Comparative Properties of Stromal Cells and Their Role in Tissue Regeneration

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UNIVERSITY OF MIAMI

COMPARATIVE PROPERTIES OF STROMAL CELLS AND THEIR ROLE IN
TISSUE REGENERATION

By
Santhosh Kumar Sivajothi

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida
May 2016
In regenerative medicine, efforts should include utilization of the safest and least invasive methods with the goal of achieving optimal therapeutic outcome. Advancements in stem cell science have triggered the use of cells to cure various disease conditions. However, the clinical success of such approaches depends largely on understanding the basic biology of the key players involved—stem cells, stromal cells, and their microenvironment. Research shows that stromal cells have key roles in protecting and regulating tissue resident stem cells and maintaining tissue homeostasis. The objective of this dissertation work is to study the role of stromal cells in tissue repair and to find properties of stromal cells relevant for use in regenerative therapy. Many clinical studies have focused on transplantation of isolated and \textit{ex vivo} expanded stem cells into patients to achieve tissue repair. This study looks at the feasibility of stromal cell mobilization by growth factors and drugs. The effect of mobilized stromal cells was tested in an animal model of liver injury. Further, the properties of stromal cells isolated from different tissue sources were compared. Injections of combinations of granulocyte colony stimulating factor (G-CSF) and AMD3100 (plerixafor) were able to mobilize stromal cells into circulation. This combination also improved survival and tissue function in mice with
CCl4 induced liver damage. Stromal cells obtained from different sources were similar in morphology, phenotype and differentiation capacity into MSCs but were functionally different. Heart derived stromal cells and heart stromal cell conditioned media both inhibited proliferation of tumor cells *in vitro*. These findings present stem cell mobilization as an alternate or adjunct treatment to current regenerative approaches. Unique properties of different stromal cells, like the tumor cell suppression ability of heart stromal cells, will increase our understanding of their biology and enable more educated choices to be made in selection of cell source for regenerative therapies.
This dissertation is dedicated to my parents who endeavored to give me the best education possible and my wonderful wife Pooja. Their love and unyielding support is my greatest strength.

I would like to specially thank my mentor Dr. Ian McNiece for his instruction, patience and guidance.
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AS</td>
<td>Adipose stroma</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>Bone marrow mesenchymal stem cell</td>
</tr>
<tr>
<td>CCl4</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony forming unit- fibroblast</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>GM-CFC</td>
<td>Granulocyte macrophage colony forming cell</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony- stimulating factor</td>
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<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
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<td>HPC</td>
<td>Hematopoietic progenitor cell</td>
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<tr>
<td>HPP-CFC</td>
<td>High proliferative potential- colony forming cell</td>
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<tr>
<td>HS</td>
<td>Heart stroma</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<td>MNC</td>
<td>Mononuclear cell</td>
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<td>MSC</td>
<td>Mesenchymal stem cell</td>
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<td>SP</td>
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CHAPTER 1: INTRODUCTION

The work described in this thesis aims to study the key characteristics of stromal cells and better understand how they can aid in regeneration of damaged tissues and organs. This section provides a review of the topics relevant to this work.

1.1 History and Overview of Stem Cells

The term ‘stem cell’ was first used by Haeckel in 1868 to describe the origin of multicellular organisms from a unicellular organism (1). The recognition that tissues vary in their capacity to regenerate and the identification of tissues that can self-renew over an organism’s complete life span are rooted in 19th-century biological and medical science. Artur Pappenheim proposed that cells of the blood were related to one another, with mature cell types descending from a single cell type in a unified view of hematopoiesis (2). The existence of a stem cell, viewed as the ultimate origin of self-renewal in self-renewing tissues, was first postulated by Regaud based on his studies of spermatogenesis (3). Till and McCulloch showed that single cells could yield multi-lineage descendants while preserving the multipotency of the parent cell (4). They gave weight to the idea of a stem cell and introduced methods to define the cardinal properties of those cells, self-renewal and differentiation. Then in 1970, Leroy Stevens noticed that the primordial germ cells that gave rise to teratomas (tumors in scrotum of mice) looked a lot like the cells of considerably earlier embryos (5). Transplanted cells from various stages of early strain 129 embryos, including inner cell mass cells in mouse bellies yielded an impressive range of tissue types. These were called “pluripotent embryonic stem cells”. In 1998,
James Thomson reported the creation of the first batch of human embryonic stem cells, which they derived from early embryos (6).

Stem cells by definition have two essential properties, i.e. the capacity of self-renewal, and the capacity to differentiate into different cell lineages (7).

i) **Stem cells are capable of dividing and renewing themselves for long periods**. Unlike muscle cells, blood cells, or nerve cells, stem cells may replicate many times, or proliferate. A starting population of stem cells can go through numerous cycles of cell division while remaining unspecialized like its parent cells.

ii) **Stem cells are unspecialized**. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, stem cells cannot carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells of a tissue or organ.

iii) **Stem cells can give rise to specialized cells**. Under the right conditions, or given the right signals, stem cells can differentiate into the many different cell types that make up an organism. Unspecialized stem cells give rise to specialized cells though a process called ‘differentiation’. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. There are many signals inside and outside cells that trigger each step of the differentiation process. The internal signals are genetic and external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in
the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division.

1.2 Types of Stem Cells

There are two main types of stem cells, embryonic and non-embryonic. Embryonic stem cells (ESCs) are totipotent and, accordingly, they can differentiate into all three embryonic germ layers. On the other hand, non-embryonic stem cells (non-ESCs), also known as adult stem cells, are just multipotent; their potential to differentiate into different cell types seems to be more limited (8). Embryonic stem cells are derived from the inner cell mass of a blastocyst (a very early embryo) and the adult stem cells are derived from mature tissue. A large variety of cell types have been used for regenerative medicine, including adult cells, resident tissue specific stem cells, bone marrow stem cells, embryonic stem cells and the recent breakthrough discovery of induced pluripotent stem cells from mature/adult cells (iPS) (9). The focus of this thesis will be on adult stem cells in regenerative medicine.

1.2.1 Embryonic Stem Cells

Human embryonic stem cells (ES cells) are primitive (undifferentiated) cells that can self-renew or differentiate into all cell types found in an adult body (10, 11). The derivation of mouse ES cells was first reported in 1981 (12, 13) but it was not until 1998 that the derivations of human ES cell lines were first reported (6). A new era in stem cell biology began in 1998 with the derivation of cells from human blastocysts and fetal tissue
with the unique ability of differentiating into cells of all tissues in the body. Embryonic stem cells are derived from embryos at a developmental stage before the time that implantation would normally occur in the uterus. Each of the cells (blastomeres) of these cleavage-stage embryos is undifferentiated. The first differentiation event in humans occurs at approximately five days of development, when an outer layer of cells committed to becoming part of the placenta (trophectoderm) separates from the inner cell mass (ICM). The ICM cells have the potential to generate any cell type of the body, but after implantation, they are quickly depleted as they differentiate to other cell types with more limited developmental potential. The ICM derived cells can continue to proliferate and replicate them indefinitely and still maintain the developmental potential to form any cell type of the body. Bongso et al. first described isolation and culture of cells of the inner cell mass of human blastocysts, and techniques for deriving and culturing stable hES cell lines were first reported in 1998 (6). The trophectoderm was removed from 5th day blastocysts consisting ICM of 30-34 cells, was placed into tissue culture.

1.2.2 Adult Stem Cells

Stem cells that are found in developed tissue, regardless of the age of the organism at the time are referred to as adult stem cells. After development of the embryo most tissues are fully differentiated, and further growth or repair of damaged sites is undertaken by stem cells residing in particular tissues. Adult stem cells encompass a variety of populations of undifferentiated cells and are found in most adult tissues, where they act as reservoirs during the normal turnover and regeneration of an organ or tissue (14). Both their potency and proliferative potential are narrower than those of their embryonic counterparts but adult stem cells possess high proliferative potential,
substantial self-renewal capacity and ability to differentiate into at least one type of mature functional progeny (15). Hematopoietic stem cells (HSCs) are one of the best characterized adult stem cell populations but stem cells have been found in many adult tissues including blood, skin, central nervous system, liver, gastrointestinal tract, and skeletal muscle (16). Adult stem cells can also trans-differentiate give rise to the cells of a very different tissue, such as nerve cells in the brain (17). Therefore, exploring the possibility of using adult stem cells for cell-based therapies has become a very active area of investigation.

1.3 Stem Cell Niche

In 1978, Schofield proposed the “niche” hypothesis to describe the physiologically limited microenvironment that supports stem cells (18). The niche hypothesis has been supported by a variety of co-culture experiments in vitro and by bone marrow transplantation, in which the niche is first ‘emptied’ through irradiation or drug treatments (19, 20). However, these studies did not resolve the issue of the exact stem cell location and niche structure in vivo. Although locating and further identifying stem cell niches in mammals has been difficult owing to their extremely complicated anatomic structures, studies regarding stem cells and their location/niche in other genetic model systems, including those of Drosophila melanogaster and Caenorhabditis elegans, have been fruitful. In mammals, the epithelial stem cell location was successfully identified in the bulge area of hair follicles, and the intestinal stem cell location was identified near the crypt base. These were based on the adult stem cell’s ability to retain the BrdU or 3H-thymidine labels (21, 22). Recently, there has been significant progress regarding stem
cells and their surrounding microenvironments in a variety of mammalian models. In 2003, two independent, simultaneous studies using genetic mutant mouse models led to the identification of osteoblastic cells, primarily those lining the trabecular bone surface, as the key component of the HSC niche (23, 24). Historically, ‘niche’ is generally used to describe the stem cell location. However, we believe “niche” is composed of the cellular components of the microenvironment surrounding stem cells as well as the signals emanating from the support cells.

1.3.1 Features, Structures, and Functions of the Stem Cell Niche

After comparison of the stem cell niches in the ovary and testis of *D. melanogaster* and in *C. elegans*, as well as in mammalian bone marrow, hair follicle, intestine, brain, and testis, the common features, structures, and functions of the stem cell niche are summarized as follows:

- The stem cell niche is composed of a group of cells in a special tissue location for the maintenance of stem cells. The niche’s overall structure is variable, and different cell types can provide the niche environment. For example, N-cadherin-positive osteoblastic lining cells in the trabecular bone form the niche for HSCs (23, 24).

- The niche functions as a physical anchor for stem cells. E-cadherin-mediated cell adhesion is required for anchoring germline stem cells (GSCs) and somatic stem cells (SSCs) in *Drosophila* (25), and N-cadherin may be important for anchoring HSC in the bone marrow niche. Other adhesion molecules, such as integrins, may help anchor stem cells to extracellular matrixes (26).
In invertebrates and mammals, the stem cell niche exhibits an asymmetric structure. Upon division, one daughter cell is maintained in the niche as a stem cell (self-renewal); the other daughter cell leaves the niche to proliferate and differentiate, eventually becoming a functionally mature cell.

The niche generates extrinsic factors that control stem cell fate and number. Many signal molecules have been shown to be involved in regulation of stem cell behavior, including Shh, Wnts, Notch and TGF-β/BMPs (27-30). Among these, the BMP and Wnt signal pathways have emerged as common pathways for controlling stem cell self-renewal and lineage fate from *Drosophila* to mammals (31). Several pathways can be utilized to control self-renewal of one stem cell type, whereas one growth factor can regulate several different stem cell types. The presence of signaling components of multiple conserved developmental regulatory pathways in stem cells supports the ideas that stem cells retain the ability to respond to these embryonic regulatory signals and that orchestration of these signals is essential for proper regulation of stem cell self-renewal and lineage commitment. The three-dimensional niche microenvironment consisting of signaling molecules, intercellular communication and the interaction between stem cells and their neighboring extracellular matrix is thought to influence/control genes and properties that define ‘stemness’ of the stem cells, i.e. self-renewal or development to committed cells. For example, it remains uncertain whether endosteal cells, such as osteoblasts and osteoclasts, influence HSC numbers in the bone marrow by promoting the maintenance of HSCs that reside in direct contact with these cells, or by secreting factors that act at a distance, directly or indirectly regulating HSCs that are localized
to other nearby microenvironments (32, 33). An interesting theory put forward is that stem cells might be terminal differentiation cells with the potential to display diverse cell types, depending on the host niche. Adult stem cells that are implanted into a totally different niche (different germ layer) can potentially differentiate into cell types similar to those found in the new environment. As such, it is important to understand the role of stromal cells which are a key component of the stem cell microenvironment or niche.

1.4 Stromal Cells and their Functions

The stromal cells are now known to constitute a group of cells that act as the supportive ‘mattress’ on which the maturing precursors of blood or in other words the stem and the progenitor cells rest directly (34). The exact nature of the stem niche is not defined but there is ample evidence that the stromal cells are the most important constituent of the niche structure (35, 36). The HSC niche in the bone marrow is one of the most extensively studied niche structures. The BM cells as a whole, when put into culture, are represented with plastic adherent cells, showing multipotent differentiation capacity in vitro and are diffusely termed as stromal and/or mesenchymal stem cells.

Subsequent to the work of Schofield in defining the niche, in 1985 Owen propounded that the marrow stromal cells had the following criteria:

- they are found in the extravascular compartment of the mammalian BM
- they participate in providing physical and functional support for the hematopoietic cells
- they are not of hematopoietic lineage and
• they are members of the marrow stromal system.

These hinted at the presence of closely related BM derived stromal/mesenchymal stem cells (MSCs) which are found to be essential components of the niche structure (37). Stromal cells in different forms also have a great interplay in the generation, release, differentiation and traffic of stem cells/progenitor cells (38). It has been shown that MSC pool comprises not only putative MSCs but also subpopulations at different steps of differentiation potential. Unlike other stem cells, MSCs may not have their own protective niche and seem not to be restricted to their tissue of origin (39). The stromal cells exert their effect on hematopoietic cell via direct cell-cell interaction as well as by releasing soluble factors. It is also presumed that stromal cells in turn also might receive signals provided by developing hematopoietic cells in the BM microenvironment. Evidence is accumulating that the stromal cells through the delicate mechanism of tight coupled crosstalk, can protect and nourish stem cells (40). The niche matrix is regulated by the stromal cells via several degrading enzymes like matrix metalloproteinases, adhesion molecules, extra cellular protein products, chemokines all guided through specified gene expression (41).

1.5 Mesenchymal Stem Cells (MSCs)

Friedenstein and colleagues reported that the bone marrow stroma, upon transplantation into different tissues in mice, can generate bone, fat cells, cartilage and reticular cells. This suggested the existence of non-hematopoietic BM multipotent precursor cells with skeletal and adipose potential (42). It was later shown that these precursors were a subset of fibroblast-like cells defined as colony-forming unit
fibroblasts (CFU-F) that could be selected by adherence to plastic surfaces and expanded in vitro (43). Further research showed the ability of cultured cells derived from single CFU-Fs to proliferate while preserving the ability to differentiate to osteoblasts, adipocytes and chondrocytes in vitro (38). The term mesenchymal stem cell (MSC) was then coined and gained acceptance to refer to these newly identified precursor cells (44). In addition to bone marrow, MSCs or MSC-like cells have also been isolated from adherent fraction of many tissues skeletal muscle, adipose tissue, umbilical cord, dental pulp, liver etc (45). Clonal studies have shown that plastic adherent populations isolated from bone marrow are functionally heterogeneous and contain undifferentiated stem/progenitors and lineage-restricted precursors with varying capacities to differentiate into connective tissue cell types (46).

1.5.1 Definition of MSCs

Despite their functional heterogeneity, MSC populations obtained from most tissues commonly express a number of surface receptors including CD29, CD44, CD49a-f, CD51, CD73, CD105, CD106, CD166, and Stro1 and lack expression of definitive hematopoietic lineage markers including CD11b, CD14, and CD45. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes the following minimal criteria to define human MSCs:

1. MSC must be plastic-adherent when maintained in standard culture conditions.
2. MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules.
3. MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro.
Recent studies have shown cells that express the aforementioned surface markers and are capable of differentiating into connective tissue cell types can be enriched from bone marrow by selection for STRO-1 (47), stage-specific embryonic antigen (SSEA)-1, SSEA-4, or the nerve growth factor receptor CD271 (48). Other studies have shown that bone marrow-derived MSCs express the pericyte-specific markers CD146 and 3G5 (49), consistent with the fact that specialized vascular pericytes in bone marrow are thought to represent the closest \textit{in vivo} approximation to MSCs. However, there is no single isolation method regarded as a standard in the field. Recent studies have shed some light on the exact identity and native distribution of MSCs, whereas controversial results are still being reported, indicating the need for further review on their definition and origin.

MSCs express trophic factors, such as growth factors, cytokines, and chemokines, which are known not only to reduce the inflammation, apoptosis and fibrosis of damaged tissues but also to stimulate angiogenesis and tissue cell regeneration (50). After MSCs move to damaged sites for repair, they are stimulated by local factors, such as inflammatory cytokines, ligands of Toll-like receptors and hypoxic conditions. These stimuli lead to the production of a large amount of growth factors that perform multiple functions to achieve tissue regeneration (51, 52).

1.5.2 Immunomodulatory Properties of MSCs

One of the most interesting aspects of MSCs is their immunomodulatory activity. MSCs are characterized by low expression of human leukocyte antigen (HLA) class I molecules and the absence of major histocompatibility complex (MHC) class II antigens, Fas ligand and the co-stimulatory molecules B7-1, mB7-2, CD40, and CD40L. These reduced immunogenic expression profiles cause MSCs to have immuno-tolerant
phenotypes, allowing them to be used in allogeneic transplantation (53). MSCs can express various soluble factors, such as nitric oxide, prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), IL-6, IL-10, and HLA-G. These soluble factors regulate the proliferation and functions of a variety of immune cells and induce Treg cells (54). *In vitro*, MSCs inhibit T cell activation, dendritic cell differentiation, B cell proliferation and impair the cytolytic potential of natural killer cells (55). In addition to the secretion of soluble factors from MSCs, these cells can suppress the activation of immune cells through direct cell-cell contact. MSCs can inhibit T-cell proliferation by inducing the apoptosis of effector T cells by promoting the association of programmed death-1 (PD-1) with its ligands PD-L1 and PD-L2, and MSCs are capable of rendering T cells anergic by down-regulating the expression of the co-stimulatory molecules CD80 and CD86 on antigen-presenting cells (56). Immunosuppression after MSC infusion *in vivo* has also been documented in diverse animal models of disease (57).

### 1.6 Applications of Stem Cells

- Stem cells can be used to study development. Stem cells may help us understand how a complex organism develops from a fertilized egg. We can follow stem cells *in vitro* as they divide and become increasingly specialized, making skin, bone, brain, and other cell types. Identifying the signals and mechanisms that determine whether a stem cell chooses to carry on replicating itself or differentiate into a specialized cell type, and into which cell type, will help us understand what controls normal development.
• Some of the most serious medical conditions, such as cancer and birth defects, are due to abnormal cell division and differentiation. A better understanding of the genetic and molecular controls of these processes may yield information about how such diseases arise and suggest new strategies for therapy. This is an important goal of stem cell research.

• Stem cells have the ability to replace damaged cells and treat disease. This property is already used in the treatment of extensive burns, and to restore the blood system in patients with leukemia and other blood disorders.

• Stem cells can be used to study diseases. In many cases it is difficult to obtain the cells that are damaged in a disease, and to study them in detail. Stem cells, either carrying the disease gene or engineered to contain disease genes, offer a viable alternative. We can use stem cells to model disease processes in the laboratory, and better understand disease mechanism.

• Stem cells may hold the key to replacing cells lost in many devastating diseases for which there are currently no cures available. Presently, donated tissues and organs are often used to replace damaged tissue, but the demand for transplantable tissues and organs outweighs supply. Stem cells, if they can be directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases including Parkinson’s, stroke, heart disease and diabetes. This prospect is an exciting one, but significant technical hurdles remain that need to be overcome through intensive research.
1.7 Regenerative Medicine

Regenerative medicine is an emerging and rapidly evolving field of research which aims to replace or regenerate human cells, tissue, or organs to restore or establish normal function (58). Regenerative medicine is multidisciplinary in nature, having roots in diverse disciplines such as surgery, organ transplantation, biomaterials science, engineering, developmental biology, and stem cell biology (58, 59).

Stocum has argued that regenerative medicine is not viable without an understanding of regenerative biology (60). It refers to the mechanisms whereby adult organisms restore form and function to damaged tissues and organs. Regenerative biology focuses on three aspects:

- elucidation of the mechanistic aspects of embryonic development- this is crucial to understanding regeneration
- studying adult tissue turnover and replacement and mechanisms of repair in an evolutionary context
- identification and use of adult and pluripotent stem cells (61)

The role of stem cells in regenerative biology has made them one of the key underpinnings of regenerative medicine. Regenerative therapies have the potential to revolutionize the practice of medicine and provide treatment for conditions where current therapies are inadequate. Advancements in stem cell biology, including embryonic and postnatal somatic stem cells, are at the core of making regenerative therapies a clinical reality.
1.7.1 Uses of Stem Cells in Regenerative Medicine

The use of stem cells as regenerative therapies is a highly active area of research. Depending on the type and source of cells, the goal may be for the applied cells to engraft and repopulate the tissue, modulate immune function, or to stimulate endogenous cell populations to heal the tissue. Cells intended for regenerative therapy may be embryonic stem cells, fully differentiated adult cells or stem cells derived from the tissue to be treated or a tissue with a related lineage.

**Table 1.1:** Examples of regenerative medicine strategies

<table>
<thead>
<tr>
<th>Type of technology</th>
<th>Examples of specific application(s) under study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced pluripotent stem cells</td>
<td>Endothelial cells, neurons, and hepatocytes for preclinical applications</td>
</tr>
<tr>
<td>Transdifferentiation of cells</td>
<td>Preclinical stage for transdifferentiation of fibroblasts into brain cells</td>
</tr>
<tr>
<td>Embryonic stem cells</td>
<td>Severe heart disease and macular degeneration</td>
</tr>
<tr>
<td>Autologous stem cells</td>
<td>Retinal conditions, reconstructive surgery, heart disease, bone damage, and populating matrices for organ transplantation</td>
</tr>
<tr>
<td>Allogeneic stem cells</td>
<td>Diabetes, cirrhosis, and other liver diseases, neurologic diseases, stroke, and critical limb ischemia</td>
</tr>
</tbody>
</table>

Stem cells are currently being tested as possible therapies for a variety of diseases. The choice of cells and methodology of treatment depends heavily on the nature of the condition being treated.
1.7.1.1 Cardiovascular Disease

Heart transplants remain the only treatment for the most advanced stages of cardiovascular disease and myocardial infarction (MI) but there is shortage of donors and complications of immunosuppression. Surgical remodeling of the left ventricle is limited in application and mechanical ventricular assistance remains a temporary solution for those waiting for a transplant. There is thus a need for new treatment solutions. Xenotransplantation has considerable immunological challenges and there are major safety considerations. While gene therapy and IPS cells are still in their infancy (62, 63), cell therapy has a place in the treatment of cardiovascular disease, but only in patients who retain a sufficient reserve of contractile cells. Numerous clinical trials are listed with a majority employing bone marrow, Wharton's jelly and adipose derived stem cells (64-67). Histologic observations in autopsy of samples of allogenic cardiac grafts in sex mismatch showed the formation of cardiomyocytes with the receiver genotype in the myocardial tissue coming from the donor (68). Y genotype cardiomyocytes have been shown in the myocardium of female mice that received an intravenous injection of bone marrow coming from male mice. Isotypic studies showed the homing of progenitor stem cells from bone marrow towards the lesion sites after a coronary ligation.

At the end of 2007, a US based stem cell company Osiris Therapeutics completed a human trial using allogeneic SC for the treatment for heart disease. An intravenous drip was used to deliver of the shelf MSC to patients that had recently suffered a heart attack. No deaths occurred, and the treatment is now widely thought as safe (69).
1.7.1.3 Nervous System and Neurodegenerative Diseases

Spinal cord repair after injury is currently the focus of a great deal of research (70, 71). In 2010, a first study from cells derived from embryo SC producing oligodendrocytes was carried out in a volunteer in connection with the GERON Company (72). Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a progressive muscle weakness that can result in paralysis and death. Numerous hypotheses have been developed about the origin of ALS, with some implication of immune system involvement. Cell transplantation therapy for ALS aims to generate a neuroprotective environment for degenerating motor neurons by transplantation of non-neuronal cells, rather than to replace lost motor neurons. Among the cell therapy approaches tested in animal models of motor neuron disease, systemic injection of human cord blood mononuclear cells has proven to reproducibly increase the life span of SOD1G93A mice, a model of familial ALS, even if only few transplanted cells were found in the damaged areas. The study showed that human cord blood (mononuclear cells) significantly enhanced symptoms progression and prolonged survival in SOD1G93A mice and were localized in the lateral ventricles, even 4 months after administration (73). They observed in this study that hCB-MNCs release a series of cytokines and chemokines with anti-inflammatory properties that could be responsible of the functional improvement of mouse models of motor neuron degenerative disorders. The ongoing clinical trials are mainly focused on Parkinson’s (74) and Alzheimer’s (75) diseases and ALS (76).
### 1.7.1.5 Pancreas and Diabetes

The first attempt of cell therapy by grafting of islets of Langerhans was published in 1990 (77). Other cell sources have also been proposed for pancreas and diabetes cell therapy like adult MSCs (78, 79) or fetal MSCs (80), embryonic stem cells (81) or even IPS (82, 83). Two studies with different therapeutic approaches are currently investigating the influence of cord blood stem cells on improving the function of pancreatic beta cells. In the first approach, children with young-onset diabetes are infused with autologous cord blood without chemotherapy. Initial results have shown that such autologous cord blood transplantation without chemotherapy did not result in adverse effects but has not significantly improved disease condition and all children were still dependent on administration of insulin. In the second approach, adult patients with newly diagnosed diabetes mellitus underwent nonmyeloablative chemotherapy after receiving reinfused stem cells from autologous bone marrow. Different trials on diabetes type 1 or 2 are mainly performed with autologous or allogenic bone marrow or Wharton's jelly MSC (84, 85).

### 1.7.1.6 Liver Diseases

In response to a variety of chronic injuries such as hepatitis, alcohol or drug abuse, metabolic diseases, autoimmune disorders and congenital abnormalities, liver fibrosis occurs and finally leads to hepatic cirrhosis and liver failure. Liver transplantation is the accepted treatment option for these end-stage liver diseases. Again, this is limited by the shortage of donor organs. Correction of hepatocyte functional deficiency is the prime goal of liver transplantation and there is mounting evidence in
support of cell therapy. As an alternative to liver transplantation, some studies used hepatocytes to treat patients with liver diseases. However, large numbers of hepatocytes are needed but are not available from donors or patients themselves. SC therapy has been accepted as one of the new approaches to recolonize liver. Several studies reported the hepatocyte differentiation potential of embryonic, fetal, or adult MSC but also iPS (86-88).

As the liver contains three different cell types, which are organized in three-dimensional structures, growth and regeneration processes are highly complex. Therefore the idea of using one-type of SC leading to these three types of cells to repair liver is acceptable. Various populations of SCs are under investigation in terms of their regenerative capabilities. Recently, studies showed that extrahepatic adult MSCs of different tissue origins have demonstrated their ability to express a hepatocyte-like phenotype after in vitro differentiation. These cells which include MSCs derived from bone marrow, umbilical cord, adipose tissue, and placenta are being used in clinical trials mainly for cirrhosis due to hepatitis, alcohol abuse, and liver transplantation (89-92)

1.8 Aims and Hypothesis

Stromal cells form one of the main components of the stem cell niche which supports and maintains adult stem cells in distinct regions of organs (34). Stromal cells in different forms also have a great interplay in the generation, release, differentiation and traffic of stem cells/progenitor cells (38). Thus, stromal cells in the niche protect and control stem cells which are essential mediators of tissue regeneration.
My overall hypothesis is that stromal cells are potentially key mediators of effective tissue repair and in order to use them successfully in stem cell therapies, we should better understand their properties. To test this hypothesis I undertook two different approaches:

- The first aim was to find a method to mobilize large numbers of stromal cells to the site of tissue injury and to test their effectiveness in repair
- The second aim was to characterize stromal cells and their properties in order to make smarter decisions on their use in regenerative therapy

This two pronged approach will help to understand the possibility and effectiveness of repairing tissue damage using autologous stem cells of the patient without external manipulation and also expand our knowledge of the basic biology of stromal cells.
CHAPTER 2: MATERIALS AND METHODS

2.1 Apheresis Products

Non-mobilized PBPC and G-CSF mobilized PBPC were purchased from Allcells LLC (Emeryville, CA):

2.2 Cells and Cell Culture

Human bone marrow MSCs were isolated by differential adhesion from bone-marrow aspirate obtained from human donors with patient consent and under IRB approval. Red cell lysis was performed on the marrow aspirates and mononuclear cells (MNCs) were washed and re-suspended in Human Mesenchymal Stem Cell Medium with supplements (Stem Cell Technologies, Vancouver, BC) and incubated in 25cm² (T-25) tissue culture flasks (Corning) and allowed to adhere for 3 days in a humidified tissue culture incubator at 37°C with 5% CO₂. On day 3 of growth, non-adherent cells were removed with full replacement of the growth media. Cells attached to the surface were expanded over another two week period with media changes at regular intervals. Cells were harvested at day 21 using 0.05% Trypsin-EDTA (GIBCO BRL, Grand Island, NY). BMMSCs were frozen in cryovials at 1,000,000 cells per vial in 1 mL of cryopreservation medium.
2.3 Generation of Stromal Cell Lines

The stromal cell lines used in experiments were generated from respective tissue samples. All tissues were obtained with patient consent and under IRB approval. Briefly, tissue samples were cut into tiny pieces with addition of 3 ml PBS supplemented with 1% fetal bovine serum (FBS; Akron Biotech, Boca Raton, FL) to keep them wet. The tissue pieces were then incubated with collagenase solution (Sigma Chemicals, St.Louis, MO) at a final concentration of 0.12% v/v for 20 min in PBS supplemented with 1% FBS to digest them. Digested tissue pieces were then seeded into tissue culture flasks and incubated with alpha-minimum essential medium (α-MEM; Mediatech Inc., Herndon, VA) containing 20% FBS and 1% Penicillin-Streptomycin-Glutamine (GPS, Gibco) at 37°C (This medium referred to as A20 will be used for all cell culture experiments). The adherent cells growing out of the periphery of the tissues pieces represent the stromal cell population. The cells were then trypsinized, passaged and maintained. Adipose stromal cells (AS) were obtained from ATCC.

Human cord blood was obtained under IRB approval. Mononuclear cells (MNCs) were isolated from the cord blood by gradient density separation using Ficoll-paque (GE Healthcare, Pittsburgh, PA) by following manufacturer’s instructions. CD34+ cell selection was performed on the CB-MNCs by magnetic separation using CD34 magnetic beads and separation columns (Miltenyi Biotec, San Diego, CA) by following the manufacturer’s instructions.
2.4 Co-Culture Assays

2.4.1 Preparation of Stromal Cell Layer

Stromal cell lines were harvested by trypsinization using 0.05% trypsin-EDTA, counted and seeded in T-25 flasks at a density of \(10^5\) cells/ml (total 5ml/flask) in A20 media and incubated at 37°C, 5% CO\(_2\). Once the flasks were 80-90% confluent, they were used to initiate co-culture.

2.4.2 Co-culture of Cells over Stroma

10^5 cells were seeded per T-25 flask containing the stromal cell layer and co-cultured for 14 days in an incubator at 37°C, 5% CO\(_2\). The flasks were supplemented with fresh media at d7 of culture. At the end of 14 days, the cells were removed and cell counts were performed using trypan blue 0.4% (Gibco, Grand Island, NY). Any tumor cells adhering to the stromal cell layer were removed by adding 0.5% trypsin-EDTA to the flask for <30 seconds with continuous gentle tapping.

2.5 Flow Cytometry

Cells to be phenotyped were harvested by trypsinization and counted. 10^5 cells were then transferred to FACS tubes. The following cell-surface antigens were marked with anti-human antibodies: CD105 (eBioscience), CD90, CD73, CD45 and CD34 (Becton Dickinson) at appropriate concentrations in a total volume of 100ul of FACS buffer (PBS supplemented with 1% FBS). They were then incubated at 4°C in dark for 30 mins. Appropriate isotype-matched, nonreactive fluorochrome-conjugated antibodies were used as controls. Cells were then washed and re-suspended in 500ul ice-cold FACS
buffer. Labeled cells were acquired using a FACSCalibur flow cytometer running CellQuest software (Becton Dickinson) and acquired data was analyzed using FlowJo (v10.1) software.

2.6 Differentiation Assays

2.6.1 Adipogenic Differentiation

To induce adipogenic differentiation, the cells were seeded at a density of $2 \times 10^4$ cells per cm$^2$ into multi-well plates and cultured in A20 medium till 100% confluence. Then the growth A20 medium was replaced by adipogenic induction medium consisting of alpha-MEM containing 1 mM dexamethasone (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma-Aldrich), 10 μg/ml recombinant human insulin (Sigma-Aldrich), 100 mM indomethacin (Sigma-Aldrich), and 10% FBS (Akron). Cells were grown in this medium for 21 days, with fresh media replacement every 3 days. The non-induced control cells were fed only with alpha-MEM medium containing 10% FBS (A10). Adipogenic differentiation was confirmed by the formation of neutral lipid-vacuoles stainable with Oil Red O (Sigma-Aldrich). For the Oil Red O staining, cells were fixed with 10% formalin (Sigma-Aldrich), washed, and stained with a working solution of 0.5% Oil Red O in methanol for 5 minutes.

2.6.2 Osteogenic Differentiation

To induce osteogenic differentiation, the cells were seeded at a density of $2 \times 10^4$ cells per cm$^2$ into multi-well plates and cultured in A20 medium till 100% confluence. Then the growth A20 medium was replaced by osteogenic induction medium consisting of alpha-MEM containing 10% FBS (Akron) 100nM dexamethasone (Sigma-Aldrich),
50µM ascorbic acid (Sigma-Aldrich) and 20mM β-glycerol phosphate (Sigma-Aldrich). Medium was changed every 3 days. After 17 days, cells were fixed in 10% formalin for 10 mins and stained with 10% Alizarin Red for 5 mins to visualize the mineral deposits formed by osteoblasts.

2.7 Colony Forming Assays

2.7.1 GM-CFC and HP-CFC Assay

Colony forming assays were used to evaluate the levels of primitive (HPP-CFC) and mature (GM-CFC) progenitor cells in the peripheral blood of mice. WBCs collected from blood by Ficoll separation were plated in 1-mL methylcellulose based assays at 500 to 10,000 cells per culture in rhSCF, recombinant human interleukin 3 (rhIL-3), rhIL-6, rhG-CSF, and rhGM-CSF (Stem Cell Technologies Inc., Vancouver, BC, Canada). Cultures were incubated for 14 days at 37°C, 5% CO₂ and GM-CFC colonies identified as colonies of ≥50 translucent cells using a dissecting microscope at 20× magnification. After scoring at day 14, the cultures were incubated for an additional 14 days and scored for HPP-CFC, defined as compact large colonies (diameter >0.5mm) that contained >50,000 cells per colony.

2.7.2 CFU-F Assay

Cells were plated in triplicate cultures (35mm culture dishes) at 2 different densities using complete media prepared with MesenCult Basal Medium and Mesenchymal stem cell Stimulatory Supplements (StemCell Technologies; Vancouver, BC, Canada). The formation of CFU-F was evaluated after 10 days of culture in a humidified 5% CO2/37°C environment. Cultures were washed with calcium and
magnesium free phosphate buffered saline (PBS) twice and then fixed with methanol for 10 minutes. Remaining methanol was removed and plates were air dried. Plates were stained with Giemsa stain for 1 minute and washed with distilled water. CFU-F colonies containing >50 cells were counted using light microscopy.

2.7.3 In vitro Hematopoietic Progenitor Cell Assay

The progenitor content of hematopoietic cell/stromal cell co-cultures was assessed using in vitro methylcellulose colony-forming assay. Recombinant murine (rm) interleukin-3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech Inc (Rocky Hill, NJ). Recombinant human (rh) IL-6, granulocyte colony-stimulating factor (G-CSF), and recombinant rat (rr) stem cell factor (SCF) were obtained from Amgen (Thousand Oaks, CA). Cells were plated in methylcellulose (M4230, StemCell Technologies) supplemented with 100 ng/ml rr SCF, 100 ng/ml rm IL-3, 100 ng/ml rh IL-6, 100 ng/ml rh G-CSF, and 100 ng/ml rm GM-CSF. Hematopoietic colonies (committed colony-forming cells granulocyte-macrophage, CFU-GM) were scored after 14 days of culture in 5% CO₂ at 37°C according to the established criteria.

2.8 Animals

Eight to 12 week-old BDF1 (C57Bl/6 x DBA₂ F₁) mice were used for all experiments unless specified otherwise.
2.9 Mobilization of Stromal Cells

Animals were injected intraperitoneally (IP) with G-CSF (250 ug/kg), Substance P (10ug/kg) and AMD3100 (100ug/mouse) either alone or in specified combinations. At various intervals blood was collected from the tail vein into microcentrifuge tubes containing heparin. Full blood counts were performed using an Advia 120 Hematology Analyzer (Bayer Corp., Norwood, MA).

2.10 Liver Injury Model

A CCl4 induced liver injury model was adopted. CCl4 was diluted to 50%v/v in olive oil. To induce injury, 2.5 ml/kg or 1 ml/kg of the 50%v/v CCl4 solution was injected intraperitoneally. Animals were sacrificed by carbon dioxide suffocation and blood was harvested by cardiac bleeding. Liver tissue was collected, washed in PBS and fixed in 4% paraformaldehyde. 5um sections were cut for histological staining with H&E.

2.11 Microarray Analysis

2.11.1 Total RNA Extraction, Microarray Hybridization, and Data Analysis

RNA was extracted from three independent cultures of each of the stromal cell lines from adult human bone marrow and fetal heart. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instruction and additionally purified with RNeasy Mini Kit (Qiagen, Cat # 74106). RNA was quantified with a Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington) and its quality was examined with a Bioanalyzer 2100 using the
RNA 6000 Nano kit (Agilent, Santa Clara, CA). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX) according to the manufacturer’s instructions starting with 400 ng total RNA. Successful cRNA generation was checked using the Bioanalyzer 2100. Samples were added to the Beadchip after randomization using the randomized block design to reduce batch effects. Hybridization to the Sentrix Human-6 Expression BeadChip (Illumina, Inc., San Diego, CA), washing and scanning were performed according to the Illumina BeadStation 500 manual (revision C). The resulting microarray data was analyzed using Illumina Beadstudio software.

2.11.2 TaqMan Real-time PCR microRNA Array

Total RNA was isolated as described above. RNA quality was assessed with a Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington) and RNA integrity and presence of the small RNA fraction was determined using a Bioanalyzer 2100 (Agilent, Santa Clara). 60ng of total RNA was reverse transcribed using the human megaplex pool A and B primers and the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City) according to the manufacturer’s instructions. Each sample was pre-amplified for 12 cycles using human pool A and B pre-amplification primers and the Taqman PreAmp Master Mix (Applied Biosystems) according to the manufacturer’s instructions. For each sample the pre-amplification reactions A and B were diluted and each reaction was combined with Taqman Gene-Expression Master Mix (Applied Biosystems) split in 8 aliquots and each aliquot added to one of the eight sample ports of the Human miRNA Taqman array A or B, respectively. Each of the ports of the Taqman array feeds 48 reaction vessels holding individual miRNA assays. Human
miRNA Taqman array A and B hold 667 different miRNA target and 4 miRNA reference assays. The real-time PCR reactions were run according to the manufacturer’s instructions. RealTime Statminer Software (Integromics, Philadelphia) was used to analyze the data. The reference miRNA assays included on the Taqman arrays did not pass the expression stability test. Therefore reference miRNA assays were chosen based on expression stability between different samples using the GeNorm algorithm.

2.11.3 Quantitative-PCR

Real-time PCR for miR-1, miR-133a and miR206 was performed on the CStrCs and BM MSC. The single tube TaqMan MicroRNA Assay was used. All reagents, primers and probes were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA). RNU6B was used as a normalizer. One nanogram of RNA per sample was used for the assays. All RT reactions, including no-template (no cDNA) controls and minus controls (no reverse transcriptase), were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Comparative real-time PCR was performed in triplicate. Expression of the microRNAs was calculated utilizing the comparative Ct method and compared with t test. P < 0.05 was considered statistically significant.

2.12 Conditioned Media

2.12.1 Generation of Conditioned Medium

Cells are plated in T-162 flasks and cultured with A20 growth medium at 37°C, 5% CO2. After the cells are 90% confluent, the growth medium is removed and cells are
washed twice with PBS. Fresh alpha MEM medium without serum is added and the cells are incubated at 37°C, 5% CO2. This media is then collected at different intervals as indicated in each experiment and centrifuged to remove any cells. The supernatant is collected and labeled as 1X conditioned medium.

2.12.2 Concentration of Conditioned Medium

Conditioned medium is collected and concentrated by dead end filtration method with constant pressure. A bioseparations stirred cell unit (Stirred Cell Model 8400, Millipore) is assembled with a 10,000 kDa ultrafiltration membrane (Ultracel Ultrafiltration YM-10, Millipore). The medium to be concentrated in filled in the unit and the unit is assembled on top of a magnetic stirring platform. The unit is connected to a nitrogen tank and flow of nitrogen is started to apply constant pressure over the medium inside the unit. The flow through solution is discarded and the volume of medium inside the unit decreases. When the desired volume is reached, the process is stopped by depressurizing the unit and the concentrated medium is collected. This medium is sterilized by filtering through a 0.45um Acrodisc syringe filter. Concentrated medium can be stored in aliquots at -20°C.
CHAPTER 3: MOBILIZATION OF STROMAL CELLS

3.1 Background

Regeneration and healing of damaged tissue is, in many cases, critical to survival. Repair refers to the restoration of tissue architecture and its function after injury. In some tissues endogenous repair occurs and damaged regions are sufficiently replaced to return the tissue to more of a normal state; this is the repair process of regeneration. Other tissues may be incapable of restoring the tissue, and repair may partly or completely occur by the laying down of connective tissue or fibrous tissue, thereby leading to healing by scar formation or fibrosis, thus reducing tissue functionality. Stem cell therapy offers the potential of tissue repair for a number of diseases. Mesenchymal stem cells (MSC) are self-renewing cells with the ability to differentiate into several cell lineages. In addition to their stem cell characteristics, MSC are easily obtainable from many tissues and can be expanded in vitro without loss of potency. Their differentiation into osteoblasts, chondrocytes and adipocytes has become a standard potency assessment for these cells and has defined their clinical applicability in musculoskeletal diseases. In vitro, MSCs have also been differentiated into cells with phenotypic features of myocytes and cardiomyocytes, neurons, Schwann cells, endothelial cells and others (93, 94). These properties have raised hopes for the application of MSC in regenerative therapies. There are various criteria to be considered for effective treatment by stem cells. The source of stem cells used for therapy, timing of administration and their mode of delivery are some of the most important determinants of their medical efficacy.
3.1.1 Modes of Stem Cell Delivery

One critical aspect for MSC transplantation and subsequent therapeutic efficacy is the delivery method. The optimal cell delivery technique should provide the most regenerative benefit with the lowest side effects. The most common routes of MSC transplantation outside of tissue engineering-based methods are by systemic infusion (intravenous or intra-arterial) or by local delivery (direct intra-tissue injection).

The most convenient mode of MSC transplantation is peripheral (systemic) infusion. The major drawback of systemic cell delivery is failure to reach the desired destination due to trapping of cells in lungs, spleen, liver, thymus, kidney and skin (95). These sites of MSC engraftment do not appear significantly different between healthy animals and disease models; however the dynamics of cell fate are different between studies and models used. In a mouse model, cells traced 48 hours after intravenous transplantation were mostly detected in the lung, liver, intestine, skin and bone marrow. Approximately 5~10% of the injected cells remained in the spleen, while no cells were detectable in lymph nodes (96). The survival or ability to detect the cells in the different homing organs was relatively short. Less than 0.01% of intravenously injected human cells were detectable after 4 weeks in the lungs of mice (97). A total body count of labeled MSCs by bioluminescence showed a decrease from the intensity measured at 10~15 minutes post intravenous infusion to about 60% after 24 hours and less than 10% after 72 hours. No cells were detectable after 7 days in the lungs, spleen, liver and kidney in an acute kidney injury model in the same study (98). Engraftment in the lungs, and in the other major target organs, is a very rapid event, cells can be detected already seconds or minutes after intravenous transplantation (99). The cause for this entrapment in the
lungs is likely a combination of mechanical and physiological conditions and may be due to the small capillary size, the large capillary network and the strong adhesion properties of MSC. It was also shown in a study of myocardial regeneration that after systemic injection, very few cells engrafted in the infracted heart (100, 101). Only 1~5% of delivered cells engrafted within the target site for regeneration. Only 3.5% of all injected cells were retained in the heart after 6 weeks (102).

Stem cell transplantation directly into the site of injury is another option. Direct injection should have the advantage of precise localization of the cells, despite its invasive nature. In this case, the spread of the cells from the injection site is also quite restricted, seriously limiting the scale of tissue repair in many situations. Engraftment of transplanted cells into tissue is also very poor (typically <3%) (103). These methods also involve varying degrees of in vitro cell manipulation which introduces extra issues of safety and cost.

MSCs have the ability to home to damaged tissue sites. In humans, mobilization of progenitor cells from the bone marrow occurs after acute myocardial infarction (AMI), suggesting a natural attempt at cardiac repair (104). When MSCs are delivered exogenously and systemically administered to humans and animals, they are always found to migrate specifically to damaged tissue sites with inflammation (105), despite many of them being trapped in the lung. Therapeutic mobilization of bone marrow progenitor cells after injury would, in theory, amplify the existing healing response. Mobilization of these progenitors is an attractive strategy because it is simple and would obviate the need for invasive harvesting or delivery procedures. The best cellular product would be an autologous product, eliminating graft versus host or rejection issues and
could be delivered in the peripheral circulation. It would be ideal if stromal cells could be
induced to migrate from their niches to the sites of tissue injury under physiological
conditions without any in vitro manipulation. This would provide a non-invasive and
potentially repeatable method to recruit stromal cells in their natural state for tissue
repair. Cytokine-induced cell mobilization offers a readily available autologous cell
source which can be delivered in a non-invasive manner for tissue repair.

3.1.2 Mobilizing Agents

The mobilization of stem cells from their niches, such as the bone marrow,
into circulation can be achieved by certain compounds called mobilizing agents.

3.1.2.1 Granulocyte Colony Stimulating Factor (G-CSF)

Granulocyte colony stimulating factor (G-CSF) was among the first cytokines to
be identified and to enter clinical trials. The identification of G-CSF followed the
development of an assay measuring the effects of G-CSF and related cytokines by two
independent groups in the 1960s: Ray Bradley and Don Metcalf at the University of
Melbourne, Australia, and Yasuo Ichikawa and Leo Sachs at the Weizmann Institute,
Israel (106, 107). These assays measured the ability of test agents to stimulate colony
formation in hematopoietic cells in semi-solid culture and gave many of these cytokines
their names, e.g. granulocyte colony stimulating factor, granulocyte/macrophage colony
stimulating factor etc. Although colony forming assays permitted the quantitation of G-
CSF, it was more than a decade before Nicos Nicola finally isolated the murine cytokine
from medium conditioned with the lung tissue obtained from endotoxin treated mice in
1983 (108). Human G-CSF was purified soon after from the conditioned medium of the
bladder carcinoma cell line 5637 by Karl Welte in 1985 (109). The concurrent development of molecular biology techniques meant that the G-CSF gene was soon cloned by Shigekazu Nagata in Japan and independently by Lawrence Souza from AMGEN in 1986, permitting the large scale production of this cytokine and its subsequent clinical application (110, 111). G-CSF is central to the production of neutrophils in health and diseased states and is responsible for the dramatic increase in neutrophil numbers in response to infection or insults affecting bone marrow function such as anti-cancer cytotoxic chemotherapy. G-CSF mediates its effects by binding to a single homodimer receptor, G-CSFR (110). Plasma concentrations of G-CSF are normally low to undetectable, but rise rapidly in response to infection and subsequently decline with recovery (112). The release of G-CSF into the bloodstream by tissues stimulates neutrophil production within, and mobilization from, the bone marrow.

Bone marrow transplantation (also termed hematopoietic cell transplantation [HSCT]) is the oldest form of anti-cancer immunotherapy in clinical use, and involves the transplantation of hematopoietic stem and progenitor cells from self (autologous) or histo-compatible allogeneic related or unrelated volunteer donors. Traditionally, hematopoietic cells for both autologous and allogeneic transplantation were obtained by collecting large volumes of bone marrow, aspirated from the pelvic crests under general anesthesia. However, pre-clinical data showed that G-CSF could mobilize hematopoietic cells in large numbers from the marrow into the circulation with increased progenitor cells of all lineages detected in the spleens of G-CSF treated mice (113). Subsequent clinical trials demonstrated that adequate numbers of these cells could be collected from cancer patients or normal donors to allow successful autologous and allogeneic HSCT
respectively (114, 115). These clinical trials have led to the widespread use of G-CSF-mobilized hematopoietic cells collected by leukapheresis in the majority of autologous and allogeneic transplants. The biology underlying the process of HSC mobilization has been extensively studied but our understanding of the process is still incomplete. G-CSF promotes proliferation and maturation of the myeloid series, while at the same time it induces substantial changes in the bone marrow stroma, leading to the rise of HSC in the circulation by 60 times compared to baseline (116). Only cells of the myelo-monocytic series, including macrophages and OMAC, express the G-CSF receptor (CD114), whereas HSC do not (117). Thus mobilization through G-CSF is indirect.

The endpoint of every mobilization attempt is the collection of adequate numbers of HSC, reflected practically by the absolute number of CD34+ cells collected per kilogram of body weight. Adequate number is considered as the dose of HSCs that ensures rapid and sustained long-term hematopoiesis after the administration of myeloablative chemotherapy and HSC infusion. The optimal dose of HSC is $5 \times 10^6$/kg, with little clinical benefit with doses $5–8 \times 10^6$/kg and no further improvement with grafts containing $>10 \times 10^6$/kg CD34+ cells (118). The lowest acceptable dose is $2 \times 10^6$/kg. There are a substantial proportion of patients who fail to mobilize and collect adequate numbers of HSC and consequently cannot proceed to autologous stem cell transplantation. The inability of adequate HSC mobilization is associated with various factors such as age, low bone marrow cellularity (<30%) and genetic polymorphisms in BM niche associated molecules (119, 120). However, nearly 5% of healthy donors fail to mobilize adequate numbers of HSC. A patient is considered a poor mobilizer if he/she collected $<2 \times 10^6$/kg CD34+ cells through three consecutive days of apheresis, after the
administration of an adequate mobilization regimen (G-CSF monotherapy at a dose of \( \geq 10 \mu g/kg/day \) or chemotherapy + G-CSF \( \geq 5 \mu g/kg/day \)) (121). However patients who fail to achieve a maximum peak of circulating CD34\(^+\) cells \(< 20/\mu L\) on the day of the expected peak are also considered poor mobilizers (122). The day of the expected peak is dependent on the mobilizing regimen. With G-SCF monotherapy at a dose of 10 \( \mu g/kg \), peak levels of CD34\(^+\) cells are expected on the 5th-6th day after the initiation of G-CSF.

### 3.1.2.2 Mozobil/ Plerixafor (AMD3100)

Plerixafor is a bicyclam molecule with the chemical name I, 1′-[1,4-phenylenebis(methylene)]-bis-1,4, 8,11-tetraazacyclotetradecane; a molecular weight of 502.79 g/M; and the chemical formula C28H54N8· 8HBr·2H2O (123). The plerixafor molecule contains 2 monocyclam rings linked by an aromatic bridge originally designed to improve on the antiviral activity of previous molecules in this class (eg, JM2763) (124).

![Figure 3a: Chemical structure of plerixafor (AMD3100)](image)

It is a reversible CXCR4 antagonist has been approved in both the US and the EU for the mobilization of patients with lymphoma and myeloma. With its use in combination with G-CSF, approximately 70% of poor mobilizers may succeed in the collection of adequate numbers of HSC in order to proceed to ASCT (125). The
superiority of plerixafor in combination with G-CSF over G-CSF alone was established by several phase II (126, 127) and two phase III multicenter randomized double-blinded placebo controlled studies (125, 128). The high cost associated with plerixafor calls for a stringent protocol with well-defined criteria for its proper use (129).

Plerixafor can be applied with three strategies: (i) identification of patients with risk factors for poor mobilization and upfront use of plerixafor. This strategy is still outside the current approved indications and is not recommended; (ii) combination of G-CSF and plerixafor in a second mobilization attempt for patients who have already failed a prior HSC collection. This is the current approved usage of the drug; G-CSF is given at a dose of 10 μg/kg/day × 4 days and plerixafor is administered at a dose of 240 μg/kg on the fourth day, 9–11 hours before collection on the 5th day; (iii) the ‘just in time’ use of plerixafor seems to be the most cost-effective strategy: the proper mobilization regimen according to the patient’s disease status is chosen. If CD34 counts in peripheral blood on the day of the expected peak are suboptimal (<20/μL), plerixafor is administered prior to HSC collection. With this latter strategy >90% of the patients can achieve grafts with >2 × 10^6/kg CD34^+ cells (130). Another practical issue with plerixafor is the optimal timing of its administration. Current data indicate that the peak of circulating CD34^+ cells can be achieved in 3–8 hours after its administration, which may be more convenient for organizing the procedure of apheresis (131).

3.1.2.3 Substance P

Substance P (SP) is a highly conserved 11–amino acid neuropeptide contained in many unmyelinated primary afferent axons that terminate in the dorsal horn of the spinal cord and caudal spinal trigeminal nuclei (132, 133). SP is released from primary afferent
terminals by noxious or painful stimuli (134-136) and is thought to be involved in pain transmission at the first central synapse. Substance P has identical protein sequences in mice, rabbits and humans. It’s diverse expression in non-neural tissues, such as BMSCs (137), epithelial cells (138), endothelial cells, macrophages, neutrophils (139) and tumor cells (140), suggests another function in addition to its role in pain perception. These roles so far include neuro-immune modulation (141), bone marrow fibrosis (142), tumor cell proliferation (143, 144), wound healing (145, 146) and substance P–stimulated alleviation of delayed healing in trigeminal denervation in the eye or in capsaicin-induced neurotrophic keratopathy (147, 148). Hong and colleagues showed that Substance P is an injury-inducible factor that acts early in the wound healing process to induce mobilization of CD29+ stromal-like cells which are similar to BMMSCs and accelerate wound healing in an alkali burn model (149).

Growth factors such as G-CSF and GM-CSF and small drugs such as AMD3100 (plerixafor) have routinely been used to mobilize hematopoietic progenitors into peripheral blood (150, 151). However, despite exciting preclinical data in animal models, the clinical studies to date have shown minimal clinical benefit (115). Many reports have shown that treatment with G-CSF leads to egress of HSCs and HPCs from the bone marrow (BM) to the peripheral circulation. This led us to hypothesize that mobilization with G-CSF does not mobilize stem cells which are capable of engrafting and repairing tissue damage. As the BM contains a second stem cell population, mesenchymal stem cells (MSCs) (66), we further hypothesized that G-CSF fails to mobilize MSCs into the peripheral circulation. Hong and colleagues have described SP induced mobilization of MSC-like CD29+ stromal cells in rabbits. This suggests that other mobilization
approaches may have the potential to mobilize MSCs into the peripheral circulation and these cells could facilitate tissue repair. We evaluated different growth factors and combinations of growth factors for the ability to mobilize MSCs into peripheral circulation and also tested the therapeutic effect of any such mobilization in a mouse model of liver injury.

3.2 Results

3.2.1 MSCs or MSC Precursors in Peripheral Blood

Recent studies demonstrating the potential improvement in cardiac function in patients experiencing an acute MI with injection of MSCs into the peripheral blood (66) led us to investigate whether normal peripheral blood (PB) or mobilized PB contains MSCs or CD271+ cells. We obtained normal PB and PB from normal donors mobilized with G-CSF and evaluated the potential of the MNCs to generate MSCs. When placed in standard culture there was development of adherent cells that appeared like adipocyte cells and endothelial cells (Figure 3.1). When passaged the adherent cells failed to continue to grow and there was no evidence of MSC formation in any PB samples tested. Some G-CSF mobilized PB products were selected for MSC precursor cells expressing CD271 but these cells failed to generate MSCs and upon flow analysis contained almost exclusively double positive cells for CD271+ and CD45+ cells. These results suggest that there are few if any MSCs or MSC precursors in steady state PB or G-CSF mobilized PB.

Figure 3.1: Culture of MNCs under MSC conditions. MNCs separated from peripheral blood of normal donors (unmobilized) (A) and donors mobilized with rhG-CSF (B) were cultured under conditions used for growing MSCs. Few adherent cells formed which did not passage further in culture.
3.2.2 Stromal Cell Mobilization In Mice:

We used a mouse model of cell mobilization to test the potential of different mobilization agents. BDF1 mice were treated with cytokines or other mobilization agents and peripheral blood was analyzed for the presence of mobilized cells.

3.2.2.1 Peripheral Blood WBC Levels in Mobilized Mice:

G-CSF mobilization increases the circulating WBC count in mice and humans, with typical counts being 25,000 ~ 30,000 WBCs/µl. We evaluated the effects of growth factors G-CSF (250 µg/kg), Substance P (10µg/kg) and AMD3100 (100µg/mouse), alone and in combination, on peripheral white blood cell (WBC) count in treated mice. G-CSF and SP were administered intraperitoneally for 5 days, both for individual and combination treatment. For treatment with G-CSF plus AMD3100, G-CSF was administered for 5 days and AND3100 was injected on the fifth day along with G-CSF. Treatment of mice with SP or AMD3100 had no significant effect on WBC levels through the 5 day treatment. The combination of SP plus G-CSF and G-CSF plus AMD3100 resulted in equivalent WBC levels as G-CSF alone (Figure 3.2).
3.2.2.2 Progenitor Cell Levels in Mobilized Mice:

MNCs from mobilized mice were cultured in methycellulose to measure mature (GM-CFC) and primitive (HPP-CFC) hematopoietic progenitor cells. SP combined with G-CSF had no effect on the levels of either progenitor cells. While G-CSF plus AMD3100 resulted in increased GM-CFCs compared to G-CSF alone, it had no effect on the levels of HPP-CFC (Figure 3.3).

Figure 3.2: Peripheral blood WBC counts for mice treated with G-CSF, SP, AMD3100, G-CSF + SP, or G-CSF + AMD3100. Treatment of mice with SP or AMD3100 had no significant effect on WBC levels through the 5 day treatment. The combination of SP plus G-CSF and G-CSF plus AMD3100 resulted in equivalent WBC levels as G-CSF alone.

Figure 3.3: Colony forming cells in the peripheral blood of mice treated with G-CSF, SP, AMD3100, G-CSF + SP, or G-CSF + AMD3100. SP combined with G-CSF had no effect on the levels of either progenitor cells, while G-CSF plus AMD3100 resulted in increased GM-CFC
compared to G-CSF alone, but had no effect on the levels of HPP-CFC. Colony numbers are presented per 200,000 peripheral blood mononuclear cells plated. The numbers are the median number of triplicate plates ± SD.

3.2.2.3 Mobilization with G-CSF and Substance P:

Mice were injected intraperitoneally with 250 µg/kg of G-CSF or normal saline for 5 days and PB harvested on the sixth day. The mononuclear (MNC) fraction was isolated and plated onto culture flasks. Cultures of MNCs from mice treated with saline failed to form adherent cells and contained few cells (Figure 3.4). In contrast, cultures of MNCs from G-CSF treated mice contained significantly more cells and formed adherent cells in culture but those adherent cells failed to proliferate.

![Figure 3.4](image1.png)

**Figure 3.4:** Culture of peripheral blood MNCs from mice. MNCs were obtained from mice treated with saline (A) or rhG-CSF (B) and cultured. No adherent cell formation was observed in A, whereas few adherent cells appeared in B but did not proliferate.

MNCs from mice treated with 10 µg/kg of Substance P for 5 days resulted in formation of adherent cells after 5 to 7 days of culture. Cytospin preparations of MNCs from the SP treated mice demonstrated an increase in blast like cells (Figure 3.5). Significantly more adherent cells were obtained from G-CSF treated mice compared to SP treated mice.
Based on the data shown above for mobilization of cells by Substance P, we hypothesized that combining Substance P with G-CSF may result in a synergistic increase of MSC precursor cells in the peripheral blood of treated animals. This was based upon the large number of cells migrating from the BM of mice treated with G-CSF to the peripheral circulation. We proposed that this increased trafficking of cells would similarly shift MSC precursor cells into the peripheral circulation at higher levels than Substance P treatment alone. Mice (n=5 per treatment arm) were injected intraperitoneally with either 10 µg/kg of Substance P, 250 µg/kg of G-CSF, or a combination of both for 4 days and PB was harvested on the fifth day. Cultures of MNCs from mice treated with G-CSF and SP generated a few adherent stromal cells after 5 to 7 days. The combination of SP plus G-CSF resulted in many foci observed in 35 mm wells. After 10 days of culture several foci were observed in culture from SP treated mice and one focus from G-CSF treated mice. Approximately 50% of the well contained MSC-like cells in culture from SP plus G-CSF treated mice. Between 2 and 3 weeks of culture the MSC-like cells in cultures from SP + G-CSF treated mice approached confluence (Figure 3.6).

Figure 3.5: Stromal cell formation from WBC from mice treated with SP (A). The WBC from SP treated animals contained increased numbers of blast cells (B).
Figure 3.6: Culture of PB MNCs from mice treated with G-CSF, SP or SP+G-CSF. Panels A, B and C represent level of MCs-like cells in 10d culture of PB MNCs from G-CSF, SP and G-CSF+SP respectively. Combination treatment of G-CSF+SP resulted in multiple foci of MSCs indicating synergistic effect of mobilization. Panel D represents PB MNC culture from G-CSF+SP group at 3 weeks. Adherent cells developed in culture and continued to proliferate and reach confluence.

MNCs from treated mice were also plated in standard hematopoietic progenitor colony forming assays. Colony formation from cultures of 200,000 cells per 35mm petri dishes were scored at 10 days of culture. As shown in Figure 3.7, cells from G-CSF treated mice and G-CSF+SP treated mice contained similar number of GM-CFC colonies. Culture of MNCs from G-CSF treated mice contained typical hematopoietic colonies while colonies containing both hematopoietic and MSC-like cells formed in MNC cultures from G-CSF + SP treated mice (Figure 3.7).
Figure 3.7: Formation of hematopoietic colonies in HP-CFC assay. Typical hematopoietic colonies (A) were formed from WBC of rhG-CSF treated mice and mixed hematopoietic cell and MSC colonies (B) were formed from WBCs of rhG-CSF + SP treated mice. (C) Count of GM-CFC colonies per 200,000 cells plated. Values represent mean number of colonies.

3.2.2.4 Mobilization with the Combination of G-CSF and AMD3100

Mice were treated with G-CSF alone (daily from D1 to D5), AMD3100 alone (single dose) or the combination of G-CSF+AMD3100 in which G-CSF was given on D1 to D5 and a single dose of AMD3100 given on D5. Culture of MNCs from mice treated with G-CSF resulted in only few adherent cells, while AMD3100 resulted in a few colonies of MSC-like cells. The combination of G-CSF+AMD3100 resulted in a synergistic increase in MSC-like cells, with confluent cultures emerging within 2 to 3
weeks. When these cultures were confluent the cells were passaged using trypsin treatment and reseeded into secondary flasks (Figure 3.8).

Figure 3.8: MNC culture from peripheral blood of G-CSF+AMD3100 treated mice. Peripheral blood MNCs from mice treated with G-CSF+AMD3100 were cultured. Many adherent cells were formed, which proliferated and continued to passage. The cells shown are from passage 6 (P6) (magnification 100X).

3.2.2.5 G-CSF + AMD3100 Treatment Mobilizes MSCs

Mice were divided into groups (n=3-5) and injected with either PBS (control) or with the G-CSF+AMD3100 regimen. Blood was drawn at the end of injections, PBMCs separated, stained with antibodies to MSC markers and analyzed by FACS. The results indicate that mobilized cells collected after injection with G-CSF+AMD3100 express higher percentages of key MSC markers CD271, CD105, CD90 and CD73 (Table 3.2, Figure 3.9). This shows that G-CSF+AMD3100 treatment is capable of mobilizing MSCs into peripheral circulation.

Table 3.1: Elevation of MSC markers in G-CSF+AMD3100 mobilized cell population. All values are percentages of the cell population analyzed which express the corresponding marker; 2x10^5 cells analyzed for each marker.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>G-CSF+AMD3100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD271</td>
<td>1.3</td>
<td>11.3</td>
</tr>
<tr>
<td>CD105</td>
<td>1.42</td>
<td>12.2</td>
</tr>
<tr>
<td>CD90</td>
<td>7.56</td>
<td>22.3</td>
</tr>
<tr>
<td>CD73</td>
<td>4.1</td>
<td>23.2</td>
</tr>
<tr>
<td>CD29</td>
<td>22.6</td>
<td>80.1</td>
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<tr>
<td>CD44</td>
<td>28.3</td>
<td>90.5</td>
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</tr>
<tr>
<td></td>
<td>6.9</td>
<td>20.6</td>
</tr>
<tr>
<td>CD45</td>
<td>19.5</td>
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<tr>
<td>CD34</td>
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<td>5.3</td>
</tr>
<tr>
<td>CD11b</td>
<td>3.1</td>
<td>15.7</td>
</tr>
</tbody>
</table>

**Figure 3.9: Phenotypic characterization of G-CSF+AMD3100 mobilized cells.** Mobilized cells collected after G-CSF+AMD3100 injection were phenotyped by staining with MSC marker panel. Figure shows representative histogram plots of expression levels of the markers (green fill) compared to isotype (no fill).

### 3.2.3 Mobilization in Cynomolgus Monkey (*Macaca fascicularis*).

We established that treatment of mice with G-CSF+AMD3100 results in mobilization of MSCs into peripheral circulation. We tested this effect further by using cynomolgus monkeys, a subhuman primate model. The animal was injected with the G-CSF+AMD3100 regimen and blood was collected on days 0, 3, 5, 6, 7 and 10. Hematological analysis of the blood was performed using ADIVA analyzer. As shown in Figure 3.10, G-CSF+AMD3100 treatment causes an increase in WBC and lymphocyte count at d5. PBMCs were collected from the blood and colony forming unit (CFU-F) assay was performed plating. No CFU-Fs are obtained during the G-CSF arm of treatment. There is a huge increase in CFU-F numbers (mean= 402) on d5 following
AMD3100 injection after which they fall back to low levels on d6 and d7. This demonstrates that G-CSF+AMD3100 treatment can mobilize CFU forming MSCs into peripheral blood.

![Figure 3.10: Mobilization by G-CSF+AMD3100 in cynomolgus monkey](image)

Figure 3.10: Mobilization by G-CSF+AMD3100 in cynomolgus monkey. (A) Numbers of whole WBC and lymphocytes increases during G-CSF+AMD3100 treatment, with peak cell counts reached on d5. (B) There is marked increase in the number of CFUs per 10^5 plated cells in the blood of cynomolgus monkey following AMD3100 administration, with CFU levels falling to pre-treatment levels in subsequent days. (Values represent mean ± SEM; CFU assay performed in triplicates).

3.2.4 Mobilization in Humans

Patients with end stage liver disease (enrolled in a Phase I clinical trial titled ‘Mobilization of Stem Cells With G-CSF and Mozobil in Patients With End Stage Liver Disease’ [NCT01711073]) were treated with G-CSF+Mozobil (Mozobil is the clinically approved version of AMD3100) regimen and blood samples obtained at intervals during and after treatment. As expected with G-CSF+Mozobil treatment in patients, there in an increase in the levels of CD34+ cells in the blood, with peak levels occurring after Mozobil injection (d6). There is also an increase in the number of CFUs in the blood following treatment (d6) (Figure 3.11). This pattern of mobilization in primates and humans suggests that G-CSF+AMD3100 treatment induces the mobilization of MSCs into peripheral circulation and this effect is consistent across different species.
Figure 3.11: Mobilization by G-CSF+AMD3100 in patients. (A) CD34+ cells in blood increase as a result of G-CSF+Mozobil treatment, peaking after Mozobil administration. (B) CFU-F assay performed by plating $10^6$ PBMCs isolated from patient blood samples. There is a large increase in the number of CFU-Fs following Mozobil injection. All values are represented as mean ± SD.

3.2.5 Differentiation Potential of Mobilized Cells

Differentiation into osteogenic and adipogenic lineages is another hallmark of MSCs. To further confirm that the stromal cells mobilized by G-CSF+AMD3100 are indeed MSCs, they were subject to differentiation assays. Mobilized cells from mice and cynomolagus monkey were cultured in MSC growth medium and confluent cultures were fed with osteogenic or adipogenic induction medium. At the end of the induction period, the cells were stained with Oil Red-O or Alizarin Red to confirm differentiation into adipocytes and osteocytes respectively (Figure 3.12). Mobilized cells from both mice and cynomolagus monkey differentiated into adipogenic and osteogenic lineages, further demonstrating that the mobilized cells are MSCs.
Figure 3.12: Differentiation of mobilized cells. Cells mobilized by G-CSF+AMD3100 treatment in mice (top panel) and cynomolgus monkey (bottom panel). (A), (C) Intracellular fat droplets stained by Oil-Red O indicates adipogenic differentiation. (B), (D) Staining of extracellular calcium deposits by Alizarin Red indicates osteogenic differentiation

3.3 Effect of Stromal Cell Mobilization in Injured Animals

The functional benefit of stromal cell mobilization was tested using an animal model of tissue injury

3.3.1 Mouse Model of Acute Liver Injury

The liver is an important metabolic organ in the body, and can be injured by various factors including viral infection, trauma, and chemical reagents. Carbon tetrachloride (CCL$_4$)-induced liver injury is a classic model of chemical liver injury in mice (152). This model was adopted to study the effect of stromal cell mobilization by G-CSF+AMD3100 on survival and tissue regeneration in injured mice. First, the appropriate dosages of CCL$_4$ were determined. CCL$_4$ was diluted in olive oil to 50% v/v and injected intraperitoneally. Several doses were tested and a dose of 2.5 ml/kg CCL$_4$ was determined to be the sub-lethal dose where at least 80% of mice died in 7-10 days.
Change in levels of serum transaminases ALT (alanine aminotransferase) and AST (aspartate aminotransferase) is an important indicator of liver injury. Our results revealed that serum ALT and AST levels were significantly elevated on day 5 after the administration of CCl4, compared with that in control group, thus establishing liver damage (Figure 3.13). Kinetics analysis of AST and ALT levels showed that their levels increased rapidly after liver injury, peaking between days 3 and 5. These levels drop by d7 but are still much significantly than control (Figure 3.13).

![Graph showing AST/ALT levels and kinetics](image)

**Figure 3.13: Establishing the CCl4 liver injury model.** (A) AST/ALT levels are significantly elevated on d5 after injury by 2.5 ml/kg CCl4, P<0.01. (B) AST/ALT kinetics from days 1-7. Values are expressed as mean±SEM, n=3.

The 2.5 ml/kg CCl4 dose was used in performing survival analysis of mice after G-CSF+AMD3100 treatment. A lower dose of 1 ml/kg CCl4 was used to induce acute liver injury.

### 3.3.2 Survival of CCl4 injured Mice After G-CSF+AMD3100 Treatment

Mice (n=20) were injected with 2.5 ml/kg CCl4 (50% v/v) (d0) and separated into two groups (n=10 each). One group was treated with PBS (control) and the other was treated with G-CSF+AMD3100 regimen and animals were followed for 3 weeks.
Following AMD3100 administration on d4, G-CSF+AMD3100 treated group showed significantly higher survival compared to control group (P<0.05). There is a clear separation of the survival curves after AMD3100 injection indicating that stromal cell mobilization by G-CSF+AMD3100 treatment is responsible for increased survival (Figure 3.14).

![Figure 3.14: Kaplan-Meier survival curve of CCl4 injured mice. Increased survival after sub-lethal CCl4 injury (2.5 ml/kg) is observed in G-CSF+AMD3100 group compared to control group, *= P<0.05. n=10 mice per treatment arm.](image)

### 3.3.3 Characterizing Response to G-CSF+AMD3100 Treatment in CCl4 Injured Mice

The effect of G-CSF+AMD3100 treatment on CCl4 injured mice was studied using the following parameters.

#### 3.3.3.1 AST and ALT Levels in Serum

Mice were injured with 2.5 ml/kg CCl4 and divided into PBS treated and G-CSF+AMD3100 treated groups (n=3 each). Levels of AST and ALT enzymes were
measured at d7 after injury. At this stage in the G-CSF+AMD3100 treated group, levels of AST and ALT were significantly lower than PBS treated animals and comparable to baseline levels in uninjured mice. Mice injected with CCl4 at a dose of 1 ml/kg were divided into two groups and treated with PBS or G-CSF+AMD3100. On d5 and d7 after injury, n=3 mice were sacrificed from each treatment arm and AST/ALT levels were measured. G-CSF+AMD3100 treatment reduces serum AST/ALT levels on days 5 and 7 after injury compared to PBS treated animals (Figure 3.15).

3.3.3.2 Body Weight and Relative Liver Weight

CCl4 injured mice (1 ml/kg CCl4) divided into PBS and G-CSF+AMD3100 treatment arms (n=4 each) and their body weight was followed for 2 weeks. G-CSF+AMD3100 treated animals maintained higher normalized body weight compared to PBS treated mice (Figure 3.15). Mice (n=36) were divided into 3 groups of 12 mice each—baseline (uninjured), PBS treated and G-CSF+AMD3100 treated (both injured with 1 ml/kg CCl4). At days 0, 5, 7 and 14, n=3 mice from each group were weighed, sacked, livers excised and weighed. Relative liver weight (r.l.w) was calculated as weight of liver tissue per 100g body weight of the animal. Initially r.l.w increased in injured mice compared to baseline. At d5 G-CSF+AMD3100 treated mice showed reduction in r.l.w compared to PBS treated mice and the difference was statistically significant by d7 and d14 (Figure 3.15). These results demonstrate that G-CSF+AMD3100 treatment contributes to the amelioration of liver injury.
3.3.3.3 Histological Analysis of Liver Sections

Three groups of mice (n=3 each) were injured with injection of 1 ml/kg CCl4; one group was treated with PBS and other two received G-CSF+AMD3100 regimen. The PBS treatment arm and one G-CSF+AMD3100 treatment group were sacrificed at d10 and the other group at d21. Liver tissue was obtained, fixed, paraffin embedded, cut into 5µm thick sections and stained with hematoxylin & eosin (H&E) to visualize areas of necrosis. PBS treated animals present with marked areas of tissue necrosis and hepatocellular damage while G-CSF+AMD3100 treated animals show reduced signs of damage on days 10 and 14 after injury. G-CSF+AMD3100 treatment is shown to reduce the extent of liver damage induced by CCl4 and promote tissue repair.

Figure 15: Characterizing parameters of liver injury in CCl4 injured mice. Serum levels of AST and ALT in mice injured with 2.5 ml/kg CCl4 (A) and 1 ml/kg CCl4 (B) (n=3 in each group). (C) Normalized body weight of control, injured (PBS treated) and injured (G-CSF+AMD3100 treated) mice (n=4 each). (D) Relative liver weight calculated as weight of liver per 100g of body weight (n=3 each). (E) Mouse liver sections (5µm) stained with H&E. White arrow indicates areas of necrosis. (All values represented as mean±SEM)
3.3 Discussion

The therapeutic applications of stem cells is a promising and rapidly emerging branch of regenerative medicine in which stem cell-based treatments could be applied to treat and cure many aggressive and lethal diseases in humans (153). The autologous or allogeneic transplantation of stem cells or their further differentiated progeny into patients may constitute a potential therapeutic strategy, alone or in combination with the conventional treatments (154). Although the use of stem cells to repair damaged tissues offers potential cures for a range of diseases, the results in clinical trials to date have shown minimal benefits (155). There are many variables that need to be optimized.
including optimal cell dosing, delivery methods to obtain engraftment of cells, optimal manufacturing for *in vivo* use, immune barriers for allogeneic cells, and the need for cytokines via the microenvironment *in situ*. Further, many of the trials to date involve harvesting BM or other tissue for production of cellular products *in vitro*. This leads to concerns over costs, safety, availability and rejection.

MSCs have the capacity to differentiate into adipocytic, chondrocytic, and osteogenic lineages and potentially other lineages including epithelial, myocardial, or neuronal lineages (38). It is thought, therefore, that MSCs may be used therapeutically to promote tissue regeneration in the treatment of diseases such as *osteogenesis imperfecta* and Parkinson's disease (156, 157). Additionally, MSCs have been reported to have immunosuppressive properties, and as such, they may be therapeutically useful for the treatment of autoimmune diseases (158). Despite the potential of MSCs in regenerative medicine, *ex vivo* manipulation and therapy using MSC have indicated serious limitations including the number of cells that could be infused without causing entrapment in the lungs, poor homing of transplanted cells and possible tumorigenesis (159-161). In order to alleviate the aforementioned limitations, investigators are evaluating methodologies of mobilization of stem cells *in vivo* in an attempt to shift stem cells to the peripheral circulation and subsequent trafficking to the damage tissue. G-CSF has been used to mobilize stem cells in many of the clinical trials to date, however as shown in this report, G-CSF only mobilizes HSCs and not MSCs. We hypothesize that this is the primary failure of the trials to date as HSCs do not repair the stromal environment and have limited ability to differentiate into non-hematopoietic tissues. We demonstrate the potential of other growth factors to mobilize both HSCs and MSCs into
peripheral blood. In particular, AMD3100 synergizes with G-CSF to effectively mobilize HSCs and MSCs in mice, cynomolgus monkey and humans. The mobilized cell fraction contained colony forming cells which passaged in culture, phenotyped as MSCs by flow cytometry (Figure 3.9) and differentiated into osteocytes and adipocytes upon induction. We thus focus our discussion on AMD3100 and G-CSF.

G-CSF promotes proliferation and maturation of the myeloid series, while at the same time it induces substantial changes in the bone marrow stroma, leading to the rise of HSC in the circulation by 60 times compared to baseline (116). The chemokine axis SDF-1α/CXCR4 is critically involved in the retention of HSCs within the bone marrow. At a molecular level, G-CSF has been shown to act by disrupting the SDF-1α/CXCR4 retention axis, both by reducing CXCR4 expression on HSCs and levels of SDF-1α in the bone marrow (162). The hyperplastic myelomonocytic series (through G-CSF) secrete a large variety of proteases, which induce proteolytic cleavage/clearance of SDF-1, leading to the release of the CXCR4 receptors on HSC and their subsequent liberation from the BM stroma. The activity of the proteases is further assisted by the cleavage of protease inhibitors (116). This same proteolytic mechanism is responsible for the degradation of VCAM-1, fibronectin, and OPN, leading to reduced cellular adhesion of HSC through their receptor VLA-4 to BM stroma (163). Co-administration of G-CSF and CXCR4 inhibitors results in additive and more potent HSC mobilization compared to G-CSF alone (116).

The clinical form of plerixafor/AMD3100 is Mozobil which is used in combination with G-CSF to mobilize HSCs in cancer patients who do not have sufficient mobilization of HSC with G-CSF alone. AMD3100 is a CXCR4 antagonist that disrupts
the SDF-1/CXCR4 axis in a synergistic way to G-CSF. The following hypotheses have been proposed for its mechanism of action (151). (a) The inhibition of CXCR4 on HSC leads to loss of their sensitivity to SDF-1. As a result, HSC are attracted to the circulation through a positive signal, most likely the sphingolipid sphingosine-1-phosphate (S1P). Alternatively HSC passively move to the blood. (b) AMD3100 targets SDF-1 that is produced by BM stromal cells, causing a decrease of its levels and leading ultimately to mobilization. (c) Bonig and Papayannopoulou have suggested that AMD3100 does not actually cause mobilization from the BM, but it traps HSC in the circulation by binding on CXCR4 and leading to loss of chemoattraction to SDF-1. This hypothesis requires a rapid steady state HSC turnover between marrow and other tissues and may explain the high HSC numbers observed in the blood within 30 minutes after plerixafor administration (164). The synergy obtained with G-CSF and AMD3100 is consistent with each growth factor acting through distinct mechanisms. AMD3100 also leads to a more rapid mobilization. Recent studies have also evaluated possible differences in cell composition between G-CSF and AMD3100-mobilized grafts (165). This is consistent with our results showing greater number of CFU-GM in G-CSF+AMD3100 mobilized cells (Figure 3.3).

There is evidence that bone-marrow-derived MSCs contribute to tissue regeneration, suggesting that these cells are also mobilized in response to tissue injury; however, the factors and mechanisms regulating the mobilization of MSCs are currently unknown. A portion of MSCs express high levels of CXCR4 in the bone marrow (166, 167). Sordi et al. showed that BM-MSCs expressed selected chemokine receptors (CXCR4, CX3CR1, CXCR6, CCR1, and CCR7) and also secreted their corresponding
ligands (CXCL12, CX3CL1, CXCL16, CCL3, CCL19, and CCL21) (168). BM-MSCs, therefore, not only express chemokine receptors but also are able to secrete some chemokines that can act in an autocrine loop (ie, CXCL12-CXCR4). CXCL12-CXCR4 and CX3CL1-CX3CR1 represented the major migration axis for BM-MSCs to tissue stimuli. Ji et al. also that CXCL12-CXCR4 and CX3CL1-CX3CR1 interactions mediate the trafficking of transplanted MSCs in a rat model of left hypoglossal nerve injury (169). These studies suggest that CXCR4/CXCL12(SDF-1) plays a key role in adhesion of MSCs in the marrow and in their migration to sites of tissue injury.

However, in our experiments administration of AMD3100, an antagonist to the CXCR4/SDF-1 axis, alone resulted in very poor mobilization of MSCs suggesting that MSC retention in the bone marrow is a tightly regulated process involving many distinct mechanisms. Treatment of mice, primates and humans with G-CSF for 5 days, followed by addition of AMD3100 on the 5th day resulted in huge increase in CFU-F forming MSCs in circulation. G-CSF alone did not mobilize MSCs but G-CSF stimulated proliferation of MSCs in the BM by promoting osteoclast resorption (170). The time-dependent increase of MSCs in the BM caused by G-CSF and subsequent interference with the anchoring CXCR4/SDF-1 axis by AMD3100, combined with yet undescribed effects, could lead to the mobilization of MSCs into circulation aided by the greatly increased flow of HSCs out of the BM. We propose this as a possible mechanism by which MSCs could be mobilized into circulation. Further, a single dose acute administration of G-CSF+AMD3100 did not lead to sufficient MSC mobilization. This suggests that the ability of G-CSF to promote the mobilization of MSCs is dependent on changes to the bone marrow environment or the progenitor phenotype that occurs over a
number of days. This further supports our working hypothesis. Studies have also shown that pre-treatment by VEGF or IGF-1 followed by AMD3100 administration leads to mobilization of endothelial progenitor cells and also MSCs (171, 172). These findings suggest that different subsets of BM cells can be mobilized by different cytokine combinations. Further studies need to be performed to clearly understand the key components and processes involved in MSC mobilization.

We established a mouse model of liver injury by CCl4 and identified parameters to measure the extent of liver injury and repair. We demonstrated that G-CSF+AMD3100 treatment was life-saving in mice injured with the sub-lethal dose of 2.5 ml/kg CCl4 (Figure 3.14) compared to control arm. There is a clear separation of the survival curves after AMD3100 administration suggesting that the mobilization of MSCs is able to ameliorate the injury. Our studies also showed that treatment with G-CSF alone could not rescue critically injured mice. The mechanisms involved in MSC homing to sites of injury likely involve both local and systemic inflammatory signals that act in concert to direct stem cells toward areas of wounding. MSCs have been demonstrated to migrate to injured tissues along chemotactic gradients of the growth factors platelet-derived growth factor-AB (PDGF-AB), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) (173). Similarly, the chemokines such as stromal-derived factor-1 (SDF-1) have shown significant capacity to induce chemotactic migration of MSCs. Interestingly, inflammatory cytokines have been shown to prime MSCs for chemotaxis likely through the upregulation of receptors for chemotactic factors. SDF-1 expression is upregulated in the injured tissues (174). This SDF-1 chemokine gradient could attract the MSCs mobilized by G-CSF+AMD3100 treatment to
the site of liver injury. We believe that the cells responsible for amelioration of liver injury are derived from the BM. We demonstrated that pre-treatment with G-CSF is required for substantial MSC mobilization and for therapeutic benefit in injured mice versus AMD3100 alone. Since G-CSF is a well-established inducer of HSC mobilization from the BM, this shows that the cells responsible for the reparative effect arise from the BM. However, lineage tracing experiments need to be performed to confirm definitively.

Upon reaching the injured tissue, MSCs can participate in tissue repair. The exact mechanisms by which MSCs contribute to tissue repair are not fully defined. A consistent finding among most studies is that the observed improvements in tissue function cannot be attributed directly to the relatively small number of delivered stem cells, which demonstrate a poor ability to differentiate into functional cells and a limited and temporal engraftment. The current explanation for the improvements observed in clinical trials is that the transplanted cells release soluble factors that have positive effects on tissue regeneration or vasculogenesis. This ‘paracrine hypothesis’ seems viable, as many stem cell types secrete molecules, like cytokines, chemokines, and growth factors which are known not only to reduce the inflammation, apoptosis and fibrosis of damaged tissues but also to stimulate angiogenesis, protection of existing functional cells, endogenous stem cell recruitment and tissue cell regeneration (50). After MSCs move to damaged sites for repair, they are stimulated by local factors, such as inflammatory cytokines, ligands of Toll-like receptors and hypoxic conditions. These stimuli lead to the production of a large amount of growth factors that perform multiple functions to achieve tissue regeneration (51, 52). Trophic factors secreted by MSCs can lead to the survival of living and dying hepatocytes via anti-apoptotic (stromal cell-derived factor 1, HGF, insulin-like growth
factor 1 [IGF-1], and vascular endothelial growth factor [VEGF]), mitogenic (EGF, HGF, nerve growth factor [NGF], and transforming growth factor α [TGF-α]), and angiogenic effects (VEGF) (175, 176). HGF, EGF and TGF-α, which are potent mitogens, are primarily associated with hepatocyte proliferation (177, 178), and VEGF enhances angiogenesis, which is responsible for liver regeneration.

MSCs can express various soluble factors, such as nitric oxide, prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), IL-6, IL-10, and HLA-G. These soluble factors regulate the proliferation and functions of a variety of immune cells and induce Treg cells (54). In particular, PGE2 increases the anti-inflammatory cytokine IL-10 and decreases tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and IL-12 in dendritic cells (DCs). PGE2 also reduces IFN-γ and IL-4 in Th1 and Th2 cells and stimulates the proliferation of Treg cells (54). Moreover, IDO and HLA-G suppress the proliferation of effector T cells, inhibit the maturation of DCs, inhibit the proliferation and immunoglobulin G secretion of B cells and reduce the cytotoxicity of NK cells (55, 179).

In addition to the secretion of soluble factors from MSCs, these cells can suppress the activation of immune cells through direct cell-cell contact. MSCs can inhibit T-cell proliferation by inducing the apoptosis of effector T cells by promoting the association of programmed death-1 (PD-1) with its ligands PD-L1 and PD-L2, and MSCs are capable of rendering T cells anergic by down-regulating the expression of the co-stimulatory molecules CD80 and CD86 on antigen-presenting cells (56, 180). Unbalanced immune cell populations or immune cell infiltration of the liver can disrupt its immune-privileged state, resulting in liver injury or fibrosis. Therefore, the immune-modulatory potential of MSCs plays an important role in the treatment of liver injury.
CCI4 stimulates reactive oxygen species (ROS) production, which results in hepatocyte damage through lipid peroxidation and the alkylation of proteins, nucleic acids, and lipids (181). MSCs have been shown to overcome CCL4-induced oxidative stress *in vitro* and to reduce liver injury through anti-oxidant activities *in vivo* (182). The up-regulation of ROS in CCl4-treated liver cells has been reported to be attenuated by co-culturing with MSCs via an increase in superoxide dismutase activity and the induction of AREs, which represents a cytoprotective response in the injured liver (182). Despite growing evidence supporting the paracrine theory, the exact nature or sequence of the numerous interactions between delivered stem cells and the injured tissue microenvironment is yet to be determined and remains a crucial question to be answered.

Serum markers AST and ALT are significantly reduced in the G-SCF+AMD3100 treated group compared to control. The treated group also maintains their body weight over two weeks after an initial drop due to injury indicating better health than the control group. Relative liver weight is increased in mice in the control group due to infiltration of immune and inflammatory cells and hypertrophy, compared to the G-CSF+AMD3100 treated group. Analysis of liver sections from both groups of mice reveals large areas of necrosis on d10 in the PBS treated group. Necrosis regions are present to a lesser extent in the G-CSF+AMD3100 treated group on the same day and on d21. These results taken together indicate that MSC mobilization by G-CSF+AMD3100 treatment aids in survival of acutely injured mice and repair of damaged tissue.

Our study focused on the combination of G-CSF plus Mozobil given the availability of these drugs for clinical applications. The goal would be to mobilize MSCs following tissue injury and traffic MSCs to the damaged site to minimize inflammation
and tissue damage. In diseases with chronic tissue damage, such as chronic cardiac ischemia and liver cirrhosis, mobilization of MSCs would aim to repair the damaged stromal environment within the tissue and facilitate recovery of endogenous stem cells to replace damaged functional cells. The majority of current clinical trials using cellular products involve invasive delivery of the cell product through catheters or other direct injection methodologies. This will limit the flexibility of delivering multiple cell products to simultaneous delivery and will limit the potential for repeat treatments. The mobilization of MSCs described in our research offers a more flexible approach with treatment that can be repeated on multiple occasions. In addition, it is possible that mobilization of MSCs may be combined with delivery of cellular products. Preparation of the stromal microenvironment within the tissue could be achieved by initial mobilization of MSCs followed by the delivery of a second stem cell population with flexible timing. The availability of off-the-shelf drugs, such as G-CSF and Mozobil, could offer a simple primary or adjunct treatment for patients in need of regenerative therapies.
CHAPTER 4: COMPARATIVE ANALYSIS OF STROMAL CELLS DERIVED FROM DIFFERENT TISSUE SOURCES

4.1 Background

Homeostasis is an important process in the structural and functional maintenance of tissues in the body and is controlled by complex genetic, molecular and cellular interactions. Stem cells residing in different organs of the adult are critical components of the homeostatic process. Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) and can self-renew and differentiate into all blood lineages (183). This property of the HSCs is maintained by the microenvironment in which they reside. Dexter et al. (184, 185) demonstrated that the stromal cells that are present in the BM microenvironment are important for hematopoiesis and that adherent stromal-like cultures could support the maintenance of hematopoiesis. The BM stroma consists of multiple cell types, including mesenchymal stromal cells (MSCs), osteoblasts, adipocytes, endothelial cells and macrophages (23, 186, 187). MSCs have been further described as cells giving rise to colonies of fibroblast-like cells in culture (43). Pittenger et al. (38) later showed that these fibroblast-like cells were capable of differentiation into adipocytes, osteoblasts and chondrocytes. MSCs also secrete a number of factors that promote tissue regeneration from the tissue cellular progenitors. MSCs are immuno-privileged cells as the result of the low expression of class II major histocompatibility complex (MHC-II) and costimulatory molecules on their surface. In addition to these properties, they are capable of immuno-regulation and exert immunosuppressive effects by means of direct cell-to-cell interactions and secretion of soluble factors (56, 188, 189). These properties have
made BM-MSCs an exciting candidate for cell therapy, leading to their evaluation in a variety of clinical trials for treating diseases such as osteogenesis imperfecta (190), articular cartilage defects (191), graft-versus-host disease (GVHD) (192) and myocardial infarction (67). Because of the success in phase I safety and phase II efficacy trials, more clinical trials are moving to phase III clinical trials specifically for osteoarthritis, GVHD, myocardial infarction and Crohn's disease (http://www.clinicaltrials.gov). Currently, bone marrow represents the main source of MSCs for both experimental and clinical studies (193). However, the harvest of BM is a highly invasive procedure and the number, differentiation potential, and the maximal life span of MSC from BM decline with increasing age (68), which makes the search for adequate alternative sources of these cells for autologous and allogenic use necessary. Recently, many researchers have reported the isolation of stromal cells from other tissues such as heart, liver, adipose, skin, pancreas, umbilical cord blood and teeth (45, 194-201). Identification of additional sources of cells which can be used for transplant therapy carries immense clinical significance and clinical trials have already begun to test these alternate sources of MSCs (202). However, similarities and differences between these cell populations are not clearly defined, and the potential impact of contaminating tissues with foreign stromal cells is not known. A detailed understanding of the properties of these different stromal cells, especially their tumorigenicity (203) and tumor permissiveness becomes even more important. Armed with such knowledge, we will be able to make better decisions about the right source of therapeutic cells for a particular type of tissue injury.

Preliminary reports have demonstrated similar phenotypes for various tissue-specific stromal cells but suggest differences in their functional properties (204-208). In
this study, we isolated stromal cells from BM, heart, adipose, and liver to compare their \textit{in vitro} morphology, phenotype and differentiation capacity. We examined their comparative functional properties in a co-culture system with tumor cell lines and umbilical cord blood (CB) mononuclear cells (MNC). We also generated conditioned media from each stromal cell population and tested its effect on tumor cell proliferation.

4.2 Results

4.2.1 Stromal Cell Morphology

BM stromal cells (BM-MSC), heart stroma (HS), adipose stroma (AS) and liver stroma (LS) were cultured in A20 standard media, and morphology was evaluated by means of light microscopy. Stromal cells from each tissue had a similar spindle-shaped, fibroblastic morphology consistent with BM MSCs (Figure 4.1). Furthermore, this morphology was maintained even after multiple passages of the cells.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{morphology.png}
\caption{Morphology of stromal cell lines. Stromal cells obtained from bone marrow, adipose tissue, liver and heart were seeded in flasks. All stromal cells have spindle-like fibroblastic morphology similar to BM-MSCs. Magnification 200X}
\end{figure}
4.2.2 Growth Rate of Stromal Cells

HS formed CFU-F colonies when plated in vitro, with a median of 26 CFU-Fs per 100 cells plated. In contrast, BM-MSCs contained fewer CFU-F (p<0.01), forming only 3 CFU-F per 100 cells plated. This data suggests that HS have a higher proliferative potential than BM-MSCs. Growth curves of stromal cells supports this data, with HS, AS and LS all having a faster growth rate than BM-MSCs (Figure 4.2).

![Graphs showing colony formation and growth potential](image)

Figure 4.2: Colony formation and growth potential. (A) Mean CFU-F frequency of HS and BM-MSCs per 100 cells plated, P<0.01. (B) Culture initiated with 20,000 cells of each stromal cell line (experiment performed thrice in triplicate) in 6-well culture plate (2000 cells/cm²). The cells proliferated at different rates with plateau being reached between d6 and d12 depending on the stromal cell line.

4.2.3 Stromal Cell Phenotype and Differentiation

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has established the phenotype of MSCs as CD105+, CD90+, CD73+, CD45− and CD34− (163). Therefore, we analyzed the stromal cell lines by use of flow cytometry for the expression of these markers (Figure 4.3). Each of the stromal cell lines had surface expression of CD105, CD90 and CD73, with levels of expression...
varying slightly between cell lines. They were also negative for expression of hematopoietic cell markers CD45 and CD34. There was also no expression of any hematopoietic lineage markers (i.e., CD19, CD3 and CD14). These results demonstrate that morphologically and phenotypically, stromal cells from various tissues have identical morphological and phenotypic expression.

Figure 4.3: Phenotypic analysis of stromal cell lines. Flow cytometry histograms of BM-MSC, AS, LS and HS cells. Plots show appropriate isotype control staining (open histogram) versus specific antibody staining (black-filled histogram). Histograms are representative of three independent experiments.
Table 4.1: Phenotypic analysis of stromal cell lines by flow cytometry. Values represent percentage expression of corresponding marker on cell surface. All values shown as mean±SEM

<table>
<thead>
<tr>
<th></th>
<th>CD45-</th>
<th>CD90+</th>
<th>CD73+</th>
<th>CD105+CD90-CD73+</th>
<th>CD105+</th>
<th>CD90+</th>
<th>CD73+</th>
<th>CD105+CD90-CD73+</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM/MSC</td>
<td>95.5±1.8</td>
<td>98.4±1.8</td>
<td>95.2±1.9</td>
<td>92.6±2.2</td>
<td>95.5±0.9</td>
<td>96±1.4</td>
<td>97.1±0.9</td>
<td>94.1±1.7</td>
</tr>
<tr>
<td>AS</td>
<td>92.5±3.2</td>
<td>93.3±0.8</td>
<td>92.9±1.1</td>
<td>90.4±1.6</td>
<td>93.1±1.2</td>
<td>94±2.1</td>
<td>89.2±2.1</td>
<td>88.5±2.8</td>
</tr>
<tr>
<td>LiS</td>
<td>97.5±1.1</td>
<td>98.6±0.5</td>
<td>98.2±1.3</td>
<td>98.3±0.5</td>
<td>98.7±0.8</td>
<td>97.7±0.7</td>
<td>98.1±0.8</td>
<td>97.2±1</td>
</tr>
<tr>
<td>HS</td>
<td>95.3±3.5</td>
<td>99.5±0.7</td>
<td>97.1±2.1</td>
<td>92.6±3.9</td>
<td>95.2±3.6</td>
<td>98.2±0.9</td>
<td>97.6±1.2</td>
<td>93.1±2.7</td>
</tr>
</tbody>
</table>

Another feature of BM-MSCs is the ability to differentiate into non-hematopoietic cells such as adipocytes and osteocytes; therefore, we tested the capacity of these stromal cells to differentiate into these lineages. As shown in Figure 4.4, all four stromal lines differentiated into adipocytes (Figure 4.4 A) and osteocytes (Figure 4.4 B) demonstrating MSC-like properties.

Figure 4.4: Differentiation potential of stromal cell lines. (A) Adipogenic differentiation. Accumulation of intracellular lipid droplets was determined by use of oil red O staining. (B) Osteogenic differentiation. Cultures had extracellular calcium deposition as demonstrated by positive alizarin red S staining. Experiments were performed in triplicate and repeated three times. Representative images are shown. Magnification 200X.
4.2.4 Functional Differences between Stromal Cells

Stromal cells derived from different tissues were similar in their morphology, phenotype and differentiation capacities. The next step in their characterization was to investigate any functional differences between them.

4.2.4.1 Gene and microRNA Expression Analysis of HS vs BM-MSCs

Genetic analysis provides an in depth look at the intrinsic differences between two types of cells. Results of such analysis can give rise to clues about differences in function between two cell types and aid in choosing relevant functional assays. The BM-MSCs and HS were culture expanded and then RNA prepared for micro array analysis. We performed a whole genome gene array analysis and a microRNA array. This analysis demonstrated distinct gene patterns between these two sources of stromal cells. In particular, our analysis has demonstrated distinct cytokine and cytokine receptor patterns (Table 4.2). The data suggests that stromal cells in different tissues secrete different cytokines and express different cytokine receptors. This is consistent with local control of tissue specific stem cells and progenitor cells through cytokines. HS also expressed increased levels of genes encoding cell adhesion molecules and focal adhesion molecules (Table 4.3 and 4.4), with an increase in a number genes associated with endothelial, vascular and muscle cells. The increased expression of myosin genes and laminin alpha 5 is consistent with cardiac expression compared to bone marrow derived cells.

**Table 4.2**: Cytokines and Cytokine Receptors Expressed at 2 fold or Higher Levels in Cardiac Stromal Cells Compared to Bone Marrow MSC

<table>
<thead>
<tr>
<th>IL10RB</th>
<th>Interleukin 10 receptor, beta</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFRSF11B</td>
<td>Tumor necrosis factor receptor superfamily, member 11b</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>Transforming growth factor, beta receptor II (70/80kDa)</td>
</tr>
<tr>
<td>TNFRSF12A</td>
<td>Tumor necrosis factor receptor superfamily, member 12A</td>
</tr>
<tr>
<td>IFNGR2</td>
<td>Interferon gamma receptor 2 (interferon gamma transducer 1)</td>
</tr>
<tr>
<td>FAS</td>
<td>Fas (TNF receptor superfamily, member 6)</td>
</tr>
<tr>
<td>PDGFR1A</td>
<td>Platelet-derived growth factor receptor, alpha polypeptide</td>
</tr>
<tr>
<td>CXCL16</td>
<td>Chemokine (C-X-C motif) ligand 16</td>
</tr>
<tr>
<td>GHR</td>
<td>Growth hormone receptor</td>
</tr>
<tr>
<td>VEGFC</td>
<td>Vascular endothelial growth factor C</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>TNFRSF14</td>
<td>Tumor necrosis factor receptor superfamily, member 14</td>
</tr>
<tr>
<td>CCL26</td>
<td>Chemokine (C-C motif) ligand 26</td>
</tr>
<tr>
<td>MET</td>
<td>Met proto-oncogene (hepatocyte growth factor receptor)</td>
</tr>
<tr>
<td>IL11RA</td>
<td>Interleukin 11 receptor, alpha</td>
</tr>
<tr>
<td>LEPR</td>
<td>Leptin receptor</td>
</tr>
</tbody>
</table>

**Table 4.3:** Cell Adhesion Molecules Expressed at 2 fold or Higher Levels in Cardiac Stromal Cells Compared to Bone Marrow MSC

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRXN2</td>
<td>Neurexin 2</td>
</tr>
<tr>
<td>ITGB2</td>
<td>Integrin, beta 2 (complement component 3 receptor 3)</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>CD99</td>
<td>CD99 molecule</td>
</tr>
<tr>
<td>HLA-F</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>HLA-E</td>
<td>Major histocompatibility complex, class I, E</td>
</tr>
<tr>
<td>ICAM3</td>
<td>Intercellular adhesion molecule 3</td>
</tr>
<tr>
<td>ICAM2</td>
<td>Intercellular adhesion molecule 2</td>
</tr>
<tr>
<td>HLA-DMA</td>
<td>Major histocompatibility complex, class II, DM alpha</td>
</tr>
<tr>
<td>NFASC</td>
<td>Neurofascin homolog</td>
</tr>
</tbody>
</table>
Table 4.4: Focal Adhesion Molecules Expressed at 2 fold or Higher Levels in Cardiac Stromal Cells Compared to Bone Marrow MSC

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK3CD</td>
<td>Phosphoinositide-3-kinase, catalytic, delta polypeptide</td>
</tr>
<tr>
<td>MYLK</td>
<td>Myosin, light polypeptide kinase</td>
</tr>
<tr>
<td>CCND2</td>
<td>Cyclin D2</td>
</tr>
<tr>
<td>LAMA5</td>
<td>Laminin, alpha 5</td>
</tr>
<tr>
<td>MYLPF</td>
<td>Fast skeletal myosin light chain 2</td>
</tr>
<tr>
<td>ITGA1</td>
<td>Integrin, alpha 1</td>
</tr>
<tr>
<td>TNC</td>
<td>Tenascin C (hexabrachion)</td>
</tr>
</tbody>
</table>

There were 26 miRNAs up regulated and 6 down regulated by more than 2 fold in HS compared to BM MSC (Figure 4.5). Three (miR-1, miR-133a and miR-206) of the up regulated miRNAs have functions in myogenesis and cardiomyocyte development (175, 176) and two (miR-20a and miR-26a) have function in stem cell differentiation (177, 178). Of particular note was miR-206, which was up regulated by more than one thousand fold in HS. miR-206 has been reported as a muscle specific miR that promotes muscle differentiation and regulates connexin 43 expression during skeletal muscle development (179, 180). In addition, miR-1 and miR-206 has been reported to be upregulated in the heart following myocardial infarction compared to normal heart (181).

We further evaluated the levels of expression of miR-1, 133a and 206 in HS by q-PCR. There was a 20 fold, 8 fold and 46 fold higher expression of miR-1, miR-133a and miR-206 respectively, in HS compared to BM MSCs. Interestingly, previous studies have implicated miR-206 in inhibition of proliferation of tumor cells (Figure 4.5).
Figure 4.5: miRNA expression analysis of HS vs BM-MSC. (A) Fold expression of miRNAs in HS compared to BM-MSCs. Red bars indicate miRs downregulated in HS. (B) RT-PCR quantification of selected miRs.
4.2.4.2 Co-Culture of Stromal Cells with Tumor Cell Lines:

To test if HS could inhibit proliferation of tumor cells, a human promyelocytic leukemia cell line, HL-60 was cultured on a confluent layer of BM-MSCs and HS and the proliferation of the tumor cells was followed. As shown in Figure 4.6, co-culture of HL-60 cells on HS inhibited proliferation of HL-60 cells, while control cultures resulted in normal proliferation. At day 7, HL-60 cell number increased from a starting level of 1 x 10^4 cells to 7.5 x 10^5 cells (viability= 97%) in control cultures with no stroma. In contrast, the same number of HL-60 cells seeded onto HS yielded only 1.9 x 10^5 cells with viability dropping to 45%. Also, HL-60 cells proliferated at an even higher rate when co-cultured on BM-MSCs, yielding 1 x 10^6 cells with viability of 93%.

Figure 4.6: HL-60 cells in culture. (A) HL-60 cells cultured alone or with HS and BM-MSCs. (B) Expansion of HL-60 cells in culture for 7 days under different conditions. (C) Viability of HL-60 cells after 7d culture. All values represented as mean.
We used the well characterized chronic myelogeneous leukemia cell line K562 to further study the effect of stromal cells from different tissues on tumor cells. As with HL-60 cells, BM-MSCs supported proliferation of K562 cells as compared to cultures without BM-MSCs. Extensive K562 cell proliferation was observed in flasks seeded with BM-MSCs, AS and LS (Figure 4.7A), with clusters of K562 cells consistent with clonal proliferation. In contrast, little proliferation was observed in K562 co-culture with HS. As seen in Figure 4.7B, the calculated total viable cell proliferation was greatest with co-culture on BM-MSCs (mean, $8.84 \times 10^6$), with similar growth in AS (mean, $7.54 \times 10^6$) and LS (mean $6.61 \times 10^6$). Total cell proliferation on HS (mean, $0.63 \times 10^6$) was 14-fold less than BM-MSCs, suggesting significant inhibition in co-cultures with HS. Of note, was the significant loss of viability in co-cultures with HS (mean, 31.8%), further suggesting that inhibition in culture might be a result of induction of apoptosis (Figure 4.7C) through a cytotoxic mechanism. K562 cells were viable in co-culture with BM-MSCs (mean 92.1%) with minimal loss of viability in AS (mean 83.5%) and LS (mean, 81%) co-cultures. These results suggest potential functional differences between the tissue-specific stromal cells. It also demonstrates the unique capability of HS in inhibiting proliferation and induction of cell death in K562 cells. This inhibitory effect of HS was further demonstrated on the following human tumor cell lines- myeloma cell line U-266, B-cell lymphoma cell line Raji, multiple myeloma cell line ARP-1 and multiple myeloma cell line RPMI-8266, indicating similar mechanisms among cancer cells.
Figure 4.7. Proliferation and viability of K562 cells cultured with stromal cell lines. Cultures were initiated with 100,000 K562 cells on confluent stromal cells. (A) K562 cells cultured on stromal cell lines (BM-MSC; AS; LS; HS) as observed under a light microscope on day 14 of culture (100x). (B) K562 total cell number enumerated with trypan blue staining after 14 days of culture. (C) K562 cell viability (indicated as percentage of viability of total cells) after 14 days of culture. Error bars represent standard error of the mean (n = 6). *P < 0.05. These data were repeated in three separate experiments.

4.2.4.3 Stromal Cell Co-Culture with CB Cells

We also examined whether the different stromal cell lines would have differential effects on proliferation of CB-Hematopoietic Stem and Progenitor Cells (HSPC).
After 14 days of culture, maximum proliferation was enumerated with BM-MSCs (mean, $7.11 \times 10^6$) (Figure 4.8A). Similar proliferation was seen with co-culture with AS (mean $3.14 \times 10^6$) and LS (mean $4.01 \times 10^6$). However, the proliferation of AS was significantly less than with BM-MSCs. To test for the functional output induced by proliferation on stromal cells, we assayed cell progeny in colony-forming assays specific for granulocyte/macrophage progenitors (CFU-GM). CB progeny co-cultured on BM-MSCs yielded $5.65 \times 10^4$ CFU-GM colonies (Figure 4.8B), whereas AS proliferated $3.31 \times 10^4$ total CFU-GM colonies. LS induced proliferation of more CFU-GM colonies ($7.81 \times 10^4$ CFU-GM) than BM-MSCs, although this was not significant. Although HS induced minimal total cell proliferation in co-cultures, proliferation of CFU-GM colonies was similar to that in BM-MSCs ($6.38 \times 10^4$ CFU-GM colonies), suggesting protection of progenitor populations. These results further validate the notion that stromal cells are functionally different though they present phenotypically and morphologically as a similar cell type.
4.2.4.4 Reversibility of the Inhibitory Effect of HS on K562 Cell Proliferation

Since HS had an inhibitory effect on tumor cells and CB cells in co-cultures compared with the other stromal lines, we investigated whether this effect was permanent or temporary in long-term culturing. To test this, we removed the hematopoietic cells from HS co-culture after 14 days and cultured in media and growth factors without the presence of stroma. We observed a steady increase of total viable cells in culture, suggesting that the inhibitory effect on CB cells was cytostatic and reversible on stromal cell removal. We performed this same experiment K562 cells. After 14 days of co-culture with stromal cells, K562 cells were collected, washed and equal number of viable cells were seeded in flasks and cultured without stroma for 14 days. As shown in Figure 4.9, K562 cells cultured on BM-MSC, AS and LS all proliferated like control K562 cells but those cultured on HS still failed to proliferate. This suggested that HS promotes a cytotoxic effect on K562 cells persisting even after removal of stromal cells. However, it is unclear how this cytotoxic mechanism is induced.

Figure 4.9: Proliferation of K562 cells on stroma followed by off stroma expansion. Panels A and B represent fold expansion and viability of K562 cells grown on stromal lines for 14 days (n=3 each). After 14 days, K562 cells are collected, washed and plated in flasks without stroma (C, D). (n=3) (All values are represented as mean±SEM)
4.2.4.5 *K562 Cell Proliferation is Inhibited by HS in a Transwell Assay*

We demonstrated that HS could inhibit the proliferation of K562 cells in co-culture assays. To test if this effect is cell contact dependent, transwell growth assay was performed. K562 cells were cultured in transwell inserts for 14 days either in presence or absence of a confluent layer of HS. Figure 4.10 shows that the fold expansion of K562 cells cultured in presence of HS is significantly lower (mean = 7.6 fold) compared to control (mean = 26 fold). However, unlike in co-culture assay, the viability of K562 cells in transwells is not adversely affected in the presence of HS (mean viability = 82%). This indicates that the inhibitory effect of HS on K562 cell proliferation does not require cell contact and that certain factors are secreted into the media by HS which can inhibit cell proliferation. Increased viability of K562 cells in presence of HS in transwell assay
compared to co-culture indicates that cell contact enhances the cytotoxic effect of HS on the tumor cells.

![Figure 4.10: Growth of K562 cells in transwell assay. (A) 14d expansion of K562 cells in transwells. (B) Viability of K562 cells after 14d culture in transwells. (n=3; all values represented as mean±SEM)](image)

4.2.4.6 Effect of HS-conditioned Media on K562 Cell Proliferation

As noted above, HS is capable of inhibiting K562 cell proliferation through certain factors secreted into the culture media. To test this, we generated conditioned media (CM) from HS (HS-CM) and concentrated it to 20X. CM was collected after 2, 7 and 10 days of conditioning by HS. K562 cells were cultured for 14 days with addition of 20% of 20X HS-CM while control K562 cells received no HS-CM. K562 cell proliferation is inhibited in the presence of HS-CM compared to control K562 cells. CM collected at different stages of conditioning (2d, 7d, 10d) by HS inhibited K562 proliferation to a similar degree. Interestingly, K562 cells cultured on top of HS layer yielded lesser fold expansion compared to culture in transwell assay or in presence of HS-CM which might indicate an additional role of cell contact in inhibition of tumor cell proliferation by HS. HS-CM inhibited K562 proliferation in a concentration dependent manner, with 1% of 20X HS-CM in growth medium being the minimum amount required.
for this inhibitory effect. At lower concentrations of HS-CM, K562 cells proliferated to control levels (Figure 4.11).

Figure 4.11: Effect of HS-CM on K562 cell proliferation. (A) Fold expansion of K562 cells with CM (20%, 20X) conditioned by HS for 2d, 7d and 10d. (B) Viability of K562 cells. (C) Fold expansion of K562 cells in transwell assay vs HS-CM. (D) K562 cell expansion with different doses of 20X HS-CM compared to control. (All values represent mean±SEM of triplicate experiments)

4.2.4.7 Characterization of HS-CM

We deduced that HS-CM contains certain factor(s) responsible for K562 inhibition. Physio-chemical analysis was performed to determine the nature of this factor(s). Alamar blue assay was adopted to assay for HS-CM activity. Living cells metabolize the alamar blue reagent and convert it into a red fluorescent molecule which
can be read on a fluorescent plater reader. HS-CM was subjected to different conditions namely high heat, rapid freeze/thaw cycles enzymatic digestion by trypsin enzyme. Tumor cell inhibitory activity of the resulting HS-CM after each treatment was analyzed. Figure shows that HS-CM activity remained intact after freeze/thaw treatment. However, heating the HS-CM to 60 or 80 degrees Celsius reduced its activity. Digestion by trypsin, a protease enzyme, completely neutralized the inhibitory activity of HS-CM and K562 cells proliferated to control levels (Figure 4.12). These results are consistent with the factor(s) of interest in HS-CM being a protein.

![Figure 4.12: Physio-chemical characterization of HS-CM. (A) K562 inhibitory activity of HS-CM (20%, 20X) subjected to different temperature conditions. (B) K562 inhibitory activity of HS-CM (20%, 20X) after protease treatment.](image)

**4.3 Discussion**

MSCs are currently being tested in a range of clinical trials to regenerate damaged tissue and treat inflammation in conditions such as myocardial infarction, brain and spinal cord injury, diabetes, cartilage and bone injury, Crohn's disease and GVHD (206). The use of BM-MSCs in clinical transplantation has been feasible and well tolerated,
with clinical studies moving to efficacy trials (209, 210). However, some phase II/III
efficacy trials have presented mixed clinical data (155). Many trials have used MSCs
isolated from BM; however, the presence of stromal cells in other tissues raises the
question of which tissue source is optimal. Our studies demonstrated that stroma from
different tissues have very similar morphology: spindle-shaped adhering cells,
characteristic of many stromal niche/basement membrane-type cells. Furthermore, they
exhibited expression of the hallmark surface markers, CD105, CD90 and CD73 and
negative for CD34 and CD45 and also underwent osteogenic and adipogenic
differentiation, which has been established for classic BM-MSCs. This finding has been
previously reported by multiple groups (211).

Many tumor cells grow rapidly on BM-MSCs, which provide support for their
continuous proliferation and growth (212). In our co-culture, stromal cells from liver and
adipose also supported the proliferation of K562 cells. Recent reports have demonstrated
that adipose tissue and associated stroma can maintain breast cancer cells and induce
pharmacological resistance (213, 214). Additionally, the links between adipose tissue and
activation of inflammation and chemokine pathways with regard to oncogenesis is
becoming more recognized (215). The establishment of metastatic cancers in the liver
along with the frequency of cancers originating in the liver further support the hypothesis
that liver stromal cells can maintain tumor cells (216). It is unclear if these stromal cells
can support all types of cancer cells or have preferential selection.

Our results demonstrate that heart derived stromal cells have a similar phenotype
to BM derived MSCs; however their gene and microRNA expression profiles are distinct.
The expression of growth factors and cytokines differ between the two cell populations.
Analysis of miRNA expression demonstrates that HS expresses cardiac related miRNAs at much higher levels than BM-MSCs. The high expression of miR-1, miR-133a and miR-206 in HS suggests that miRNAs play an important role in the function of stromal cells in the heart. These miRNAs are necessary for proper skeletal and cardiac muscle development and function, and have a significant influence on multiple myopathies, such as hypertrophy, dystrophy and conduction defects. MiR-206 had a 1,000 fold higher level of expression in HS compared to BM-MSCs. In addition to the role of miR-206 in skeletal and cardiac muscle development, it has been reported to be downregulated and exert tumor suppressive function in several malignancies such as breast cancer (217), lung cancer (218), gastric cancer (219), colorectal cancer (220), renal cell carcinoma (221), endometrioid adenocarcinoma (222), glioma and neuroblastoma (223) and osteosarcoma (224). It also suppresses breast cancer metastasis (225, 226) and blocks human rhabdomyosarcoma growth by promoting myogenic differentiation (226).

Interestingly, we demonstrated significant inhibition of proliferation of K562 and other tumor cells on HS and loss of viability. This observation is consistent with in vivo physiology. Most cardiac and pericardial tumors are metastatic lesions and primary tumors are rare. In addition, 70% of primary tumors of the heart are benign, including myxomas. Almost all malignant tumors of the heart are sarcomas (227). Given the high levels of blood circulation in the heart, metastatic tumor formation would be expected. Our findings suggest that stromal cells in the heart create a non-supportive micro-environment for tumor cells, thus preventing ‘seeding’ or induction of carcinogenesis. High expression of miR-206 may contribute to the inhibitory effects of HS but its exact role and mechanism of action are yet to be determined.
It is unclear to what extent direct interaction of tumor cells with HS and secretion of cytokines or other factors into the micro-environment milieu is responsible for the inhibition of proliferation and loss of viability. K562 cells were inhibited by HS when grown in transwells without contact with each other, suggesting contact between stroma and tumor cells is not essential for this effect. However, a larger fraction of the K562 cells are viable when contact is absent. We also observed prolonged inhibition of proliferation even after removal of tumor cells from HS, suggesting that the effect is cytotoxic but the mechanism of cytotoxicity is unclear. The requirement for stroma-tumor cell contact for this effect suggests the delivery of cytotoxic factors may occur through cell-cell channels. Additionally, with the use of conditioned media prepared from HS cells, we have demonstrated that the inhibitory effect is in part due to secreted molecules which inhibit tumor cell proliferation. This is consistent with the currently widely accepted view that stromal cells, both naturally and in transplant therapies act through paracrine mechanisms by secreting various cytokines. Initial characterization of the conditioned medium revealed a reduction of inhibitory activity after heating to high temperatures and a total loss of activity after protease digestion of CM. This is consistent with the secreted factor(s) being a protein. Furthermore, MSCs can secrete micro-vesicles or exosomes which contain certain pre-microRNAs (228). The released exosomes can facilitate cell-to-cell communications and thus, can alter cell activities in target cells. In the case of HS, release of tumor suppressing miRNAs via exosomes could mediate the inhibition of tumor cell proliferation. Further studies are in progress to define the responsible molecule(s) and to identify the mechanism of action as well as their potential effect across multiple cancer types.
The support of CB hematopoietic stem/progenitor cells by BM-MSCs has been reported in many publications, whereas the potential of other stromal sources remains to be defined (204). Similar proliferation of CB was observed with AS and LS as compared with BM-MSCs, whereas HS significantly inhibited CB proliferation. The potent tumor cell inhibition by HS did not affect stem/progenitor populations of CB as detected with the maintenance of CFU-GM colonies compared to BM-MSCs. The progenitor populations were maintained but other mononuclear cells were not. This finding further highlights the differences in function that HS has on normal cells compared with tumor cells. The effects with tumor cells were primarily cytotoxic with continuous loss of proliferation even upon removal of HS, whereas normal hematopoietic cells exhibited cytostatic responses with survival and subsequent proliferation even after long term exposure on HS cells. Further work is currently being undertaken to address the mechanism of action of the secreted inhibitory factor from HS.

Although initial applications for MSC therapy focused on multi-lineage differentiation capacities, recent clinical trials have focused almost entirely on the ability of MSCs to exert their biological function through the secretion of cytokines, serving both paracrine and endocrine functions (229-231). The range of molecules and signaling pathways that mediate the effects of MSC therapy are currently unknown. Our own unpublished data and the work of other researchers have demonstrated different gene expression profiles of various stromal cells with a wide range of cytokines and growth factors (232). Rossini et al. (208) recently demonstrated different functional properties between cardiac and BM stromal cells. It is possible that infusion of non-homologous stromal cells may alter the microenvironment, for example, infusion of BM-MSCs in
cardiac tissue, might alter the tumor suppressive microenvironment to support tumor growth. Alternatively, it is possible that stromal cells infused into a different tissue may adapt and alter their genetic composition to the local tissue environment. Further studies are needed to better understand the similarities and/or differences of stromal cells from various tissues and their ability to cross-talk with primary cells and local environments. This would lead to the optimization of better and potentially safer cellular therapies for treatment of disease.
CHAPTER 5: CONCLUSION

The objective of regenerative medicine is to develop novel therapies to replace or restore function to tissues and organs within the human body. Since the 1960s and the therapeutic use of hematopoietic stem cells of bone marrow origin, there has been an increasing interest in the study of stem cells that have the ability to proliferate and differentiate into various tissues and their potential use in regenerative therapy. In that regard, most of the therapeutic attention has focused upon stem-cell-based transplantation approaches to disorders that range from type I diabetes to myocardial infarction. Currently, several elusive questions remain to be addressed: [1] optimal delivery approach, [2] best stem cell type, [3] most effective dose, and [4] timing of administration. Mode of stem cell delivery is one of the key factors determining cost, feasibility and medical efficacy. Researchers are evaluating various methods of delivery of stem and stromal cells (both autologous and allogeneic) for tissue repair (233). However, a different therapeutic approach has been suggested by the evolving body of work showing that many adult tissues contain resident stem cell populations. It is now clear that these resident stem cells function to maintain and, in some cases, repair tissues (234). These findings have led to the idea that recruiting endogenous stem cells could enhance tissue repair or regeneration. Stromal cells are components of stem cell niches where they perform key structural and functional roles (235). Mesenchymal stem cells, identified as key components of the HSC niche, have awakened a great deal of interest in regenerative medicine due to their plasticity, and immunomodulatory and anti-inflammatory properties and as such are currently the subject of more than 200 clinical
trials aimed at treating a broad range of degenerative conditions (236). Many studies raised the intriguing possibility that if we could understand how physiological cues enhance stem cell function then we could manipulate these same mechanisms to promote repair (237, 238). G-CSF has been used successfully for decades to mobilize HSCs from bone marrow. Many studies have looked at the possibility of mobilizing MSCs by G-CSF treatment. Recent reports also suggest the mobilization of distinct progenitor cell populations from the marrow dependent on the combination of growth factors used (171, 239). Our research demonstrates the mobilization of MSCs from the bone marrow using a combination treatment of G-CSF and plerixafor (AMD3100). This combination is clinically approved for safe use in HSC transplant and thus does not suffer from adverse off target effects like with many other growth factors (240). Following successful mobilization of MSCs, the key question to answer is their effect on the repair of damaged tissues. Using a model of chemically induced liver injury, we showed that G-CSF+AMD3100 treatment significantly improves survival of critically injured mice and stabilizes liver health after acute injury compared to control treated mice. Other researchers have studied the use of this combination in different disease models have not demonstrated the mobilization of MSCs (241, 242). The mechanisms by which these mobilized stromal cells conduct or aid in tissue repair in still unknown. Paracrine secretions of MSCs which help in reducing inflammation, promote angiogenesis and regulate immune cell activity have been suggested as the main effector of repair at the damaged tissue sites (243). The microenvironment of the damaged tissue also exerts huge influence on the outcome of repair efforts. Stromal cells in the tissues are important for maintaining tissue specific stem cells, thus repairing the stromal environment could be
key factor to ensure effective repair. A healthier bed of stromal cells could ensure better survival of both endogenous and transplanted stem cells and could be a more effective treatment strategy. Indeed, pre-clinical studies in pig have showed that delivery of a combination of cardiac stem cells (CSC) and MSCs to damaged cardiac tissue post-infarct led to 7 fold higher CSC engraftment, significantly decreased infarct size and increased LV function versus either cell type alone (244, 245). Regenerative therapy by mobilization may not replace stem-cell-based transplantation, because in many situations the endogenous repair capacity will not be sufficient and/or the endogenous stem cells will themselves be impaired. In such cases, mobilization can be used as an adjunct therapy to transplantation either to prepare injured tissues to receive cell transplant or as maintenance thereafter. Nonetheless, this approach can provide hope for many disorders that are currently untreatable. Before any stem cell based therapeutics can be applied in the clinic, more research is necessary to understand their behavior upon transplantation as well as the mechanisms of their interaction with the diseased microenvironment. Our research provides an example of the potential of endogenous cell therapy by mobilization and offers encouragement for the continued search for new bioactive molecules or chemical entities which help increasing our knowledge of endogenous cell regulatory mechanisms and move us closer to the goal of effective regenerative therapies.

MSCs represent an important stem cell population with multipotent capabilities which are extremely useful for clinical applications. Recently, isolations of adult stromal cells from different sources have been reported. Stromal cells derived from adipose tissue (246), peripheral blood (247), the lung (248) or the heart (249) have also shown promising potential for proliferation and differentiation into different cell types. Many of
these sources of adult stem and stromal cells are being evaluated for clinical transplant into various tissues (250, 251). Thus it is important to study the properties of these varieties of stromal cells and discern their similarities and differences and understand how they interact with cells of other tissues. This will enable us to determine the optimum source, dosage and manipulations of cells required for safer and more effective therapies in clinic. We isolated stromal cells from heart, liver, adipose and bone marrow. We showed that these stromal cells are morphologically and phenotypically similar to BMMSCs but they vary in their functional properties. There are certain reported discrepancies within the MSC literature resulting in differing descriptions of the biological properties of MSC, however these effects may be explainable in part by the existence of distinct subpopulations within a tissue-derived culture that exhibit some functional variation (252-254). We discovered that heart stromal cells (HS) were able to inhibit the growth and reduce viability of a variety of tumor cells in direct co-culture, non-contact transwell co-culture and through the addition of media conditioned by HS cells. This effect was not reproduced with stromal cells from other tissues. HS also had a similar effect on the proliferation of CBMNCs but in this case the effect was reversible suggesting a cytostatic effect as opposed to the cytotoxic effect of HS on tumor cells. The mechanisms of this cytotoxic effect need to be elucidated but contact between HS and tumor cells appears to be important. The genetic make-up of these HS cells gives us clues as to the factors responsible for this effect. A comparative gene and microRNA array analysis of HS and BMMSCs revealed a host of genes (cytokines, cytokine receptors and adhesion molecules) and miRNAs that are differentially expressed in HS, including some which have been implicated in tumor suppression. A previous report by Rossini and
colleagues comparing cardiac derived stromal cells to BMMSCs also showed variation in the expression of a number of miRNAs (208). We observed over 1000 fold overexpression of miR-206 in HS compared to BMMSCs. In addition to its role in cardiac development, miR-206 has been implicated as a tumor suppressor in a variety of cancers (217, 220). This may explain the ability of HS to suppress tumor cell proliferation and also the fact that primary tumors in the heart are extremely rare. This observation may have significant impact on deciding which source of stromal cells to employ for regenerative therapy. It is possible that introduction of large numbers of tumor permissive stromal cells into non-native tissues, as is the case with transplantation of BMMSCs into other tissues, may have negative consequences in the long run. Our novel findings regarding HS highlight clearly the need to properly characterize each stromal cell source under consideration for clinical use.

In summary, stromal cells are an essential part of the microenvironment of adult tissue stem cells and contribute to tissue structure, function and homeostasis. They are likely to play a key role in the tissue regeneration process but their exact roles and mechanisms of action are not yet clear. Our research demonstrated clearly how endogenous stromal cells can be recruited by a clinically approved, non-invasive treatment regimen to aid in repair of damaged tissues. We also described how stromal cells derived from different tissues are different and cannot be assumed to function in a uniform way in stem cell therapy applications. With the increasing interest in stem cell based therapies to treat various diseases, it is essential to properly understand the essential factors governing effective stem cell use. The novel effect of cardiac stromal
cells described in this research is only one example of the many properties that need to be thoroughly studied if we are to successfully use stromal cells in therapies. The future studies outlined above will provide answers to key questions about delivery of stromal cells, their mechanisms of action and their basic biology. We believe that these answers will help to make more educated decisions about the use of stem and stromal cells and design safer and more effective regenerative therapies.
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