biochemical and biophysical cues capable of stimulating native-like cellular activities is crucial in developing successful functional tissue grafts.

The hydroxyapatite- collagen composite scaffolds, which mimic the ECM of bone, are considered as a good option for the biomimetic approach of BTE. Collagen type I promotes cell adhesion and proliferation, and hydroxyapatite acts as a chelating agent for mineralization of osteoblasts in bone tissue regeneration (Landis \textit{et al}.., 1993). Hydroxyapatite has shown to induces biomineralization of apatite by triggering an electrostatic interaction, with positive calcium ions and then with negative phosphate ions (Kim \textit{et al}.., 2004). Biologically inspired biocomposites of collagen type I and nano-HA are bioactive, osteoconductive and osteoinductive, thus ideal for BTE.

In their recent study, Zhang \textit{et al} (2010) incorporated collagen and the resulted nanofibrous hydroxyapatite/collagen/chitosan composite scaffold significantly increased osteoblast proliferation, alkaline phosphatase expression, and mineral deposition. Chitosan is a natural polymer with structural similarity to hyaluronic acid and chondroitin sulphate found in bone (Yang \textit{et al}.., 2009). Chitosan is reported to accelerate wound healing, influence tissue regeneration and osteogenesis (Kuo \textit{et al}.., 2006; Guzmán-Morales \textit{et al}.., 2009). Excellent properties of chitosan such as osteoconductivity,
biocompatibility, tailorable biodegradability, low immunogenicity and better mechanical property make it an interesting biopolymer for bone tissue engineering applications (Francesko and Tzanov, 2011). Chitosan scaffolds alone, cannot imitate all the properties of natural bone, while composite scaffolds of chitosan with hydroxyapatite show promise in mimicking the organic portion, as well as the inorganic portion, of natural bone (Venkatesan and Kim, 2010). Chitosan when incorporated with hydroxyapatite showed significant increases in the osteoblastic cell growth (Manjubala et al., 2008).

**Biomimetic modification of scaffolds for bone tissue engineering**

Biomimetic approach also proposes the modification of existing scaffolds materials with ECM components and bioadhesive motifs like RGD or short peptides derived from extracellular matrix components. Such modifications are aimed at generating biofunctional surfaces with improved biological performance (Garcia and Reyes, 2005). Biomimetic modification of biomaterial or scaffolds with RGD peptide has enhanced osteogenic cell adhesion, proliferation, and differentiation (Zhang et al., 2011).

To improve the osteogenic property of a silk-fibroin scaffold, Zho and co workers (Zhao et al., 2009) used the biomimetic strategy and pre-mineralized the scaffold in a CaCl₂ buffer solution, which resulted in a biomimetic apatite coating. The apatite coating reduced fibrous encapsulation, improved the bone bonding, more bone tissue formation in short time period and better osteointegration.

Another strategy for improving the performance of biomaterials is biomimetic functionalization with bioactive agents. Liu et al (2010) used a biomimetic BMP-2-functionalized calcium phosphate coating capable of releasing BMP-2 gradually in a cell-mediated, physiological-like manner. They observed that significantly higher volume
densities of newly formed bone tissue were consistently induced by coating-incorporated BMP-2 than by the adsorbed BMP-2 of similar amounts.

To summarise, in this chapter, we have discussed about the basic concept, the status and future applications of regenerative therapy for bone tissue regeneration. The *in vivo* microenvironment surrounding the cells play very crucial role in regulating cell adhesion, proliferation and differentiation. Thus, the extracellular matrix based biomimetic approach of tissue regeneration holds great promise in developing functional bone tissue construct.