CHAPTER 2

REVIEW OF LITERATURE

1. Introduction

Stem cells have recently generated more public and professional interest than almost any topic in biology. One reason stem cells capture the imagination of so many is the promise that understanding their unique properties may provide deep insights into the biology of cells as well as a path toward treatments for a verity of degenerative illness.

2. Definition of Stem cells

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells (Weissman *et al.,* 2001). More explicitly, stem cells can generate daughter cells identical to their parented cells (self-renewal) as well as produce progeny with more restricted potential (differentiated cells).

3. Properties of stem cells (Bianchi *et al.,* 2001; Sandhu *et al.,* 2001)

Stem cells differ from other kinds of cells in the body. All stem cells, regardless of their source, have 3 general properties:

1. *Unspecialized*: Stem cell does not have any tissue-specific structures that allow it to perform specialized functions.
2. *Self-renewal*: Stem cell has an ability to go through numerous cycles of cell division while maintaining the undifferentiated state.
3. *Differentiation*: Stem cells can give rise to specialized cells**.**

4. Potency of stem cells (Gardner, 2002).

Potency specifies the differentiation potential (the potential to differentiate into different cell types) of the stem cell.

1. Totipotent: Stem cells that can differentiate into embryonic and extraembryonic cell types. Such cells can construct a complete, viable, organism. These cells are produced from the fusion of an egg and sperm

cell. Cells produced by the first few divisions of the fertilized egg are also totipotent (Fig. 2.1).

2. *Pluripotent*: Stem cells that are the descendants of totipotent cells (Fig. 2.1) and can differentiate into nearly all cells, i.e. cells derived from any of the 3 germ layers.
3. *Multipotent*: Stem cells that can differentiate into a number of cells, but only those of a closely related family of cells (Fig. 2.1).
4. *Oligopotent*: Stem cells that can differentiate into only a few cells, such as lymphoid or myeloid stem cells.
5. *Unipotent*: Stem cells that 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 (Fig. 2.1).

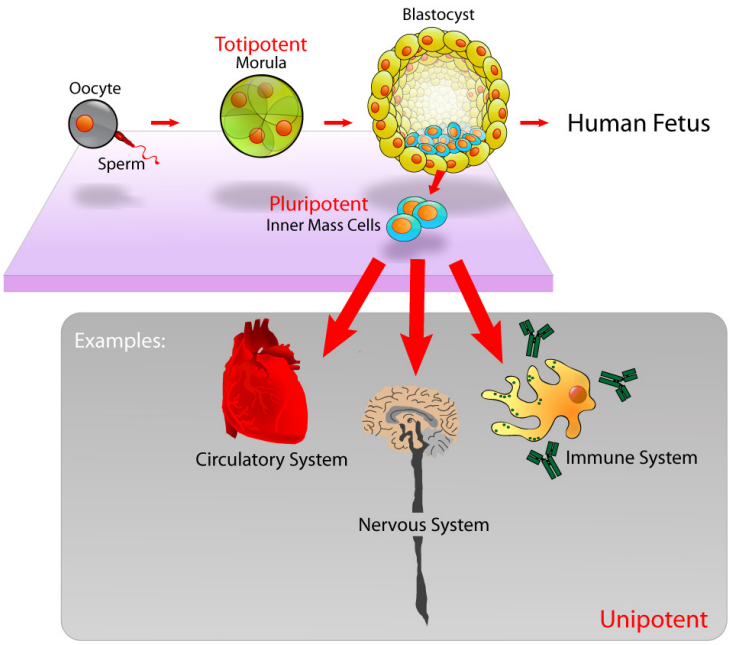

Figure 2.1 Potency of stem cells

(Source: http://upload.wikimedia.org/wikipedia/commons)

5. Types of stem cells

There are 2 main types of stem cells

1. Embryonic stem cells
2. Adult stem cells

6. Embryonic stem cells

Embryonic stem (ES) cells are cultures of cells derived from the epiblast tissue of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryos (Fig. 2.1) (Thomson *et al.,* 1998; Henningson *et al.,* 2003). A blastocyst is an early stage embryo-approximately four to five days old in humans and consisting of 50–150 cells.

ES cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. They do not contribute to the extra-embryonic membranes or the placenta.

Nearly all research to date has taken place using mouse embryonic stem cells (mES) or human embryonic stem cells (hES). Both have the essential stem cell characteristics, yet they require very different environments in order to maintain an undifferentiated state. Mouse ES cells are grown on a layer of gelatin and require the presence of leukemia inhibitory factor (LIF). Human ES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic fibroblast growth factor (bFGF or FGF-2). Without optimal culture conditions or genetic manipulation, ES cells will rapidly differentiate. After nearly ten years of research, there are no approved treatments or human trials using ES cells. ES cells, being pluripotent cells, require specific signals for correct differentiation. If injected directly into another body, ES cells will differentiate into many different types of cells, causing a teratoma (Thomson *et al.,* 1998; Henningson *et al.,* 2003).

7. Adult stem cells

Adult stem cells are thought to be the undifferentiated cells, found among differentiated cells in a tissue or organ that can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Scientists also use the term somatic stem cell instead of adult stem cell, where somatic refers to cells of the body (not the germ cells, sperm or eggs) (Perin *et al.,* 2003).

Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyst), the origin of adult stem cells in some mature tissues is still under investigation. Research on adult stem cells has generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led researchers and clinicians to ask whether adult stem cells could be used for transplants. In fact, adult hematopoietic, or blood-forming, stem cells from bone marrow have been used in transplants for 40 years. Scientists now have evidence that stem cells exist in the brain and the heart. If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies (Raff, 2003).

The history of research on adult stem cells began about 50 years ago. In the 1950s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells), were discovered a few years later. These non-hematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow, and can generate bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue. In the 1960s, scientists who were studying rats discovered two regions of the brain that contained dividing cells that ultimately become nerve cells. Despite these reports, most scientists believed that the adult brain could not generate new nerve cells. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types astrocytes and oligodendrocytes, which are non-neuronal cells and neurons or nerve cells (Filip *et al.,* 2003; Perin *et al.,* 2003; Tuan *et al.,* 2003).

A great deal of adult stem cell research has focused on clarifying their capacity to divide or self-renew indefinitely and their differentiation potential (Raff 2003). Most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, etc. (Barrilleaux *et al.,* 2006; Gimble *et al.,* 2007). Adult stem cell treatments have been successfully used for many years to treat leukemia and related bone/blood cancers through bone marrow transplants. The use of adult stem cells in research and therapy is not as controversial as embryonic stem cells,

because the production of adult stem cells does not require the destruction of an embryo. Additionally, because in some instances adult stem cells can be obtained from the intended recipient, (an autograft) the risk of rejection is essentially non-existent in these situations.

Source of adult stem cells

Adult stem cells have been identified in many organs and tissues (Fig. 2.2), including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis (Douay *et al.,* 2009; Skutella, 2009).

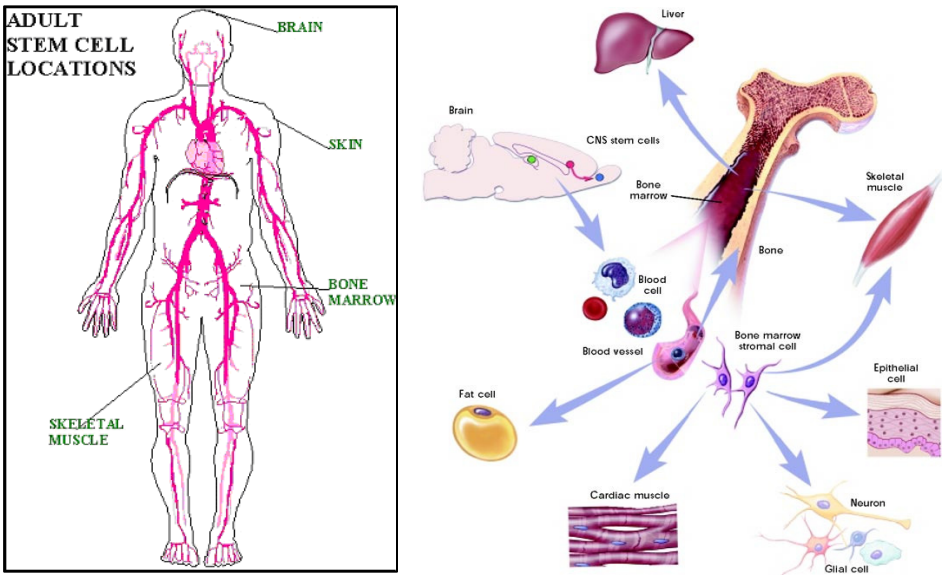

Figure 2.2 Source of adult stem cells

(Source: www.topnews.in/health/files/Adult-Stem-Cells.jpg)

8. Hematopoietic stem cells (HSCs)

Hematopoietic stem cells (HSCs) are multipotent stem cells that give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells). The hematopoietic tissue contains cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent, and unipotent progenitors (Muller-Sieburg *et al.,* 2002).

Source of HSCs

HSCs are found in the bone marrow of adults (Fig. 2.2), which includes femurs, hip, ribs, sternum, and other bones. Cells can be obtained directly by removal from the iliac crest using a needle and syringe, or from the blood following pre-treatment with cytokines, such as G-CSF (granulocyte colony-stimulating factors), that induce cells to be released from the bone marrow compartment. Other sources for clinical and scientific use include umbilical cord blood (Muller-Sieburg *et al.,* 2002)**.**

Morphological Characteristics of HSCs

As stem cells, they are defined by their ability to form multiple cell types (multi-potency) and their ability to self-renew. It is known that a small number of HSCs can expand to generate a very large number of progeny HSCs. Stem cell self-renewal is thought to occur in the stem cell niche in the bone marrow, and it is reasonable to assume that key signals present in this niche will be important in self-renewal. There is much interest in the environmental and molecular requirements for HSC self-renewal, as understanding the ability of HSC to replenish themselves will eventually allow the generation of expanded populations of HSC *ex vivo* that can be used therapeutically (Muller-Sieburg *et al.,* 2002; Sieburg *et al.,* 2006).

Physical characteristics of HSCs

With regard to morphology, HSCs resemble lymphocytes. They are non-adherent, and rounded, with a rounded nucleus and low cytoplasm-to-nucleus ratio. Since HSCs cannot be isolated as a pure population, it is not possible to identify them in a microscope. The above description is based on the morphological characteristics of a heterogeneous population, of which HSCs are a component (Muller-Sieburg *et al.,* 2002; Sieburg *et al.,* 2006).

Specific markers of HSCs

In reference to phenotype, hematopoeitic stem cells are identified by their small size, lack of lineage (Lin) markers, low staining with vital dyes such as rhodamine 123 (rhodamine^{dull}, also called rho^{lo}) or Hoechst 33342, and presence of various antigenic markers on their surface, many of which belong to the cluster of

differentiation series, like: CD34, CD38, CD45 and also c-kit the receptor for stem cell factor. The hematopoietic stem cells are negative for the markers that are used for detection of lineage commitment, and are, thus, called Lin-; and, during their purification by FACS, a bunch of up to 14 different mature blood-lineage markers, e.g., CD13 & CD33 for myeloid, CD71 for erythroid, CD19 for B cells, CD61 for megakaryocytic, etc. for humans; and, B220 (murine CD45) for B cells, Mac-1 (CD11b/CD18) for monocytes, Gr-1 for Granulocytes, Ter119 for erythroid cells, Il7Ra, CD3, CD4, CD5, CD8 for T cells, etc. for mice antibodies are used as a mixture to deplete the lin+ cells or late multipotent progenitors (MPPs) (Muller-Sieburg *et al.,* 2002; Sieburg *et al.,* 2006).

9. Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are non-hematopoietic cells, which reside in the bone marrow together with better known and characterized class of stem cells hematopoietic stem cells. They were first described by Fridenstein *et al.* in 1976, as the clonal, plastic adherent cells, being a source of the osteoblastic, adipogenic and chondrogenic cell lines (Fig. 2.3) (Friedenstein, 1976). The interest in MSCs rapidly grows with expanding knowledge about their exceptional characteristics and usefulness in the clinical application.

Sources of MSCs

The main source of MSCs is the bone marrow. These cells constitute, however, only a small percentage of the total number of bone marrow populating cells. Pittenger *et al.* (2002) showed that only 0.01% to 0.001% of mononuclear cells isolated on density gradient (Ficoll/Percoll) give rise to plastic adherent fibroblast like colonies. The number of MSCs isolated from this tissue may vary in terms of the yield and the quality, even when the cells are obtained from the same donor (Phinney *et al.,* 1999). Apart from the bone marrow, MSCs are also located in other tissues of the human body. There is an increasing number of reports describing their presence in adipose tissue (Gaetani *et al.,* 2008), umbilical cord blood, chorionic villi of the placenta (Igura *et al.,* 2004), amniotic fluid (Tsai *et al.,* 2004), and peripheral blood (Zvaifler *et al.,* 2000). The amount of MSCs decreases with age and infirmity (Fibbe *et al.,* 1996). The greatest number of MSCs is found

in neonates including umbilical cord, placenta and amnion (Igura *et al.,* 2004; Tsai *et al.,* 2004).

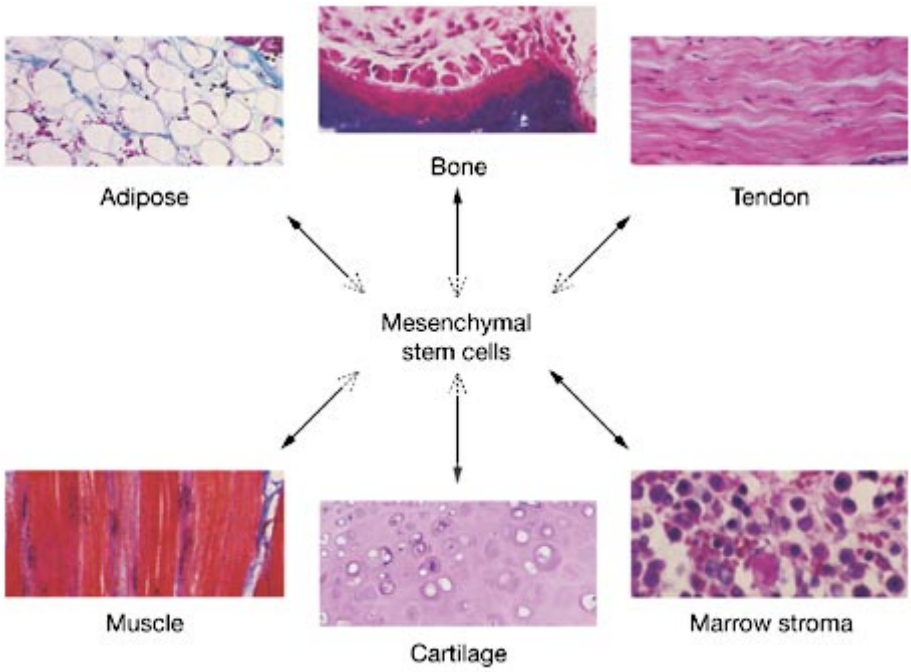

Figure 2.3 Derivations of mesenchymal stem cells
(Source: www.nature.com/n7/images/ncprheum0216-f3.jpg)

Morphological characteristics of MSCs

MSCs are characterized morphologically by a small cell body with a few cell processes that are long and thin (Fig. 2.4). The cell body contains a large, round nucleus with a prominent nucleolus which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. The remainder of the cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. The cells, which are long and thin, are widely dispersed and the adjacent extracellular matrix is populated by a few reticular fibrils but is devoid of the other types of collagen fibrils (Takagi *et al.,* 2008).

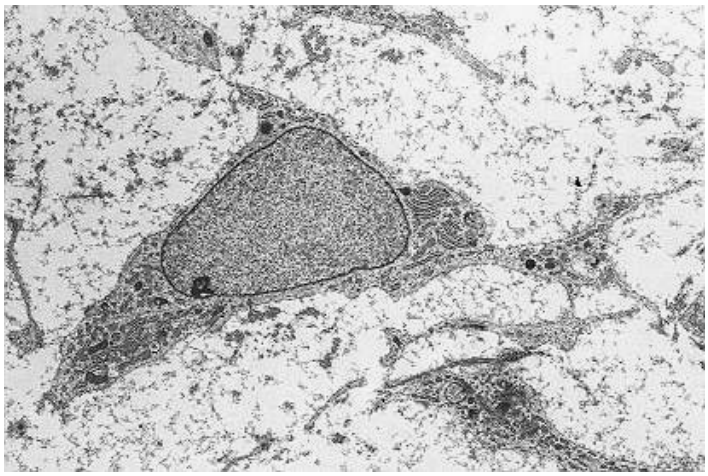

Figure 2.4 Transmission electron micrograph demonstrated the morphology of mesenchymal stem cells
(Source: http://upload.wikimedia.org/wikipedia/commons/f/f4)

Cell surface markers of MSCs

MSCs constitute a heterogeneous population of cells, in terms of their morphology, physiology and expression of surface antigens. Up to now, no single specific marker has been identified. MSCs express a large number of adhesion molecules, extracellular matrix proteins, cytokines and growth factor receptors, associated with their function and cell interactions within the bone marrow stroma (Devine *et al.,* 2000). They also express a wide variety of antigens characteristic for other cell types. The population of MSCs isolated from bone marrow express: CD44, CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD166, CD29, CD73 (SH3 and SH4), CD90 (Thy-1), CD117, STRO-1 and Sca-1 (Cognet *et al.,* 1999; Baddoo *et al.,* 2003; Gronthos *et al.,* 2003; Boiret e*t al.,* 2005). MSCs do not possess markers typical for hematopoietic and endothelial cell lineages: CD11b, CD14, CD31, CD33, CD34, CD133 and CD45. The absence of CD14, CD34 and CD45 antigens on their surface create the basis to distinguish them from the hematopoietic precursors (Baddoo *et al.,* 2003).

There have been studies to find an accurate combination of a limited number of antigens in order to isolate pure population of MSCs from a tissue. From the data available up to now, several options have been proposed in this context. One of them suggests that the co-expression of CD105 and CD73 could be sufficient

(Pittenger *et al.,* 2002). Another one implies that the expression of CD166 and CD105 makes it possible to separate the earliest precursors of MSCs from more mature cells (Alsalameh *et al.,* 2004). In turn, examination of the CFU-F obtained from bone marrow stroma demonstrated that the MSCs fraction may by identified by several markers, including STRO-1, Thy-1, CD49a, CD10, Muc18/CD146, as well as with the antibodies to receptors for PDGF (platelet derived growth factor) and EGF (epithelial growth factor) (Pittenger *et al.,* 2002; Baddoo *et al.,*. 2003; Gronthos *et al.,* 2003). Although MSCs have been described by a subset of surface antigens, little is known about fresh or non-expanded MSCs, mostly because of their very low frequency in adult bone marrow (Boiret *et al.,* 2005). The findings by Boiret *et al.* showed that the most discriminative markers for MSCs examined after short time of adherence (1-3 days) were: CD73 and CD49a, as all the CFU-F-colonies (100%) were CD73 and most (95.2%) were CD49a- positive (Boiret *et al.,* 2005). Interestingly, these data did not confirm that CD105 and CD90 could be selective markers for MSCs, as only 45.4% and 49% of the CFU-F were positive for these molecules, respectively. Furthermore, the authors checked the surface protein expression on freshly isolated bone marrow MSCs, showing, as found previously, that CD73 and CD49a were the most extensively expressed antigens in CFU-F-enriched subset. These results stand in opposition with the popular description of MSC phenotype, which postulated the STRO-1 antigen to be exclusively expressed by primitive mesenchymal precursors. However, the presence of some antigens may change *in vitro*, due to specific culture conditions and the duration prior to individual passages. Interestingly, some antigens may be found on freshly isolated MSCs, but their expression disappears in culture. Such a phenomenon was observed in case of CD34 antigen. This molecule was expressed by MSCs obtained from mouse fetal lungs, but could not be found in *in vitro* cultures of MSCs. (Fibbe and Noort, 2003) This would suggest that the expression of that molecule vanishes during the maturation process. Similar results were obtained in case of chemokine receptor expression on human MSCs (Honczarenko *et al.,* 2006). The second passage BM-MSCs expressed: CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6. At the 12-16 passage, there was no expression of any of those molecules, which was also confirmed by a disability of the cells to migrate towards specific chemokine attractants. Moreover, the loss of these

receptors expression was accompanied by a decrease in the expression of adhesion molecules: ICAM-1, ICAM-2, VCAM-1 and CD157. Moreover, the alteration in BM-MSCs phenotype was associated with increasing cell cycle arrest and induction of the apoptotic pathway (Honczarenko *et al.,* 2006). The change in antigen expression has been also described for MSCs undergoing differentiation process.

Biology and functions of MSCs

Human MSCs are known to constitute a heterogeneous population of cells and their properties and functionality depend on the environmental characteristics. MSCs can be expanded in culture and give rise to fibroblastic colonies (CFU-F). The CFU-F units are well documented to possess an extended proliferative potential *in vitro* (Dazzi *et al.,* 2006). The number of colonies obtained from bone marrow aspirates differs among species, as well as throughout the culture conditions used in each individual experiment. Colony formation by MSCs derived from adult human BM is feeder cell independent. The cultures of MSCs are, however, not completely explored. Former studies claimed that MSCs isolated from bone marrow comprise a single phenotypic population forming symmetric, spindle-shaped colonies (homology up to 98%). More recent studies, however, indicate that single-cell derived colonies are morphologically heterogeneous, containing at least two different cell types: small spindle shaped cells and large cuboidal or flattened cells (Colter *et al.,* 2000). These cells were, thus, proposed to represent an *ex vivo* subset of recycling uncommitted MSCs (Colter *et al.,* 2000). Nevertheless, the latest findings show that MSC colonies contain as much as three types of cells. The third fraction was described to be composed of very small rapidly self-renewing cells (Colter *et al.,* 2001), which are reported as the earliest progenitors and possess the greatest potential for multilineage differentiation. The examination of these cells revealed that they were about 7 μm in diameter and had a high nucleus-to-cytoplasm ratio. They could be also distinguished from more mature cells by the presence of specific surface epitopes and expressed proteins, like vascular endothelial growth factor receptor-2, tyrosine kinase receptor and transferrin receptor and annexin II (lipocortin 2). Some of the rapidly renewing cells contained also other markers, like c-kit (CD117), multi-drug resistance epitope and epithelial membrane antigen. Interestingly, these cells were negative for STRO-1, an antigen

originally MSCs play a significant role in bone marrow microenvironment. The major function of these cells is to create a tissue framework, which assures a mechanical support for hematopoietic cell system. They secrete a number of extracellular matrix proteins, including fibronectin, laminin, collagen and proteoglycans. Moreover, MSCs produce hematopoietic and non-hematopoietic growth factors, chemokines and cytokines, thereby participating in the regulation of hemopoiesis. MSCs secrete: IL-1a, IL-1b, IL-6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor, stem cell factor (SCF), fetal liver tyrosine kinase-3, thrombopoietin and hepatocyte growth factor (HGF) (Colter *et al.,* 2000; Gronthos *et al.,* 2003; Boiret *et al.,* 2005; Dazzi *et al.,* 2006). Some of these proteins are produced by quiescent cells, whereas the others after stimulation.

The involvement of MSCs in hematopoiesis is additionally consolidated by their presence in fetal liver and bone marrow just prior to the onset of definitive hemopoiesis at those sites (Campagnoli *et al.,* 2001) A study in animal model confirmed that human MSCs marked with Green fluorescent protein (GFP) and transplanted into the tibia of NOD/SCID mice, integrated into the functional components of hematopoietic microenvironment and actively participated in the hematopoietic cell development (Muguruma *et al.,* 2006). During 4 to 10 weeks after transplantation, GFP-MSCs differentiated into pericytes, myofibroblasts, stromal cells, osteocytes and endothelial cells. This led to the increase in the number of functionally and phenotypically primitive human hematopoietic cells in murine bone marrow microenvironment. The engrafted cells supported human hematopoiesis via secreted factors and by physical interactions with primitive hematopoietic cells (Muguruma *et al.,* 2006). Other studies showed that co-transplantation of human MSCs and HSCs resulted in increased chimerism or/and accelerated hematopoietic recovery in animal models and in humans (Fibbe *et al.,* 1996). Moreover, MSCs are known to produce a variety of cytokines that are involved in homing (stromal derived factor-1; SDF-1) or proliferation and differentiation of hematopoietic cells (GM-CSF, SCF, IL-6) (Hoffmann *et al.,* 2002). It has been proposed that several chemokine axes are involved in maintaining bone marrow homeostasis, and that some chemokines, which MSCs possess the receptors for, like CCR9 and CXCR4 may operate in an autocrine

manner, similarly as it is in case of HSCs (Honczarenko *et al.,* 2006). Among other well known biological activities of MSCs, it is worth to emphasize their immunomodulatory functions. These cells are able to inhibit responses of alloreactive T lymphocytes. They express neither MHC class II molecules nor co-stimulatory receptors (CD80, CD86) on their surface, therefore they do not addition of interferon-γ (IFN-γ) to the cultures of MSCs enhances the expression of MHC class I and triggers the expression of MHC class II, but not of the co-stimulatory molecules (Fibbe and Noort, 2003). It has been well established that MSCs from various species can exert profound immunosupression by inhibiting T-cell responses to polyclonal stimuli and to their cognate peptide (Krampera *et al.,* 2003). The inhibition did not seem to be antigen specific and targeted both primary and secondary T cell responses (Krampera *et al.,* 2003). The inhibitory effect was shown to be directed mostly at the level of cell proliferation. T cells stimulated in the presence of MSCs were arrested in the G1 phase as a result of cyclin D down regulation (Glennie *et al.,* 2005). The suppression, however, was not apoptotic and could be reversed. In the absence of MSCs and with appropriate stimuli, T cells continue to proliferate. The precise mechanism by which MSCs modulate immunological response is still to be clarified, but overall data suggest that soluble factors as well as cell contact mediated mechanisms are involved. Blocking experiments with the use of neutralizing monoclonal antibodies against transforming growth factor-β (TGF-β) and suggest that these factors are at least in part responsible for the inhibitory effects caused by MSCs. Moreover, MSCs can affect other cells participating in immune response like B cells (Glennie *et al.,* 2005) and dendritic cells (Jiang *et al.,* 2005).

Growth and expansion of MSCs

Various protocols have been developed to grow and expand BM-MSCs. Cells which initially adhere to the tissue culture plastic, display fibroblastic appearance and develop into symmetrical colonies between 5 and 7 days after plating. Human MSCs proliferate most rapidly and maximally retain their multipotential ability when cultured at relatively low densities (Tropel *et al.,* 2004). These cells may be seeded at the range from 1×10^4 to 4×10^5 cells/cm^2. The initial culture concentration affects not only growth of MSCs but also their morphology

(Tropel *et al.,* 2004). When the cells are grown at a low density, they mostly display a spindle-like shape, but when they reach confluence and start to grow in several layers, the cells become flat with torn ends. *In vitro* growth of MSCs is characterized by the occurrence of three phases, similarly to other progenitor cells: (i) an opening lag phase, which lasts for 3-4 days, followed by (ii) a rapid expansion (log phase) and closes with (iii) a stationary phase (Colter *et al.,* 2001). The last stage does not rely on cell contact inhibition and replating the cells triggers their growth for approximately five more passages (Colter *et al.,* 2001). Prockop *et al.* (2003) suggests that the shift between different stages is regulated mainly by the expression of Dickkopf-1 (Dkk-1) and Wnt5a genes, which play opposite roles. The greatest expression of Dkk-1 appears during the log phase and shortens the former stage by inhibition of Wnt5a expression, whereas Wnt5a protein level becomes maximal during the stationary phase. Under optimal conditions, MSCs can be maintained in culture for 20-30 population doublings and still retain their capacity for differentiation (Tropel *et al.,* 2004). More recent studies show that these cells are able to grow and divide for even more than 50 population doublings. This indicates a great proliferative potential of these cells. Examination of the cell cycle profile of MSCs revealed that about 10% of these cells occur in phases S, G2 and M of the cell cycle, while the vast majority of the cells remain in the G0/G1 phase (Cognet *et al.,* 1999). However, extensive subcultivation of MSCs impairs their functionality and the cells display evident signs of senescence and/or apoptosis. Proliferation of MSCs is influenced by a number of cytokines and growth factors. The list of hormones and other molecules involved in the regulation of CFU-F proliferation *in vitro* is growing (Wang *et al.,* 2000). PDGF and fibroblast growth factor-2 (FGF-2) have been shown to be potent mitogens for CFU-F , and EGF exerts the same effect on STRO-1 enriched population of MSCs. Opposite results can be obtained after addition of interferon-alpha and interleukin 4 to the culture. Both cytokines inhibit colony formation stimulated by the combination of EGF and PDGF in a dose-dependent manner. Additionally, it was demonstrated that binding of heparin-binding epidermal growth factor (HB-EGF) to its receptor HER-1 on MSCs, consolidates proliferation and prevents differentiation of these cells induced by conditioning. Thus, it can be speculated that the HB-EGF/HER-1 axis is important for MSC renewal and differentiation. The proliferative activity of MSCs

was shown to be directly proportional to their differentiation potential (Prockop, 2003).

Differentiation potential of MSCs

It is still not clear if there is one multipotent MSCs that gives rise to each cell of mesenchymal origin, or a mixture of progenitor cells committed to different cell lineages. In earlier studies it was believed that MSCs could differentiate only into tissues of mesodermal origin. Recently, according to large-scale studies on MSC biology, this dogma has been changed. Successful differentiation has been achieved in a variety of cell lineages, including osteoblasts, chondrocytes, adipocytes (Fig. 2.5), fibroblasts, myoblasts and cardiomyocytes, hepatocytes, and even neurons (Egusa *et al.,* 2005; Kosmacheva *et al.,* 2008) .

However, some scientists hypothesize that generating cells of origin different than mesodermal, is due to specific reprogramming process of gene expression in MSCs (Kim *et al.,* 2007) or occurs as a result of particular soluble factor activity. According to the former hypothesis, it was believed that MSCs undergo a process called 'stem cells plasticity; changing their lineage commitment. One of such theories, termed stochastic repression/induction model, claims that differentiation potential observed for various sets of MSCs arises from a series of gene silencing events occurring during development (Dennis, 2002). This results in the appearance of diverse MSC populations capable of expressing different cell-commitment genes. However, the data from other investigators rebut a statement about MSC plasticity (Ratajczak *et al.,* 2004). Pittenger *et al.* (2002) reported that approximately one-third of them might be successfully directed to the osteogenic, chondrogenic and adipogenic lineages. *In vitro* differentiation into particular cell lineage demands treating the cells with a proper mixture of specific differentiating factors. It must be mentioned that basal nutrients, cell density, spatial organization, mechanical forces, growth factors and cytokines, all play a role in MSC differentiation. It has also been reported that the differentiation potential may differ in the relation to the source of MSCs.

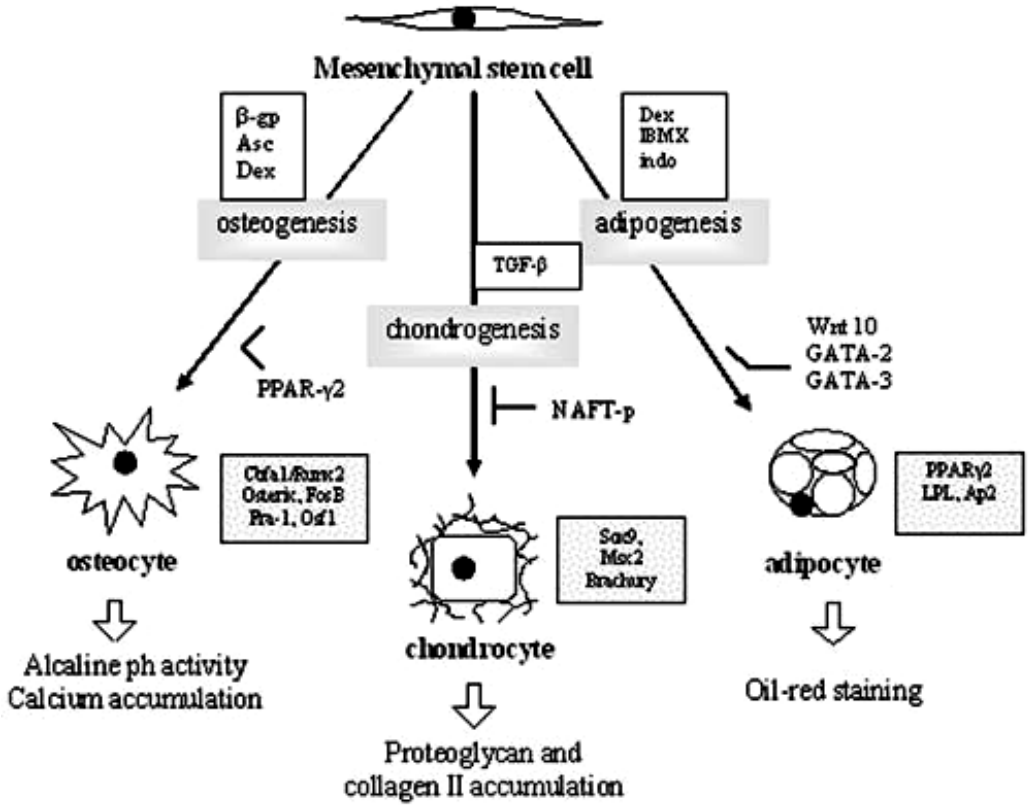

Figure 2.5 The scheme of MSC differentiation into the three mesenchymal lineages: osteocytes, chondrocytes and adipocytes. The upper boxes contain inducing factors for each of these pathways, and the lower ones - the major transcription factors (shadowed). Ways to identify differentiated cells are pointed by empty arrows. Abbreviations: β-gp - β-glycerophosphate; Asc - ascorbic acid; Dex - dexamethasone; TGF-β - transforming growth factor-β; IBMX - isobutylmethylxanthine; indo-indomethacin; PPARγ2 -peroxisome proliferation-activated receptor γ2; NAFT–nuclear factor of activated T cell; LPL - lipoprotein lipase; Ap2 - fatty acid binding protein.

In order to obtain osteoblastic cell line (Fig. 2.5), the confluent monolayer of MSCs should be incubated with a mixture containing β-glycerophosphate, ascorbic acid and dexamethasone, throughout the period of 2-3 weeks (Dennis and Charbord, 2002). Participation of bone morphogenetic proteins (BMPs) in bone formation process has been also postulated, although different BMPs play different roles (Jaiswal *et al.,* 2000; Cheng *et al.,* 2003; Noel *et al.,* 2004; Krampera *et al.,* 2005). Other important factors involved in osteogenic regulation are: TGF, insulin-like growth factor (IGF), brain-derived growth factor (BDGF), FGF-2, leptin and parathyroid hormone related peptide (PTHrP) (Lou *et al.,* 1999). These proteins regulate secretion of matrix proteins and the expression of signals necessary for bone remodeling through osteoclast activation. Among the transcription factors committed to osteogenesis, pivotal roles are attributed to Cbfa1/Runx2, Osterix, ΔFosB, Fra-1, Aj18 and Osf1. Apart from them, Msx2, Dlx5 and TWIST were shown to take part in this process (Zelzer and Olsen, 2003). As it was documented, Cbfa1/Runx2 is necessary for osteoblast formation, but only Dlx5 allows distinguishing the mineralized osteoblasts. Progression of osteogenesis might be measured through alkaline phosphatase activity and calcium accumulation (Pittenger *et al.,* 1999). Human MSCs were shown to possess a great potential to differentiate into osteoblasts, which was maintained for up to 40 doublings in culture, even after cryopreservation .

Chondrogenesis (Fig. 2.5) in turn is classically carried out in micro-mass cultures of MSCs after addition of TGF-β. Among TGF-β family members, the most important role in chondrogenesis play BMPs and cartilage derived morphogenetic proteins (CDMPs) (Dennis and Charbord, 2002). Apart from BMP signaling, cooperation between BMPs and members of Hedgehog family (Hh) has been reported (Yamaguchi *et al.,* 2000). A regulatory role in this process has been attributed to the proteins from Wnt family. Among them, Wnt-4 and Wnt-14 were shown to display high expression at sites of future joint development, whereas Wnt-7a was shown to inhibit chondrogenesis (Hwang *et al.,* 2004). Additionally, as recent data indicate, the signaling triggered by the FGF receptor 3 is sufficient to induce chondrogenic differentiation (Hoffmann *et al.,* 2002). TGF-β and related cytokines exhibit the ability to induce signal transduction pathways specific for chondrogenesis, mostly via activation of mitogen-acivated protein (MAP) kinases

such as: ERK-1, p38, PKC and Jun (Sekiya *et al.,* 2002), whereas FGF receptor acts through Smad protein signaling. The activation leads to induction of specific transcription factors expression. The most important roles play Sox9, Msx2 and Brachury (Hoffmann *et al.,* 2002). They were shown to activate the expression of chondrocyte-specific genes, like aggrecan and collagen II. Participation in this process has been also shown for Hox, Pax and Forkhead. Chondrogenic formation, except from morphological changes, may be verified by histological testing for the presence of proteoglycan in the extracellular matrix and collagen type II chains, which are typical of articular cartilage (Pittenger *et al.,* 1999). Inhibitory effect on chondrogenesis may be achieved through nuclear factor of activated T cell -NAFT-p activity (Ranger *et al.,* 2000).

In vitro adipogenesis (Fig. 2.5) can be induced by treating MSCs with a hormonal cocktail containing dexamethasone, isobutyl methyl xanthine (IBMX) and indomethacin. The differentiation might be confirmed using Oil-red O staining technique and controlling the expression of specific proteins, such as peroxisome proliferation-activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL), and the fatty acid binding protein (Ap2). Inhibition of adipogenesis can by accomplished by the induction of Wnt10b (Ross *et al.,* 2000), GATA-2 and GATA-3. An interesting role in MSCs differentiation toward osteoblastic versus adipogenic cell lineage is played by BMP proteins. The BMP-2 as well as bFGF have been shown to synergistically enhance *in vivo* bone formation by MSCs . Selective blocking of the BMP receptor type 1B (BMPR-1β) resulted in the differentiation into adipocytes, which would likewise suggest that the expression of this receptor is required for osteocyte formation. Conversely, over expression of BMPR-1A blocked adipogenic differentiation and prompted osteoblastic generation. The findings indicate that changes in the BMP receptor levels are intrinsic factors for the commitment into adipogenic or osteoblastic cell line. Additionally, adipocyte transcription factor - PPARγ2 was demonstrated to repress osteogenesis. Apart from factors inducing differentiation into the three cell lines described above, the molecules promoting other cell lineage formation, like myocardium and even neurons, have been identified, but they are not completely defined so far (Heng *et al.,* 2004; Egusa *et al.,* 2005).

Clinical application of MSCs

The specific characteristics of MSCs, including their extensive proliferative potential and the ability to differentiate into various cell types, like bone, fat and cartilage, make them an attractive tool in regenerative medicine. This is especially evident in such fields as cellular biology and gene therapy, resulting in considerable increase in the number of clinical trials based on the use of MSCs.

Apparently, these cells might be simply isolated from various tissues and expanded in culture in large numbers that gives the opportunity to create a tissue-engineered constructs containing these cells and reintroduce them into a patient (Sakai *et al.,* 2003). Full healing is a complex process and demands integration of the regenerated tissue with the surrounding host tissues and differentiation through the natural signaling pathways. As it was documented, MSCs possess the capacity to engraft into various tissues and organs when infused systematically, and this engraftment has been shown to be stable in the long term (Devine and Hoffman, 2000). Even more, MSCs infused to the peripheral circulation have the ability to migrate to a specific site of injury. This phenomenon has been reported in animal models of bone fracture, cerebral ischemia and myocardial infarction. Previous study, the authors managed to localize MSCs transplanted to neonatal mice, using the whole body imaging technique (Niyibizi *et al.,* 2004). On the 7^{th} day post injection, the cells presented a wide distribution throughout the body of the recipient mice. Eighteen days later, the majority of infused cells were found in lungs and liver, and a very small population was present in other tissues. Finally, 35 days post infusion, a significant number of the cells was located in bones, indicating that these cells may participate in bone formation . Interesting results were delivered by Prockop *et al.* (2003), who examined the MSC engraftment efficiency in various tissues in immunodeficient mice, using a sensitive RT-PCR method. The engraftment appeared to be at a very low level, and varied in different tissues.

Interestingly, the survey revealed the presence of a subpopulation of small size MSCs- rapidly self renewing MCSs (RS-MSCs), which engrafted preferentially in comparison to a larger, slowly renewing MSCs (SR-MSCs). The two subpopulations varied not only in terms of differentiation potential but also in the surface epitopes. The more effective engraftment of RS-MSCs might be partially

explained by their expression of CXCR4 and CXCR1, which are known to be involved in the trafficking of MSCs (Lecka-Czernik *et al.,* 1999).

MSCs have been also proposed to be an excellent potential tool for gene therapies. They can be subjected to various genetic modifications, such as transduction with viral vectors carrying a therapeutic gene or cDNA for special proteins, serving as molecular transmitters. In a mouse model, the genetically modified MSCs implanted in an ectopic site and subsequently transplanted to a secondary donor, showed about 74% stable gene transfer efficiency. They could be therefore useful in delivering particular genes into organs or a tissue of special need. Furthermore, there have been clinical studies in humans with MSCs transfected with viral vectors containing the gene for coagulation factor VII or IX, in case of haemophilia treatment. These cells are also metabolically active and may serve as a suitable source secreting therapeutic proteins, such as defective enzymes. When successful, this approach could bring outstanding results in tissue and body repair.

One of the fields for MSCs use in regenerative medicine is the treatment of bone defects. First approach to bone repair relied on biodegradable scaffolds impregnated with recombinant BMPs, and was designed to induce bone formation through the recruitment of local MSCs (Lane *et al.,* 1999). This project was successfully accomplished in an animal model (Lewis rats), showing that MSCs attracted to BMP-2 are able to regenerate the injured bone. As another example, MSCs were activated through the intramuscular injection of adenovirus-mediated hBMP-2 gene transfer in nude mice, which resulted in local MSC proliferation and differentiation. Furthermore, a portion of implanted cells were competent themselves to respond to the factors in an autocrine or paracrine way. The bone healing using MSCs might be improved with the use of other specific cytokines, like IGF, PDGF and FGF. With reference to numerous clinical trials using MSCs, a special attention ought to be paid toward osteogenesis imperfecta (OI) treatment. This is a genetic disorder resulting from mutations in collagen I gene, causing many abnormalities especially in bone structure. There have been over 150 mutations responsible for the OI outcome identified, affecting COL1A1 and COL1A2 genes. As collagen is the major protein of the extracellular matrix of the bone, the patients with OI suffer from frequent and numerous fractures, progressive deformities of

limbs and spine, retarded bone growth and short stature (Horwitz *et al.,* 2001). Therefore, a treatment strategy for OI is mainly aimed at improving bone strength through ameliorating the structural integrity of collagen. Among therapies applied to OI, only cell and gene regimens brought positive effect and seem to be the only reasonable tools.

The cell therapy approach targeted to osteoblast formation from MSCs was first investigated on murine models. MSCs isolated from transgenic mice were transplanted into irradiated recipient mice. The location of these cells was inspected 1-5 months after cell infusion. According to the results, 1.5%-12% of the cells were found in various tissues, including bones. Other studies were performed using immunodeficient SCID mouse model, confirming the homing capacity of hMSCs to the bone marrow and the ability to differentiate into osteoblasts *in vivo* (Horwitz *et al.,* 2001). The first steps in therapeutic approach using MSC transplantation in OI patients were done by Horwitz *et al.* in 1999. Allogenic unmanipulated bone marrow from HLA-identical or single-antigen-mismatched siblings was transplanted to three children with OI. The therapeutic outcome was successful (1.5%-2% of engraftment), showing donor-derived MSCs located in the bone marrow of the recipient. Bone marrow MSCs were able to give rise to properly functioning osteoblasts, resulting in the increase in bone mineral content, as well as the improvement in growth velocity and the reduction of bone fracture frequencies. Encouraged by the results, the authors performed next trials. Bone marrow was obtained from allogenic, HLA-compatible, sibling donors and was given twice to each patient. Among the five children enrolled in this study, three appeared chimeric and showed donor osteoblast engraftment. As a result, those children gained significant increase in total body length with a median of 7.5 cm, measured 6 months after transplantation, in comparison to 1.25 cm for control patients. Moreover, the bone mineral content improved by 45% to 77% of the baseline values. The number of fractures, visualised by radiography, declined from an average of 10 during 6 months before treatment, to 2. Unfortunately, the follow-up study demonstrated that the growth ratio either decreased or remained unchanged. In contrast, bone mineralization continued to increase. Better results were obtained when purified population of MSCs was used for grafting. Such a survey was performed by Horwitz *et al.* in 2001, demonstrating the successful engraftment of

MSCs (Horwitz *et al.,* 2001). The study enrolled six children, each of them received two infusions of the allogenic cells. MSCs were transduced with the LNc8 or G1PLII retroviral vectors, in order to localize the engrafted cells in patients. The vectors contained either the neomycine phosphotransferase gene (neoR) or nonexpressing β-galactosidase (β-gal) and neoR sequences, respectively. The transduction efficiency was in a range from 2% to 25%. The cells expressing G1PLII marker were detected in five patients, at least at one site. The localization included bones, skin and marrow stroma and brought a positive healing effect expressed as the acceleration of growth velocity, in a range from 60% to 94% of the predicted values for age- and sex-matched healthy children . Furthermore, there has been a novel clinical trail of *in utero* MSC transplantation in patient with severe OI. Allogenic, HLA-incompatible MSCs obtained from a human male fetal liver, were injected to the umbilical vein at the week 32 of gestation, in a total number of 6.5×10^6 cells. After a baby-girl delivery, a centromeric XY-chromosome-specific probe revealed 0.3% of the donor cells. Interestingly, when examining whole male genome, the detection of Y chromosome positive cells showed 7.4% of the engraftment. There was no immunoreactivity against transplanted cell detected, indicating the safety of the study. The outcome was outstanding, demonstrating the improvement of bone mineralization from 48% at 3 months to 56% at 12 months and 76% at 22 months, in comparison to age-matched controls. However, this increase may be partially attributed to pamidronate treatment, started from the 4^{th} month. The follow-up revealed only 3 fractures during the first two years, normal psychomotor development and correct growth tendency. A new approach toward OI treatment has been developed with the occurrence of gene therapy. In the picture of the disease, the product of mutant allele interferes with the peptide produced by normal allele, resulting in abnormal collagen fibril formation. The gene therapy therefore, should be first directed toward silencing of the mutant allele expression, and then replacing the mutated gene. This can be achieved either by degradation of the mutant mRNA or by disruption of the mutant gene. However, the treatment strategy might be complicated by the genetic heterogeneity of the disease and the fact, that most OI mutations are dominant-negative. Gene therapy trial combined with the use of MSCs was performed by the Russel's group, who performed *ex vivo* genetic modification of autological MSCs from OI patients. The cells were targeted

with viral vector AAV-COLe1INpA that was designed to disrupt exon 1 of the chromosomal *COL1A1* gene, by inserting an inactivating cassette. This would change the mutated gene into a null form, eliminating the production of abnormal collagen chains, thus leading to mild disease symptoms. The results demonstrated that 31% to 90% of the positively selected MSC clones (0.06% to 0.23% of unselected MSCs) underwent gene targeting at one allele of *COL1A1* gene. There were very similar targeting frequencies at mutant and wild type alleles, suggesting that there was no allele preference in this process. Furthermore, very similar targeting frequencies in a range of 90% were observed in polyclonal, as well as in monoclonal cell population. Gene modification improved collagen processing, stability and structure, thus preventing pro-collagen peptide retention within the cells. Moreover, the diameter of collagen fibrils, as well as the melting temperature was dramatically improved, resembling the values obtained for wild-type cells. The targeted cells were also tested for bone and fat formation ability *in vivo*, demonstrating their multilineage potential.

Another great challenge for tissue engineering using MSCs is the treatment of cartilage lesions. The first reports handling this issue come from Wakitani *et al.* (1994), who filled mechanically induced full-thickness lesions in New Zealand white rabbits with collagen sponges saturated with MSCs. These cells differentiated into active chondrocytes that produced cartilagineous matrix. However, there were some drawbacks in the first experiment: a discontinuity between the host tissue and the new tissue, as well as the progressive thinning of the repaired tissue was observed. Other scientists successfully performed the cartilage differentiation in knee joints, using MSCs stimulated with BMP-2 and IGF-1, whereas unstimulated MSCs failed to induce chondrogenesis under the same circumstances. It is also worth to itemize that pro-inflammatory cytokines, which are expressed in abundance in pathological situations, effectively inhibit BMP-mediated chondrocyte response. Nevertheless, there have been reports of MSC differentiation into tendon, as well as trials for vertebral disc regeneration with the use of scaffolds (Sakai *et al.,* 2003). Those animal model results seem to be very promising; however, further studies are needed before their application to humans.

Further example of potential clinical MSC usefulness is the possibility to accelerate the reconstitution of hematopoiesis in patients after myeloablative

chemotherapy or radiotherapy. Such approach seems to successfully attenuate graft versus host disease (GvHD) after hematopoietic stem cell transplantation. The stromal support has been well documented to be essential for hematopoiesis and the cell-cell interactions in the marrow microenvironment are critical for normal hematopoietic function. In a mouse model, MSC infusion not only prevented the occurrence of graft failure, but also had an immunomodulatory effect (Gao *et al.,* 2001). Moreover, preliminary reports of cotransplantation of MSCs and HSCs from HLA-identical siblings showed the reduction in acute and chronic GvHD. It was demonstrated that addition of MSCs to the grafting material significantly accelerated reconstitution of hematopoiesis in autologic and allogenic transplantations. This was observed especially in umbilical cord blood transplantation, both haploidentical and from unrelated donors. In one case report, a patient with acute lymphoblastic leukemia, who developed severe GvHD after allogenic HSC transplantation and did not respond to the applied therapy, was cured by the use of haploidentical MSCs. The cells were given twice and no toxicity after infusion was observed. The outcome indicated that MSCs had a striking immunosuppressive effect and caused a rapid healing of damaged gut epithelium. Additionally, the patient had no minimal residual disease in blood and bone marrow one year after transplantation.

In addition, there are also observations indicating the usefulness of MSC transplantation in myocardium regeneration after myocardial infarction. Among all bone marrow-derived cell populations, only MSCs were shown to be able to differentiate into cardiomyocytes *in vitro*. Murine model studies using 5-azacytidine to induce cardiomyocyte differentiation confirmed at the molecular level that this cell type could originate from MSCs. The cells not only contained myotube-like structures and myofilaments, but were also positively stained for the cardiomyocyte specific markers, such as sarcomeric myosin, desmin and actinin, and showed the expression of cardiomyocyte-specific genes and transcription factors. The same effect was obtained for human MSCs. Prompted by *in vitro* studies, scientists performed *in vivo* experiments. Wang *et al.* (2007) demonstrated that murine MSCs participate in the formation of new cardiomyocytes in the normal, uninjured heart. Immunohistochemistry executed 4 weeks after injection proved that donor-derived MSCs were present in the heart, expressing cardiac markers. The same potential

was demonstrated for human MSCs, which were injected into the heart of SCID mice. Although the cells engrafted in small percentage (0.44%), they were positive for cardiac markers. When used in animal models for cardiac damage, MSCs successfully colonized the injured tissue and transformed into properly active cardiac cells. Spectacular results were obtained when MSCs transplanted into injured heart were transduced with a virus encoding Akt-an antiapoptotic gene prolonging cell survival, which prevented the pathological remodeling of the left ventricle after infarction. Approximately 80% of the injured myocardium regenerated and the cardiac function was completely restored. Besides improving cardiac function, MSCs were shown to be able to increase the ventricular wall mass. Furthermore, local administration of MSCs to the heart generated *de novo* myocardial formation, giving the hope of the use of these cells in the treatment of coronary heart disease. The injection of MSCs into infarct zone of patients with myocardial infarction appeared to be beneficial for the general heart functionality (Stamm *et al.,* 2003).

Promising results have been also obtained when using MSCs in neuronal lesion treatment. Previous studies showed that MSC transplantation improves recovery after stroke or traumatic brain injury. Additionally, in *in vitro* co-cultures of MSCs and neural stem cells, preferential neuronal differentiation has been observed. Moreover, grafts of MSCs in animal models have been shown to promote remyelination as well as partial recovery of function. After direct injection of MSCs into rodent brain, the cells migrated within the brain and differentiated into GFAP+ glial populations. The transplantation of MSCs into infarcted brain led to the reduction of cell death and the increase in cell proliferation. Moreover, MSCs were demonstrated to be able to produce even myelinating Schwann-like cells, with the typical spindle-shaped morphology and the expression of specific markers, such as LNGFR, Krox-20, CD104 and S100 (Kim *et al.,* 2007). Testing these cells *in vivo*, by means of transplantation to autologous muscle conduit with 2 cm gap in rat sciatic nerve, showed their capacity to colonize the lesion site and regenerate the damaged nerve. The cells were able to myelinate more than one axon in some cases, similarly as it is in CNS. In a different set of experiments, MSCs transplanted into a subtotal cervical hemisection in adult female rats, were able to integrate efficiently into the injury site. Moreover, immunohistochemical analysis showed marked

axonal growth, indicating that these cells enhance axonal growth after spinal cord injury. Interestingly, the recovery levels strongly depended on the human donor and even varied from lot to lot of MSCs isolated fraction.

The list of reports indicating that MSCs contribute to tissue repair *in vivo* enlarges. There are examples of MSC utilization in the repair of kidney (Herrera *et al.,* 2004). The cells were also found to promote angiogenesis, and were used in chronic skin wound treatment. The implantation of MSCs together with occlusive dressing and subsequent epidermal grafts significantly accelerated wound healing and decreased the risk of amputation in endangered patients.

Clinical trials based on MSCs can omit many of the limitations associated with the use of embryonic stem cells (ES). Unlike ES, MSCs are not immunogenic, when used autologically, they do not induce immune rejection and are also less probable to trigger teratoma formation, not to mention the ethical concerns. Unfortunately, there are also some drawbacks concerning the use of MSCs. Firstly, according to some observations MSCs fused with endogenous differentiated cells and formed tetraploid cells *in vivo*, although such an event seems to be extremely rare. Secondly, MSCs were shown to permit tumor growth in allogenic recipients in animal models. A further question arises, whether the grafted MSCs can maintain their undifferentiated state, thus supporting the therapeutic effect on a long term basis. This statement, however, have as many pros as cons. According to study, MSCs derived from postnatal tissue including umbilical cord (Lu *et al.*, 2006), Wharton's jelly (Ma *et al.,* 2005), amnion (Alviano *et al.,* 2007) and placenta (Fukuchi *et al.,* 2004) possess the impaired ability to differentiate into both osteoblasts and adipocyte. Other scientists, on the contrary, demonstrated that MSCs isolated from fat display the same characteristics as MSCs from bone marrow and might be alternatively used for clinical trials.

10. Umbilical cord

The umbilical cord (Fig. 2.6) is the connecting cord from the developing embryo to the placenta. It develops from the yolk sac and allantois. It forms by the fifth week of fetal development, replacing the yolk sac as the source of nutrients for the fetus. The cord is not directly connected to the mother's circulatory system, but

instead joins the placenta, which transfers materials to and from the mother's blood without allowing direct mixing.

The umbilical cord in a full term neonate is usually about 50 centimeters long and about 2 centimeters diameter. This diameter decreases rapidly within the placenta. The umbilical cord is composed of Wharton's jelly, a gelatinous substance made largely from mucopolysaccharides. It contains one vein, which carries oxygenated, nutrient-rich blood to the fetus and two arteries that carry deoxygenated, nutrient depleted blood away. Occasionally, only two vessels (one vein and one artery) are present in the umbilical cord. This is sometimes related to fetal abnormalities, but it may also occur without accompanying problems. It is unusual for a vein to carry oxygenated blood, and for arteries to carry deoxygenated. However, this naming convention reflects the fact that the umbilical vein carries blood towards the fetus's heart, while the umbilical arteries carry blood away (Lu *et al.,* 2006).

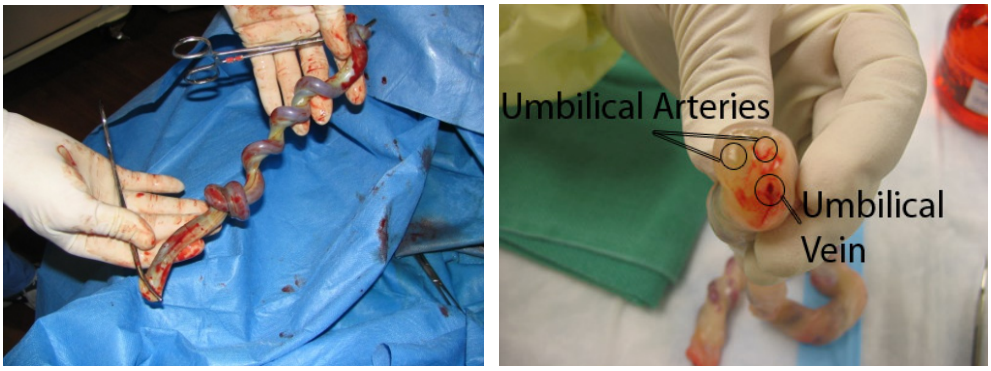

Figure 2.6 The structure of umbilical cord
(Source: homepages.cae.wisc.edu/~bme300/umbilical_f07/)

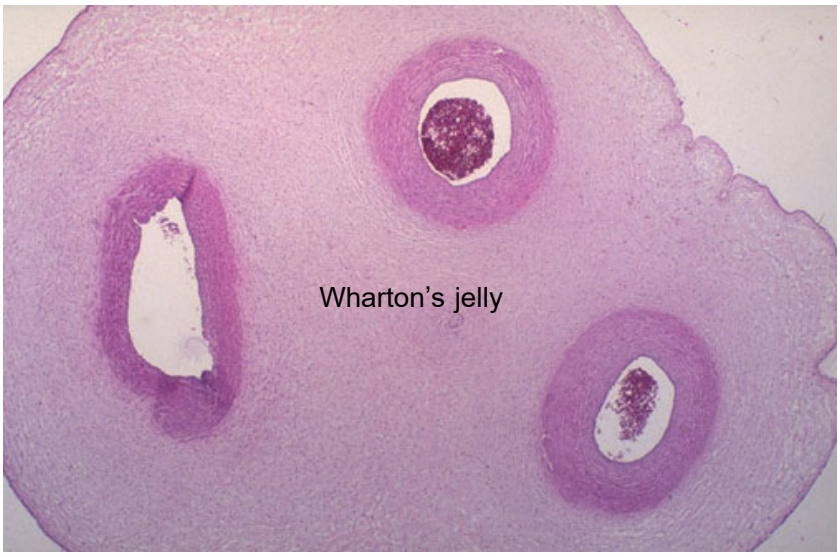

Figure 2.7 The structure of Wharton’s jelly
(Source: http://library.med.utah.edu/WebPath/jpeg2/PLAC074.jpg)

11. Wharton's jelly

Wharton's jelly is a gelatinous substance within the umbilical cord (Fig. 2.7), largely made up of mucopolysaccharides (hyaluronic acid and chondroitin sulfate). It also contains some fibroblasts and macrophages. As a mucous tissue it protects and insulates umbilical blood vessels. Wharton's jelly, when exposed to temperature changes, collapses structures within the umbilical cord and thus will provide a physiological clamping of the cord, an average of 5 minutes after birth. In some cases, such as in water birth with the cord immersed, the Wharton's jelly reaction will occur much later (Troyer and Weiss, 2008).

12. Placenta

The placenta is a highly vascularized ephemeral organ present in eutherian mammals that connects the developing fetal tissues to the uterine wall. The placenta supplies the fetus with maternal nutrients, and allows fetal waste to be disposed of via the maternal kidneys.

In humans, the placenta averages 22 centimeters in length and 2-2.5 centimeters in thickness (Fig. 2.8). It typically weighs approximately 500 grams. It

has a dark reddish/blue or maroon color. It connects to the fetus by an umbilical cord of approximately 55-60 centimeters in length that contains two arteries and one vein. The umbilical cord inserts into the chorionic plate (has an eccentiric attachment). Vessels branch out over the surface of the placenta and further divide to form a network covered by a thin layer of cells. This results in the formation of villous tree structures. On the maternal side, these villous tree structures are grouped into lobules called cotelydons.

In humans the placenta usually has a disc shape (Fig. 2.8) but different mammalian species have widely varying shapes. The placenta begins to develop upon implantation of the blastocyst into the maternal endometrium. The outer layer of the blastocyst becomes the trophoblast which forms the outer layer of the placenta. This outer layer is divided into two further layers; the underlying cytotrophoblast layer and the overlying syncytiotrophoblast layer. The syncytiotrophoblast is a multinucleate continuous cell layer which covers the surface of the placenta. It forms as a result of differentiation and fusion of the underlying cytotrophoblast cells, a process which continues throughout placental development. The syncytiotrophoblast, thereby contributes to the barrier function of the placenta. The placenta grows throughout pregnancy. Development of the maternal blood supply to the placenta is suggested to be complete by the end of the first trimester of pregnancy (approximately 12-13 weeks) (Huppertz, 2008).

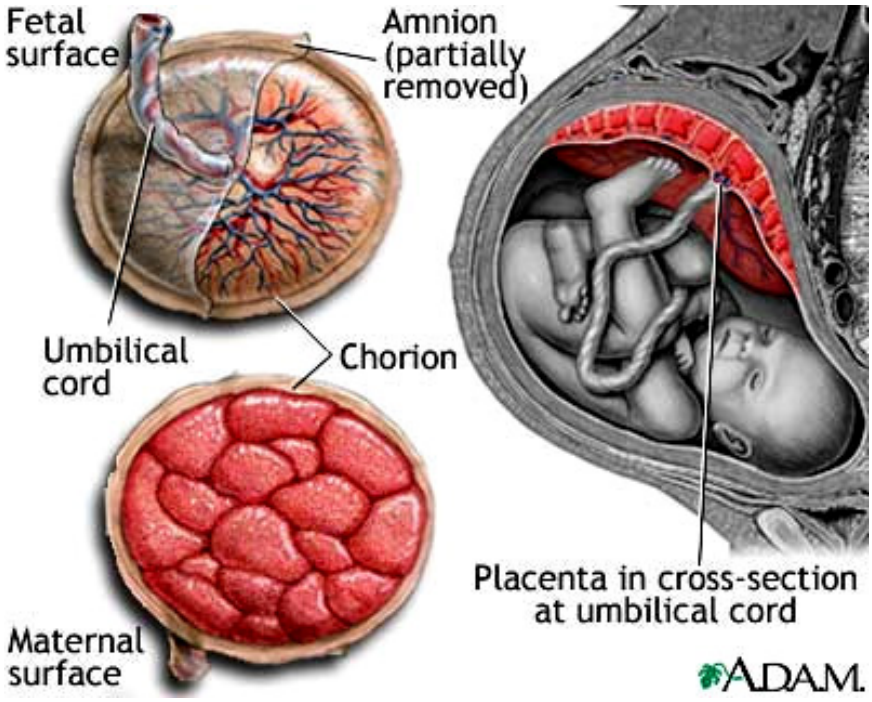

Figure 2.8 The structure of placenta
(Source: www.clarian.org/graphics/images/en/17010.jpg)

13. Amnion

The amnion is a membranous sac that surrounds an embryo. The primary function of this is the protection of the embryo for its development. In the human embryo the earliest stages of the formation of the amnion have not been observed; in the youngest embryo which has been studied the amnion was already present as a closed sac, and appears in the inner cell mass as a cavity. This cavity is roofed in by a single stratum of flattened, ectodermal cells, the amniotic ectoderm, and its floor consists of the prismatic ectoderm of the embryonic disk- the continuity between the roof and floor being established at the margin of the embryonic disk. Outside the amniotic ectoderm is a thin layer of mesoderm, which is continuous with that of the somatopleure and is connected by the body-stalk with the mesodermal lining of the chorion. When first formed the amnion is in contact with the body of the embryo, but about the fourth or fifth week, fluid begins to accumulate within it. This fluid increases in quantity and causes the amnion to expand and ultimately to

adhere to the inner surface of the chorion, so that the extra-embryonic part of the coelom is obliterated. The fluid increases in quantity up to the sixth or seventh month of pregnancy, after which it diminishes somewhat; at the end of pregnancy it amounts to about 1 liter. The amniotic fluid allows the free movements of the fetus during the later stages of pregnancy, and also protects it by diminishing the risk of injury from without. It contains less than two percent solids, consisting of urea and other extractives, inorganic salts, a small amount of protein, and frequently a trace of sugar. That some of the fluid is swallowed by the fetus is proved by the fact that epidermal debris and hairs have been found among the contents of the fetal alimentary canal (Han *et al.,* 2008)**.**

14. Bone marrow transplantation

Bone marrow transplantation or hematopoietic stem cell transplantation (HSCT) is the transplantation of blood stem cells derived from the bone marrow or blood. Stem cell transplantation is a medical procedure in the fields of hematology and oncology, most often performed for people with diseases of the blood, bone marrow, or certain types of cancer. Stem cell transplantation was pioneered using bone marrow derived stem cells by a team at the Fred Hutchinson Cancer Research Center from the 1950s through the 1970s led by E. Donnall Thomas, whose work was later recognized with a Nobel Prize in Physiology or Medicine. Thomas' work showed that bone marrow cells infused intravenously could repopulate the bone marrow and produce new blood cells. His work also reduced the likelihood of developing a life-threatening complication called graft versus host disease (GvHD). The first physician to perform a successful human bone marrow transplant was Robert A. Good at the University of Minnesota in 1968. With the availability of the stem cell growth factors GM-CSF and G-CSF, most hematopoietic stem cell transplantation procedures are now performed using stem cells collected from the peripheral blood, rather than from the bone marrow. Collecting peripheral blood stem cells provides a bigger graft, does not require that the donor be subjected to general anesthesia to collect the graft, results in a shorter time to engraftment, and may provide for a lower long-term relapse rate. Hematopoietic stem cell transplantation remains a risky procedure with many possible complications; it has traditionally been reserved for patients with life-threatening diseases. While

occasionally used experimentally in nonmalignant and nonhematologic indications such as severe disabling auto-immune disease and cardiovascular disease, the risk of fatal complications appears too high to gain wider acceptance. Many recipients of HSCT are multiple myeloma or leukemia patients who would not benefit from prolonged treatment with, or are already resistant to chemotherapy. Candidates for HSCT include pediatric cases where the patient has an inborn defect such as severe combined immunodeficiency or congenital neutropenia with defective stem cells, and also children or adults with aplastic anemia who have lost their stem cells after birth. Other conditions treated with stem cell transplants include sickle-cell disease, myelodysplastic syndrome, neuroblastoma, lymphoma, Ewing's Sarcoma, Desmoplastic small round cell tumor and Hodgkin's disease. More recently non-myeloablative, or so-called "mini transplant," procedures have been developed that require smaller doses of preparative chemo and radiation. This has allowed HSCT to be conducted in the elderly and other patients who would otherwise be considered too weak to withstand a conventional treatment regimen (Ash *et al.,* 2009; Panaroni *et al.,* 2009; Prochazka *et al.,* 2009).

15. Graft types

1. Autologous graft

Autologous HSCT requires the extraction (apheresis) of haematopoietic stem cells (HSCs) from the patient and storage of the harvested cells in a freezer. The patient is then treated with high-dose chemotherapy with or without radiotherapy with the intention of eradicating the patient's malignant cell population at the cost of partial or complete bone marrow ablation. The patient's own stored stem cells are then returned to his/her body, where they replace destroyed tissue and resume the patient's normal blood cell production.

Autologous transplants have the advantage of lower risk of infection during the immune-compromised portion of the treatment since the recovery of immune function is rapid. Also, the incidence of patients experiencing rejection is very rare due to the donor and recipient being the same individual. These advantages have established autologous HSCT as one of the standard second-line treatments for such diseases as lymphoma. However, for others such as acute myeloid leukemia, the reduced mortality of the autogenous relative to allogeneic HCST may be

outweighed by an increased likelihood of cancer relapse and related mortality, and therefore the allogeneic treatment may be preferred for those conditions (Zalom *et al.,* 2009).

2. Allogeneic graft

Allogeneic HSCT involves two people: the (healthy) donor and the (patient) recipient. Allogeneic HSC donors must have a tissue (HLA) type that matches the recipient. Matching is performed on the basis of variability at three or more loci of the (HLA) gene, and a perfect match at these loci is preferred. Even if there is a good match at these critical alleles, the recipient will require immunosuppressive medications to mitigate graft versus host disease. Allogeneic transplant donors may be *related* (usually a closely HLA matched sibling), *syngeneic* (a monozygotic or 'identical' twin of the patient - necessarily extremely rare since few patients have an identical twin, but offering a source of perfectly HLA matched stem cells) or *unrelated* (donor who is not related and found to have very close degree of HLA matching). Allogeneic transplants are also performed using umbilical cord blood as the source of stem cells. In general, by transplanting healthy stem cells to the recipient's immune system, allogeneic HCST appear to improve chances for cure or long-term remission once the immediate transplant-related complications are resolved. A compatible donor is found by doing additional HLA-testing from the blood of potential donors.

The HLA genes fall in two categories (Type I and Type II). In general, mismatches of the Type-I genes (i.e. HLA-A, HLA-B, or HLA-C) increase the risk of graft rejection. A mismatch of an HLA Type II gene (i.e. HLA-DR, or HLA-DQB1) increases the risk of graft versus host disease. In addition a genetic mismatch as small as a single DNA base pair is significant so perfect matches require knowledge of the exact DNA sequence of these genes for both donor and recipient. Leading transplant centers currently perform testing for all five of these HLA genes before declaring that a donor and recipient are HLA-identical. Race and ethnicity are known to play a major role in donor recruitment drives, as members of the same ethnic group are more likely to have matching genes, including the genes for HLA.

To limit the risks of transplanted stem cell rejection or of severe graft versus host disease in allogeneic HSCT, the donor should preferably have the same human leukocyte antigens (HLA) as the recipient. About 25 to 30 percent of allogeneic HSCT recipients have an HLA-identical sibling. Even so-called "perfect matches" may have mismatched minor alleles that contribute to graft-versus-host disease (Zalom *et al.,* 2009).

16. Bone marrow collection

In the case of a bone marrow transplant, the HSCs are removed from a large bone of the donor, typically the pelvis, through a large needle that reaches the center of the bone (Fig. 2.9). The technique is referred to as a bone marrow harvest and is performed under general anesthesia.

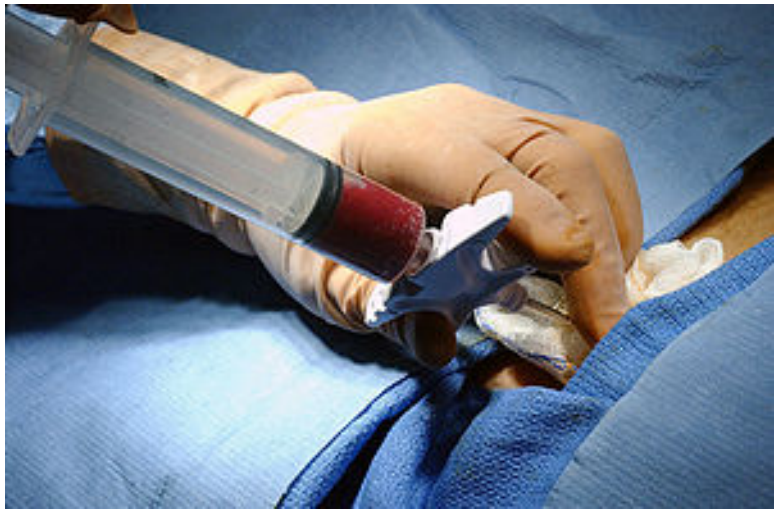

Figure 2.9 Bone marrow harvestment
(Source:http://en.wikipedia.org/wiki/Hematopoietic_stem_cell_transplantation)

17. Alternative sources of stem cells for allogeneic HSCT

1. Peripheral blood stem cells

Peripheral blood stem cells are now the most common source of stem cells for allogeneic HSCT. They are collected from the blood through a process known as apheresis. The donor's blood is withdrawn through a sterile needle in one arm and passed through a machine that removes white blood cells. The red blood cells

are returned to the donor. The peripheral stem cell yield is boosted with daily subcutaneous injections of Granulocyte-colony stimulating factor (G-CSF), serving to mobilize stem cells from the donor's bone marrow into the peripheral circulation (Bian *et al.,* 2009).

2. Umbilical cord blood

Umbilical cord blood is obtained when a mother donates her infant's umbilical cord and placenta after birth. Cord blood has a higher concentration of HSCs than is normally found in adult blood. However, the small quantity of blood obtained from an umbilical cord (typically about 50 mL) makes it more suitable for transplantation into small children than into adults. Newer techniques using ex-vivo expansion of cord blood units or the use of two cord blood units from different donors are being explored to allow cord blood transplants to be used in adults (Hayashi *et al.,* 2009; Hill *et al.,* 2009).

18. Conditioning regimens of HSCT

1. Myeloablative transplants

The chemotherapy or irradiation given immediately prior to a transplant is called the conditioning or preparative regimen, the purpose of which is to help eradicate the patient's disease prior to the infusion of HSCs and to suppress immune reactions. The bone marrow can be ablated with dose-levels that cause minimal injury to other tissues. In allogeneic transplants a combination of cyclophosphamide with busulfan or total body irradiation is commonly employed. This treatment also has an immunosuppressive effect which prevents rejection of the HSCs by the recipient's immune system. The post-transplant prognosis often includes acute and chronic graft versus host disease which may be life-threatening; however in certain leukemias this can coincide with protection against cancer relapse owing to the graft versus tumor effect. Autologous transplants may also use similar conditioning regimens, but many other chemotherapy combinations can be used depending on the type of disease (Hari *et al.,* 2008).

2. Non-myeloablative allogeneic transplants

This is a newer treatment approach using lower doses of chemotherapy and radiation which are too low to eradicate all of the bone marrow cells of a recipient. Instead, non-myeloablative transplants run lower risks of serious infections and transplant-related mortality while relying upon the graft versus tumor effect to resist the inherent increased risk of cancer relapse. Also significantly, while requiring high doses of immunosuppressive agents in the early stages of treatment, these doses are less than for conventional transplants. This leads to a state of mixed chimerism early after transplant where both recipient and donor HSCs coexist in the bone marrow space. Decreasing doses of immunosuppressive therapy then allows donor T-cells to eradicate the remaining recipient HSC and to induce the graft versus tumor effect. This effect is often accompanied by mild graft versus host disease, the appearance of which is often a surrogate for the emergence of the desirable graft versus tumor effect, and also serves as a signal to establish an appropriate dosage level for sustained treatment with low levels of immunosuppressive agents. Because of their gentler conditioning regimens, these transplants are associated with a lower risk of transplant-related mortality and therefore allow patients who are considered too high-risk for conventional allogeneic HSCT to undergo potentially curative therapy for their disease. These new transplant strategies are still somewhat experimental, but are being used more widely on elderly patients unfit for myeloablative regimens and for whom the higher risk of cancer relapse may be acceptable (Tsirigotis *et al.,* 2006).

19. Complications and side effects of HSCT

HSCT is associated with a high treatment-related mortality in the recipient (10% or higher), which limits its use to conditions that are themselves life-threatening. Major complications are veno-occlusive disease, mucositis, infections (sepsis) and graft versus host disease.

1. Infection

Bone marrow transplantation usually requires that the recipient's own bone marrow is destroyed. Prior to "engraftment" patients may go for several weeks without appreciable numbers of white blood cells to help fight infection. This puts a

patient at high risk of infections, sepsis and septic shock, despite prophylactic antibiotics, and accounts for a large share of treatment-related mortality. The immunosuppressive agents employed in allogeneic transplants for the prevention or treatment of graft versus host disease further increase the risk of opportunistic infection. Immunosuppressive drugs are given for a minimum of 6-months after a transplantation, or much longer if required for the treatment of graft versus host disease. Transplant patients lose their acquired immunity, for example immunity to childhood diseases such as measles or polio. For this reason transplant patients must be re-vaccinated with childhood vaccines once they are off of immunosuppressive medications.

2. Veno-occlusive disease

Severe liver injury is termed hepatic veno-occlusive disease (VOD). Elevated levels of bilirubin, hepatomegaly and fluid retention are clinical hallmarks of this condition. There is now a greater appreciation of the generalized cellular injury and obstruction in hepatic vein sinuses, and it has thus been referred to as sinusoidal obstruction syndrome (SOS). Severe cases are associated with a high mortality. Anticoagulants or defibrotide may be effective in reducing the severity of VOD but may also increase bleeding complications. Ursodiol has been shown to help prevent VOD, presumably by helping the flow of bile.

3. Mucositis

The injury of the mucosal lining of the mouth and throat and is a common regimen-related toxicity following ablative HSCT regimens. It is usually not life-threatening but is very painful, and prevents eating and drinking. Mucositis is treated with pain medications plus intravenous infusions to prevent dehydration and malnutrition.

4. Graft versus host disease (GvHD)

GvHD is an inflammatory disease that is unique to allogeneic transplantation. It is an attack of the new bone marrow's immune cells against the recipient's tissues. This can occur even if the donor and recipient are HLA-identical because the immune system can still recognize other differences between their

tissues. It is aptly named graft versus host disease because bone marrow transplantation is the only transplant procedure in which the transplanted cells must accept the body rather than the body accepting the new cells.

20. Types of GvHD

Clinically, GvHD is divided into acute and chronic forms (Arora *et al.,* 2009).

1. Acute graft versus host disease

Acute GvHD typically occurs in the first 3 months after transplantation and may involve the skin, intestine, or the liver, and is often fatal. High-dose corticosteroids such as prednisone are a standard treatment; however this immuno-suppressive treatment often leads to deadly infections.

2. Chronic graft versus host disease

Chronic GvHD may also develop after allogeneic transplant. It is the major source of late treatment-related complications, although it less often results in death. In addition to inflammation, chronic graft versus host disease may lead to the development of fibrosis, or scar tissue, similar to scleroderma; it may cause functional disability and require prolonged immunosuppressive therapy. Graft versus host disease is usually mediated by T cells when they react to foreign peptides presented on the MHC of the host.

21. Prevention of GvHD

1. DNA-based tissue typing allows for more precise HLA matching between donors and transplant patients, which has been proven to reduce the incidence and severity of GvHD and to increase long-term survival.

2. The T-cells of umbilical cord blood (UCB) have an inherent immunological immaturity, and the use of UCB stem cells in unrelated donor transplants has a reduced incidence and severity of GvHD.

3. Methotrexate, cyclosporin A and tacrolimus are common drugs used for GVHD prophylaxis.

4. GvHD can largely be avoided by performing a T-cell depleted bone marrow transplant.

22. Treatment of GvHD

Intravenously administered corticosteroids, such as prednisone, are the standard of care in acute GvHD and chronic GvHD. The use of these corticosteroids is designed to suppress the T-cell mediated immune onslaught on the host tissues; however in high doses this immune-suppression raises the risk of infections and cancer relapse. Therefore it is desirable to taper off the post-transplant high level steroid doses to lower levels, at which point the appearance of mild GvHD may be welcome, especially in HLA mis-matched patients, as it is typically associated with a graft versus tumor effect (von Bonin *et al.,* 2009).

23. MSCs and GvHD

MSCs have been consistently shown to exert a potent immunosuppressive effect superior in magnitude to any other immunosuppressive cell type thus far described (Uccelli *et al.,* 2006). MSCs inhibit the responses of T cells to mitogenic and polyclonal stimuli (Bartholomew *et al.,* 2002), as well as to their cognate peptides. Such an effect is not cognate dependent because it can still be observed using MSCs from third-party donors fully mismatched for the MHC haplotype of the responder T-cell or MSCs which are constitutively negative for MHC molecule expression. Not only is MSCs induced unresponsiveness not antigen-specific, it also lacks any selectivity. In fact, MSCs are equally effective at inhibiting proliferation of memory and naive T-cells (Krampera *et al.,* 2003) do not preferentially affect CD4+ or CD8+ subsets and have similar effects on B cell proliferation. There is also evidence that MSCs can inhibit the IL-2 induced proliferation of natural killer (NK) cells.

The characterization of MSC-induced anergic T-cells has shown that whilst MSCs do not affect T-cell activation they potently interfere with cell proliferation. T-cells, stimulated in the presence of MSCs, are arrested at the G0/G1 phase of cell cycle as a result of inhibition of cyclin D2. Since the effector functions are only partially impaired, MSCs induce an unresponsive T cell profile that is fully consistent with that observed in division arrest anergy, whereby cell proliferation is

profoundly inhibited despite partial conservation of effector function. In accordance with these data, MSCs do not seem to interfere with NK cytotoxicity because MSCs can be lysed by NK cells. The same effects on cell cycle progression have been documented with monocytes and cells of different tissue origin when in contact with MSCs. Overall, these data clearly indicate that the 'immunosuppressive' effect is the consequence of a non-specific anti-proliferative effect (Glennie *et al.,* 2005; Spaggiari *et al.,* 2006).

One of the most challenging conditions for the use of MSCs is graft versus host disease (GvHD) after allogeneic HSCT. The first experience with the use of MSCs for the prevention of GvHD was reported in 2002 but the first documented observation of their clinical efficacy in GvHD was reported by the Karolinska Transplant Centre which successfully treated a 9-year-old boy suffering steroid-resistant grade IV acute GvHD by using haploidentical third-party MSCs. Further studies were carried out producing different outcomes. The prophylactic administration of MSC to HLA-identical siblings did not show any effect on the incidence of GvHD in an American multicentre study, whilst a more recent open-label randomised study in a similar setting reported a substantial reduction in the incidence of GvHD (Le Blanc *et al.,* 2004; Lazarus *et al.,* 2005).

The study by Polchert *et al.* (2008) not only has implications for designing therapeutic approaches to GvHD, but also contains information for understanding the biology of MSCs. In fact, the need for the correct timing is related to the necessity for the appropriate inflammatory environment to allow MSCs to acquire their immunosuppressive properties. When donor T cells from interferon-γ (IFN-γ) knock-out mice were utilized as effectors of the GvHD, MSCs were unable to affect the survival of the recipients regardless of the time at which they were infused. Furthermore, IFN-γ treated MSCs could confer on MSCs the capacity to become effective if injected at time of BMT.

The role of IFN-γ in augmenting the immunosuppressive activity of MSCs in vitro had already been described as well as the necessity for MSCs to be activated or 'licensed' before being able to exert their anti-proliferative effect; however, the study by Polchert *et al.* (2008) now provides direct *in vivo* evidence for such MSCs 'licensing'. Whether MSCs become directly responsible for the overall immunosuppression remains to be established and several questions are now

open. Unfortunately, the paper by Polchert *et al.* does not address the issue of MSCs localization and in particular whether IFN-γ is a key molecule in routing MSCs to the site of injury. Previous experience in animal models has described a link between GvHD and recruitment of MSCs and a similar pattern has been reported concerning the use of MSCs for tissue repair, thus suggesting a prominent role for inflammation in recruiting MSCs. This concept is consistent with that which has been shown in conditions such as experimental arthritis whereby inflammation results in the recruitment of mesenchymal cells to the joint. A small proportion of MSCs express a restricted set of chemokine receptors, such as CXCR4 and CXCR3, the expression of which, at least on phagocytes, is regulated by IFN-γ. The selective migration of antigen-specific T cells under inflammatory conditions would greatly facilitate the effect of MSCs in controlling immune responses (Sordi *et al.,* 2005; Krampera *et al.,* 2006).

Therefore, IFN-γ stimulation could play a dual role in enhancing the efficacy of MSCs immunomodulation, by directing the localization of both MSCs and effector T cells. First, the induction of chemotactic factors inducing MSCs migration wouldpromote selective accumulation of MSCs at the inflammatory site, thus reducing the need for large number of cells needed to elicit immunosuppression. Second, by inducing MHC molecule expression on the local endothelium, IFN-γ may favour the selective recruitment of antigen-specific primed T cells at the same site as the MSCs. In summary, the instrumental effect of IFN-γ activity on MSC-mediated immunomodulation relies upon its ability to gather and retain suppressive and effector cells in the same anatomical compartment (Fig. 2.10). This hypothesis is corroborated by the evidence that the number of MSCs detectable at any site even shortly after their injection is barely measurable.

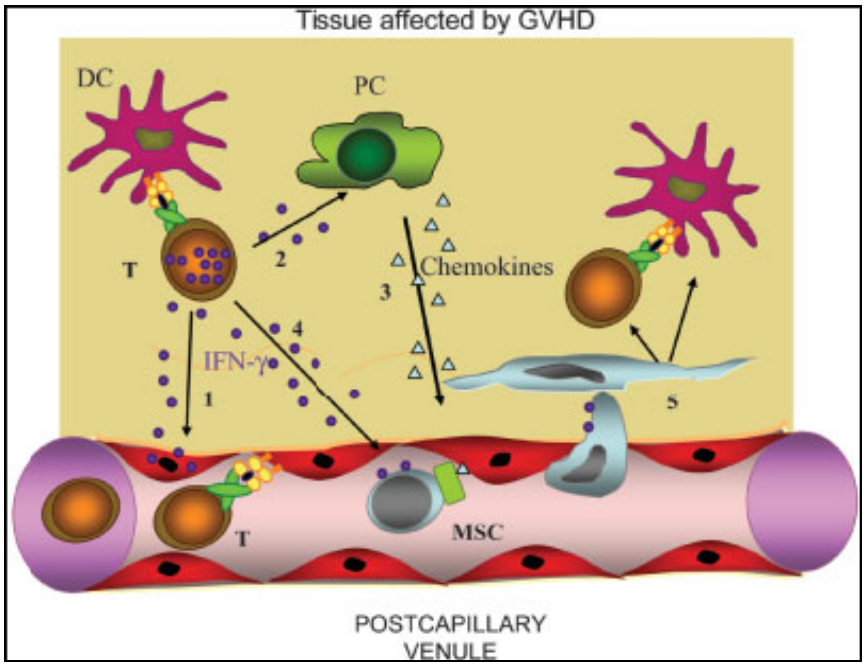

Figure 2.10 IFN- γ regulates the anatomy of MSC-induced T cell tolerance.
A brief summary of the proposed effects of IFN- γ on the localization of MSCs and effector T-cells leading to efficient immunomodulation. IFN- γ secreted by activated T cells induces antigen-display on the local endothelium (1) leading to the accumulation of activated T-cells in the tissues targets of GvHD. In parallel IFN- γ induces chemokine secretion by parenchymal cells (2) leading to enhanced recruitment of circulating MSC into the tissue (3) IFN- γ 'licenses' MSCs (4) to effectively mediate immunosuppression either directly on the pathogenic T cells or indirectly by generating other immunosuppressive networks via tolerogenic DC or regulatory T-cells (5). DC: dendritic cells, PC: parenchymal cells, MSCs: mesenchymal stem cells, T: T lymphocytes.

Although MSC-mediated immunosuppression requires an initial cell contact phase, the ultimate signal is mediated by several factors including TGF-β, indoleamine 2,3-dioxygenase, prostaglandin E2, nitric oxide, hemoxygenase and insulin-like growth factor binding protein (Dazzi *et al.,* 2006). Considering that the same molecules are involved also in the recruitment and activation of other cellular networks mediating immune suppression, like natural killer T cells and myeloid-derived suppressor cells, it seems reasonable to configure the existence of an innate type of tolerance whereby effector cells are rapidly recruited in conditions of acute inflammation. MSCs and as recently demonstrated, all stromal cells would be a component of this family of tolerance inducers (Dazzi *et al.,* 2006).