Chapter 1: Introduction and Review of Literature

<table>
<thead>
<tr>
<th>Quadri</th>
<th>Four</th>
<th>Mesenchymal progenitor cell</th>
<th>Cartilage cells, fat cells, stromal cells, bone forming cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri</td>
<td>Three</td>
<td>Glial restricted precursor</td>
<td>2 types of astrocytes, oligodendrocytes</td>
</tr>
<tr>
<td>Bi</td>
<td>Two</td>
<td>Bipotential precursor from murine fetal liver</td>
<td>B cells, macrophages</td>
</tr>
<tr>
<td>Uni</td>
<td>One</td>
<td>Mast cell precursor</td>
<td>Mast cells</td>
</tr>
<tr>
<td>Nullipotential</td>
<td>None</td>
<td>Terminally differentiated cell e.g. Red blood cell</td>
<td></td>
</tr>
</tbody>
</table>

Those from the inner cell mass of blastocyst have been attributed with a ‘pluripotent’ potential and therefore with the capacity to generate all or most cell lineages derived from the three embryonic germ layers: ectoderm (skin and neural lineages), mesoderm (blood, fat, cartilage, bone and muscle) and endoderm (digestive and respiratory systems) (Gardner and Beddington 1988, Li M et al., 2001). During development, ESC divides and originates distinct subpopulations, including non-self regenerating progenitors that undergo terminal differentiation. The embryonic germ cells or the primordial germ cells, the embryonal carcinoma cells show the pluripotency as well. The fetal tissue stem cells, the cord blood and placental stem cells, the adult stem cells are all showing either pluripotency or multipotency depending on their function and physiological states. The properties of embryonic stem cells are illustrated in Table 1.2.
Table 1.2: Properties of ES cells

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Origin</td>
<td>Derived from the inner cell mass/epiblast of the blastocyst</td>
</tr>
<tr>
<td>Long-term self-renewal</td>
<td>Capable of undergoing an unlimited number of symmetrical divisions without differentiating</td>
</tr>
<tr>
<td>Karyotype</td>
<td>Exhibit and maintain a stable, full (diploid, normal complement of chromosomes), normal complement of chromosomes</td>
</tr>
<tr>
<td>Potentiality</td>
<td>Pluripotent ES cells can give rise to differentiated cell types that are derived from all the three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm). Capable of integrating into all fetal tissues during development. Mouse ES cells maintained in culture for long periods can still generate any tissue when they are reintroduced into an embryo to generate a chimeric animal. Capable of colonizing the germ line and giving rise to egg or sperm cells.</td>
</tr>
<tr>
<td>Clonogenicity</td>
<td>A single ES cell can give rise to a colony of genetically identical cells, or clones, which have the same properties as the original cell. EC cells express the transcription factor Oct-4, which then activates or inhibits a host of target genes and maintains ES cells in a proliferative, non-differentiating state.</td>
</tr>
<tr>
<td>Cell fate</td>
<td>Can be induced to continue proliferation or to differentiate.</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>Lack the G1 checkpoint in the cell cycle. ES cells spend most of their time in the S phase of cell cycle, during which they synthesize DNA. Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication.</td>
</tr>
</tbody>
</table>
1.5.2. Germinal Stem Cells

Early in embryogenesis a few cells are designated to become germinal cells (Meachem et al., 2001). These cells migrate into primitive gonad (genital ridge) and differentiate into the female or male germ cell precursors, depending on the presence of two X chromosomes (female) or one X and one Y chromosome (male). They can be recognized by expression of the transcription factor Oct4 and of alkaline phosphatase (Anderson et al., 2000). Studies beginning in the 1970s involving the transplantation of germinal cells clearly demonstrated the totipotency and tumorigenicity of the germinal cells.

1.5.3. Stem Cells from Umbilical Cord Blood:

The blood that remains in the umbilical cord is a plentiful and potentially exhaustible source of pluripotent stem cells that can be used in a number of cell therapies. The cord blood is collected after a baby is born and the cord has been clamped and cut. The collection is painless and safe and families can choose to save and store or donate blood (www.cordblood.org). These are primitive cells with clinical potential matching that of the far more controversial embryonic stem cells ESC. They appear to be much more versatile than “adult stem cells” such as those found in bone marrow which repair damaged tissue during life.

There is an abundance of clinical applications using human umbilical cord blood (HUCB) as a source for stem cell populations. Other than haematopoietic progenitors, there are mesenchymal, endothelial stem cells and neuronal precursors, in varying quantities, which are found in human umbilical cord blood. These may be useful in diseases such as immune deficiency and autoimmune disorders. Considering issues of safety,
availability, transplant methodology, rejection and side effects, it is contended that a therapeutic stem cell transplant, utilizing stem cells from HUCB, provides a reliable repository of early precursor cells that can be useful in a great number of diverse conditions. Drawbacks of relatively smaller quantities of mononucleated cells in one unit of cord blood can be mitigated by in-vitro expansion procedures, improved in-vivo signalling, and augmentation of the cellular milieu, while simultaneously choosing the appropriate transplantation site and technique for introduction of the stem cell graft (Ghen et al., 2006).

Tissue-engineered living blood vessels (TEBV) with growth capacity represent a promising new option for the repair of congenital malformations. TEBV with tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord progenitor cells. Thus, blood-derived progenitor cells obtained before or at birth may enable the clinical realization of tissue engineering constructs for pediatric applications (Schmidt et al., 2006).

Mesenchymal stem cells (MSC) could be isolated from human umbilical cord Wharton’s Jelly. They were capable of differentiating into nerve-like cells using beta-mercaptoethanol. The induced MSC not only underwent morphologic changes, but also expressed the neuron-related genes and neuronal cell markers. They may represent an alternative source of stem cells for central nervous system cell transplantation (Ma et al., 2005).

1.5.4. Adult Stem Cells:

Adult stem cells, somatic stem cells, or organ-specific adult stem cells are small subpopulations of quiescent slow-cycling-undifferentiated resident cells, with high proliferative and pluripotent potentiality and the ability to self-
renew and to originate daughter cells, which finally differentiate into functionally mature cells, regenerating all the cell types of the tissue where they are located. Their proliferative reserve exceeds an individual lifetime. These adult stem cells present few organelles and a large nuclear cytoplasmic ratio and may express specific antigens, (Spangrude et al., 1988, Welm et al., 2002), integrins (Collins et al., 2001). Table 1.3 showing the properties of adult stem cells.

Table 1.3: Properties of Adult SC.

<table>
<thead>
<tr>
<th>Tissue Origin</th>
<th>Present in many tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term</td>
<td>Capable of maintaining homeostatic of SC</td>
</tr>
<tr>
<td>Self-renewal</td>
<td>Compartment for the entire life time of the organism</td>
</tr>
<tr>
<td>Karyotype</td>
<td>Exhibit and maintain a stable, full (diploid, normal complement of chromosomes)</td>
</tr>
<tr>
<td>Potentiality</td>
<td>The large majority of adult stem cells are not pluripotent, like ES, since they have a limited differentiation capacity. They can be multipotent, such as hematopoietic SC or unipotent such as skin SC. Experimental evidence suggests that the only exception are MAPc since these can give rise to differentiated cells of all the three types of primary germ layers of the embryo (endoderm, mesoderm and ectoderm).</td>
</tr>
<tr>
<td>Clonogenicity</td>
<td>A single adult SC, in vitro can only give rise to a colony of differentiated cells lacking the properties of the original cell. The molecular mechanisms that maintain adult SC in</td>
</tr>
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</table>
a proliferative, non-differentiating state are almost completely unknown.

<table>
<thead>
<tr>
<th>Cell fate</th>
<th>Can be induced to differentiate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>The large majority of adult SC are in a quiescent state. Adult SC requires an external stimulus from the microenvironment to enter the cycle and initiate DNA replication (Stem cell niche).</td>
</tr>
<tr>
<td>Plasticity</td>
<td>Adult SC may have the ability to generate specialized cells of other tissues. The mechanism is still debated (Cell fusion? Transdifferentiation?)</td>
</tr>
</tbody>
</table>

There are various sources of adult stem cells including bone marrow, peripheral blood, liver, kidney. Adult SC has been identified in many animal models and human tissues. The list of adult tissues reported to contain SC is growing and included bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelium of the skin and digestive system, cornea, retina, liver, pancreas, heart and the CNS (Table 1.4).

Table 1.4: Adult stem cells from various tissues

<table>
<thead>
<tr>
<th>Type of adult stem Cell</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic stem cells</td>
<td>Bone marrow</td>
<td>Zhao et al., 2003</td>
</tr>
<tr>
<td>Mesenchymal Stem cells</td>
<td>Bone marrow, amniotic fluid, peripheral blood, adipose tissue, dermis, articular</td>
<td>Friedenstein et al., 1974, Pittenger et al., 1999, Zvaifler et al., 2000, Campagnoli et al., 2001,</td>
</tr>
</tbody>
</table>
### Table: Tissues Identified to Contain Stem Cells

<table>
<thead>
<tr>
<th>Stem Cell Type</th>
<th>Tissue/Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Monocytes</td>
<td>Peripheral blood</td>
<td>Zhao et al., 2003</td>
</tr>
<tr>
<td>Epidermal Stem Cells</td>
<td>Skin</td>
<td>Alonso and Fuchs et al., 2003</td>
</tr>
<tr>
<td>Hair follicle Stem Cells</td>
<td>Hair follicle</td>
<td>Hoffman et al., 2006</td>
</tr>
<tr>
<td>Corneal epithelial stem cells</td>
<td>Limbus</td>
<td>Schermer et al., 1986, Lavker et al., 2004</td>
</tr>
<tr>
<td>Respiratory Tract stem cells</td>
<td>Respiratory Tract</td>
<td>Delplanque et al., 2000, Kotton et al., 2001</td>
</tr>
<tr>
<td>Dental Stem Cells</td>
<td>Dental Pulp</td>
<td>Shi et al., 2005</td>
</tr>
<tr>
<td>Gastrointestinal Tract Stem Cells</td>
<td>Neck/isthmus region</td>
<td>Modlin et al., 2003</td>
</tr>
<tr>
<td>Hepatic Stem Cells</td>
<td>Intraheptic biliary tree of liver</td>
<td>Thorgeirsson and Grisham, 2003</td>
</tr>
<tr>
<td>Pancreatic Stem Cells</td>
<td>Duct cells of pancreas</td>
<td>Bonner-Weir et al., 2000</td>
</tr>
<tr>
<td>Salivary Gland stem cells</td>
<td>Intercalated duct of salivary glands</td>
<td>Kishi et al., 2006</td>
</tr>
<tr>
<td>Renal Stem Cells</td>
<td>Renal papilla of kidney</td>
<td>Oliver et al., 2004</td>
</tr>
<tr>
<td>Mammary gland Stem Cells</td>
<td>Mammary gland ducts</td>
<td>Stingl et al., 2006</td>
</tr>
<tr>
<td>Prostatic Stem Cells</td>
<td>Proximal region of ducts of prostate</td>
<td>Tsujimura et al., 2002</td>
</tr>
<tr>
<td>Myogenic Progenitors</td>
<td>Adult skeletal and Cardiac Muscle</td>
<td>Beauchamp et al., 2000,</td>
</tr>
</tbody>
</table>

Among tissues identified to harbor stem cells throughout postnatal life, bone marrow has been studied for many years. There are two major types of SC
found in the BM: HSC which generate blood cells, and MSC that support hematopoiesis.

1.5.4.1. Mesenchymal Stem Cells:

MSCs reside in the stromal fraction of the bone marrow, which provides the cellular microenvironment supporting hematopoiesis. Mesenchymal stem cells were first described as bone-forming progenitors from the stromal fraction of rats by Friedenstein and Petrakova in 1966 (Friedenstein et al., 1966) and Friedenstein went on to pioneer in vitro culture methods for the isolation and differentiation of MSCs (Friedenstein et al., 1987). MSCs have subsequently been shown to differentiate into a number of mesenchymal cell types including osteoblasts, chondrocytes and adipocytes (Pittenger et al., 1999) (Figure 1.5).

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**THE MESENGENIC PROCESS**

- **Mesenchymal Stem Cell (MSC)**
- **Proliferation**
- **Osteogenesis**
- **Chondrogenesis**
- **Myogenesis**
- **Marrow Stromal**
- **Tendogenesis**
- **Capillarogenesis**
- **Other**
- **Bone Marrow/Periostum**
- **Lineage Progression**
  - **Transient Osteoblast**
  - **Transient Chondrocyte**
  - **Myoblast**
  - **Transient Stromal Cell**
  - **Transitory Fibroblast**
- **Osteoblast**
- **Chondrocyte**
- **Myoblast Fusion**
- **Unique Micro-niche**
- **Maturation**
  - **Osteocyte**
  - **Hypertrophic Chondrocyte**
  - **Myotube**
  - **Stromal Cells**
  - **TIL Fibroblast**
  - **Adipocytes, Dermal and Other Cells**
- **Bone**
- **Cartilage**
- **Muscle**
- **Marrow**
- **Tendon/ Ligament**
- **Connective Tissue**

**Figure 1.5:** The mesengenic process of MSC
1.5.4.1.1 Sources of Primary MSC

MSCs are typically isolated from the stromal fraction of adult bone marrow. In fresh bone marrow, MSCs account for only 0.01-0.0001% of nucleated marrow cells (Dazzi et al., 2006). Murine MSCs are classically obtained from the femurs and tibias of mice by flushing the marrow out of the bones with culture medium and transferring the resultant cell suspension in culture. Human MSCs can be similarly obtained from healthy volunteers by taking aspirates of bone marrow from the iliac crest and expanding on tissue-culture plastic (Risbud et al., 2006). Over recent years, MSC-like cells have also been identified in a number of different tissues (Friedenstein et al., 1987). Cells exhibiting MSC morphology and cellular characteristics have been isolated from adult peripheral blood (Zvaifler et al., 2000), adipose tissue (Zuk et al., 2001) skin tissue (Chunmeng et al., 2004), trabecular bone (Sottile et al., 2002) as well as fetal blood, liver, bone marrow (Campagnoli et al., 2001), lung (in ‘t Anker et al., 2003) and even in exfoliated deciduous teeth (Miura et al., 2003) Further MSC-like populations have been discovered in umbilical cord blood (Erices et al., 2000) and within the chorionic villi of the placenta (Igura et al., 2004) Amniotic fluid has also been cited as a source of MSCs, with potential far-reaching implications for such areas as prenatal diagnosis and gene therapy (In ‘t Anker et al., 2003).

1.5.4.1.2 Isolation of Primary MSC

It is notable that, contrary to most biological systems, human MSCs are better characterized than animal MSCs. This is probably due to the fact that MSCs are easily isolated and expanded from adult human tissue collected from
healthy volunteers. MSCs have nevertheless been isolated from a number of other species. Along with human MSCs, the better characterized cultures are those of rat (Santa Maria et al., 2004) and mouse (Baddoo et al., 2003) origin, although therapeutic potential in large animal models has been investigated with MSCs from horse (Smith et al., 2003), cow (Bosnakovski et al., 2005), pig (Moscoso et al., 2005) dog (Silva et al., 2005), sheep (Rhodes et al., 2004) and baboon (Devine et al., 2001).

Three main approaches have been described for the isolation of MSCs and can either be used independently or combined together to obtain a more homogeneous culture. The traditional isolation method relies on the fact that MSCs selectively adhere to plastic surfaces, whereas hematopoietic cells do not and can therefore be removed through medium changes (Luria et al., 1971). Whilst this eliminates most contaminating cells, the remaining heterogeneity of the culture progressively decreases by serial passaging and after a number of passages the culture is enriched in the self-renewing fraction, the stem cells. Another published isolation protocol involves centrifugation over a Percoll gradient, which separates cell populations based on their density and allows the enrichment of nucleated cells (Dazzi et al., 2006). However, both methods are quite nonspecific and an approach that is now increasingly being used, resorts to sorting of bone marrow populations by flow cytometry (FACS), based on MSC reactivity to a number of antibodies. This can either be achieved by positively selecting for expressed antigens or by a process of immunodepletion of cells expressing hematopoietic and/or other lineage antigens. For instance, antibodies against CD34, a surface marker found on hematopoietic cells, are frequently used to identify and
remove nonmesenchymal cells from a marrow culture (Pittenger et al., 1999). Since there is no single specific marker available to unequivocally identify the MSC, different groups have opted for a variety of marker combinations. MSCs appear relatively stable as primary cultures (Mareschi et al., 2006, Bernardo et al., 2006) although spontaneous transformation events have been observed in long-term cultures (Rubio et al., 2005).

1.5.4.1.3 Surface Markers on MSC

There are various markers used by different groups to identify the MSC fraction from human bone marrow include, but are not limited to, CD13, CD29, CD31, CD44, CD54, CD63, CD73, CD105, CD106, CD140b, CD166 and Stro1 (Pittenger et al., 1999, Bruder et al., 1998, Gronthos et al., 1994, Vogel et al., 2003, Mitchell et al., 2006, Covas et al., 2003). Comparisons of the various combinations used by different groups show that the majority of subsets include either CD29, CD105 or both. Although various groups have used these markers, there is still no general consensus on the optimal marker combination for MSCs. Some of this conjecture may be due to variations in sample origin, culture techniques and media composition among laboratories or differences in the age of the donors from which the MSCs were obtained and used for immunophenotyping. Because different antibody subsets are likely to selectively isolate slightly different cell types, comparison and evaluation of published data arising from different groups can be difficult. For instance, some groups report a degree of heterogeneity in their cultures after isolation and purification, with occasional description of a subset of small rounded cells among the more common fibroblast-like phenotype (Colter et al., 2000). This ambiguity begs the question of what MSCs are: do they purely
represent the proliferating fibroblastic-like progenitors from the bone marrow stroma or do they include all cells capable of forming mesenchymal tissue? In the absence of a specific cell marker, MSCs may well incorporate a number of different cell populations all potentially variable in their phenotypic and growth characteristics, with mesenchymal differentiation as a common denominator.

1.5.4.1.4 Basic Biology and Function of MSC

1.5.4.1.4.1 Basic Biology

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 were they 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). Studies in rodents with 3[H]-thymidine labelling demonstrated that CFU-F are essentially in a noncycling state in vivo (Zvaifler et al., 2000). 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, while the rodent cells require a source of irradiated feeder cells to achieve maximal plating efficiency (Prockop et al., 1997, Bruder et al., 1998). 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%) (Pittenger et al., 1999). 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 (Bruder et al., 1998, Im et al., 2005). In terms of proliferative potential, the cells have been also described as small rapidly-renewing, and large slowly-renewing (Reyes et al., 2001). Contrarily, the work performed by Colter et al. (Colter et al., 2000) describes the population of small and agranular cells (RS-1) within stationary culture of MSCs with a low capacity to generate colonies and non-reactive to the cell cycle-specific antigen Ki-67. That cell subpopulation was shown, however, to be responsible for the capacity of the whole population of MSCs to expand in culture. Furthermore, it was speculated that RS cells may cycle under stimulation by factors secreted by the more mature MSCs. These cells were, thus, proposed to represent an ex vivo subset of recycling uncommitted mesenchymal stem cells (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, transferrin receptor and annexin II (lipocortin 2). Some of the rapidly renewing cells contained also other markers, like c-kit (CD117), multidrug resistance epitope and epithelial membrane antigen. Interestingly, these cells were negative for STRO-1, an antigen originally considered as a marker for MSCs (Dennis et al., 2002)
1.5.4.1.4.2 Function of MSC:

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-1α (Interleukin), IL-1β, 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) (Boiret et al., 2005, Colter et al., 2001, Dazzi et al., 2006, Gronthos et al., 2003, Katz et al., 2005). Some of these proteins are produced by quiescent cells, whereas the others after stimulation. The involvement of MSCs in hemopoiesis 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). An animal model study confirmed that human MSCs marked with 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. Other studies showed that cotransplantation of human MSCs and HSCs resulted in increased chimerism or/and accelerated hematopoietic recovery in animal models and in humans (Fibbe et al., 2003, Koc et al., 2000, Lane et al., 1999). 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) (Hoffman A 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 costimulatory receptors (CD80, CD86) on their surface, therefore they do not exhibit antigen-presenting cell activities (Angoulvant et al., 2004, Fibbe et al., 2003]. The 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 costimulatory molecules (Fibbe et al., 2003). It has been well established that MSCs from various species can exert profound immunosupression by inhibiting T-cell responses to polyclonal stimuli (Di Nicola et al., 2002) and to their cognate peptide (Krampera M 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 downregulation (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 (Di Nicola et al., 2002). 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-α (TGF-α) and HGF suggest that these factors are at least in part responsible for the inhibitory effects caused by MSCs (Di Nicola et al., 2002). Moreover, MSCs can affect other cells participating in immune response like B cells (Glennie et al., 2005) and dendritic cells (Jiang et al., 2005).

1.5.4.1.5 Circulation and Niche of MSCs

Little is known about the nature and localization of undifferentiated multipotent MSCs. These cells may be found in various tissues in special places called 'stem cell niches', which serve as stem cells reservoirs. They remain quiescent and possess the capacity for self-renewal after an injury, disease or aging (Pittenger et al., 1999). The stem cell niche hypothesis for the bone marrow cells (Figure 1.6) was developed by Schofield, who suggested that certain microenvironmental conditions of the marrow stroma could maintain the stem cells in a primitive, quiescent state (Shofield et al., 1978). The investigation of anatomical distribution of MSCs within bone marrow revealed that the cells are located in a close association with endosteum (Gronthos et
al. , 2003). Such places, therefore, could be regarded as potential niches for MSCs. The findings are, however, based on the STRO-1+ stromal cell population, and the identification of MSCs expressing other specific markers, may change this picture. The question how MSCs maintain their undifferentiated state within the niche is not completely resolved. However, there are some findings indicating that MSC decision to differentiate or to stay quiescent is regulated by Wnt family members, which support undifferentiated state of MSCs, as well as their inhibitors, like: Dkk1, Frzb-1 (Sato et al., 2004). Wnt signaling is known to prevent differentiation process by inducing high levels of oct-3/4, rex-1 and the homeodomain transcription factor Nanog (Sato et al., 2004). Apart from Wnt- and Dkk1-mediated signaling, also Notch, Hedgehog and BMP-pathways play a role in proliferation and differentiation of stem cells. Therefore, it can be speculated, that at least some of these factors are also important for MSCs growth in their niche. After particular stimuli, a stem cell may leave its niche and circulate in blood (Fernandez et al., 1997). The cell must afterwards be attracted to another site, where under specific microenvironmental circumstances is able to enter its differentiation program (Watt et al., 2000).
Figure 1.6: The mesenchymal stem cell niche is shown in Figure 1.6. MSCs are shown in their putative perivascular niche (BV, blood vessel), interacting with (1) various other differentiated cells (DC₁, DC₂, etc.) by means of cell adhesion molecules such as cadherins, (2) extracellular matrix (ECM) deposited by the niche cells mediated by integrin receptors, and (3) signaling molecules, which may include autocrine, paracrine, and endocrine factors. Another variable is $O_2$ tension, with hypoxia associated with MSCs in the bone marrow niche. (Source: Kolf et al., 2007)

The study on MSC homing indicates that the expression of chemokine receptors, as quoted previously, help them in trafficking to various tissues, including bone marrow (Lee et al., 2006). Among them, a pivotal role is played by CXCR4, the receptor for SDF-1, inter alia, produced by stromal cells. Many findings confirm the extensive multi-organ homing ability of MSCs. In murine model, circulating mesenchymal progenitors, detected in blood stream, were able to migrate and colonize various tissues (Gao et al., 2001). Similar results were obtained in humans (Reading et al., 2000). Moreover, these cells were present in the blood of breast cancer patients after growth factor-induced mobilization of hematopoietic stem cells. These data suggest that adequate stimuli may mobilize and release quiescent MSCs.
r esiding in a tissue. Additionally, a subset of quiescent cells (5-10%) was identified in cultures of mesenchymal cells isolated from cord blood, suggesting that uncommitted mesenchymal progenitors circulate during gestation, and travel from fetal sites into other tissues early during development (Makino et al., 1999). As another example, MSCs were described to locally migrate to injured sites, to support the regeneration process. Such cases were documented in cartilage repair (Caplan et al., 1997), muscle (De Barri et al., 2003) and heart regeneration, migration throughout forebrain and cerebellum (Kopan et al., 1999) and differentiation into osteoblasts in regenerating bone (Horwitz et al., 2002). The homing capacity of MSCs may decrease after extensive culturing in vitro. A study based on syngeneic mouse model revealed that primary bone marrow derived MSCs were able to home efficiently to the bone marrow and spleen, whereas culture-expanded MSCs had lost this capacity after 24-48 hours in culture (Fibbe et al., 2003). It might be speculated, therefore, that in vitro propagation of bone marrow-derived MSCs dramatically decreases their homing to bone marrow and spleen.

1.5.4.1.6 Differentiation:

Cell differentiation proceeds from unspecialized cells to tissue specific cells through selectively environmentally induced protein expression. Almost all organs and tissues have a pool of progenitor cells that can respond to normal cell turnover demands, or during injury or damage response. To effectively function in the organ or tissue where progenitor cell differentiation is required, differentiation must result in a phenotypically matched cell. Therefore through targeting specific genes, many signaling possibilities exist that can...
different cell phenotype. Several in vitro studies have been conducted to assess the differentiation potential of MSCs, as well as to set up culture conditions, differentiation stimuli, and methods for the identification of each differentiated phenotype. These are supported by in vivo studies demonstrating that bone marrow-derived MSCs develop into terminally differentiated phenotypes, like those forming bone (Goshima et al., 1991; Kadiyala et al., 1997), cartilage (Kadiyala et al., 1997), tendon (Young et al., 1998), muscle (Ferrari et al., 1998), neural (Parr et al., 2007), and adipose tissues (Maueny et al., 2007).

1.5.4.1.7 Clinical Applications of MSC

The availability of autologous MSCs, which are easily accessible from patients, makes them a promising source of cells for many clinical applications in the evolving field of regenerative medicine. As well as providing the stromal (scaffold) fraction of the bone marrow for HSCs to proliferate on, MSCs are thought to play a role in hematopoiesis itself. (Dazzi et al., 2006). MSCs have been shown to significantly improve hematopoietic recovery in patients receiving high-dose chemotherapy when compared with autologous blood stem cell transfusion alone (Koc et al., 2000). Koc and coworkers (Koc et al., 2000) co-infused culture-expanded MSCs with autologous blood stem cells in breast cancer patients and observed accelerated hematopoietic recovery.

Furthermore, MSCs represent an advantageous cell type for allogeneic transplantation as evidence suggests that MSCs are immune-privileged with low MHC (Major Histocompatibility Complex) I and no MHCII expression, (Uccelli et al., 2006) therefore reducing risks of rejection and complications for
transplantation. In utero transplantation of human MSCs into sheep have shown that grafted cells could integrate a variety of host tissues without any specific immune response (Lichty et al., 2000). MSCs have also been found to be immunosuppressive, through a mechanism thought to involve paracrine inhibition of T- and B-cell proliferation (Di Nicola et al., 2002) as such have been used in trials investigating their effect on autoimmune diseases and GvHD (Le Blanc et al., 2004). Co-infusion of donor-derived MSCs together with HSCs has been shown to reduce the incidence and severity of GvHD in sibling allografts (Lazarus et al., 2005). It was reported that a nine-year-old patient suffering from progressive severe GvHD that was unresponsive to classical therapy was treated with a MSC intravenous transplant from his mother and demonstrated a complete recovery (Le Blanc et al., 2004). The hypo-immunogenic properties of MSCs are considered by some to be sufficient to allow transplantation even between individuals who are not HLA-compatible (Le Blanc et al., 2003).

(i) Potential Application of MSC for Osteochondral repair

One of the fields for MSC use in regenerative medicine is the treatment of bone defects. The osteogenic potential of MSCs has been utilised to treat cases of defective fracture healing, both alone and in combination with scaffolds to repair large bone defects with a high degree of success (Quarto et al., 2001). MSCs have also been used for cartilage repair. Autologous MSCs were expanded ex vivo, embedded in a collagen gel and reimplanted into areas of articular cartilage defect in osteoarthritis patients (Wakitan et al., 2002). In this study, formation of hyaline cartilage-like tissue was improved in the experimental group compared to control. Although most applications for
tissue repair involves local transplantation of MSCs has been in place for a long time. Hematopoietic stem cell transplants. Recently, children suffering from osteogenesis imperfecta were treated systematically with allogenic MSCs. Transplanted MSCs were shown to migrate to the bone and produce collagen, thus providing a new and efficient route to alleviate the debilitating consequences of this genetic condition (Horwitz et al., 1999).

(ii) Potential Application of MSC for Myocardial Repair

Current clinical trials are investigating the potential of MSCs for the treatment of myocardial infarction (Stamm et al., 2003). As previously discussed, a number of groups have reported MSC differentiation in vitro. The current in vivo approach consists of injecting undifferentiated MSCs or whole bone marrow directly into the heart and although the underlying mechanisms remain to be elucidated, significant improvement has been detected (Wollett et al., 2004, Fuchs et al., 2003). The report by Chen and coworkers (Chen et al., 2004) demonstrated a significant and sustained improvement in global left ventricular ejection fraction, suggesting that MSC infusion triggers the formation of new cardiomyocytes and neangiogenesis in the human heart (Nagaya et al., 2004). It is still unclear whether MSCs act directly by in situ differentiation or fusion with resident myocytes (Lee et al., 2005) or indirectly through secretion of pro-myogenic factors promoting endogenous myocardial repair, such as VEGF and FGF (Xu et al., 2006).

(iii) Potential Applications of MSCs for Neurological Disorders

Promising results have also been obtained when using MSCs in neuronal lesion treatment. Previous studies showed that MSC transplantation...
improves recovery after stroke or traumatic brain injury (Chopp et al., 2002). Additionally, in vitro cocultures of MSCs and neural stem cells, preferential neuronal differentiation has been observed (Lou et al., 2003). Moreover, grafts of MSCs in animal models have been shown to promote remyelination (Akayama et al., 2002) as well as partial recovery of function (Chopp et al., 2000). After direct injection of MSCs into rodent brain, the cells migrated within the brain and differentiated into GFAP+ glial populations (Azizi et al., 1998). 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 (Keilhoff et al., 2006). 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 (Keilhoff et al., 2006). 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 (Neuber et al., 2005). The list of reports indicating that MSCs contribute to tissue repair enlarges. There are examples of MSC utilization in the repair of kidney...
(Herrera et al., 2004) muscle (De Bari et al., 2003) and lung (Ortiz et al., 2003). The cells were also found to promote angiogenesis (Hernigou et al., 2002) and were used in chronic skin wound treatment (Badiavas et al., 2003). 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 (Yamaguchi et al., 2005). Clinical trials based on MSCs can omit many of the limitations associated with the use of embryonic stem cells (ES). Unlike ES, MSC are not immunogenic, when used autologously, 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 (Spees et al., 2003). Secondly, MSCs were shown to permit tumor growth in allogenic recipients (Djouad et al., 2003) 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.

1.5.4.2 Limbal Stem Cells

1.5.4.2.1 The Cornea

Cornea is the main structure for refraction of light penetrating the eye; hence its transparency is essential for vision. The cornea is made up of five layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium (Figure 1.7). The corneal epithelium, which represents 10% of the total corneal thickness, and is responsible for protecting the eye from
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Section 1.5.4.2.2 Limbal Epithelial Stem Cells

A self-renewing tissue must contain a stem cell pool, which provides an unlimited supply of proliferating cells. This is true for the corneal epithelium with a large body of research indicating these cells reside in the limbal basal region and are aptly named limbal epithelial stem cells (LESC; Figure 1.8). LESCs share a number of features with other adult somatic stem cells. These include having small cell size (Roman et al., 2003), the lack of expression of differentiation markers such as cytokeratin 3/12 (Schermer et al., 1986) and high nuclear to cytoplasmic ratio.
Figure 1.7: **Localization of corneal stem cells**. A: Histological section and tissue layers of cornea. B: The corneal limbus is localized to the corneoscleral border. The upper and lower regions most protected by the eyelids contain the Vogt’s palisades that apparently host most of the corneal epithelial stem cells. C: Cross-section of the corneoscleral transition. The corneal epithelium is contiguous with the conjunctiva, the corneal stroma transitions into the sclera, whereas the corneal endothelium is linked with the trabecular meshwork. These transitional zones together contain the majority of stem cells in the adult cornea.

Figure 1.8: A cross-sectional diagram of the human corneal limbus. Limbal epithelial stem cells reside in the basal layer of the epithelium (Ep), which undulates at the limbus. Daughter transient amplifying cells divide and migrate towards the central cornea (arrowed) to replenish the epithelium, which resets on Bowman’s layer (BL). The stroma (St) of the limbal epithelial stem cell niche is populated with fibroblasts and melanocytes and also has a blood supply.
LESC are considered to be primitive cells as they are slow cycling and therefore label retaining under normal conditions but have the ability to highly proliferate in response to injury (Costarelis et al., 1989, Laveker et al., 2003). Stem cells have the ability to divide asymmetrically to repopulate the stem cell pool. Barbaro et al. (2007) found expression of C/EBP in a subset in vivo and in vitro and have suggested it is involved in the regulation of self-renewal and cell cycle length of LESC. Other pathways have been linked to stem cell renewal, such as Notch-1. Corneal specific inducible ablation of Notch1 demonstrated differentiation of LESC into hyperplastic, keratinised skin like epithelium (Vaurclair et al., 2007). Furthermore LESC express progenitor markers, including, p63 (Pelligrini et al., 2001) ABCG2 (Watanabe et al., 2004) and more recently N-cadherin.

1.5.4.2.3 Evidence for the Location of LESC to the Limbus:

The first experimental evidence for the location of LESC to the limbus was the movement of pigment from the limbal region towards an epithelial defect in rabbit corneas following wounding (Mann et al., 1944). Some years later Davanger and Evanson (Davanger et al., 1971) observed a similar migration of pigment from limbus to central cornea and proposed that the Palisades of Vogt (PV) situated in the corneal limbus provided the source of LESC (Huang et al., 1991). This movement from limbal to central cornea has been described as centripetal migration. This was demonstrated by gradual replacement of donor epithelium with host cells following lamellar keratoplasty, by looking at the dilution of sex chromatin using a female donor graft in a male recipient in rabbits (Kinoshita et al., 1981). Furthermore, the complete removal of the...
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Limb results in impaired corneal function, neovascularization and conjunctival growth (Huang et al., 1991). As stem cells are slow cycling they divide occasionally and therefore can be identified as label retaining cells (LRCs) (Bickenbach et al., 1986). Exposing cells to DNA precursors such as triitated thymidine and bromodeoxyuridine followed by a chase period of 4-8 weeks, the slow cycling stem cells retain this label whereas the more differentiated transient amplifying cells (TAC) undergo dilution of the label through multiple divisions. Through the use of triitated thymidine, Cot sarrel et al. found slow cycling or LRCs located in the limbal basal region of the mouse cornea and postulated that 10% of limbal basal cells were stem cells. This population of limbal basal cells phenotypically appear to be more primitive as they are small and round (Roman et al., 2003). The limbal basal region has areas lacking in differentiation markers. For example, the 64kDa cytokeratin 3 (CK3) was found in all layers of the corneal epithelium and the suprabasal layer of the limbal epithelium, however it was absent from the limbal basal cells and the adjacent conjunctiva (Schermer et al., 1986). A similar pattern was found with the corneal specific 55kDa protein, cytokeratin 12 (CK12) (Chaloin-Dufau et al., 1990). Furthermore, there is a lack of markers such as connexin 43 (Matic et al., 1997) and involucrin (Chen et al., 2004), both associated with cells destined for differentiation. Interestingly, the limbal basal region expresses progenitor cell markers such as the transcription factor p63 (Pelligrini et al., 2001), especially the ΔNp63α isoform (Di Iorio et al., 2005), the ATP-binding
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In vivo and in vitro studies have found that limbal basal cells have a higher proliferative potential when compared to peripheral and central cornea. Large epithelial wounds in rabbits have been shown to heal faster than smaller central defects, implying that the proliferative capacity of the peripheral cornea is greater than that of the central (Lavker et al., 1991). In the human, limbal explant cultures have a greater proliferative potential when compared to central explants (Ebatto et al., 1987). Based on human epidermal studies (Barran et al., 1987), supporting clonogenic studies found cells isolated from the limbus produced the larger holoclones (stem cell derived) compared to the less clonogenic meroclones and paraclones found elsewhere in the cornea (Pelligrini et al., 1999). Furthermore, LESCs proliferation is resistant to inhibition by tumor-promoting phorbol esters (Krusel et al., 1993, Lavker et al., 1998).

Supplementary to experimental studies the clinical evidence also points to the limbus as the location of corneal stem cells. In normal maintenance, the limbal epithelial cells are thought to act as a barrier to the conjunctival epithelial cells (Tseng SC, 1989). Ambati and coworkers recently shown that soluble vascular endothelial growth factor receptor 1 (sFlt 1) plays an important role in corneal avascularity (Ambati et al., 2006).

Further to this, expression of sFlt 1 was found in the corneal epithelium of normal individuals with less seen in vascularized patients (Ambati et al., 2007). When the limbus is non-functional, the conjunctiva can invade the corneal epithelium leading to chronic inflammation, neovascularization and...
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... cornel opacity. This phenomenon is known as limbal stem cell deficiency and can be attributed to both hereditary and acquired conditions. Further clinical evidence suggesting the location of LESCs was shown by Kenyon and Tsen, where they transplanted two limbal explants taken from the contralateral healthy eye of patients onto the other damaged eye. This resulted in epithelisation of the cornea and regression of persistent epithelial defects and neovascularisation (Kenyon et al., 1989). This initial work has led to the use of expanded LESCs grown on amniotic membrane (Tsai et al., 2000) and the use of autologous mucosal epithelial cell grafts (Nakamura et al., 2003).

1.5.4.2.4 The LESC Niche

The surrounding microenvironment or niche of a stem cell, which consists of cellular and extracellular components, is hypothesised to prevent them from differentiating and thus determines their fate (Schöflein et al., 1983, Watt et al., 2000). Once a stem cell divides asymmetrically and leaves its niche it enters a differentiation pathway under the influence of different environmental stimuli. Interestingly, the mechanism by which this occurs still remains unclear. This theory is in keeping with the LESC niche as it differs from the remaining corneal stroma both anatomically and functionally.

The hypothesised presence of a limbal niche is supported by the following reported in vivo studies:

a) The stromal support for the limbal epithelium determines the final phenotypic outcome of the epithelium proves the fact that when embryonic (Coulombe and Coulombe, 1971) or adult (Ferraris et al., 2000) rabbit corneal epithelium is recombinated with embryonic murine dermis and subsequently transplanted into nude mice, there is a change in expression...
of the corneal specific keratin pair CK3/CK12 to the epidermal specific keratin pair CK1/CK10 with the formation of hair follicles and sweat glands. This process of transdifferentiation from corneal to epidermal phenotype is controlled by the embryonic dermal stroma.

b) Esparza et al. (2003) have shown in the rabbit corneal epithelium that the limbal or corneal stroma can modulate stem cells and transiently amplify cells by influencing epithelial differentiation and lineage commitment. They showed that the limbal stroma promotes less epithelial differentiation and protects the epithelial cells from apoptosis, while the corneal stroma promotes increased epithelial differentiation and apoptosis. Like other stem cell niches, it is proposed that soluble factors, matrix components, and cell adhesion molecules probably mediate these processes within the limbal stem cell niche.

c) Clinically, the limbal 'niche' theory is supported by the fact that pathological conditions that affect the limbal stroma (e.g. inflammation, neurotrophic disorders, hormonal deficiencies and developmental anomalies such as pax6 gene mutations) can lead to limbal stem cell deficiency (Puangdeecharoen and Tsen, 1995).

d) The presence of a specific limbal stem cell niche that functions to maintain these cells in a quiescent state while communicating differently with their non-stem cell neighbors is supported by three studies: i) Esparza and colleagues (Esparza et al., 2002) showed that the limbal stroma enhances corneal epithelial stem cell survival; ii) the fact that the basement membrane beneath the limbal basal is distinct in terms of matrix composition (Ljubimov et al., 1995) and iii) the work of Steppe MA et al. that
1.5.4.2.4.1 Factors that Maintain the Limbal Stem Cell Niche (Figure 1.9):

A) Intrinsic Factors

Asymmetrical Division - It can be hypothesized that limbal epithelial stem cells like other stem cells probably undergo asymmetrical cell division, giving rise to a determined transient amplifying cell and stem cells to maintain and preserve the longevity of the epithelial population throughout the entire lifespan of the individual. It has still not been proven that the corneal epithelial stem cells can asymptomatically divide.

Positional - The unique position of limbal stem cells within the undulation of the limbal palisade makes the corneal stem cells respond better to signals from the neighboring conjunctival cells, transient amplifying cells, limbal fibroblasts, limbal capillaries and to cytokines and growth factors made available by the vascular tissue.

Interactions of Proteoglycans and Matrix Molecules - Proteoglycans and matrix molecules within the limbal basement membrane react with the various cytokines released from the neighboring tissue to initiate stem cell division and their subsequent differentiation.

Cell Receptors and Protein Expression - Strong expression of the TrkA receptor in basal limbal epithelial cells suggests that the nerve growth factor (NGF) signaling is involved in the control of limbal stem cell compartment ( Touhami et al., 2002 ).
Figure 1.9: Limbal stem cell niche. Limbal epithelial stem cells (SC) are located at the limbal basal layer. In this epithelial level, there are several other cell types in the vicinity such as the immediate progeny, i.e., early transient amplifying cells (eTAC), melanocytes (M), and Langerhans cells (LC). It remains to be determined whether these cell types act as niche cells. It is believed that eTAC will be destined for progeny production by differentiating into late TACs (lTAC) located at the corneal basal layer, then into suprabasal post-mitotic cells (PMC), and finally into superficial terminally differentiating cells (TDC). The limbal basement membrane (BM) separating the epithelium from the underlying stroma has several unique components. The subjacent limbal stroma contains mesenchymal cells (MC), which may also serve as niche cells. Because the limbal stroma is highly innervated and vascularized, the respective role of nerves (N) and blood vessels (BV) in niche remains to be defined. (Li W et al., 2007)

B) Extrinsic Factors

The microenvironment or niche of stem cells maintains "stemness" (Schöfl, 1983). Tissue culture studies have shown that limbal epithelial cells line
senece with time, indicating that stem cells are maintained by factors other than intrinsic properties alone. The therapeutic success of transplanted cultured limbal epithelial cells may be due to the persistence of TACs, rather than stem cells alone, and long-term follow-up is required to determine the duration over which this presumed "stemness" is maintained (Lindberg et al., 1993). The limbus acquired its blood supply from the palisades of Vogt, which provide nutrition and a greater scope for interaction with blood-borne cytokines (Gibson, 1989; Zieske, 1994).

1) Extracellular matrix role in functioning as the niche – Integrins have been postulated to regulate the onset of differentiation and morphogenesis in the stratified epidermis. High levels of β1 integrin have been postulated to maintain the stem cells in their presumed niche via the mitogen activated protein kinase (MAPK) pathway (Zhu et al., 1999). Such a role of integrins may also be important in the limbal epithelium.

2) Contribution of stem cells to an establishment of their own niche – Fuchs and coworkers on grafting individually cultured epidermal stem cells onto the skin of mutant hairless mice reported the regeneration of new hair follicles and supporting cells, by creating a niche de-novo (Blankpain et al., 2004). This role is yet to be investigated in the limbal stem cells.

3) Epithelial-mesenchymal cytokine interactions in the limbal stem cell niche – Stem cell behaviour is determined by interactions of the corneal epithelium with its underlying stroma via extracellular matrix, cell membrane associated molecules and cytokines. Li et al. have
implied different patterns of cytokine crosstalk between the epithelial cells and stromal keratocytes by showing that: a) epithelial cells produce: TGF α (transforming growth factor), IL-1 β (interleukin), and PDGF-B (platelet derived growth factor), b) epithelial and stromal cells produce: IGF-1, TGF-β1, TGF-β2, LIF and bFGF, and c) stromal fibroblasts produce KGF and HGF (Li and Tsen, 1997). KGF, produced by limbal stromal fibroblasts modulates stem cell proliferation by a mitogenic effect. HGF, produced by central corneal fibroblasts initiates differentiation and migration signals in epithelial cells. KGF production by limbal fibroblasts can be stimulated by IL-1 β and inhibited by TGF-α, PDGF-B and IL-1 β, all of which are expressed by corneal epithelial cells (Brazzell et al., 1991). IL-1 β, produced by epithelial cells at times of cellular stress, stimulates limbal stromal fibroblasts to release KGF, which in turn stimulates limbal epithelial cell proliferation.

4) Contribution by conjunctival epithelial cells - Besides producing various defense factors that help protect the corneal epithelium, the conjunctival epithelium expresses much lower Id1, Id3 and Id4 (inhibitor of differentiation proteins) when compared to the limbal epithelium. This may indicate that there are genes like the Id group that are involved in domain segregation and determination, or phenotype maintenance of the distinct lineages such as the corneal and the conjunctival epithelium (Wolosin et al., 2004). These findings were reported during the development stages of these two epithelial lineages.
Contribution by the vascular endothelial cells in the niche

The vascularized limbal stroma provides the limbal epithelium with nutrition and a scope for epithelial interaction with blood and fibroblast derived cytokines (Krusse and Tsen 1993b; Kruse and Volk, 1997). The limbus acquires its blood supply from the capillaries within the Palisades of Vogt.

6) The melanocyte-epithelial unit in the limbal niche

The dendritic melanocytes with the limbal basal epithelium forms a melanocyte epithelial unit, that functions in: i) protecting the limbus against UV light and ii) antioxidant activity (Protas, 1980), which assists in quenching UV-induced oxidant formation in the corneal epithelium.

1.5.4.2.5 Stromal Stem Cells:

In 2005, isolation of murine and bovine corneal stromal stem cells by sphere forming assay was reported by two independent groups (Du Y et al., 2005, Yoshida et al., 2005). In the same year, isolation of stromal stem cells from human cornea was also reported (Du Y et al., 2005). In this latter study, some stromal cells have shown ABCG2 positivity. Based on this observation, the side population was selected by digestion with collagenase and hyaluronidase. In culture, these side population cells showed clonal growth and could be differentiated to express keratocyte, chondrogenic and neurogenic markers (Du Y et al., 2005). The same group has concurrently showed that while these undifferentiated corneal stromal cells predominantly express stem cell related genes (Bmi-1, kit, Notch-1, Six2, Pax-6, ABCG2, Spp10, p62/OSIL) in adherent cultures, when passed in suspension in serum free medium with FGF2 and insulin, they form spheroid pellets, in
which keratocyte-like cells secrete an ordered ECM and express mRNAs of known (keratocan, PTGDS, ALDH3A1) and potential (FLJ30046/SLAIN, CxA4R, PDK4, MTTAC2D1, F13A1) keratocyte markers (Du Y et al., 2007).

Multipotent, fibroblast-like cells were isolated from limbal stroma by other groups as well (Draiva et al., 2005). In the earlier study, after enzymatic digestion of de-epithelialized stroma of limbal explants, stage specific embryonic antigen 4 (SSEA-4) positive cells were sorted by magnetic activator cell sorter (MACS). The isolated multipotent fibroblast-like cell showed a unique marker profile (CD34, CD45, CD123, CD14, CD106, HLA-DR+, SSEA4, CD73, CD105+) different from that of bone marrow mesenchymal (Jung et al., 2009) or other adult stem cells but similar to that of embryonic stem cells (Oct-4, Sox-2, Tra1-60, Tra1-80+) (Draiva et al., 2005). This marker profile is quite similar to that of very small embryonic-like cells of the adult humans (Zuba-Surma et al., 2009).

The presence of bone marrow derived cells was shown when irradiated wild type mice were transplanted with bone marrow or hematopoietic stem cells of GFP expressing transgenic mice. Most of these cells differentiated into antigen presenting cells in the host's cornea and only a small percentage of BM derived cells represented other (unidentified) cell types (Sosnova et al., 2005). Bone marrow derived cells formed approximately half of the pericytes but none of the endothelial cells of new vessels in a mouse model of experimental corneal neovascularization (Ozerdem et al., 2005). Recently, bone marrow derived progenitor cells were shown to promote wound healing and re-epithelialization in alkali injured rabbit corneas (Ye et al., 2006).
Yoshida and coworkers (Yoshida et al., 2005) isolated a subset of cells termed neural crest derived corneal precursors (COPs) from adult mice. These cells showed side population characteristics, were multipotent, clonogenic (sphere forming), and expressed various adult stem cell markers (nestin, notch-1, musashi-1, ABCG2). Experiments with transgenic mice proved that limbal bone marrow derived cells and COPs are two distinct cell populations and that COPs have a neural crest origin, which was also confirmed by the expression of the embryonic neural crest marker Twist, Snail, Slug and Sox-9. COPs expressed surface marker Sc-1 and CD34 and were negative for CD45 and c-kit.

Altogether, these results indicate that bone marrow derived cells mainly act as enhancers of wound healing and neovascularization, and take part in the immunological defense of the cornea. On the other hand, corneal stromal stem cells and COPs may serve as stem cells in the maintenance of the mesenchyma-derived parts of the cornea. As both cell types are located mainly in the peripheral cornea, interactions between them are possible. Understanding these interactions, as well as elucidating the behavior of these cell types under physiological and pathological conditions will greatly increase our knowledge on corneal wound healing and regeneration.

1.6. Scope and Aim of the Study

1.6.1. Scope of the Study

The field of stem cell biology is gaining a lot of importance in the therapeutics and the role of these cells in regenerative medicine is being explored in a number of clinical trials worldwide. Various sources of cells that are being evaluated
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With the belief that adult autologous cells have a better acceptance in clinical trials, we explored the potential of bone marrow derived stromal cells to transdifferentiate into neural lineage. Our initial aim was to establish and characterize the BMSCs of rat and human origin and explore their stemness and plasticity. Around the same time, our lab has reported a new observation of finding stromal cells in limbal cultures, which showed features similar to BMSC. So I pursued the objective of comparing the phenotype of these mesenchymal cells by various techniques.

1.6.2. The Focus of the Thesis

1. Rat Bone Marrow Stromal Cells - Isolation, characterization and differentiation of rat bone marrow stromal cells

2. Human Bone Marrow Stromal Cells - Isolation, characterization and differentiation of human bone marrow stromal cells

3. Limbal Stromal Cells - Isolation, characterization and differentiation of limbal stromal cells

4. Gene Expression Profile - Gene expression profile of limbal explant culture derived cells in comparison to bone marrow derived mesenchymal stem cells.