A self-renewing multi-potent population of cells and their progeny maintain homeostasis of the mesenchymal compartment

By

Rahul Sarugaser

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Rahul Sarugaser, Ph.D. (2008)

Institute of Biomaterials & Biomedical Engineering, University of Toronto

Abstract

Recent evidence suggests that “mesenchymal stem cells” (MSCs) are resident in the perivascular compartment of connective tissues. However, since the definition of a stem cell assumes that these progenitors have clonal self-renewal and multi-lineage differentiation potential, the term “MSC” has been criticised, as it has been impossible to isolate definitive clonally derived “MSCs.” To test for this most basic definition of a stem cell, here it is shown that human umbilical cord perivascular cells (HUCPVCs) are capable of multilineage differentiation in vitro and, more importantly, in vivo, displaying the ability to differentiate into functionally synthetic cells that direct and contribute to rapid connective tissue healing by producing bone, cartilage and fibrous stroma in a mouse injury model. Uniquely, these cells can be enriched to >1:3 clonogenic frequency in early passage culture, making it possible to isolate clones and daughter sub-clones from mixed gender suspensions, determined to be definitively single-cell-derived by Y-chromosome fluorescent in situ hybridization (FISH) analysis. Each clone was assayed for multi-lineage differentiation capacity into the five mesenchymal lineages: myogenic, adipogenic, chondrogenic, osteogenic and fibroblastic (stroma). The observation that daughter sub-clones possess equal or lesser differentiative potential to their respective parent clones demonstrated the two intrinsic properties of stem cells in vitro: clonal self-renewal and multi-lineage differentiation. This evidence provides a new hierarchical structure of robust MSCs self-renewing to produce more restricted progenitors that gradually lose differentiation potential until a state of complete restriction to the fibroblast is reached. The methods described herein combined with recognition of this lineage hierarchy provides a significant advance to the understanding of MSC biology, and will enable interrogation of the properties of robust self-renewal and differentiation of MSCs in serially transplanted living recipients.
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# List of Abbreviations

<table>
<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% SM</td>
<td>2% FBS-supplemented media</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Anxa5</td>
<td>Annexin A-5</td>
</tr>
<tr>
<td>AS</td>
<td>Adipogenic supplements</td>
</tr>
<tr>
<td>BLM</td>
<td>Bone like mineral</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BN</td>
<td>Bone nodule</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CIC</td>
<td>Colony initiating cell</td>
</tr>
<tr>
<td>CS</td>
<td>Chondrogenic supplements</td>
</tr>
<tr>
<td>CVC</td>
<td>Calcifying vascular cell</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSMLC</td>
<td>Fast skeletal myosin light chain</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>HuNu</td>
<td>Human nuclear antigen antibody</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>LAC</td>
<td>Later-adhering cell</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MACOF</td>
<td>CFU with muscle, adipose, cartilage, bone and fibroblast potential</td>
</tr>
<tr>
<td>MAPC</td>
<td>Mesenchymal adult progenitor</td>
</tr>
<tr>
<td>MD</td>
<td>Multilineage differentiation</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex (Chapter 2), Myosin heavy chain (Chapter 3 &amp; 4)</td>
</tr>
<tr>
<td>Micro CT</td>
<td>Micro computed tomography</td>
</tr>
<tr>
<td>MPC</td>
<td>Mesenchymal progenitor cell</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron specific enolase</td>
</tr>
<tr>
<td>OI</td>
<td>Ostogenesis imperfect</td>
</tr>
<tr>
<td>OS</td>
<td>Osteogenic supplements</td>
</tr>
<tr>
<td>P0-P12</td>
<td>Passage 0-12</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>P(SCD)</td>
<td>Probability of single cell derivation</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
</tr>
<tr>
<td>SCD</td>
<td>Single cell derived</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>SR</td>
<td>Self-renewal</td>
</tr>
<tr>
<td>SSC</td>
<td>Somatic Stem Cell</td>
</tr>
<tr>
<td>s.c.</td>
<td>Sub-cutaneous</td>
</tr>
<tr>
<td>T-75</td>
<td>75cm² tissue culture flask</td>
</tr>
<tr>
<td>TD</td>
<td>Therapeutic dose</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>UC</td>
<td>Umbilical cord</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical cord blood</td>
</tr>
<tr>
<td>WJ</td>
<td>Wharton’s jelly</td>
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1. CHAPTER 1: Introduction
1.1. **Stem Cells**

By analyzing the morphological ontogeny of haematopoiesis in bone marrow (BM), Pappenheim\(^1\) postulated the existence of an undifferentiated stem cell ("gemeinsame Stammzelle") that gives rise to the plethora of blood cells via an intermediate state of progenitor cells (Figure 1-1).

![Figure 1-1. Pappenheim’s drawing of the concept of a common undifferentiated mesenchymal precursor cell for all blood lineages and endothelium, the “Retikuloendothelzelle.”](image)

He observed undifferentiated mesenchymal reticuloendothelial cells producing mature lymphocyte and myeloid cells through a transitionary state of immature parenchymal cells, but it was not until Till & McCulloch’s\(^2\) pioneering work in this same haematopoietic system, that stem cells were first experimentally validated. They demonstrated that limiting dilutions of precursors from BM of adult mice, when transplanted intravenously, formed haematopoietic spleen nodules in a dose-dependent manner\(^2-6\). This finding gave rise to the hypothesis that, a small population of multi-potent, self-renewing, clonogenic stem cells divides to generate progeny, each progressively more restricted in developmental potential, until ultimately mature tissue-specific cells are formed (as reviewed in\(^7\)).

1.1.1. **Stem Cell Properties**

Based on these observations, as well as those of other stem cell systems (discussed below), it has been determined that stem cells function through extensive self-renewal capacity such that they maintain homeostasis of their surrounding tissue, and robustly contribute to repair and homeostasis upon transplantation. As such, stem
cells have been defined to possess two critical properties: clonal self-renewal and differentiation capacity\(^8\): the ability of a cell to robustly give rise to an identical daughter that maintains the potential to differentiate into the cognate cells of its tissue. Accordingly, during development and tissue repair, stem cells propagate their pool by symmetric and/or asymmetric division (self-renewal); and, in the processes of aging and disease, they become extinguished during clonal exhaustion (differentiation) (Figure 1-2).

![Diagram](image)

**Figure 1-2.** During development and repair, stem cells propagate their pool by symmetric division forming identical progeny. During tissue maintenance and repair, they divide asymmetrically to form one identical daughter and one differentiated cell, the latter of which is responsible for regenerating the tissue. During aging and disease, the stem cell population is depleted due to clonal exhaustion of the stem cell pool. (SR = self-renewal).

In order to describe the concepts of stem cell self-renewal and differentiation, the example of embryonic development is used here. During embryogenesis (Figure 1-3) the single-celled zygote divides producing two identical daughter cells. These cells continue to divide symmetrically to produce the 8-cell morula, each cell of which is identical to the original zygote as it can contribute to the development of an entire organism\(^9,10\). The next few divisions are characterized by a restriction step, specification by clonal exhaustion, as the totipotent morula cell population is extinguished. In this case clonal exhaustion is a normal process of development, as the purpose of the totipotent stem cells is to eventually give rise to the fully developed organism along with its associated extra-embryonic tissue. In doing so, the cells organize themselves into

1-3
the blastocyst, which is composed of three distinct cell types, the inner cell mass (ICM), the underlying primitive embryonic endoderm, and outer trophoblast. The former exclusively gives rise to the embryonic tissues, while the latter two exclusively produce the extra-embryonic tissues. The ICM cells then produce progeny through a process of sequential restriction eventually giving rise to the variety of tissue-specific stem cells that produce the fully formed organism. These adult, or somatic stem cells (discussed below) function differently in homeostasis and repair, as rather than clonally exhausting their supply, they self-renew by symmetric and/or asymmetric division so as to maintain their pool throughout the life of the organism. Careful regulation of the stem cell pool's (a)symmetric self-renewal or clonal exhaustion cell fate decisions influences the production of mature cells which maintain homeostasis and enable rapid and robust responses to physiologic stresses, infection and injury.

![Diagram showing stages of embryogenesis](image)

Figure 1-3. Self renewal (SR) and multilineage differentiation (MD) steps during embryogenesis.

### 1.1.2. Stem Cell Ontogeny

The fundamental stem cell properties of self-renewal and multilineage differentiation influence mammalian development through a process of alternating proliferation and progressive restriction (specification). As previously described, the zygote proliferates into the morula that subsequently undergoes its first round of restriction into two distinct lineages: embryonic and extra-embryonic cells. This represents the first differentiation event in mammalian development. The ICM cells...
proliferate and become further restricted, producing the hypoblast and epiblast, and the latter further differentiates into the embryonic epiblast that is responsible for generating the embryo proper. This process of progressive restriction continues through formation of the primitive streak and gastrulation, in which specification into the three germ layers (ectoderm, mesoderm and endoderm) occurs.

As the embryonic cells become more and more restricted during tissue specification, it is believed that small pools of somatic stem cells (SSCs) remain undifferentiated in the developing tissue\textsuperscript{14}. (NB. SSC is a term used here to classify all stem cell types within the developed organism). For example, it has been shown that mesenchymal and haematopoietic progenitors co-localize in the embryonic mesoderm-derived aorta-gonad-mesonephros (AGM) region of developing mice\textsuperscript{15}. Both SSC types expand their pools during development to a ‘plateau’ found in adult BM, and accordingly, it has been suggested that these cells act in concert to provide a supportive micro-environment for each other’s survival and proliferation\textsuperscript{16}. It is these cells that are eventually responsible for maintaining homeostasis and coordinating tissue repair in the developed organism.

1.1.2.1. Stem Cell Types

Conventionally, two main types of stem cells are recognized. Those isolated from the embryo’s ICM, ‘embryonic stem cells’ (ESCs)\textsuperscript{17,18}, are considered pluripotent as they present the ability to contribute to the development of an entire organism by giving rise to all its cell types\textsuperscript{19,20}. While they retain the properties of their origin in terms of developmental potential, ESCs are considered an artifact of culture and do not to exist \textit{in vivo}\textsuperscript{19}. Alternatively, SSCs, stem cells found in fully developed organisms, are present in many if not all tissues. They are usually considered multipotent, providing the tissue is comprised of multiple parenchymal cell types, but cannot reconstitute the organism as a whole. Indeed, while ESCs are capable of generating all the cell types of the embryo, and SSCs are responsible for tissue (re)generation and maintenance, conventional wisdom dictates that the latter are the progeny of the former. As it was found that a very specifically isolated SSC population, mesenchymal adult progenitor cells (MAPCs), were able to contribute to all germ layers of an embryonic mouse\textsuperscript{21,22}, it
had been suggested that a small population of ESC-like cells are retained in the adult organism, although this work has not been repeatable to date.

1.1.3. Stem Cell Niche

Schofield first introduced the concept of a stem cell ‘niche’\(^{23}\) in 1978 when he observed that a “stem cell is seen in association with other cells that determine its behaviour.” Recent evidence has demonstrated that stem-cell populations are established in ‘niches’\(^{24-26}\) - specific anatomic locations that regulate how they participate in tissue generation, maintenance and repair\(^{27}\). Essentially, the niche encompasses all of the elements immediately surrounding the stem cells when they are in their naïve state, including the non-stem cells that might be in direct contact with them as well as extracellular matrix (ECM) and soluble molecules found in that locale\(^{28}\) (Figure 1-4).

![Figure 1-4](image)

Figure 1-4. Inputs feeding back on stem-cell function in the niche. Elements of the local environment that participate in regulating the system of a stem cell in its tissue state are depicted. These include the constraints of the architectural space, physical engagement of the cell membrane with tethering molecules on neighbouring cells or surfaces, signaling interactions at the interface of stem cells and niche or descendent cells, paracrine and endocrine signals from local or distant sources, neural input and metabolic products of tissue activity. (From Scadden)\(^{27}\).

All of these components act in concert to maintain the stem cells in their undifferentiated state. It is likely that certain cues then find their way into the niche in
order to signal the stem cells that their differentiation is required for regeneration or repopulation of a given tissue. It has been hypothesized that niches inhabit cloistered locations, donating long distance recruitment of stem cells; or, are sequestered in most if not all tissues as a repository of stem cells which behave to maintain homeostasis and repair injury of the tissue within which they reside.

1.1.3.1. **In vitro Stem Cell Niche**

It has recently been demonstrated that human ESCs create their own ‘niche’ *in vitro* (Figure 1-5) by producing a population of supportive differentiated cells\(^{29,30}\).

![Proposed model of human ES cell paracrine regulation.](image)

> Figure 1-5. Proposed model of human ES cell paracrine regulation. To maintain culture homeostasis within its autologously produced niche, human ES cells (hESCs) spontaneously and continuously differentiate into hDFs, providing a continuous source of endogenous human ES cell supportive factors. (From Bendall et al.\(^{29}\)).

These spontaneously generated cells provide a host of supportive paracrine factors required for ESC self-renewal, thus maintaining the pool of resident progenitors. However, it can be argued that the concept of an ‘*in vitro* niche’ is contrary to the definition of a niche which requires a specific ‘anatomical’ location with all of the elements described above contributing to tissue (re)generation. As well, ESC cultures do not attain a homeostatic balance, as they require passaging upon a certain confluence, whereas a niche is a putative homeostatic environment that maintains its own equilibrium. Nevertheless, this is an important concept, as, while this has not yet been demonstrated in other adherent stem cell systems *in vitro*, it is likely that a similar
mechanism of action is required to generate and maintain their stem cell pools in culture.

1.2. **Somatic Stem Cells**

Subsequent to Pappenheim’s initial observations of human BM, several groups have corroborated the existence of BM-resident haematopoietic precursors by showing haematopoietic recovery from transplanted BM after irradiation damage. Since then, BM has been the focus of intense study and investigators have determined the presence of two SSC-types therein: haematopoietic and mesenchymal. More recently, these SSC-types have been identified in several other organs. Haematopoietic stem cells have been found in the fetal liver (as cited in) and umbilical cord blood, while mesenchymal progenitors have been isolated from skin, fat, muscle, fetal liver, placenta, and umbilical cord; while other SSCs which are neither haematopoietic nor mesenchymal have been found in the brain and intestinal crypt.

The primary responsibility of SSCs is to maintain homeostasis of the tissues within which they reside. Throughout the life of an organism, four tissue types require constant turnover: skin, intestinal lining, blood and bone. Skin and intestinal lining are ectodermal and endodermal in origin respectively; and the SSCs that constantly renew these tissues are of the same respective germ-layer origin. Similarly with blood and bone, both are mesodermally derived, although each has distinct lineage-specific precursors. It had been suggested that these SSCs could cross germ lineage boundaries, but more recent evidence has shown that these observations were either artifact of culture or the result of cell fusion. Haematopoietic SSCs are exclusively responsible for maintaining homeostasis of the haematopoietic system, and it is believed that mesenchymal SSCs perform a similar function for the specific connective tissues within which they reside.

1.2.1. **Haematopoietic Stem Cells**

Haematopoietic stem cells (HSC) are rare cells that can both self-renew and differentiate to form all of the major blood cell lineages.
Since their initial discovery\(^2\), the HSC pool has been characterized phenotypically and is identified by distinct subpopulations based on both phenotype and function\(^{63,66}\). These observations have provided a definitive hierarchy of HSC self-renewal and differentiation in which long-term reconstituting HSCs\(^{66-68}\) have the greatest self-renewal capacity and give rise to all haematopoietic lineages; while their immediate progeny, short-term HSCs\(^{69}\) also generate all haematopoietic lineages, but do so for only 8-10 weeks. Immediately downstream are lineage-restricted progenitor cells, including common lymphoid progenitors and common myeloid progenitors. The latter progenitors in turn produce two more restricted progenitor types: granulocyte-monocyte progenitors, and megakaryocyte-erythrocyte progenitors\(^{70,71}\). Indeed, the HSC system is the most well defined hierarchy of stem cell self-renewal and differentiation that has been identified to date in any human\(^72\) SSC system.

1.2.2. The Mesenchymal Compartment

1.2.2.1. The CFU-F

The mesenchymal compartment was first identified as a source of specific mesenchymal tissue progenitors when Friedenstein and colleagues\(^{33,73}\) isolated
fibroblast colonies in culture from guinea-pig spleen and BM. When cultures of these cells were placed in diffusion chambers \textit{in vivo}, they formed osteoblasts and bone matrix with embedded osteocytes. These adherent fibroblastic colony-forming cells, identified as precursors\textsuperscript{74-76} \textit{in vitro}, were termed “fibroblast colony forming units” (CFU-Fs)\textsuperscript{77,78}. Determination of CFU-F frequency within a given tissue was dependent on limiting dilution, the minimum number of cells required to produce a single CFU-F in culture. Friedenstein and colleagues determined this frequency to be \(~1:[2.5\times10^5]\) in guinea-pig BM\textsuperscript{73}, and \(~1:[5\times10^4]\) in C\textsubscript{57}Bl\textsubscript{6} mice\textsuperscript{77}, while Castro-Malaspina et al.\textsuperscript{78} found the CFU-F frequency in adult human BM to be relatively variable at \(1:[7.5\times10^4 \pm 1.1\times10^4]\); and that the number of colonies formed was linearly correlated with the number of cells seeded, although these cells formed larger colonies when plated at higher densities suggesting paracrine signaling systems that potentiate colony growth.

Importantly, Friedenstein et al.\textsuperscript{73} further attempted to assay the clonal purity of CFU-F-derived colonies. By mixing suspensions of male and female BM-derived cells, they identified presence/absence of X- and Y-chromosomes in metaphase cells. From this analysis they determined 10 colonies to be gender-pure; although, their analysis was restricted to only 3 or 4 metaphase cells within each colony. They determined the probability (P) of single cell origin of the 39 cells they analyzed to be \(1/[5\times10^{11}](\sim2\times10^{-12})\) (using statistical analysis of: if the P of the second donor in each colony is k, then the P of finding a mixed colony after identification of 39 cells is \((1-k)^{39}\), where k=0.5). Unfortunately this calculation is incorrect, as use of this statistical analysis should be restricted to each colony, i.e. the P of finding a mixed colony after identification of 4 cells is \((1-k)^4\), determining the probability of each colony being single cell derived = 0.06.

Several investigators have termed limiting dilution-produced CFU-F-derived colonies “clones.” In this case, the use of the term “clone” is an approximation, as it has yet to be shown that colonies produced in this way are definitively single cell derived (the definition of a clone). Accordingly, it is necessary to be conservative with the use of this term, and restrict its use to exclusively describe definitive single cell-derived populations.
1.2.2.2. The CFU-O

Ashton et al.\textsuperscript{79} determined the osteo/chondrogenic potential of rabbit BM CFU-F-derived cells \textit{in vivo} as either, formation of bone surrounding cartilage, or intramembranous bone unassociated with cartilage. These initial observations suggested that postnatal marrow contains osteogenic precursors with the potential to differentiate via the endochondral or intramembranous embryonic skeletal development pathways. Friedenstein et al.\textsuperscript{80} then demonstrated that 45\% of limiting dilution-derived large CFU-Fs produced bone and cartilage in diffusion chambers when implanted intraperitoneally. This was the first evidence that certain CFU-F-derived cells serve as common precursors of both bone and cartilage. Owen and colleagues\textsuperscript{81} supported these observations, as they showed that under osteogenic induction, individual rabbit CFU-F’s displayed differential osteogenic (CFU-O) capacity \textit{in vitro}. When stained for alkaline phosphatase (ALP) expression, each colony displayed a distinct level of expression. This observation provided evidence of heterogeneity within the CFU-F pool, further suggesting that each CFU-F is at a different stage of tissue development. Subsequently, Kuznetsov et al.\textsuperscript{82} using a similar limiting dilution technique (7 to 14,000 cells per 176cm\(^2\) dish), isolated individual human BM-derived CFU-Fs in culture from dishes with 10-30 distinct colonies, and showed that 20 out of 34 (59\%) CFU-F ‘strains’ produced bone when implanted in a hydroxyapatite/tricalcium phosphate vehicle \textit{in vivo}. This CFU-O frequency increased to 68\% when the CFU-Fs were pre-induced with Dexamethasone (Dex) in culture before implantation. Furthermore, they showed that each ‘strain’ displayed differential osteogenic capacity, as some displayed abundant bone formation, while others produced ‘poor bone.’

By analyzing the osteogenic differentiation of cells derived from the rat calvaria, Turksen & Aubin\textsuperscript{83} demonstrated the possible existence of at least two distinct populations of osteoprogenitors. One population appeared to spontaneously differentiate into bone nodules in culture without the addition of a stimulating factor such as Dex. These data led them to conclude that this first population represented a committed osteoprogenitor compartment within the calvarium suggesting that “the mature osteoblast phenotype appears to be a default pathway.” The other population appeared to be less differentiated, as it would undergo osteoblastic differentiation only
following the addition of specific inductive stimuli such as Dex or other steroids (e.g. progesterone)\textsuperscript{84}, suggesting the presence of an inducible osteoprogenitor cell population in calvaria-derived and BM cell cultures. Together, these data served to illustrate the heterogeneity within the CFU-F pool, which consists predominantly of fibroblast precursors and sub-populations of committed and uncommitted CFU-Os.

1.2.2.3. The CFU-C

While the BM compartment had been identified as a source of stromal fibroblast and osteogenic precursors, initial evidence suggested that chondrocytic\textsuperscript{79,80,85} differentiation of these cells was closely associated with CFU-F and CFU-O formation. Johnstone et al.\textsuperscript{86} demonstrated that rabbit BM CFU-F-derived cells could produce chondrocytes (CFU-C) in micromass cultures supplemented with Dex and transforming growth factor-β3 (TGF-β3); while Mackay et al.\textsuperscript{87} confirmed this finding with human BM CFU-F-derived cells. These micromass cultures of CFU-F-derived cells did not provide a quantitative measure of the number of individual CFU-Fs that were able to differentiate into cartilage, nor did they present the normal morphology associated with hyaline cartilage. Rather than forming organized matrix within which the four chondrogenic cell morphologies exist, they produced collagen I-expressing fibrocartilage more characteristic of endochondral cartilage formation. This correlated with Fell’s\textsuperscript{88} observations that the aggregation of chondroprogenitor mesenchymal cells into precartilage condensations eventually formed bone in embryonic chick limbs. This process is dependent upon signals initiated by cell-cell and cell-matrix interactions and the current view is that a series of patterning systems function sequentially over time\textsuperscript{89}. Figure 1-7 illustrates the sequential upregulation of Sox9\textsuperscript{90} and Runx2\textsuperscript{91} (chondrogenic and osteogenic master regulatory genes respectively) during chondrogenesis and endochondral ossification. Moreover, a recent study\textsuperscript{92} has shown that Sox9 is dominant over Runx2 during skeletogenesis, and it is not until Sox9 downregulation that endochondral ossification can occur. This close interrelationship between Sox9 and Runx2 explains the interdependence of CFU-O and CFU-C formation both \textit{in vitro} and \textit{in vivo}. 

1-12
1.2.2.4. The CFU-A

While the relationship between Runx2, Sox9, and adipogenic gene upregulation has yet to be delineated, Nuttall and colleagues\textsuperscript{94} have found that MPCs, when supplemented with Dex and 3-isobutyl-1-methylxanthine, could produce adipocytes (CFU-A) after 21 days in culture. Interestingly, when Conget and Minguell\textsuperscript{95} compared osteogenic and adipogenic potential of early and late passage MPCs, they found that osteogenesis was maintained independently of passage number, however, adipogenesis was lost after only three passages. Majors et al.\textsuperscript{96} have described a marked decrease in ALP-expressing CFU-O's after 30 days of culture (approx 5 passages), although Bruder and colleagues\textsuperscript{97} have only described this decrease after 9-10 passages. Alternative analysis of these results could be interpreted as the sequential forfeiture of adipogenic and osteogenic capacity of MPCs due to extended culture. It is unclear whether individual populations of CFU-A and CFU-O were being sequentially extinguished; or whether a population of tripotential CFU-A/O/F cells was sequentially losing each lineage capacity by clonal exhaustion through intermediates of CFU-O/F and CFU-F. Accordingly, in order to dissect this mechanism, a putative CFU-A/O/F needs to be clonally isolated and assayed for forfeiture of one or more lineages in extended culture.
1.2.2.5. The CFU-Myo

It had been suggested early on that adult bone marrow contains cells capable of differentiation into skeletal muscle\(^98\), although this was initially disputed\(^99\). More recently, Ferrari et al.\(^100\) demonstrated that unfractionated BM cells from C57/MLC3F-\(nlacZ\) transgenic mice (in which \(\beta\)-Gal expression is under control of the muscle-specific myosin light chain 3F promoter), when injected into the tibialis anterior muscle of scid/bg mice, produced muscle fibers (CFU-Myo) containing \(\beta\)-Gal\(^+\) nuclei. Interestingly, the number of BM-derived \(\beta\)-Gal\(^+\) nuclei were less numerous than those observed in the satellite cell-injected contralateral leg. From the data presented, it is not clear whether the \(\beta\)-Gal\(^+\) nuclei represented new muscle fibres, or fusion of a BM cell with the existing muscle. In a subsequent study, Gussoni et al.\(^101\) intravenously transplanted whole BM from C57BL/10 mice into female \(mdx\) mice (an animal model of Duchenne’s muscular dystrophy). By Y-chromosome FISH analysis, the authors observed donor cells present in multiple haematopoietic tissues as well as fused with host myofibres, in which the donor cells were expressing dystrophin. Although this was a clinically important outcome, the authors did not address whether the donor cells had actually differentiated, or simply upregulated dystrophin within the host myofibres with which they had fused. Accordingly, definitive in vivo myogenic differentiation of BM-derived cells has yet to be demonstrated. However, using an in vitro assay, Wada et al.\(^43\) demonstrated that downregulation of MyoD (master myogenic regulatory gene) in muscle-derived MPCs (see 1.3.3.1) facilitated osteogenesis in vitro, suggesting a yet to be delineated interdependence of CFU-Myo and CFU-O differentiation.

Together, these data serve to illustrate that heterogeneity of the CFU-F pool is not restricted to a subset of CFU-O progenitors, but includes subsets of CFU-C, CFU-A and CFU-Myo progenitors that display interdependent relationships.

1.3. Mesenchymal Stem Cells (MSCs)

Based on the work of Friedenstein\(^33,73-77\), Ashton\(^79,102\), Castro-Malaspina\(^78\) and Owen\(^81\) et al., Caplan\(^103,104\) postulated the existence of a “mesenchymal stem cell” (MSC). While it was not until the late 1990’s that the distinct chondrogenic, adipogenic and myogenic lineages of CFU-F-derived cells were determined, Caplan proposed that
an MSC resides in the marrow compartment, and is the precursor responsible for producing, rejuvenating and repairing these skeletal tissues. He further suggested that, in response to injury, MSCs proliferate at the repair site to produce a large pool of cells, where they undergo commitment and enter a specific lineage pathway. These cells repair the damaged tissue by fabricating unique tissue components in a sequence of synthesis and assembly events comparable to that observed during embryonic development. Accordingly, he proposed a structure of MSC differentiation (Figure 1-8a) in which a self-renewing MSC produces parallel development of single lineage-specific restricted progenitors. This model has been the dogma in MSC literature to date.

1.3.1. Proposed Hierarchies of MSC differentiation

More recently, by observing ‘transdifferentiation’ of committed osteogenic cell lines and rat calvarial cells into cartilage and adipose in vitro, Aubin postulated that MSCs differentiate according to a lineage hierarchy in which a “multipotential cell gives rise to more restricted bi- or tripotential cells, and these ultimately give rise to monopotential progenitors.” In her proposed hierarchy (Figure 1-8b), she outlined transition of theoretical bipotential CFU-A/O and CFU-C/O progenitors into a default CFU-O lineage. She further offered that MSCs with unlimited self-renewal capacity were also responsible for producing CFU-F and CFU-Myo, but differentiated by a distinct pathway from the CFU-A, CFU-C and CFU-O lineages. She also suggested that this array of theoretical tri-, bi- and monopotential cells in culture complicate the ability to unambiguously discriminate between these cells, and that prospective isolation combined with rigorous assessment of the functional capacity of individual progenitors is required to define the nature of a MSC.

Given Caplan and Aubin’s theories, in an attempt to experimentally delineate the ontogeny of putative MSCs, Muraglia and colleagues isolated “clonal” human BM MSC populations by plating 2×10^3 cells/well in 96-well plates and isolating wells with only one CFU-F. As described in 1.2.2.1 above, limiting dilution does not definitively produce clonal populations; accordingly, the term ‘limiting dilution-produced colonies’ (LDPCs) will be used here. The authors assayed 185 LDPCs for capacity to differentiate into bone, cartilage and adipose in vitro as determined by osteocalcin, collagen II and
Sudan black staining respectively. They determined that 17% of these LDPCs could differentiate into all three lineages (OCA), and addition of FGF-2 increased this frequency to 34%. Interestingly, the majority of remaining LDPCs were bipotential and maintained exclusive OC (80% and 60% in FGF- and FGF+ conditions respectively), but neither OA nor CA capacity; while the remaining 3-5% of clones showed only monopotential osteogenic capacity, displaying neither chondrogenic nor adipogenic potential. These data suggest that FGF-2 largely prevents the differentiation of tripotential OCA’s into bipotential OC’s in culture; and accordingly, the authors proposed a hierarchy of MSC differentiation (Figure 1-8c). An OCA progenitor first loses its adipogenic capacity to give rise to a bipotential CA progenitor, which in turn loses its chondrogenic capacity, eventually producing a monopotential osteogenic progenitor, as its default lineage. Similarly to that proposed by Aubin105, the default lineage in this hierarchy was determined to be osteogenic.

![Figure 1-8.](image)

By postulating the existence of intermediate progenitors with the propensity to differentiate into more than a single lineage, proposal of these hierarchies has contributed significantly to MSC biology. Unfortunately, non-clonal limiting dilution analysis of only three of the five mesenchymal tissues (these authors failed to include CFU-F and CFU-Myo in their analyses) limits the impact of this work. More importantly, these hierarchies neither explain the emergence of nor interrelationships between the connective tissues of the body. While adipose, cartilage and bone are intimately linked in the marrow, these hierarchies neglect the stromal tissue that provides a supportive
environment for these cells. It is possible that the fibroblasts that produce this ECM are responsible for creating a ‘niche’ for these cells and/or their precursors, as they likely interact through all three ‘niche’ properties, paracrine signaling, cell-cell contact and cell-matrix interactions. As well, the myogenic lineage is also linked with these tissues, as it is closely associated with bone and adipose in situ, and is dependent on fibroblasts to produce its underlying ECM of collagen, elastin, laminin, etc. Accordingly, a hierarchy should account for all five mesenchymal lineages when postulating the steps of differentiation from MSC to end phenotype.

1.3.2. MSC Phenotype

The term “MSC” has been adopted as the common nomenclature for tissue culture adherent fibroblastic BM CFU-F-derived cells. As had been done in the haematopoietic system, several investigators examined an array of existing and novel cell-surface and intracellular antigens to prospectively identify MSCs. Caplan’s group identified SH2 (CD105)\textsuperscript{107}, SH3(CD73)\textsuperscript{108}, SB-10, SB-20 and SB-21\textsuperscript{109,110} as possible candidate markers, while STRO-1\textsuperscript{111-113} was also identified as a potential MSC marker. It was further determined, that these cells expressed several adhesion proteins including CD44 and VCAM-1 (CD106)\textsuperscript{95}; but completely lacked the HSC markers CD14, CD45 and CD34\textsuperscript{95,114}, identifying them specifically as a non-haematopoietic progenitor pool. More recently, Shi et al.\textsuperscript{115} have suggested the use of the endothelial marker CD146 marker to define a sub-set of perivascular progenitor cells (see 1.3.3.6), and this marker has subsequently been identified as present on several multipotential mesenchymal populations\textsuperscript{115-118}. While different combinations of these markers have been used to prospectively identify pure MSC populations, none have succeeded, resulting in reliance on retrospective analysis of CFU-O, CFU-C and CFU-A to determine the ‘stemness’ of a prospective mesenchymal tissue-derived cell.

Accordingly, due to the inability to prospectively isolate putative MSCs that exist at a low frequency in BM, resulting in the reliance on limiting dilution to produce an approximation of ‘clonal’ colonies; the fundamental properties of stem cells, clonal self-renewal and multilineage differentiation of MSCs, have never been demonstrated. Accordingly, the term ‘mesenchymal stem cell’ has been disputed by the International
Society for Cellular Therapy\textsuperscript{119}. They have suggested refinement of the term ‘MSC’ to define multipotent ‘mesenchymal stromal cells.’ As a result, Keating\textsuperscript{120} has identified three conditions by which these cells can be identified: 1) they are plastic adherent under standard culture conditions, 2) they express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR, and 3) they must differentiate into osteogenic, chondrogenic and adipogenic cells \textit{in vitro}.

For purposes of clarity, these CFU-F-derived MSCs will hereinafter be referred to as ‘mesenchymal progenitor cells’ (MPCs).

\subsection*{1.3.3. Mesenchymal Cell Sources}

As discussed above, Friedenstein and colleagues first isolated MPCs from haematopoietic tissues, spleen, thymus and BM\textsuperscript{33,73,76,77}; and accordingly, the majority of subsequent work has utilized BM as the primary source for MPC interrogation. More recently, several other tissues have been identified as sources of MPCs.

\subsubsection*{1.3.3.1. Muscle Tissue}

Satellite cells have been identified as unilineage stem cells that are located between the basal lamina and the sarcolemma of muscle fibres\textsuperscript{121} and maintain the ability to repair damaged muscle fibers\textsuperscript{122-124}. Initial evidence suggested that these cells are heterogeneous, as newly generated myofibres in transplanted mice were formed by the progeny of a small percentage of satellite cells\textsuperscript{125}, while in other studies, they became quiescent, persisting in the muscle of mice for >1 year\textsuperscript{126}. Very recently, Kuang et al.\textsuperscript{127} definitively demonstrated that there exist two distinct populations of satellite cells within the muscle, Pax7\textsuperscript{+}/Myf5\textsuperscript{+} and Pax7\textsuperscript{+}/Myf5\textsuperscript{-} populations. Intriguingly, they found that the Pax7\textsuperscript{+}/Myf5\textsuperscript{-} satellite cells divided asymmetrically through a basal-apical oriented division to produce a Pax7\textsuperscript{+}/Myf5\textsuperscript{+} cell, and that this cell formed apically to its basal precursor. Prospective isolation and transplantation of these two cell types into the muscle showed that the Pax7\textsuperscript{+}/Myf5\textsuperscript{-} population robustly contributed to the satellite cell pool, while the Pax7\textsuperscript{+}/Myf5\textsuperscript{+} population exhibited limited differentiation. Based on these observations, it has been further suggested that the asymmetric division of the basal Pax7\textsuperscript{+}/Myf5\textsuperscript{-} unipotent stem cell to produce an apical Pax7\textsuperscript{+}/Myf5\textsuperscript{+} daughter
provides that the latter will fuse with the muscle fiber during regeneration, while the former will remain in the niche to generate other stem cells or re-enter quiescence\textsuperscript{128}.

Multipotent MPCs have also been isolated from skeletal muscle, although it is still not clear whether these cells are distinct from unipotential satellite stem cells. Lee et al.\textsuperscript{42} showed that a CD34\textsuperscript{+}Bcl-2\textsuperscript{+} osteo/myo-progenitor population was resident within the basal lamina of muscles. By using limiting dilution, they isolated ‘clonal’ populations of these cells, that when transduced with recombinant human bone morphogenetic protein-2 (BMP-2), could accelerate healing of skull defects in SCID mice. Levy et al.\textsuperscript{129} demonstrated that osteoprogenitors isolated from mature human skeletal muscle could, under osteogenic conditions, upregulate ALP and osteocalcin in vitro, although they did not demonstrate elaboration of bone matrix in their cultures. Wada et al.\textsuperscript{43}, using myogenic human muscle-derived cells demonstrated downregulation of MyoD (myogenic master regulator) and upregulation of ALP, Runx2 and osteocalcin under osteogenic induction with exogenous BMP-2. They further demonstrated that these cells could elaborate bone matrix in vitro as identified by alizarin red staining.

While multipotential MPCs can be isolated from muscle tissue, their ontogeny remains undetermined. Qu-Petersen et al.\textsuperscript{130} attempted to prospectively identify satellite cells by labeling Sca-1\textsuperscript{+} cells in murine muscle sections. They determined that Sca-1\textsuperscript{+} cells were present beneath the basement membrane (satellite cells), but also in association with capillaries. More recently, Brachvogel and colleagues\textsuperscript{131} determined that an annexin A5 (Anxa5)-expressing population was present in adult vascular and skeletal tissues. Importantly, they determined that Anxa5 was specifically expressed on cells located parallel to the lining endothelial cells (perivascular) of both large and small vessels in these tissues; and that these cells could produce bone, cartilage and adipose tissue in vitro, as well as muscle tissue in vivo. Accordingly, given the cumulative evidence of a perivascular Anxa5\textsuperscript{+} Sca-1\textsuperscript{+} skeletal muscle-derived cell maintaining multipotential capacity both in vitro and in vivo, combined with the observation that mesenchymal progenitor self-renewal is affected by Sca-1 deficiency\textsuperscript{132}, it is interesting to speculate that muscle-derived MPCs arise from the vasculature embedded therein.
1.3.3.2. Adipose Tissue

Since Van et al.\textsuperscript{133} isolated culture-adherent adipogenic fibroblastic cells from the ‘stromal-vascular fraction’ of human omental adipose tissue, this connective tissue has also been identified as source of precursor cells. More recently, Sen et al.\textsuperscript{41} determined that, like BM, there appears to be significant heterogeneity/variability within human lipoaspirate-derived cell populations. Using an optimized adipogenic induction protocol, they observed that preadipocytes isolated from different donors displayed varying degrees of adipogenic differentiation. Hedrick’s group subsequently demonstrated that these human lipoaspirate samples contained not just adipogenic precursors, but multipotential MPCs as well. By RT-PCR analysis of lineage specific gene upregulation, they showed that adipose tissue-derived MPCs differentiated into adipose, bone, cartilage\textsuperscript{40} and muscle\textsuperscript{134} \textit{in vitro}.

Indeed, since Van et al.\textsuperscript{133} identified the stromal-vascular fraction of lipoaspirates to be a source of precursor cells, Gronthos and colleagues\textsuperscript{117} determined expression of several mesenchymal markers on these cells including CD105, CD44 and, importantly, the endothelia/perivascular cell marker CD146. More recently, Yoshimura et al.\textsuperscript{135} confirmed presence of these markers and further illustrated expression of CD90, CD133 and CD117 (c-kit). While it has yet to be shown in adipose tissue, given that muscle, another mesenchyme-derived tissue houses MPCs in a perivascular compartment, it is most likely that adipose-derived MPCs are located around the adipose vasculature.

1.3.3.3. Vascular Tissue

In 1863, Virchow\textsuperscript{136} observed that the mineral component of atherosclerotic arterial lesions was “an ossification, and not a mere calcification” (as cited in\textsuperscript{137}). More recently, the Demer group has been investigating the process of intravascular osteogenesis. They observed that BMP-2a was upregulated in human atherosclerotic lesions, and that when these cells were transferred into culture, they produced mineralized bone nodules \textit{in vitro}\textsuperscript{137}. They further observed positive staining for a pericyte marker, 3G5, present on these cultured cells as well as in cross sections of human thoracic aorta. By isolating normal sub-endothelial cells in culture, and exposing them to 25-hydroxycholesterol and TGF-\(\beta\)1, both known to be present in atherosclerotic.
lesions, these cells elaborated bone matrix as determined by ALP and von Kossa staining\textsuperscript{138}. Due to this osteogenic capacity, they termed these cells “calcifying vascular cells” (CVCs), and further determined chondrogenic but surprisingly not adipogenic capacity of CVCs\textsuperscript{139}. As CVCs are constantly under mechanical stress, Canfield et al. have supported this observation by showing that mechanical stimulation of multipotential perivascular cells inhibits adipogenesis (personal communication). As previously discussed, it is unclear whether this lack of adipogenesis is the result of downregulation of an exclusively adipogenic population; or whether a CFU-A/O/F progenitor is being triggered to bypass its adipogenic capacity and preferentially upregulate its osteogenic potential due to mechanical stimulation. Clonal analysis of said CFU-A/O/F progenitor would reveal this underlying mechanism.

1.3.3.4. Embryonic Tissue

More recently, investigators have found evidence of MPCs present in several embryonic and extra-embryonic tissues. The fetal BM, blood and liver were analyzed for presence of multipotential MPCs\textsuperscript{140}. First trimester fetal BM and liver generated CFU-Fs at similar frequencies of ~1:\[8.5x10^4\], remarkably similar to that seen in adult BM. The CFU-F frequency in first trimester blood was slightly lower at ~1:\[1.2x10^5\], although interestingly, no MPCs could be derived from second or third trimester blood. The authors illustrated that these MPCs could differentiate into bone, cartilage and adipose by von Kossa, collagen II and oil red O staining respectively.

The observation that CFU-Fs could only be derived from first trimester blood suggests that at later points in gestation, MPCs that have migrated to their final tissue-resident repositories, no longer require the blood system for migration. While the phenomenon of HSC homing to the haematopoietic compartment has been shown\textsuperscript{44,141-145}, investigators have suggested that MPCs also ‘home’ to the marrow compartment following systemic infusion of irradiated hosts\textsuperscript{146}. 1 in \(10^5\) cells “engrafted” in the BM while the majority became enlodged in microvessels of the lung and liver. The authors suggested that it is this ‘migratory’ and ‘homing’ phenomenon that enables BM-resident MPCs to migrate to sites of tissue damage where they are then responsible for coordinating tissue repair. This idea was further carried out in an experiment by Orlic et
al.\textsuperscript{147} who intravenously injected two cytokines, stem cell factor and granulocyte-colony stimulating factor into mice with acute myocardial infarcts. They observed a significant improvement in heart function due to the formation of 15x10\textsuperscript{6} new myocytes, as identified by BrdU labeling, that expressed \(\alpha\)-sarcomeric actin, cardiac myosin, and connexin 43 \textit{in situ}. While these data did demonstrate improvement in overall cardiac function, their conclusion that the cells contributing to this significant healing were BM-derived was unfounded, as they neither demonstrated the origin of the repairing/differentiating cells nor assayed whether their observations were due to cell fusion. Another, more likely, possibility would be for MPCs already resident in the vascular fraction of respective tissues to be recruited for tissue repair, rather than ectopically-derived ‘homing’ cells. More recently though, investigators have demonstrated homing of MPCs to solid tumor chronic wound sites, as intravenous infusion of eGFP-labeled MPCs localized specifically to xenografted tumors in mice\textsuperscript{148}. The cancer cells stimulated secretion of the chemokine CCL5 from the MPCs that then acted in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis. Whether CCL5 was responsible for recruitment of infused MPCs was not assessed; although, if CCL5 does attract non-resident MPCs, it is possible that this mechanism is restricted to cancer pathologies, as MPCs did not express the chemokine until in direct contact with the CCR5-expressing (CCL5 receptor) tumorigenic cells. Nevertheless, it has yet to be shown that MPCs are recruited to/from the marrow for repair of (non-tumor) damaged connective tissues.

1.3.3.5. Extra-Embryonic Tissues

1.3.3.5.1. Placenta

The placenta also appears so be a repository for osteogenic MPCs. Igura and colleagues\textsuperscript{149} isolated MPCs from explants of placental choronic villi, and determined their ability to differentiate into bone, cartilage and fat in culture by ALP, Von Kossa, collagen II and oil red O staining; although they only analyzed upregulation of cartilage genes Sox9, collagen II and aggrecan by RT-PCR. More recently, Miao et al.\textsuperscript{150} isolated placenta-derived MPCs by mincing and enzymatically digesting placenta tissue. Under
induction, they demonstrated differentiation of these cells into putative neurogenic and endothelial cells as determined by “neuron-like cells” expressing GFAP and neuron specific enolase, and (fibroblastic) “endothelial-like cells” that expressed vWF in culture. These data are unconvincing, as the staining patterns observed appeared dystrophic, and the cells did not express the stated morphologies (i.e. endothelial cells maintain a polygonal cobblestone morphology in culture).

In another study by Takahashi et al.\textsuperscript{45}, cells explanted only from the ‘fetal part’ of the placenta displayed osteogenic differentiation by upregulating osteopontin, osteocalcin and ALP as well as producing mineralized bone matrix \textit{in vitro}; while cells explanted from the ‘maternal parts’ only displayed gene upregulation but not bone tissue elaboration. These data in comparison to the previously published data\textsuperscript{150} highlight the necessity for analysis of both lineage specific gene upregulation as well as morphological tissue-specific differentiation and ECM production to determine the nature of a prospective MPC population.

1.3.3.5.2. Umbilical Cord

MPCs have also been isolated from human umbilical cords (UC). As the embryo folds from a disc into a cone, it connects the primitive midgut (embryonic origin) to the primitive yolk sac (extra-embryonic origin)\textsuperscript{151}. The forming UC consists of three layers: an inner endodermal layer, an outer mesothelial layer, and an intermediate mesenchymal tissue layer\textsuperscript{152}. The outer mesothelial cells develop to form the amniotic epithelium of the UC, while the intermediate mesenchymal layer forms its vitelline and allantoic vessels. These vessels, which are internally lined by the inner endodermal cells, eventually form the three umbilical vessels\textsuperscript{153}, and as a result, their viscous surrounding matrix is a primitive mesenchymal tissue of both embryonic and extra-embryonic origin. This primitive connective matrix termed “Wharton’s jelly” (WJ)\textsuperscript{154}, primarily functions to provide structural support to the three vessels. Two types of cells have been identified within this WJ matrix: fibroblasts and mast cells\textsuperscript{155}. The mast cells are predominantly confined to the immediate vicinity of the vessels\textsuperscript{156}, while the “smooth muscle”\textsuperscript{157} “fibroblast-like”\textsuperscript{158} cells dispersed in the matrix have subsequently been described as “myofibroblastic” as they express high levels of $\alpha$-sm-actin, myosin,
vimentin and desmin\textsuperscript{159-161}. This myofibroblastic phenotype is very similar to that observed in pericytes\textsuperscript{162-169} (described in 1.3.3.6.1 below), multipotential cells\textsuperscript{170} which are found abluminal to vessel walls in the bone remodeling environment.

UC matrix cells first isolated\textsuperscript{171} in culture maintained their fibroblastic morphology. Naughton\textsuperscript{172} and Purchio et al.\textsuperscript{173} demonstrated that by dissecting away the vessels before explanting the remaining tissue, these cells were pre-chondrocytic, as they could produce cartilage tissue \textit{in vitro}. Also by removing the umbilical vessels and explanting the remaining tissue, Mitchell et al.\textsuperscript{174,175} isolated cells that expressed c-kit and telomerase in culture. Telomerase expression was assayed using a fluorescent telomerase detection kit that assayed telomerase activity by amplification of telomeric repeats using fluorescence energy transfer primers that produced measurable fluorescence when incorporated into TRAP products. They determined that WJ cells had approximately 1/10\textsuperscript{th} telomerase activity of a control carcinoma cell line, and that these progenitors could derive putative neurons and glia that expressed neuron-specific enolase, GAP-43, GFAP and CNPase in culture. While not stated in their methods, these authors have confirmed (personal communication) that the amniotic epithelium was left intact during their explants. As neural tissue is ectodermally derived, it is certainly possible that this amniotic epithelium was the source of ectodermal precursors that differentiated into putative neurons \textit{in vitro}. Alternatively, as MPCs have been shown to arise from neuroepithelial cells during development\textsuperscript{14}, it is possible that residual neuroepithelial cells were responsible for the neural differentiation observed. Subsequently, Wang et al.\textsuperscript{47}, using a method similar to that used by Mitchell et al.\textsuperscript{174}, differentiated the WJ-derived MPCs into putative cardiomyocytes as determined by staining of cardiac troponin I and N-cadherin in culture. They further determined differentiation of these cells into osteogenic, chondrogenic and adipogenic lineages. Chondrogenic cell pellets stained positively with Alcian blue and collagen II, von Kossa-positive osteogenic cultures upregulated osteopontin, and adipocytes stained with oil red O and upregulated PPAR-\textgamma\textsubscript{2}.

Romanov et al.\textsuperscript{176} further derived osteo/adipogenic MPCs from the sub-endothelial layer of umbilical cord veins. By using a collagenase digest combined with gentle massage, they collected sub-/endothelial cells from the umbilical vein. They
determined that these suspensions produced adherent fibroblastic cells after 2 weeks in culture, and that under osteogenic and adipogenic induction they observed cells that stained positively for ALP and oil red O respectively. Covas and colleagues\textsuperscript{177} who used an identical method to isolate MPCs made similar observations. These results were confirmed by Kim et al.\textsuperscript{48} who also used this method to isolate MPCs, but were more rigorous in their analysis of osteogenic and adipogenic phenotypes, as they also confirmed upregulation of Runx2, osteopontin, lipoprotein lipase and PPAR-\(\gamma\).

This more recent analysis underscores the requirement for rigorous endpoint phenotypic analyses that were lacking in the previous studies. For determination of a prospective MPC population, analysis of not only morphological lineage specific changes, but also gene and protein upregulation by RT-PCR and/or protein localization by antibody staining are imperative. This is especially important when analyzing the yet to be determined myogenic potential of UC-derived cells, as these cells already express myogenic markers including \(\alpha\)-sm-actin, myosin, vimentin and desmin\textsuperscript{159-161} in situ.

1.3.3.6. Perivascular Tissue

Based on populations described herein (see Chapter 2), Baksh et al.\textsuperscript{116} have demonstrated multipotential capacity of MPCs derived from the perivascular region of the human umbilical cords. These cells displayed a myofibroblastic phenotype as determined by maintenance of \(\alpha\)-sm-actin, desmin and vimentin expression in culture, and importantly, also expressed the pericyte cell surface epitope 3G5\textsuperscript{178}. The observation of multipotential cells populations derived from the perivascular region of vessels, along with that observed in CVC populations (see 1.3.3.3) suggests that these cells are possibly homologous with pericytes.

1.3.3.6.1. Pericytes

Eberth\textsuperscript{179} and Rouget\textsuperscript{180,181} first described the presence of perivascular cells closely associated with capillaries more than 100 years ago (as cited in\textsuperscript{182}). In 1923, Zimmerman\textsuperscript{183} introduced the term “pericyte” to describe these cells that are found “adjacent to capillaries,” “continuous with vascular smooth muscle cells of arteries and veins,” and are “distinctively shaped with many cytoplasmic processes that encircle
capillaries" (as cited in\textsuperscript{182}). Pericytes are a class of mural cells that develop on the lumen side of the developing dorsal aorta. The aorta, which is initially a single endothelial layer forms ‘meso-angioblasts’\textsuperscript{184} around the endothelial cells, which then accumulate additional layers of smooth muscle cells characteristic of thicker blood vessels. Recently, Esner et al.\textsuperscript{185} showed that periendothelial and vascular smooth muscle cells of the aorta are the developmental origin of paraxial mesoderm-derived skeletal muscle cells. Meso-angioblasts have been shown to give rise to a number of mesodermal derivatives, including skeletal muscle and smooth muscle\textsuperscript{186}, and it is possible that what are observed as pericytes in the developed organism are residual meso-angioblasts.

Schor et al.\textsuperscript{162} demonstrated that pericytes synthesize and deposit extracellular matrix during \textit{in vitro} bone nodule formation, while a subsequent study\textsuperscript{187} demonstrated that pericytes form CFU-Fs that produced an ECM rich in ALP and synthesize large amounts of osteocalcin. More recently, the chondrogenic and adipogenic potential of these cells was determined\textsuperscript{170}. RT-PCR analysis of \textit{in vitro} pericyte-derived cartilage pellet cultures and oil red O-staining adipocytes illustrated upregulation of Sox9, aggregan and collagen II as well as PPAR-\(\gamma\)\textsubscript{2} respectively. When inoculated into diffusion chambers and implanted \textit{in vivo}, these cells produced chondrocytes within mineralized cartilage, fibrocartilage, and a non-mineralized cartilaginous matrix, as well as small clusters of cells that morphologically resembled adipocytes.

The majority of ‘MSC’ literature has, until very recently, paid little attention to the possible pericytic provenance of MPCs. The growing body of evidence (described above) illustrating MPCs being derived from the highly vascular tissues BM, muscle and adipose as well as several embryonic and extra embryonic tissues dictates that these cells are most likely derived from the one microenvironment common to all, the perivascular ‘niche.’

\subsection*{1.3.3.6.2. The Perivascular ‘Niche’}

Predominantly unacknowledged to date, the perivascular origin of MPCs is not a new idea\textsuperscript{162,168,170}, and recent evidence has given credence to this concept. Shi and Gronthos\textsuperscript{115} determined that BM and dental pulp STRO-1-selected MPC populations
both expressed perivascular markers 3G5 and CD146. Despite their diverse ontogeny and developmental potentials, this evidence proposes a common function and possibly a common ancestor. A more recent study\textsuperscript{188} has illustrated that MPCs can be derived from most adult tissues including BM, spleen, muscle, aorta, vena cava, kidney, lung, liver, brain and thymus. The authors observed consistently high expression of CD29 (integrin $\beta$-1 chain) and CD44 across all the tissues, variable levels of CD49 (integrin $\alpha_5$ chain), CD90 (Thy-1) and CD117 (c-kit), and no expression of the haematopoietic markers CD45, CD11b, CD13, CD18, CD19 or the endothelial marker CD31. They also observed differential ability of these cells to differentiate into bone or adipose tissue \textit{in vitro}. Generally, the vasculature and BM-derived cells displayed more osteogenic, but less adipogenic capacity than muscle, while the remaining tissues produced moderate amounts of both. Taken together, the authors suggest that these data illustrate the common perivascular origin of these cells (Figure 1-9) in the basement membrane apposed to endothelial cells.

![Figure 1-9. A proposed model of MPC contribution to tissue maintenance. MPCs lie in the basement membrane (red line), apposed to endothelial cells. (Composite from da Silva Meirelles et al.)\textsuperscript{188}.](image)

This model is similar, but not identical, to the putative perivascular ‘niche’ of MPCs described by Kolf et al.\textsuperscript{28} (Figure 1-10). While da Silva Meirelles\textsuperscript{188} et al. describe MPCs located specifically in the sub-endothelial space of medium to large size vessels where they interact directly with endothelial cells, Kolf and colleagues\textsuperscript{28} describe cells in the perivascular space of microvessels, with some attachment to endothelial cells, but also maintaining other ‘niche’ interactions.
Figure 1-10. The mesenchymal stem cell niche. MSCs are shown in their putative perivascular niche (BV, blood vessel), interacting with (1) various other differentiated cells (DC1, DC2, etc.) by means of cell-adhesion molecules, such as cadherins, (2) ECM deposited by the niche cells mediated by integrin receptors, and (3) signaling molecules, which may include autocrine, paracrine, and endocrine factors. Another variable is O2 tension, with hypoxia associated with MSCs in the bone marrow niche. (From Kolf et al.)

Kolf et al. describe three basic interactions that maintain the MPCs within their ‘niche.’ First, other differentiated cells surround the MPCs and signal them either by direct cell-cell contact, or through paracrine factors. These cells can either be smooth muscle adventitial cells of the vessel itself, or other differentiated connective tissue cell types such as stromal fibroblasts. Second, collagens, fibronectin, elastin, proteoglycans and a host of other ECM molecules interact with MSCs predominantly through integrin receptors. Finally, exogenous signaling molecules including paracrine and endocrine factors have been proposed to be the main source of ‘recruiting’ molecules that activate either migration or division (symmetric or asymmetric) of these cells in response to injury. As well, the gradient of oxygen concentration may be a determining factor of MPC fate. It is still not clear what intracellular signaling events any of these interactions cause, nor how they influence MPC fate decisions. It is necessary to clarify that the niche concept is a hypothesis, and has yet to be definitively demonstrated. For the purposes of clarity, unless specifically discussing this concept, the term ‘microenvironment’ will be used here when discussing ‘niche’ environments.

While the growing body of evidence described above provides support for the
perivascular provenance of MPCs, it is not clear whether the MPCs reside in the sub-
endothelial space, or in the perivascular compartment. It is also not clear whether these
two possibilities are mutually exclusive, or if MPCs reside in/on small, medium and large
vessels. Given the cumulative evidence of Canfield’s group\textsuperscript{162,163,166,168,170}, Demer’s
\textsuperscript{137-139,189,190}, Shi et al\textsuperscript{115} and now da Silva Mereilles and colleagues\textsuperscript{188}, it would
appear that MPCs reside in virtually all vascular tissue. Based on the evidence of CVCs
forming bone within vessels, it is possible that MPCs resident in large vessels are
responsible for the normal maintenance of the vessel within which they reside, and form
bone only as a result of some pathological process. While the functionality of pericytes
has yet to be determined in direct contact with a mesenchymal compartment \textit{in vivo}, the
ability of pericytes to produce ectopic bone and adipose tissue in diffusion chambers
suggests that MPCs surrounding microvessels probably serve to repair and maintain
homeostasis of their surrounding tissues. It has yet to be determined whether the MPCs
in medium-size vessels, such as those found in the umbilical cord, are resident in the
sub-endothelial space, or in the perivascular space around the vessels. Based on the
cumulative data of CVCs and pericytes it is possible that MPCs resident in these
medium-size vessels possibly serve to both maintain homeostasis of the vessel as well
as contribute to maintenance of the surrounding connective tissue. It can be further
hypothesized that two distinct populations reside in medium-sized vessels, those in the
sub-endothelial space that are responsible for vessel maintenance, and those in the
perivascular space that are responsible for maintaining the surrounding tissue. Careful
analysis of specifically isolated cells from these vessels would be required to elucidate
the differential or similar functionality of these cells. While the evidence described herein
(see Chapter 2) suggests that MPCs reside in the perivascular compartment, it does not
exclude the possibility of the sub-endothelial provenance of these cells.

\subsection{1.3.4. Do MSCs Exist?}

Since Pappenheim’s\textsuperscript{1} original observations of undifferentiated cells, different
types of definitive stem cells have been described. It has been conclusively shown that
ESCs are pluripotent stem cells, as a single ESC can contribute to all germ layers of an
organism\textsuperscript{19,20}. As well, HSCs have been shown to be definitive multipotent stem cells. In
the definitive experiment\textsuperscript{63, 65}, a prospectively isolated single HSC was shown to repopulate the entire haematopoietic system of an irradiated host; and, identical daughter cells isolated from this host could in turn repopulate the haematopoietic system of a secondary irradiated recipient. Since Friedenstein and colleagues\textsuperscript{73} first isolation of cells from the mesenchymal compartment, it has yet to be definitively demonstrated that multipotential MPCs can be prospectively isolated as single cells and interrogated for their capacity to repopulate the entire mesenchymal systems of serially transplanted animals. Accordingly, it has never been shown that MPCs possess the two defining properties of stem cells: clonal self-renewal and multilineage differentiation, as the definitive experiment has been refractory to robust experimental evidence.

Accordingly, the first step in determining the existence of a putative ‘MSC’ would be to rigorously isolate clonal populations of MPCs, determine their differential multipotential capacity, and identify whether their clonally isolated daughters maintain this potential in extended culture. If this assay can be done, it will facilitate examination of the definitive assay: long-term serial repopulation in an \textit{in vivo} model.
Objectives

The above is a summary of the cumulative evidence to date for the existence of MPCs. Indeed, conventional wisdom dictates that a subpopulation of MPCs represent, in fact, stem cells that clonally self-renew and differentiate in order to maintain homeostasis of mesenchymal tissues. Yet, the inability to prospectively isolate MPCs resulting in exclusive reliance on retrospective analysis has made determination of the two stem cell properties of self-renewal and multilineage differentiation capacity refractory to robust experimental evidence. Below are five objectives that were designed to address this paucity within the body of knowledge.

1. To isolate MPCs from the perivascular region of human umbilical cords.
2. To determine the multilineage potential human umbilical cord perivascular cells (HUCPVCs) in extended culture.
3. To determine the ability of HUCPVCs to differentiate into multiple connective tissues in a long bone defect model.
4. To design a method to isolate definitive single cell derived (SCD) populations from HUCPVC suspensions in vitro.
5. To assay individual SCD HUCPVC populations for differential differentiation potential and self-renewal capacity in culture.

Hypothesis

A population of self-renewing multi-potent stem cells is responsible for repair of the mesenchymal compartment by producing self-renewing tissue-restricted progenitors.
2. CHAPTER 2: Human Umbilical Cord Perivascular Cells (HUCPVCs): A Source of Mesenchymal Progenitors*

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2.1. Introduction

Since it was first used to treat haematological malignancies in patients after radiation and chemotherapy\textsuperscript{191}, bone marrow (BM) has been one of the most common source of cells for cell-based therapies. The mesenchymal population of BM is targeted for a variety of therapeutic approaches affecting a wide range of tissues, including those of the musculoskeletal system: bone\textsuperscript{192,193}, cartilage\textsuperscript{194,195}, and tendons and ligaments\textsuperscript{196,197}. BM cell therapy has also been suggested for repair of the myocardium\textsuperscript{147,198,199} and is being pursued clinically for applications in hematology and oncology such as aplastic anemia\textsuperscript{200} and malignant lymphoma\textsuperscript{201}. Following encouraging results in NOD-scid mice, Koç \textit{et al.}\textsuperscript{202} have shown beneficial clinical outcomes by coinfusion of culture-expanded mesenchymal cells with hematopoietic stem cells in patients treated with high-dose chemotherapy for solid tumors. Other promising therapeutic approaches include MSCs as carriers of the therapeutic genes\textsuperscript{203} or the infusion of allogenic BM for the treatment of osteogenesis imperfecta (OI)\textsuperscript{204,205}. In the latter, BM was from an HLA (human leukocyte antigen)--identical or single mismatched sibling. However, since immune rejection and donor number limitations\textsuperscript{206} are major constraints to common use, there is an acute need to find alternative cell sources for such cell-based therapies. As cells are a fundamental requirement for tissue engineering\textsuperscript{207}, cell sourcing also remains a major challenge for human tissue-engineering strategies.

One potential alternative source of mesenchymal cells became feasible with the report by McElreavey \textit{et al.}\textsuperscript{171} of the culture of cells from Wharton’s jelly (WJ), the primitive connective tissue of the human umbilical cord (UC), first described by Thomas Wharton in 1656\textsuperscript{154}. Thus, Naughton \textit{et al.}\textsuperscript{172} and Purchio \textit{et al.}\textsuperscript{173} derived “prechondrocytes,” from explant cultures of UC WJ, and Mitchell \textit{et al.}\textsuperscript{208}, using a similar approach, reported that the fibroblast-like cells of WJ could be induced to differentiate into “neural-like” cells expressing neuron-specific enolase (NSE), as well as other neural cell markers. Romanov \textit{et al.}\textsuperscript{209}, using a different approach, enzymatically digested mesenchymal precursor cells from the UC vasculature endothelial surface, and Kadner \textit{et al.}\textsuperscript{210} minced either UC vessels or whole cord to derive an autologous cell source of
myofibroblasts for cardiovascular tissue engineering. Chacko and Reynolds\textsuperscript{157} described the cells residing in WJ as “smooth muscle cells,” but Takechi \textit{et al.}\textsuperscript{159} refined the description to “myofibroblasts” after \textit{in situ} labeling of vimentin, desmin, α-actin, and myosin, which has been recently confirmed by Kadner \textit{et al}\textsuperscript{211}. The human UC is embryologically derived at day 26 of gestation, and it grows to form a 30- to 50-cm-long helical organ at birth. Given this expansion, during the 40 weeks of gestation, there must be a mesenchymal precursor cell population within the UC that gives rise to the WJ connective tissue. We postulated that these cells would most likely be located closest to the vasculature, and thus to their source of oxygen and nutrients. Consequently, we reasoned that human umbilical cord perivascular cells (HUCPVCs), which were either discarded, or not specifically isolated, in the previously described studies, should contain a subpopulation that, when isolated, would be capable of exhibiting a functional mesenchymal phenotype.

Thus, we report a novel harvesting protocol designed to isolate HUCPVCs and show that the resultant cell population possesses a high frequency of colony-forming unit-fibroblast (CFU-F) cells that proliferate and differentiate rapidly to form bone nodules (BNs). Furthermore, we show that the isolated cell population includes an expanding subpopulation that expresses neither class I nor class II major histocompatibility (MHC) antigens, suggesting a potential role as a human allogeneic cell source for cell-based therapies.

2.2. Methods

2.2.1. Cell Isolation

Ethical approval was obtained from both the University of Toronto and Sunnybrook and Women’s College Health Sciences Centre, Toronto. The UCs from consenting full-term caesarian section patients were provided immediately upon delivery in a previously supplied vessel containing 80% α-MEM (Gibco) and 20% antibiotics (penicillin G at 167 units/ml; Sigma-Aldrich), gentamicin (50 µg/ml; Sigma-Aldrich), and amphotericin B (0.3 µg/ml; Sigma-Aldrich). Pieces of UC, 4–5 cm long, were dissected
by first parting the epithelium of the UC section along its length to expose the underlying WJ. Each vessel, with its surrounding WJ matrix, was pulled away, and the ends of each dissected vessel were tied together with a suture creating “loops” that were placed into a 50-ml tube (Falcon) containing a solution of 1 mg/ml collagenase (Sigma-Aldrich) with phosphate buffered saline (PBS). The remaining two vessels were dissected in a similar fashion, then looped and placed in the collagenase solution. After 18–24 hours, the loops were removed from the suspension, which was then diluted with PBS to reduce its viscosity, and centrifuged. Following the removal of the supernatant, the cells were resuspended in 10 ml PBS and counted using a hemocytometer. The suspended cells were run through an EasySep magnetic bead conjugated-CD45 depletion protocol (StemCell Technologies) to remove any hematopoietic cells, then observed by flow cytometry for expression of CD45 and cell-surface antigens (see 2.2.3.2).

2.2.2. Cell Culture

The cells were plated in T-75 cm² tissue culture polystyrene dishes (Falcon) in supplemented medium (SM) (75% α-MEM, 15% fetal bovine serum [FBS]; StemCell Technologies, Lot # S13E40), and 10% antibiotics, which was changed every 2 days. At day 7, adherent cells, judged 80%–90% confluent by phase contrast microscopy, were passaged using 0.1% trypsin solution (Gibco). They were then plated in T-75 tissue culture polystyrene flasks at 4x10³ cells/cm² in SM.

2.2.2.1. Limiting Dilution & CFU-F Assays

Dilutions of 1x10⁵, 5x10⁴, 2.5 x10⁴, 1 x10⁴, 5 x10³, and 1x10³ HUCPVCs were seeded onto six-well tissue culture plates (Falcon) and fed every 2 days with SM. The number of colonies, comprising >16 cells, were counted in each well on day 10 of culture and confirmed on day 14 by staining with crystal violet (Sigma-Aldrich). CFU-F frequency, the average number of cells required to produce one colony, was consequently determined to be 1 CFU-F per 300 HUCPVCs plated. Based on this frequency, the unit volume required to provide 300 HUCPVCs (done in triplicate from each of three cords) was calculated, and eight incremental unit volumes of HUCPVCs were seeded into individual wells on six-well plates. Again, colonies comprising >16
cells (CFU-Fs) were counted on day 10 of culture to assay CFU-F frequency with incremental seeding.

2.2.2.2. Cell Proliferation Assay

To obtain the cell-proliferation growth curve, aliquots of 4 \( \times 10^4 \) P2 HUCPVCs were plated into five six-well tissue culture polystyrene dishes. On days 1 through 5 of culture, one of the six-well plates was trypsinized, and the cells were counted. The total number of live cells was obtained at each time point by observing 0.4% Trypan blue (Sigma-Aldrich) exclusion on a hemocytometer. Mean doubling time of the HUCPVCs was calculated using the obtained cell counts from day 1 through day 5, and the procedure was repeated with cells from three separate cords. Doubling time of the HUCPVCs for passages 1–9 was determined by seeding 3\( \times 10^5 \) cells into T-75 flasks, which were fed with SM every 2 days, then trypsinized and counted using a hemocytometer (live cells were identified by Trypan blue exclusion) after 4 days. Mean doubling time was calculated from day 0 to day 4 for three separate cords.

2.2.3. HUCPVC Characterization

2.2.3.1. Immunocytochemistry

HUCPVCs were prepared for antibody staining following culture for 5 days on four-well glass chamber slides (Nunc, Rochester, NY). The cells were fixed in 3.7% formalin for 5 minutes, permeabilized by incubation with 100% methanol for 2 minutes at room temperature, and washed three times in 2% FBS/PBS. They were blocked with 10% FBS/PBS for 60 minutes, then incubated for a further 60 minutes with the following primary mouse-anti-human antibodies: \( \alpha \)-smooth muscle actin, desmin, vimentin (all Sigma and used at 1:100 dilution), neuron-specific enolase (Cymbus Biotechnology), and a 3G5 monoclonal antibody to microvascular pericytes (kind gift from Dr. A. Canfield, Manchester, U.K.). The cells were then washed three times in 2% FBS/PBS and incubated with two drops of AlexaFluor 488 goat anti-mouse immunoglobulin G (IgG) 2mg/mL secondary antibody (Molecular Probes) for 20 minutes, then washed again three times in 2% FBS/PBS. The cells were finally counterstained with nuclear Hoechst 33258 (observed as blue fluorescence). The primary antibody was omitted to
produce negative controls. The labeled samples were mounted on glass slides, and positive staining was observed as green fluorescence.

2.2.3.2. Flow Cytometry

Test cell populations of $>1\times10^5$ cells were washed in 2% FBS/PBS and suspended in 2% FBS/PBS with saturating concentrations (1:100 dilution) of the following conjugated mouse-antihuman antibodies: HLA-A,B,C-phycoerythrin (PE) (MHC I), HLA-DR,DP,DQ-fluorescein isothiocyanate (FITC) (MHC II), CD45-PE, CD34-PE, CD235a (Glycophorin A), CD90-PE (Thy-1), CD44-PE, CD106-FITC (VCAM-1), CD117-PE (c-kit), and CD123-PE (IL-3) (all BD Biosciences), and the following unconjugated antibodies: HLA-G, CD105 (SH2), CD73 (SH3), Oct3 (all BD Biosciences), and STRO-1 (hybridoma cell line secreting STRO-1 antibody was a kind gift from Dr. S. Gronthos, Adelaide, Australia) for 30 minutes at 4°C. Unconjugated primary antibodies were treated with a goat anti-mouse-FITC-conjugated secondary antibody (BD Biosciences) for 20 minutes at 40°C after washing with 2% FBS/PBS. The cell suspensions were then washed twice with 2% FBS/PBS and resuspended in 2% FBS/PBS for flow cytometric analysis (XL; Beckman Coulter) using the ExpoADCXL4 software (Beckman Coulter). Positive staining was defined as the emission of a fluorescence signal that exceeded levels obtained by $>99\%$ of cells from the control population stained with matched isotype antibodies (FITC-conjugated and PE-conjugated mouse IgG1κ monoclonal isotype standards), which was confirmed by positive fluorescence of human BM samples. For each sample, at least 10,000 list mode events were collected. All plots were generated in EXPO 32 ADC Analysis software. Serially passaged HUCPVCs ($0.5–1\times10^6$) were also assayed for expression of MHC I and MHC II cell-surface antigens by flow cytometry. Additional aliquots of $1\times10^6$ serially passaged HUCPVCs were frozen using an isopropanol freezing container (Nalgene) and stored at $-150°C$ for 1 week in a 90% FBS, 10% dimethyl sulphoxide (DMSO) solution (Sigma-Aldrich). After 1 week of cryopreservation, the HUCPVCs were thawed and analyzed ($\sim2.5\times10^5$ cells) by flow cytometry (see 2.2.3.2), by gating on the live cell population and observing them for expression of MHC I and MHC II cell-surface antigens.
2.2.3.3. **Bone Nodule Assay**

At the weekly passage, aliquots of 4x10^5 cells per cm^2 were plated on 35-mm tissue culture polystyrene dishes in osteogenic medium comprising SM with osteogenic supplements (OSs) (Dex at 10^-8M; Sigma-Aldrich), β-glycerophosphate (at 5mM; Sigma-Aldrich), and L-ascorbic acid (at 50µg/ml; Sigma-Aldrich). Control cultures were maintained in SM without OS. Cultures were re-fed every 2 days for a period of 7 days. The cultures were maintained until bone nodules (BNs) were observed (usually after 3–6 days), at which point the cultures were re-fed once with SM containing 9 µg/ml tetracycline (Sigma-Aldrich), then fixed after 24 hours in Karnovsky fixative (25% by volume 8% paraformaldehyde, 10% by volume 25% glutaraldehyde, 50% by volume 0.2M cacodylate buffer, 15% by volume distilled H2O), and prepared for analysis by light microscopy, phase contrast microscopy, fluorescence microscopy, and scanning electron microscopy (SEM). The BNs formed fluoresced under UV light, and were counted macroscopically.

2.3. **Results**

2.3.1. **HUCPVC Isolation**

Figure 2-1a shows the SEM appearance of the perivascular WJ matrix which, by routine hematoxylin and eosin light microscopy (Figure 2-1b), was seen to possess more densely packed cells in the perivascular region, while further out there appeared to be a relatively homogeneous distribution of cells. The harvested cells exhibited a morphologically homogeneous “fibroblast-like” appearance (Figure 2-1c) with a stellate shape and long cytoplasmic processes extending between 100 and 300 µm.
Figure 2-1. Scanning electron micrograph of an umbilical artery (a) that has been excised from a human umbilical cord as part of the HUCPVC harvesting procedure. H&E-stained umbilical cord cross section of the vessel embedded in Wharton’s jelly (b) that has densely packed cells in the perivascular tissue. The dotted line represents the outer margin of the vessel and thus illustrates the perivascular tissue from which the HUCPVCs are harvested. HUCPVCs display a fibroblastic morphology in culture (c) (fw = 660 µm).

2.3.2. HUCPVC Expression Analysis

2.3.2.1. Myo-Fibroblastic Phenotype

These fibroblast-like cells labeled positively for α-actin, desmin, vimentin, and the 3G5 monoclonal antibody (Figure 2-2), but we found no evidence of NSE. The α-actin displayed characteristic cytoskeletal morphology of traversing the cell, while the desmin possessed a fibrillar intra-cellular structure. The vimentin presented a strong punctate morphology, and the 3G5 was observed as diffuse membrane-bound staining.
2.3.2.2. Mesenchymal Expression Profile

Figure 2-2. HUCPVCs express α-actin (a), desmin (b), vimentin (c) and 3G5 (d) in culture. (All fw = 86 µm).

Figure 2-3. Flow cytometry results of HUCPVCs labeled for several cell-surface and intracellular markers. Data gained from a total of 11 umbilical cords in which n ≥ 3. Data refer to cells at P0 through P5. Values are means ± s.d.
All analyzed HUCPVCs labeled positively for CD105 (SH2), CD73 (SH3), CD90 (Thy-1), and CD44, but negatively for CD45, CD34, CD235a (glycophorin A), CD106 (VCAM1), CD123 (IL3), SSEA-4, HLA-DR,DP,DQ (MHC II), HLA-G, and Oct4 (Figure 2-3). HUCPVCs did not label with the hybridoma-derived STRO-1 antibody, although the latter did label a 35% subpopulation of a human BM positive control. Subpopulations of HUCPVCs labeled positively for other cell-surface proteins, including 15% CD117 (c-kit<sup>low</sup>) and 75% HLA-A,B,C (MHC I<sup>low</sup>).

![Flow cytometry results of MHC–/– expression on HUCPVCs with serial passaging, and the change of MHC–/– expression with Cryopreservation and thawing of serially passaged HUCPVCs which reached 95% at P5 (n ≥ 12). Values are means ± s.d.](image)

Figure 2-4. Flow cytometry results of MHC–/– expression on HUCPVCs with serial passaging, and the change of MHC–/– expression with Cryopreservation and thawing of serially passaged HUCPVCs which reached 95% at P5 (n ≥ 12). Values are means ± s.d.

Figure 2-4 illustrates the MHC I/II (MHC<sup>–/–</sup>) expression of serially passaged HUCPVCs and cryopreserved HUCPVCs. The input cell population contained 20.8% ± 3.1% which were MHC<sup>–/–</sup>. This subpopulation increased to 31.2% ± 1.7% at P5. Following cryopreservation, HUCPVCs demonstrated an increased MHC<sup>–/–</sup> population, rising from 65.2% ± 5.4% at P0 to 96.0% ± 3.9% at P5. Upon rapid thawing of the frozen aliquots of cells in a 37°C water bath, cell survival at P0 was 49.2% ± 23.8 (n = 12), while thawing of cells from P1 through P9 resulted in a survival of 62.6% ± 19.7 (n = 30).
2.3.3. Culture Kinetics

2.3.3.1. Proliferation

The digestion procedure yielded an average of 2–5×10⁶ HUCPVCs per UC (depending on the length of UC harvested, which can vary from 10–30 cm). Normalized to a unit length of cord, this represents a harvesting yield of 2.5–25×10⁴ cells/cm of cord and a harvesting efficiency of 100% since every cord yielded cells (n = 72). P0 through P7 HUCPVCs demonstrated a decreasing doubling time of 59.4 ± 42.4 hours (P0) to 19.71 ± 12.4 hours (P2), and this remained approximately constant until P8 (Figure 2-5a), by which time over 50 population doublings had already been achieved. The HUCPVCs demonstrated a growth curve with an initial lag phase (0–24 hours) and subsequent log phase (24–120 hours) (Figure 2-5b). Figure 2-7b shows that from day 0 to the end of the second passage (30 days of culture) the number of HUCPVCs increased from 6.6×10³ to 1.4×10⁷.

![Figure 2-5. Doubling time of HUCPVCs with successive passaging (a), demonstrating increasing proliferation to a 20-hour doubling time from P2 to P7 (n = 3). Proliferation of P2 HUCPVCs from 0–120 hours (b), illustrating a normal growth curve with a lag phase of 0–24 hours and a log phase of 24–120 hours (n = 3). Values are means ± s.d.](image)

2.3.3.2. CFU-F Frequency

Counting the number of cell colonies at passage 0 (P0) established a CFU-F frequency of 1:[300±136]. Seeding multiples of this number of cells demonstrated an increase in CFU-F frequency with increasing cell-seeding densities (Figure 2-6), indicative of some paracrine signaling between HUCPVCs, which may potentiate CFU-F formation.
2.3.3.3. CFU-O Frequency

Within this CFU-F population, frequencies of CFU-O (Figure 2-7a) were determined to be $2.6/10^5$ cells and $0.75/10^5$ cells in the absence of OSs, and $1.20/10^4$ cells and $1.29/10^4$ cells at P1 and P2, respectively, with the addition of OS. No BNs were found in P0 cultures in either osteogenic or non-osteogenic conditions. Thus, after 30 days of culture, $1.8x^6$ CFU-O cells were resident in the whole population in OS conditions (Figure 2-7b).

Passaged HUCPVCs in the presence of OS demonstrated markers of osteogenic expression within 4–5 days of culture. Colonies of cells with high alkaline phosphatase (ALP) expression that was positive for mineralization with von Kossa staining and
fluoresced when labeled with tetracycline were indicative of osteogenic differentiation. The colonies were characterized by an accumulation of fibroblast-like cells in direct contact with one another. The colonies expanded in size, to between 300 and 800 µm in diameter and approximately 100 µm in height (Figure 2-8a).

Figure 2-8. A bone nodule seen by scanning electron microscopy (a) (fw = 590 µm), and sectioned horizontally (parallel to culture dish surface) stained with Masson trichrome (b) (fw = 720 µm). Note the cells surrounded by abundant collagenous extracellular matrix (blue). A similar nodule labeled with tetracycline observed by (c) phase microscopy and (d) fluorescence microscopy (fw = 832 µm).

Figure 2-8b illustrates a demineralized Masson trichrome-stained transverse section of a BN. The areas in blue represent the collagen that makes up the bulk of the BN in which were embedded round nucleated cells, putatively identified as osteocytes. The cells bordering the nodules (Figure 2-8c) were of a fibroblastic morphology, while those toward the interior of a nodule were more polygonal. Ultraviolet fluorescence of the tetracycline-labeled nodules (Figure 2-8d) illustrated the variation of mineralization
associated with their structure. Mineralization appeared to be relatively heavy in the middle of the nodule, as seen by an intense fluorescence, while the periphery of the nodule had less fluorescence intensity.

2.4. Discussion

The method we describe depends on isolation of the UC vasculature and enzymatic digestion of its perivascular tissue to rapidly harvest a highly proliferative HUCPVC population. This is a significantly different approach from previous reports in which the vasculature and its surrounding tissue have been discarded.\(^{171,173,208}\) This distinction in the isolation procedure not only provided a cell harvest more rapidly than hitherto described but may also explain the lack of neuron specific enolase (NSE)-positive cells and the low number (15%) of c-kit\(^{low}\)-expressing cells in our cultures compared with those described by Mitchell et al.\(^{208}\) Indeed, contrary to the latter authors, we found no evidence of a neural phenotype even if we cultured the cells in neural inductive medium, consisting of low-serum media cultures with basic fibroblast growth factor, butylated hydroxyanisole, and DMSO. Recently, Kogler et al.\(^{213}\) also reported the isolation of somatic stem cells from umbilical cord blood (UCB). However, they achieved a mesenchymal cell harvest in only 94 of 233 cord bloods (40.3%), while we isolated mesenchymal cells from every cord received (100%). Furthermore, the frequency of the MSC-like cells derived from UCB was 1:200 million, while the harvesting method reported herein results in a CFU-F frequency of 1:300. Therefore, we conclude that our harvesting procedure is considerably more consistent and yields a greater number of relevant cells than can be achieved from UCB.

Although neither pre- nor post-CD45 sorted isolates of HUCPVCs demonstrated CD45 expression, we nevertheless negatively sorted for the CD45\(^{-}\) population to eliminate any possible contamination by hematopoietic precursors from UCB, and we tested the resultant population for a series of markers that are characteristic of embryonic and mesenchymal phenotypes. Thus, immunohistochemistry demonstrated the presence of three specific cytoskeletal markers—\(\alpha\)-actin, desmin, and vimentin—which correlates with the in situ characterization of WJ cells by Takechi et al.\(^{159}\),
Kobayashi et al.\textsuperscript{160}, and Kadner et al.\textsuperscript{210,211} Furthermore, due to their reactivity with the 3G5 monoclonal antibody\textsuperscript{178}, HUCPVCs appear to be similar to another perivascular mesenchymal precursor, the pericyte\textsuperscript{162-164}. In addition, flow cytometry illustrated that HUCPVCs present several cell-surface antigens commonly found on BM-derived so-called MSCs. Although no STRO-1 expression was observed, the cells were SH2, CD44, and Thy-1 positive. Thy-1 is commonly associated with cells of haematopoietic origin, but we were careful to exclude haematopoietic contamination during harvesting. Thy-1 is also known to be expressed in connective tissue and various fibroblast and stromal cell lines\textsuperscript{214}, including multi-potent adult progenitor cells (MAPCs)\textsuperscript{215}. A small subpopulation that expressed c-kit\textsuperscript{low} was also present, and this contrasts with MAPCs\textsuperscript{216}, which show no c-kit expression. As discussed above, Mitchell et al.\textsuperscript{208} demonstrated “very high” expression of c-kit on cells extracted from WJ, which also expressed NSE even in uninduced culture conditions. In contrast, our HUCPVCs exhibited spontaneous BN formation in non-osteogenic culture conditions. These differences suggest that our harvesting protocol resulted in a cell population that is distinct from those described by both Mitchell et al.\textsuperscript{208} and Kadner et al.\textsuperscript{210,211}, who showed no differentiated phenotype other than the myofibroblast markers found in WJ cord cells \textit{in situ}.

We found that the harvested HUCPVC population was highly ALP positive and, in addition to a subpopulation that can spontaneously elaborate BNs after P0, contains a subpopulation that may be induced to express an osteogenic phenotype and elaborate bone matrix in culture by the addition of Dex. Notably, CFU-O frequency in the OS+ cultures was twice that of the OS– cultures. Committed osteo-progenitors have been described as progenitor cells restricted to osteoblast development and bone formation\textsuperscript{105}. Since we are unaware of any reported pathologies associated with mineralization of the UC, we suggest that it is the culture conditions—environment and manipulation—that are causing this restricted induction of these early osteo-progenitors. However, committed BM-derived populations have also been shown to give rise to both adipogenic\textsuperscript{217} and chondrogenic\textsuperscript{218} lineages; thus, we may reasonably expect, through further culture manipulation, to derive these, and other, mesenchymal phenotypes. The frequency of 1 CFU-F per 300 HUCPVCs, shown by the limiting dilution assay, is
significantly higher than that observed in neonatal BM, which has been shown to possess approximately 1 MSC per 10,000 BM stromal cells\textsuperscript{104}. Our results show that these CFU-F–derived HUCPVCs proliferate rapidly in culture, demonstrating a changing doubling time during the first 30 days of culture of approximately 60, 30, and 20 hours for P0, P1, and P2, respectively (average 33.5 hours). In contrast, a 36-hour doubling time has been reported in ongoing cultures of human embryonic stem cells\textsuperscript{219}, and a longer, 60-hour, average doubling time can be calculated for the first 30 days of adult BM culture (from the 21- to 36-day data reported by Suva \textit{et al.}\textsuperscript{220}). The latter, 4-day doubling, corresponds to the report of Bruder \textit{et al.}\textsuperscript{97}, who showed that, on average, MSCs achieved two population doublings for each 9-day culture from passages 1 through 10.

Thus, HUCPVCs represent a population of cells that can be rapidly expanded for potential clinical applications. The rapid doubling time of HUCPVCs raises the question of whether a therapeutic mesenchymal cell dose could be achieved more rapidly than from currently employed marrow sources. With an average infusion of 4.3x10\textsuperscript{9} nucleated cells, Horwitz \textit{et al.}\textsuperscript{204} injected 1.7x10\textsuperscript{5} MSCs (based on 1 MSC : 2.5 x10\textsuperscript{4} mononuclear BM cells\textsuperscript{192,221}) that successfully ameliorated the condition of three patients with OI. As a result, if approximately 2 x10\textsuperscript{5} MSCs are required for a therapeutic dose (TD), Figure 2-7b illustrates that a single such dose can be derived from HUCPVCs within 10 days of harvest—and 1,000 TDs after 24 days of culture expansion. This compares favorably with the expansion of MAPCs that, given the data of Reyes \textit{et al.}\textsuperscript{215}, require 14 days to establish a culture containing approximately 10\textsuperscript{4} cells with a doubling time of 48 hours, which would result in a single TD within 22 days— and 1,000 TDs after 42 days of culture expansion. Furthermore, our data show an increase, with both passage and, particularly, freeze-thawing, of a HUCPVC population that expresses neither class I nor class II MHC antigens (MHC\textsuperscript{−/−}). Although the majority of cells at P0 were MHC I positive, the MHC\textsuperscript{−/−} phenotype increased modestly from 20%–30% during the first five passages. Specifically, in the 15%–30% of the HUCPVCs that survived vitrification, the MHC\textsuperscript{−/−} phenotype increased considerably to 65% at P0, 90% at P3, and 95% at P5. While these percentages of potentially allogeneic cells are unattainable in adhesion-dependent BM-derived cells, which retain
class I expression, they also exceed those recently published for BM-derived cells expanded in non-contact suspension conditions. Since Horwitz et al. have shown that systemically infused marrow-derived mesenchymal populations have a clear clinical potential, our selective and rapid proliferation of MHC–/– HUCPVCs is of particular clinical relevance. Although some authors have found little evidence of an immunogenic response using allogeneic, and indeed xenogenic, MSC therapy, it has been noted that histocompatibility of the cell source is a significant hurdle to be addressed for safe and effective application of cell-based therapies. In this context, an MHC–/– cell population as described herein may represent a promising avenue to surmount the potential hazards of alloreactive T cells or a host immune response leading to graft versus host disease.

**Conclusion**

Although the *in vivo* function of HUCPVCs still needs to be studied, we believe these cells represent a population of normal, rapidly expandable, MHC–/– cells that can potentially generate multiple therapeutic doses of cells for cell-based therapies, and thus they represent a significant alternative to BM in the treatment of pathologies associated with the connective tissues of the human body.
3. CHAPTER 3: HUCPVCs Exhibit Multi-Potential Capacity
   In Vitro & In Vivo
3.1. Introduction

Tissue resident progenitor cells that give rise to connective tissue have been isolated from a variety of sources including bone marrow\textsuperscript{77,78,82,226}, fat\textsuperscript{40,41}, muscle\textsuperscript{42,43}, placenta\textsuperscript{45}, umbilical cord\textsuperscript{46-48} and fetal liver\textsuperscript{140}; are generally thought to be resident in the perivascular compartment in these tissues\textsuperscript{162,168,170,188}, and are commonly called “mesenchymal stem cells” (MSCs)\textsuperscript{103} or mesenchymal progenitor cells (MPCs). While no definitive marker for these cells exists, most investigators have used the presence of CD105 (SH2)\textsuperscript{107}, CD73 (SH3)\textsuperscript{108}, and STRO-1\textsuperscript{111} combined with the absence of haematopoietic CD34, CD45 and CD235a (GlyA) markers to determine the mesenchymal phenotype\textsuperscript{120} of these cells. However, most attempts at analyzing the differentiation potential of MPCs have relied on a few simple assays that determine the ability of these colony forming-fibroblast (CFU-F)\textsuperscript{78} cells to form bone, cartilage and adipose tissue \textit{in vitro}.

In determining the osteogenic potential\textsuperscript{227} of MPCs, \textit{in vitro} induction with Dex\textsuperscript{228,229} results in the formation of mineralized bone nodules that are normally analyzed by alkaline phosphatase and Von Kossa (AgNO\textsubscript{3} labels the mineralized collagen) staining. Similar culture conditions with a PPAR-\(\gamma\)\textsuperscript{95,230,231} inducer results in the formation of adipocytes that stain with oil red O; while three-dimensional pellet cultures treated with TGF-\(\beta\)\textsuperscript{218} produce cartilage-like matrix that labels positively for collagen II and proteoglycans. In addition to analysis of the cell-surface expression profile described above, many investigators\textsuperscript{47,114,176,177,188,220,232} use these differentiation assays to classify mesenchymal tissue-derived cells as MPCs. More recently, the myogenic potential\textsuperscript{233-235} of MPCs have been demonstrated, although to date not in combination with bone, cartilage and adipose potential. Furthermore, very few investigators have analyzed up-regulation of lineage specific genes\textsuperscript{43,236,237}, and even fewer have determined MPC multi-lineage capacity \textit{in vivo}\textsuperscript{79,170}.

Here we show for the first time that a previously described mesenchymal progenitor population, human umbilical cord perivascular cells (HUCPVCs), maintains a stable progenitor pool in culture with low serum dependence; and that these cells are capable of differentiation into all five mesenchymal lineages \textit{in vitro}: muscle, adipose,
cartilage, bone and stroma (fibroblastic). We have further shown that these cells can survive for at least 6 weeks when transplanted in vivo where they not only contribute to repair of multiple damaged mesenchymal tissues, but play a larger role in recruiting and coordinating repair by the autologous cells.

3.2. Methods

3.2.1. Cell Culture

Cells isolated from the perivascular region of the UC were isolated as previously described (see 2.2.1).

3.2.1.1. Primary culture

Briefly, the UC was cut into 4 cm sections and washed in phosphate buffered saline (PBS -Mg⁺, -Ca²⁺). The epithelium was stripped away with forceps, exposing the underlying matrix. The vessels were then pulled out with their surrounding extra cellular matrix intact. The ends of the vessels were tied off with a surgical suture, and placed into a 0.5mg/ml collagenase I (Sigma-Aldrich) digest on a rotisserie in a dry 37°C incubator for 18-24 hours. The HUCPVC suspension was then washed with PBS and seeded into a 75cm² tissue culture flask (T-75) with supplemented medium (SM) comprising 5% (5% SM) or 2% (2% SM) fetal bovine serum (FBS) (HyClone, Lot KPF21344), 85% α-MEM, and 10% antibiotics (fungizone, penicillin & streptomycin), and incubated at 37°C with 5% humidified CO₂. After 24 h, non-adherent cells were removed and placed into a new culture dish (see 3.2.1.3). The adherent cells were washed twice with (PBS) (-Mg⁺, Ca²⁺) and fresh SM was added. The medium was replaced every 2 days and the cells were sub-cultured at 70-90% confluence.

3.2.1.2. Sub-culture and cell proliferation

Upon reaching 70-90% confluence as estimated by phase microscopy, the cells were washed with PBS and removed from the culture dish surface by 5 ml 0.02% trypsin and 200nM EDTA (Gibco) for 5 min at 37°C. The total number of live cells was ascertained with the use of a ViCell-XR automated cell counter (Beckman Coulter). To
expand the cells through successive passages, they were plated at 4,000 cells/cm², allowed to reach 70-90% confluence and sub-cultured as described above.

### 3.2.1.3. Later Adhering HUCPVCs

We discovered that the non-adherent cells after 24 hours of HUCPVC primary culture could be placed into a new culture dish, i.e. a T-75 in which they would attach and proliferate. These cells could be expanded and sub-cultured (as described in 3.2.1.2), and we further determined their proliferation rate and CFU-F frequency (as described in 3.2.2).

### 3.2.1.4. Cryopreservation of cells

Residual cells from sub-culture (0.5 to 5 x 10⁶) were cryopreserved using 80% FBS and 20% dimethyl sulphoxide (DMSO) (Sigma-Aldrich), and transferred into 1.5 ml polypropylene cryo-vials. The vials were placed into a -70°C freezer overnight, and transferred the following day to a liquid N₂ (-196°C) freezer for long-term storage.

### 3.2.2. HUCPVC Culture Kinetics

#### 3.2.2.1. Cell Proliferation Assay

Doubling time of the HUCPVCs for passages 1–12 was determined by seeding 3x10⁵ cells into T-75 flasks (Falcon), which were fed with SM (containing either 2% SM or 5% SM) every 2 days then trypsinized after 4 days. The total number of live cells was ascertained with the use of a ViCell-XR automated cell counter (Beckman Coulter). Mean doubling time of the cell population was calculated from day 0 to day 4 using the formula N=N₀eᵏᵗ from 9 different cord samples.

#### 3.2.2.2. Limiting Dilution & CFU-F Assays

HUCPVCs expanded in culture with 2% SM to 70-90% confluence were harvested with trypsin-EDTA and counted cells were diluted in SM and plated at 1, 5, 10, 50, 100, and 500 cells per well of a 6-well culture dish. After incubation for 5-7 days at 37°C in 5% humidified CO₂, the cells were washed with PBS and stained with 0.5% Crystal Violet (Sigma-Aldrich) for 15 min at room temperature. Cells were washed with
PBS twice, and visible colonies were counted from 7 different samples. The minimum number of cells required to produce a single colony determined the CFU-F frequency. This was repeated at every sub-culture until passage 12 (P12).

### 3.2.3. **Induction of *In vitro* Differentiation**

HUCPVCs were assayed at P3 (~10 doublings) and P6 (~15 doublings) for differentiation capacity into the five mesenchymal lineages. Results were identical for both P3 and P6 cells.

#### 3.2.3.1. **Cartilage**

2.5x10⁵ cells were placed into a 15ml tube and centrifuged at 1150rpm for 5 minutes to form a cell pellet. The media was removed and replaced with 0.5ml chondrogenic supplemented (CS) media comprising 90% α-MEM, 10% antibiotics, and 10ng/ml transforming growth factor-β1 (TGF-β1) (Chemicon). The CS medium was replaced every 4 days for 21 days. The chondrogenic pellets were then removed from the CS medium and cut in half with a scalpel blade. One half was fixed in 3.7% formalin for Alcian blue staining and collagen II immunostaining with mouse-anti-human collagen II (Chemicon), while RNA from the remaining half was harvested with 0.5ml Trizol reagent for RT-PCR analysis (as described in 3.2.3.5).

#### 3.2.3.2. **Bone**

2x10⁴ HUCPVCs were seeded in 6 well plates with 2% SM, and upon reaching ~60% confluence, the media was replaced with osteogenic supplemented (OS) media comprising 2% FBS, 88% α-MEM, and 10% antibiotics, supplemented with 10nM Dex (Sigma-Aldrich), 5mM β-glycerophosphate (Sigma-Aldrich), and 50µg/ml L-ascorbic acid (Sigma-Aldrich). The OS medium was replaced every 2 days until bone nodules were observed (usually after 7-14 days), at which point the cultures were treated with SM containing 9µg/ml tetracycline (Sigma-Aldrich), and visualized 24 hours later for fluorescent foci of mineralization. One well was then fixed in 3.7% formalin for alkaline phosphatase and Von Kossa staining along with its un-induced negative control, while another was harvested with 0.5ml Trizol reagent for RT-PCR analysis (as described in
3.2.3.5).  

3.2.3.3. Adipose

2x10^4 HUCPVCs were seeded in 6 well plates with 2% SM, and upon reaching ~60% confluence, the media was replaced with adipogenic supplemented (AS) media comprising 87% α-MEM, 10% antibiotics, 3% FBS, 33µM biotin (Sigma-Aldrich), 17µM Pantothenate (Sigma-Aldrich), 5µM Rosiglitazone (Cayman Chemical), 100nM bovine insulin (Sigma-Aldrich), 1µM dexamethosone (Dex) (Sigma-Aldrich), and 200µM isobutyl methylxanthine (Sigma-Aldrich). The AS medium was replaced every 2 days until colonies of adipocytes could be observed. One well was stained with Oil Red O (Sigma-Aldrich) along with its un-induced negative control, while another was harvested with 0.5ml Trizol reagent for RT-PCR analysis (as described in 3.2.3.5).

3.2.3.4. Muscle

2x10^4 HUCPVCs were seeded in 6 well plates with 2% SM, and upon reaching ~95% confluence, the media was replaced with myogenic supplemented media comprising 88% α-MEM, 10% antibiotics, 2% horse serum (Gibco, Lot# 480116), 1 nM Dex (Sigma-Aldrich), and 2 µM hydrocortisone (Sigma-Aldrich). The culture medium was replaced every 2 days until multinucleated myotubes could be observed (28 days). One well was harvested with 0.5ml Trizol reagent for RT-PCR analysis (as described in 3.2.3.5), while another was fixed in 3.7% formalin for immunostaining staining along with its uninduced negative control. For immuno-histochemistry, fixed cells were first permeabilized with 100% methanol for 2 minutes, and blocked with 10% FBS/PBS for 1 hour. The cells were then incubated with 1µl each of rabbit-anti-human MyoD primary antibody (Santa Cruz biotech) and mouse-anti-human fast skeletal myosin light chain primary antibody (Sigma-Aldrich) overnight, then washed thoroughly. Goat-anti-rabbit Alexa Fluor 488 secondary antibody (Molecular Probes) and goat-anti-mouse Alexa Fluor 555 secondary antibody (Molecular Probes) were then incubated with the cells for 1 hour and washed thoroughly. As a negative control, the primary antibody was excluded. A Leica DMIIRE2 inverted fluorescent microscope (Wetzlar, Germany) was used to visualize fluorescence at 519nm (green) and 565nm (red) wavelengths.
3.2.3.5. **RNA Isolation and Reverse Transcription (RT)**

Total RNA was isolated from cell cultures by treatment with 0.5ml Trizol reagent (Invitrogen). Four micrograms of total RNA was used in each RT reaction with specific primer pairs (Figure 3-1) used to amplify first strand cDNA. The amplified PCR product was fractionated on a 1.2% agarose gel and visualized by ethidium bromide staining.

<table>
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<th>F/R</th>
<th>Sequence (5'-3')</th>
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**Figure 3-1.** Primer sequences used for RT-PCR. Abbreviations are: lipoprotein lipase (LPL), fast skeletal myosin light chain (FSMLC) and myosin heavy chain (MHC).
3.2.4. Intra-Femoral Injection Model

3.2.4.1. Lentiviral transduction of HUCPVCs with eGFP

HUCPVCs were permanently labeled with eGFP by lentiviral transduction (see Appendix A for full protocol). Briefly, 2.5M CaCl$_2$ was used to precipitate the following plasmids: 15µg of GFP vector DNA, 10µg of GagPol expressing plasmid, 10µg of Rev expressing plasmid, 10µg of Tat expressing plasmid and 5µg of Vsv-G expressing plasmid (a kind gift from Dr. J. Ellis, MaRS, University of Toronto, Canada) in HeBS buffer, and used to transfect 293T cells. After 3 days of viral production on this cell line, the viral supernatant was filtered, collected and ultracentrifuged at 25,000rpm to a final volume of 1ml. The virus was then titred on both 293T cells and HUCPVCs such that a 1:10 dilution transduced HUCPVCs with 70% eGFP expression efficiency as measured by flow cytometry.

3.2.4.2. Mice

Ethical consent for intra-femoral transplantation of human mesenchymal cells into NOD-scid mice was obtained from the Ontario Cancer Institute (OCI) research ethics board. 36 NOD-scid mice were obtained from Jackson Laboratories, and were housed under sterile conditions at the OCI animal facility at Princess Margaret Hospital, Toronto, Canada.

3.2.4.3. Intra-Femoral Transplantation

0.5 x 10$^6$ eGFP-labeled HUCPVCs were injected in 20µl of α-MEM into the intrafemoral space of 26 NOD-scid mice with an additional 10 mice receiving 20µl of α - MEM (sham) (with kind help from Ms Monica Doedens in Dr J.E. Dick’s lab, MaRS, University of Toronto, Canada) using a previously published method$^{238}$. Briefly, after anesthetizing the mouse, the knee was flexed and 20µl of cells were injected with a 28.5-gauge needle through the joint into the right femur. The mice were provided with an analgesic in their drinking water for 7 days. 5 mice died during transplantation due to travel fatigue combined with the trauma of surgery. After 2, 4 and 6 weeks, the mice were sacrificed by cervical dislocation, and both femurs were excised from 7 HUCPVC-
transplanted and 3-sham transplanted mice at each time point. The contralateral femur was used as an un-transplanted control.

3.2.4.4. Bone Marrow Flush

At each time point the marrow from 3 HUCPVC-transplanted mice was flushed into 2% SM which encouraged adherence and expansion of human cells but limited adherence and proliferation of mouse cells. Briefly, after washing 3 times in a 10x antibiotic solution, the femoral head and knee were aseptically removed. A 25-guage needle was then used to flush the marrow with SM into a 6-well culture dish. After 24 hours the cultures were observed for the presence of eGFP labeled cells among the mouse mesenchymal cells.

3.2.4.5. Micro Computed Tomography Analysis

The femurs from the remaining 4 cell-injected and 3 sham-injected mice were fixed for 24 hours in 10% neutral buffered formalin. The fixed femurs were analyzed by micro computed tomography (micro CT) (General Electric Health Care). One-way blind analysis was achieved, as the operator was unaware of each sample's identity. A pixel threshold was determined by the higher pixel intensity of outside the femur or the marrow space, and software (GE MicroView) was then used to determine all pixel intensities above the threshold as bone mineral density (BMD).

3.2.4.6. Histology & Immunohistochemistry

The fixed femurs were decalcified in 20% formic acid and embedded in paraffin for sectioning into 4µm thin sections. Longitudinal sections were obtained through the entire width of each femur. From every 15 sections, 6 serial sections were labeled with Masson’s Trichrome, anti-GFP (1:100, Sigma-Aldrich), biotin-conjugated (Lightning-Link Biotin, Innova Biosciences) mouse-anti-human nuclear-antigen (HuNu) (1:300, Chemicon) anti-human-osteocalcin (1:100, Peninsula), anti-human collagen II (1:100, Chemicon), and anti-human PPARγ (1:200, Abcam) respectively, and observed by light microscopy.
3.3. Results

3.3.1. HUCPVC and Late Adherent Cell (LAC) Culture Kinetics

In culture with 5% FBS, from P2-P10 there was no significant difference between HUCPVC and LAC proliferation (Figure 3-2), as the populations doubled every 41±7 and 42±6 hours respectively. Similarly, there was no significant difference between the CFU-F frequencies in culture displaying 1:[2.6±0.5] and 1:[2.2±0.6] respectively. At low serum concentrations (5% or 2%), the progenitor pool is not affected by serum concentration (Figure 3-3), as P5 HUCPVC doubling time (41±7 and 46±9 hours respectively) and clonogenic frequency of >1:3 (1:[2.6±1.4] and 1:[2.8±1.6] respectively) were maintained.

Figure 3-2. Proliferation and CFU-F frequency of HUCPVCs (a) and LACs (b) are similar and remain stable from P2 to P10 (n=8). Values are means ± s.d.

Figure 3-3. 5% nor 2% serum concentration significantly effects frequency (a) or proliferation (b)
of HUCPVCs in culture (n=7). Values are means ± s.d.

3.3.2.  In vitro Multilineage Differentiation

3.3.2.1.  Cartilage

Under serum-free induction with TGF-β1, three-dimensional pellet cultures of HUCPVCs produced collagen II and proteoglycans (Figure 3-4), characteristic of in vitro cartilage. These pellets did not possess the organized structure of hyaline cartilage, but rather a disorganized distribution of chondrocytes within the cartilage-like extracellular matrix.

![Figure 3-4. HUCPVCs produced cartilage under inducing conditions that labeled for collagen II (negative control inset) (a, fw = 1678µm), and glycosaminoglycans as evidenced by Alcian blue staining (b, fw = 315µm).](image)

3.3.2.2.  Bone

Under inducing conditions, HUCPVCs differentiated into osteoblasts that produced mineralized bone matrix in vitro (Figure 3-5). These bone nodules fluoresced when labeled with tetracycline and displayed the hallmarks of osteogenic differentiation\(^{212,239}\), including formation of the cement line and mineralization of the osteoid.
3.3.2.3. Adipose

Figure 3-6. Under inducing conditions HUCPVCs differentiated into adipocytes that stained with oil red O (a), which sometimes formed spontaneously in culture (b). (All fw = 52 µm).

HUCPVCs formed adipocytes (Figure 3-6) that stained with oil red O when
cultured under adipogenic conditions. Occasionally, adipocytes formed spontaneously in culture, usually in close proximity to bone nodules.

### 3.3.2.4. Muscle

![Figure 3-7](image)

Figure 3-7. Muscle-induced HUCPVCs expressed nuclear-localized MyoD (a) and fast skeletal myosin light chain (b) in multi-nucleated myotubes. (Negative controls are inset, all fw = 315µm).

When cultured for 28 days under myogenic induction conditions, HUCPVCs readily formed multinucleated myotubes (Figure 3-7) that expressed nuclear-localized MyoD and cytoplasmic fast skeletal myosin light chain.

### 3.3.2.5. Upregulation of lineage-specific genes

![Figure 3-8](image)

Figure 3-8. RT-PCR analysis of un-induced (a) and induced (b) HUCPVCs demonstrated up-regulation of the following lineage specific genes: bone: Runx2 (1), collagen IA1 (2), osteopontin (3), osteocalcin (4); cartilage: Sox9 (5), collagen II (6), aggrecan (7); adipose: LPL (8); and muscle: MyoD (9), Myf5 (10), desmin (11), MHC (12), FSMLC (13). (RT+ control is GAPDH (14) and RT-control (15)).

To confirm that the morphological changes observed were due to lineage-specific differentiation, we analyzed the differentiated cells by RT-PCR. Collagen I, MyoD and desmin were present in un-induced cultures, while the remainder of lineage-specific genes analyzed demonstrated up-regulation upon differentiation (Figure 3-8).
3.3.3.  *In vivo* Potential of HUCPVCs

3.3.3.1.  HUCPVC Enlodgement

Figure 3-9. HUCPVCs survived at least 6 weeks *in vivo* as evidenced by larger GFP-expressing cells (a) among small rounded mouse mesenchymal cells (b) from flushed transplanted-mouse marrow. (All fw = 86µm).

Figure 3-10. HUCPVCs displayed nuclear-specific staining with HuNu (human nuclear-specific antigen) in transplanted mouse femurs (a-d). (d is magnification from a with sham control inset). (a,b,c fw = 140µm, d fw ~ 36µm).
When the BM of transplanted femurs was flushed into culture, larger GFP cells were evident among the smaller mouse cells (Figure 3-9) from all time points. We further determined that these cells were present in the femoral growth plate by positive staining with HuNu (Figure 3-10) that was not seen in the sham controls. Together, these observations confirm that HUCPVCs survive at least 6 weeks in vivo.

3.3.3.2. Cartilage

Figure 3-11. HUCPVC-transplanted mouse femurs displayed human-specific collagen II (a-d) in the femoral growth plate at 2 (a), 4 (b) and 6 weeks (c), not seen in the sham control (inset in d which is a magnification from b). (a,b,c fw = 140µm, d fw ~ 70µm).

Human-specific collagen II was observed in the growth plate of HUCPVC-transplanted mice (Figure 3-11), but not the sham-transplanted mice. Interestingly, the majority of the cartilage matrix was found distal to the human collagen II, illustrating that the more metabolically active mouse mesenchymal cells regenerated the majority of the tissue.
3.3.3.3. Bone

Figure 3-12. HUCPVC-transplanted mouse femurs displayed human-specific osteocalcin (a-d) on the surface of newly forming bone at 2 (a), 4 (b) and 6 weeks (c), not seen in the sham control (inset in d which is a magnification from a). (a,b,c fw = 140µm, d fw ~ 70µm).

Human-specific osteocalcin was observed on the surface of newly forming bone in transplanted femurs (Figure 3-12). Most specifically, it could be seen lining the osteoid, as the collagen became mineralized. While some background staining was present in the sham controls, the difference between HUCPVC-transplanted and sham-transplanted sections was clear. This difference was further evident upon micro CT analysis of the femurs (Figure 3-13). The distal end of the HUCPVC-transplanted femurs had significantly more bone mineral density (BMD) than sham-transplanted femurs at all time points. These differences were not as great when whole femurs were analyzed, suggesting that the majority of mesenchymal tissue repair occurred in the distal femur, the site of most significant injury.
Figure 3-13. One-way blind micro CT analysis of HUCPVC-transplanted and sham-transplanted femurs shows that there was significantly more bone mineral density (BMD) in the distal end of the HUCPVC-transplanted femurs at all time points (a), whereas this difference was only significant at 2 and 4 weeks when whole femurs were compared (b). n=4 for HUCPVCs and n=3 for sham at each time point. Values are means ± s.d and considered significant using a paired t-test with P<0.05.

### 3.3.3.4. HUCPVCs Repair Damaged Mesenchymal Tissue

Figure 3-14. Micro CT analysis (a,c) and Masson’s trichrome staining (b,d,e-h) of longitudinal sections of mouse femurs illustrate that HUCPVCs caused significantly more repair to injured bone and cartilage (a,b) compared to sham controls (c,d) at 2 weeks, although there was no observable difference at 4 and 6 weeks. (All fw = 1820 µm).

Having determined that the majority of repair occurred in the distal end of the femur, we analyzed longitudinal cross-sections of HUCPVC and sham-transplanted femurs. At 2 weeks, it was grossly evident that the sham-injected femurs still had significant damage to the hyaline cartilage, growth plate and trabecular bone, while the
HUCPVC-injected femurs appeared to be completely healed. These femurs displayed normal presence of cartilage and trabecular bone. This gross morphological difference was not seen at 4 and 6 weeks, suggesting complete autologous repair in the sham control femurs. These data, combined with the data illustrating human bone and cartilage-producing cells \textit{in vivo}, suggest that HUCPVCs have a greater role in coordinating mesenchymal tissue regeneration and produce small amounts of human tissue only as a secondary response.

3.4. Discussion

We have shown here that HUCPVC cultures perpetuate a stable progenitor pool in continued culture, and that these cells continue to maintain multi-lineage capacity both \textit{in vitro} and \textit{in vivo}.

We previously showed that HUCPVC populations exhibit a doubling time of 20 hours when cultured in 15% fetal bovine serum (FBS) (Figure 2-5a)\textsuperscript{46}. When 5% or 2% serum concentrations are employed the doubling time (41±7 and 46±9 hours respectively) increases (but is still stable over many passages) while the clonogenic frequency of >1:3 (1:2.6±1.4] and 1:2.8±1.6] respectively) is maintained (Figure 3-2) indicating that maintenance of doubling and frequency are both independent of low serum concentration. Thus, it follows that as the clonogenic frequency increases and stabilizes, so does the proliferation rate, suggesting that a significant progenitor population persists in continued culture. This is an important finding because previous reliance on unknown exogenous factors, such as those in FBS, for culture and differentiation of MSCs has been a confounding factor. Investigators have used autologous, allogeneic, and xenogeneic serum in an attempt to reduce this variability observed between BM derived MSC samples\textsuperscript{240}. They have also tried to reduce serum-dependence by culturing these cells with a variety of growth factors\textsuperscript{112,226}. To date, none of these strategies have provided consistent results. Nevertheless, we show here that under low serum conditions, the proliferation kinetics of HUCPVCs maintain a stable state and thus we have been able to test for the lineage differentiation capacity of these progenitors within the overall mesenchymal cell population.
Having established stable HUCPVC cultures in low serum conditions, utilizing serum concentrations ranging from 2-3%, HUCPVCs were assayed at passage 3 (P3) (~10 doublings) and P6 (~15 doublings) for differentiation capacity into the five mesenchymal lineages: myogenic (M), adipogenic (A), chondrogenic (C), osteogenic (O) and fibroblastic (F). Under osteogenic culture conditions, the cells changed from a fibroblastic to a more tessellated osteoblast-like morphology, and formed bone nodules by elaborating mineralized bone matrix as evidenced by the presence of positive tetracycline staining, alkaline phosphatase and positive Von Kossa staining. These bone nodules laid down characteristic proteinaceous cement line on which mineralizing collagen was deposited. Osteogenic lineage differentiation was confirmed by analysis of Runx2, collagen IA1, osteopontin and osteocalcin up-regulation. Under chondrogenic culture conditions, up-regulation of Sox9, collagen II and aggrecan was observed. The cells elaborated collagen II matrix and sulphated glycosaminoglycans, verified by immuno-histochemistry and Alcian blue staining respectively. HUCPVCs could also be induced to differentiate into lipoprotein lipase-expressing adipocytes in culture that stained with oil red O, occasionally forming spontaneously in association with bone nodules. Furthermore, as un-induced HUCPVCs express myogenic markers including desmin, vimentin and α-smooth muscle actin, myogenically-induced cells were analyzed for differential expression of MyoD and fast skeletal myosin light chain (FSMLC). Uninduced cells expressed low levels of MyoD, but no FSMLC, while induced cells formed long multinucleated myotube-like structures that expressed high levels of MyoD and FSMLC. This was confirmed by RT-PCR analysis, in which up-regulation of MyoD, Myf5, desmin, myosin heavy chain (MHC), and FSMLC were observed. Together, these data confirm that HUCPVC populations contain progenitors that can be induced to differentiate into each of the five mesenchymal lineages.

We found that the LACs were able to differentiate into all the same lineages and did not have significantly different culture kinetics from the HUCPVCs. These data, suggest that they were a similar, if not identical cell population. Interestingly, their occurrence was not a function of cell seeding density, as they would still be produced even after the initial HUCPVCs had been seeded at very low densities. We suggest that these cells up-regulated adhesion proteins only in later culture, possibly due to
paracrine inhibitory factors produced by the adherent cells. This remains to be tested.

An important criticism of MSC multi-lineage differentiation assays is their reliance on retrospective in vitro analysis; namely oil red O or Sudan Black for adipose differentiation, Alcian Blue or Toluidene Blue for chondrogenic differentiation, and Alizarin Red, Von Kossa or alkaline phosphatase staining for osteogenesis. Most studies have not augmented histochemical stains of differentiation assays with either RT-PCR or antibodies to assay up-regulation of lineage specific genes or proteins. Regardless, many well respected investigators in the MSC field argue that in vitro analysis is inadequate for determining multilineage potential, rather, lineage specific synthetic activity must be demonstrated in vivo. Accordingly, subsequent to demonstration that HUCPVCs up-regulate lineage specific genes and become synthetically active upon directed differentiation in vitro, we assayed their ability to enlodge and differentiate in an in vivo mesenchymal injury model. HUCPVCs were constitutively labeled with eGFP by lentiviral transduction and injected into the intrafemoral space of NOD-scid mice, a model that has been used to assay haematopoietic stem cell differentiation. After 2 weeks, there was significantly more healing of bone and cartilage in the HUCPVC-injected femurs than contralateral sham-injected controls (Figure 3-14). By flushing the marrow and recovering culture-adherent GFP-labeled cells, we showed that HUCPVCs survived for at least 6 weeks in vivo. By 4 weeks, complete repair was achieved in both the experimental and control femurs; although, there was significantly more bone mineral density (BMD) at 2 and 4 weeks when whole HUCPVC injected femurs were compared to controls (Figure 3-13). When the distal region of each femur was compared, this difference was larger and observed at all three time points, demonstrating that the majority of repair occurred in the knee, the site of greatest injury. In order to determine whether these cells contributed to healing by becoming synthetically active, we labeled them with monoclonal antibodies for human-specific osteocalcin, collagen II and PPARγ. As the GFP fluorescence had been eliminated by decalcification of the tissues during histology preparation, we labeled the cells with HuNu, an antibody that specifically labels human nuclei and not those of mice. While the PPARγ labeled the femurs non-specifically (data not shown), HuNu labeled cells in the femoral growth plate that were co-localized, in serial sections,
with human-specific collagen II staining. We further observed osteocalcin present in the cytoplasm of cells as well as on the contributed osteoid being produced on the surface of newly forming bone. Interestingly, the majority of cells did not label with the anti-human antibodies, suggesting that although the human cells did produce some tissue, they possibly had a greater role in recruiting and directing the mouse mesenchymal progenitors to repair the damaged tissue. This is strikingly evident in the cartilage repair, as the mouse cells rapidly produced new collagen II distal to the more slowly produced human collagen II (Figure 3-11). Together, these data demonstrate that HUCPVCs possess multilineage potential in vitro and an ability to survive and contribute to healing in vivo.

Conclusion

Having established stable low serum-dependent culture conditions that provide a clonogenic frequency of >1:3, and determined that bulk HUCPVC populations can differentiate into multiple mesenchymal lineages both in vitro and in vivo, we have demonstrated, for the first time, that a population of perivascular MPCs has true multilineage capacity. This finding provides us with a unique opportunity to further dissect MPC biology by isolating single-cell-derived populations, and assaying their individual differentiation capacity into the five mesenchymal lineages.
CHAPTER 4: Human Mesenchymal Stem Cells Self-Renew & Differentiate According to a Deterministic Hierarchy
4.1. Introduction

Mesenchymal cells have been shown to differentiate into five mesenchymal lineages in vitro: muscle, adipose, cartilage, bone and fibroblasts (see Chapter 3). While conventional wisdom dictates that clonally self-renewing mesenchymal stem cells (MSCs) do exist\textsuperscript{114,242} it has yet to be definitively demonstrated whether multipotential mesenchymal cell cultures are the result of a group of oligopotential progenitors or putative MSCs. In order to determine the clonal self-renewal and multilineage differentiation potential of MSCs, two approaches have been employed to attempt to assay single bone marrow (BM)-derived MSCs for clonal tri-lineage differentiation capacity. To date, since the progenitor frequency in human BM is low (1:10\textsuperscript{4} to 1:10\textsuperscript{6}), researchers have relied on limiting dilution\textsuperscript{106,243} and significant culture expansion before plating of 10\textsuperscript{3}-10\textsuperscript{6} cells to generate individual clones. As this method assumes single-cell-derivation of CFU-Fs, these studies could not provide definitive evidence that “clones” were single cell derived. While a study\textsuperscript{244} using single cell seeding by fluorescence activated cell sorting provided evidence of clonality, it failed to concurrently assay each clone for more than one phenotype. As well, neither \textit{in vivo} function nor self-renewal has ever been assayed in MSC “clones,” thus, demonstration of a definitive MSC remains.

The definition of a stem cell requires that mesenchymal progenitors have clonal self-renewal and multi-lineage differentiation potential to be termed \textit{stem cells}. As these properties have yet to be shown from currently established mesenchymal cell sources, the term “mesenchymal stem cell” has been criticised\textsuperscript{119}. To test for this most basic definition of a stem cell, we have used a tractable mesenchymal progenitor cell population, human umbilical cord perivascular cells (HUCPVCs) (CD45-, CD34-, CD105+, CD73+, CD90+, CD44+, CD106+)\textsuperscript{46,178}, that is capable of multilineage differentiation \textit{in vitro} and, more importantly, \textit{in vivo}, displaying the ability to differentiate into functionally synthetic cells that direct and contribute to rapid connective tissue healing by producing bone, cartilage and fibrous stroma in a mouse injury model (see Chapter 3). Uniquely, as these cells can be enriched to >1:3 clonogenic frequency in early passage culture, we show here that it is possible to isolate clones and daughter
sub-clones from mixed gender suspensions, determined to be definitively single-cell-derived by Y-chromosome fluorescent in situ hybridization (FISH) analysis; and assay each clone for multi-lineage differentiation capacity into the five mesenchymal lineages: myogenic, adipogenic, chondrogenic, osteogenic and fibroblastic (stroma). Our observation that daughter sub-clones possess equal or lesser differentiative potential to their respective parent clones demonstrated the two intrinsic properties of stem cells: clonal self-renewal and multi-lineage differentiation. These robust, single cell derived, data provide a new hierarchical mechanism of MSC self-renewal and differentiation in which a definitive self-renewing multi-potent MSC gives rise to more restricted self-renewing progenitors that gradually lose differentiation potential until a state of complete restriction to the fibroblast is reached. Recognition of this lineage hierarchy provides a significant advance in our understanding of MSC biology.

4.2. Methods

4.2.1. Traditional Mesenchymal Cell Cloning

We previously determined the CFU-F frequency of primary HUCPVCs to be 1:300\textsuperscript{46}. 300 passage 0 (P0) HUCPVCs were seeded into individual wells of 6-well tissue culture plates by limiting dilution. After 5-7 days, wells with single distinct colonies as determined by light microscopy were marked for isolation. The colonies were isolated by one of two methods.

4.2.1.1. Cloning Cylinder

The first strategy used to isolate clones was performed with the use of a ‘cloning cylinder.’ A sterile 8 mm diameter polystyrene cylinder was dipped in sterile vacuum grease, and placed around the pre-marked colony. The colony was then washed and removed from the culture dish with the use of 100µl of trypsin/EDTA, and transferred to an individual well of a new 6-well plate with fresh SM.

4.2.1.2. Mechanical Removal

Alternatively, the ‘mechanical removal’ method was used. Distinct colonies were
marked on the underside of the well with a permanent marker. A cell-scaper was then used to wipe the surface of the entire well with the exception of the marked colony. The well was then washed twice with PBS and replaced with fresh SM. Light microscopy was used to determine that no other adherent cells outside of the colony remained in the well. In both methods, the colony was then allowed to proliferate until 70-90% confluence before sub-culture and seeding into a T-75.

4.2.1.3. **Agarose**

NuSieve GTG low-melting temperature agarose (BioWhittaker) was made up at the following concentrations: 2.5%, 2.0%, 1.5%, 1.0% and 0.5%, in 85% α-MEM, 5% FBS and 10% antibiotics. 300 HUCPVCs were then cultured either on the surface of the agarose, or in suspension. Any growing colonies were observed after 10 days of culture.

4.2.1.4. **MoFlo® Fluorescence Activated Cell Sorting**

P0 HUCPVCs were plated for 24 hours, and removed from the culture surface by trypsin/EDTA. The cells (approx. 5x10⁴) were then stained with propidium iodide (Invitrogen) to label dead cells. A MoFlo® fluorescent activated cell sorter (Dako) was calibrated to deposit 1 live cell per well in 96-well tissue culture plates with 100µl SM in each well. To determine rigorous single-cell seeding, cells were only seeded when the drops immediately preceding and succeeding it were vacant.

4.2.2. **Cloning by Sub/Single-Cell Seeding**

4.2.2.1. **Clonal isolation of HUCPVCs by single cell seeding**

Isolates of male and female P0 HUCPVCs were plated separately for 24 hours, removed from the culture surface by trypsin/EDTA, washed and collected as independent suspensions. Each suspension, approximately 5x10⁴ cells (P1), were then passed through a 70µm cell strainer to ensure single cell suspension, and counted on the ViCell-XR automated cell counter. 5x10³ cells from each suspension were mixed together, and an aliquot of the mixed suspension was then diluted in SM to a concentration of 1 cell per 50µl. 50µl of the mixed suspension was placed into each well.
of 35 x 96-well tissue culture plates. After 24 hours, an additional 50µl of SM was added to each well and the SM was replaced every 5 days. After 10 days in culture, each well of the plates was observed by light microscopy for the presence of cells. Only wells with cells were maintained by replacement of SM every 3 days until they reached 70-90% confluence. The cells were then sub-cultured and seeded (P2) into an individual well of a 6-well culture dish. The media was replaced every 2 days until the cells reached 70-90% confluence, at which point they were sub-cultured and seeded (P3) into individual T-75s, in which the media was replaced every 2 days. Once the cells reached 70-90% confluence, they were removed from the culture surface (P4) by trypsin/EDTA, counted with the Vi-Cell (approximately 1-3 x 10^6 cells) and seeded as required for differentiation assays (see 4.2.4), or stored in liquid N₂.

4.2.2.2. Clonal isolation of HUCPVCs by sub-single cell seeding

Isolates of male and female P0 HUCPVCs were plated separately for 24 hours, removed from the culture surface by trypsin/EDTA, washed and collected as independent suspensions. Each suspension, approximately 5x10⁴ cells (P1), was then passed through a 70µm cell strainer to ensure single cell suspension, and counted on a ViCell-XR automated cell counter. 5x10³ cells from each suspension were mixed together, and an aliquot of the mixed suspension was then diluted in SM to a concentration of 0.5 or 0.2 cells per 50µl respectively. 50µl of the mixed suspension was placed into each well of 35 x 96-well tissue culture plates. After 24 hours, an additional 50µl of SM was added to each well and the SM was replaced every 5 days. After 10 days in culture, each well of the plates was observed by light microscopy for the presence of cells. Only wells with cells were maintained by replacement of SM every 3 days until they reached 70-90% confluence. The cells were then sub-cultured and seeded (P2) into an individual well of a 6-well culture dish. The media was replaced every 2 days until the cells reached 70-90% confluence, at which point they were sub-cultured and seeded (P3) into individual T-75s, in which the media was replaced every 2 days. Once the cells reached 70-90% confluence, they were removed from the culture surface (P4) by trypsin/EDTA, counted with the Vi-Cell (approximately 1-3 x 10^6 cells) and seeded as required for differentiation assays or stored in liquid N₂.
4.2.2.3. Sub-clonal isolation of HUCPVCs by sub-single cell seeding

P4 clonal HUCPVC populations (see 4.2.2.2) derived from 0.2 cell/well seeding were obtained as single suspensions by passing them through a 70μm cell strainer. They were then diluted in SM to 0.2 cells per 50μl and seeded into 160 x 96 well plates (5 per clone). The cells were expanded as into 6-well plates (P5), and T-75s (P6). Once they reached 70-90% confluence they were removed from the culture surface, and seeded as required for differentiation assays (see 4.2.4), or stored in liquid N₂.

4.2.3. Double blind analysis of clonal formation by FISH

As all clonal and sub-clonal isolation of HUCPVCs were done from equal suspensions of mixed male and female cells, single cell isolation was confirmed by fluorescent in-situ hybridization (FISH) analysis for the presence or absence of a Y-chromosome. 5x10³ cells from each of the 32 clones and 11 sub-clones, both derived from 0.2 cells/well seeding, were plated into independent wells on 4-well glass chamber slides. One slide was prepared with known controls including, 5x10³ mixed male/female, 5x10³ male, and 5x10³ female (two wells) cells in separate wells. The slides were then provided to an independent FISH analyst who was only given identification of the control wells. Importantly, the analysis was double blind, as neither the person who plated the cells, nor the independent analyst knew the gender of any of the 43 clones. The CEP Y Sat III-32-112024 DNA FISH probe was obtained from Vysis. Application of the probe was done following the Vysis protocol (see Appendix B), and the cells were counterstained with DAPI. The independent analyst then analyzed the 43 clones for presence/absence of Y-chromosome nuclear localization by fluorescent confocal microscopy. Wells with a Y-chromosome signal equal or less than the negative control (known female cells), or a signal equal or greater than that of the positive control (known male cells) were identified as single-cell derived (SCD) cells.

4.2.4. Multilineage Differentiation of Clones

Each clone was seeded into 12 wells of a 24 well plate. 9 wells were seeded with 10⁴ cells. Of these, 2 of each were induced with medium containing either osteogenic,
adipogenic, or myogenic supplemented media (see 3.2.3), and the remaining 3 were treated with SM containing 2% FBS as negative controls. The remaining 3 wells of the plate were seeded with 100, 50 and 10 cells, and treated with SM containing 5% FBS to assess the CFU-F frequency of each clone. As well, 2.5x10^5 cells from each clone were induced with CS medium in a 15 ml tube (see 3.2.3). Differentiation into each lineage was determined by lineage-specific morphological changes (see 3.2.3), or by RT-PCR analysis of lineage-specific genes (see 3.2.4.6).

4.2.4.1. Self-Renewal Analysis of Clones

Each daughter sub-clone was analyzed for multilineage and self-renewal differentiation potential as above, and compared to its parent clone.

4.2.5. Statistical Analysis

CFU-F frequencies and proliferation were compared using un-paired 2-tailed t-tests and micro CT data was compared using a paired 2-tailed t-test. T-test values were considered significant with a probability of less than 5% (P<0.05). For FISH analysis, if the admixture of cells of the second donor in each colony is k, then the probability of not finding a mixed colony after identification of 22-54 cells in each colony is (1-k)^22 to (1-k)^54. For k=0.5 the probability of single cell clonal formation was 10^-7 to 10^-17.

4.3. Results

4.3.1. Cloning Efficiency & Purity

4.3.1.1. Cloning Methods

5 different methods (Figure 4-1) were used to isolate single-cell-derived (SCD) clones. By seeding cells at their limiting dilution, and using a cloning cylinder to isolate individual CFU-Fs, 21% of clones survived expansion to ~10^6 cells. By using an alternative method of removing all cells around a CFU-F, this efficiency was increased to 60%. By using different densities of agarose gel, we were completely unsuccessful at isolating any SCD cells, as the HUPVCs migrated to the bottom of the dish and did not proliferate to form CFU-Fs that could be 'picked.' Regardless, all of these methods

4-7
relied on the assumption that a CFU-F is a SCD population, and no evidence to date has shown this.

<table>
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<th>Cloning technique</th>
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<th>Observed # of clones</th>
<th># surviving to induction</th>
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</tbody>
</table>

Figure 4-1. Frequency of clone formation due to different cloning techniques. †Represents daughter sub-clones seeded at 0.2 cells/well.

To isolate definitive SCD populations, we utilized a strategy of mixing equal numbers of male and female cells, and seeded them into 96-well plates. Due to the >1:3 CFU-F frequency of HUCPVCs in culture (Figure 3-2a) single cell dilution produced 117 clones, although the probability that each was a SCD population was only 0.5. Seeding 0.5 cells/well (1 cell per 2 wells) only increased the odds to 0.75. By further reducing the dilution to 0.2 cells/well, (1 cell per 5 wells), this provided a SCD cell isolation probability of 0.96. From a total of 6720 wells seeded, 45 clonal populations were produced, 32 of which survived expansion to ~10^6 cells. These 32 clones were re-seeded at the same dilution of 0.2 cells/well into 480 wells each (15,360 wells total), which produced 23 clonal daughter populations, 11 of which survived expansion to ~10^6 cells. The probability that these 11 daughter sub-clones were SCD cells was 0.9999.

4.3.1.2. Clonal Purity by FISH

While using statistical analyses to determine clonal purity at a 95% confidence
interval would be reasonable, to definitively determine the single cell origin of both the 32 parental and 11 daughter clones, double blind FISH analysis was used to determine whether the clones were of either male or female single-cell origin, or of mixed origin. Positive, negative and mixed FISH controls determined \( \geq 85\% \) or \( \leq 0\% \) thresholds for determination of single-cell-derivation. All clones and sub-clones, except one clone, had a Y-chromosome signal above the threshold for single-cell determination (Figure 4-2).

![Figure 4-2. FISH analysis of clones and daughter sub-clones derived from seeding at 0.2 cells/well. Daughter clones are 1-11 and parental clones are 12-44.](image)

As a result, 42 of the 43 isolated clones were determined to be definitively single-cell-derived with the exception of #39 (not a parent of any of the 11 daughter clones). While it could be argued that the clones were derived from doublets or triplets of same-sex cells, we analyzed 22-54 cells within each of the 32 clones and determined that the probability that each was formed from more than one cell between \( 10^{-7} \) and \( 10^{-17} \). As a result, they were considered definitive SCD clones. Importantly, our finding that single-gender clones arose from a mixed gender cell suspension suggests that only an exceptionally rigorous strategy to isolate single cells, such as the one we used, could generate reliable clonal data.

4.3.1.3. Clonal CFU-F frequency

We determined whether the clonogenic potential of the SCD populations was changed by the prolonged period in culture by analyzing their CFU-F frequency. Figure 4-3 illustrates that the parental and daughter SCD clones maintained a CFU-F
frequency of ~1:4 which was not significantly different from the CFU-F frequencies of clones produced by 0.5 or 1 cell/well seeding nor the ~1:3 frequency seen in bulk HUCPVC cultures. These data illustrate that the cloning process did not affect the overall clonogenic frequency of SCD clones.

Figure 4-3. CFU-F frequencies from clones derived from seeding densities of 1, 0.5, 0.2 and 0.04\textsuperscript{†} cells/well. \textsuperscript{†}Represents daughter sub-clones seeded at 0.2 cells/well. (n=117, 56, 32, 11 resp.)

4.3.1.4. Clonal Differentiation & Self-Renewal

Figure 4-4. Lineage frequencies of multi-lineage clones derived from seeding densities of 1, 0.5 and 0.2 cells/well. The 32 parent clones assayed were derived from seeding at 0.2 cells/well. \textsuperscript{†}Represents daughter sub-clones seeded at 0.2 cells/well.
Figure 4-5. A single HUCPVC daughter sub-clone derived from 0.2 cells/well seeding differentiated into bone, cartilage, adipose and muscle in vitro. Under induction, bone nodules were observed in culture (a) that stained with Von Kossa (black), and were surrounded by alkaline phosphatase-high expressing cells (b). Cartilage pellet cultures of HUCPVCs expressed collagen II (c) and glycosaminoglycans that stained with Alcian blue (d). HUCPVC-derived adipocytes stained with Oil Red O (e) and formed as distinct colonies (F). Myogenically-induced HUCVPCs expressed high levels of MyoD (g) and FSMLC (h) in multinucleated HUCPVC myotubes. Negative controls are uninduced cells stained with Von Kossa and alkaline phosphatase (i), and secondary-only antibody staining for collagen II (j), MyoD (k) and FSMLC (l) stained cultures. (Field widths: a,c,f = 628mm; b = 3.5mm; d,g,h = 315mm; e = 86mm). RT-PCR analysis (m) demonstrated up-regulation of the following genes: Runx2 (1), collagen IA1 (2), osteopontin (3), osteocalcin (4), Sox9 (5), collagen II (6), aggrecan (7), LPL (8), MyoD (9), Myf5 (10), desmin (11), MHC (12), FSMLC (13), GAPDH (14) and RT-control (15).

Each clone produced by the single and sub-single cell seeding was assayed for its ability to differentiate into one or more of the mesenchymal lineages: muscle, adipose, cartilage, bone and fibrous stroma. Although we did not determine that the
clones produced by 1 and 0.5 cells/well were definitive SCD clones, they, along with the definitive SCD clones demonstrated differential ability to differentiate into the 5 phenotypes (Figure 4-4). Figure 4-5 illustrates one such single daughter clone that possessed the capacity to differentiate into all five lineages, as determined by rigorous analyses of lineage-specific morphological changes, protein expression, and gene up-regulation.

Figure 4-6. 32 parent clones (a & self-renewing circles in b) produced 11 daughter clones (b) with equal or lesser differentiative potential. MACOF represents a clone that was able to differentiate into all 5 phenotypes assayed.
Importantly, as the clonal isolation became more stringent, several trends became apparent (Figure 4-4). First, the number of quinti-, quadri- and tri-lineage clones decreased, while the proportion of single lineage clones increased, suggesting that seeding densities higher than 0.2 cells/well did not produce SCD cells. Second, the relative proportion of multi-lineage SCD clones either stayed constant or increased from parent to daughter clones, suggesting that the mono-lineage SCD clones reduced in proportion, most likely because they were less robust and began to be selected out of the progenitor pool in extended culture. These data provided a map of parental and daughter clone potential (Figure 4-6). The 11 daughter sub-clones, when assessed for multi-lineage capacity, showed that 3 were uni-potent colony unit forming-fibroblast (CFU-F) clones, while one possessed the capacity to differentiate into all five lineages assayed (CFU-MACOF). Although the latter was derived from a multiligneage parental clone, two of the CFU-F clones were the progeny of uni-potential CFU-F parents, while one was derived from a bi-potent osteoblast/fibroblast (CFU-OF) parent. This pattern was observed in all the other restricted clones in which the two quadri-potent adipocyte/chondrocyte/osteoblast/ fibroblast (CFU-ACOF) clones were produced by one quinti-potent CFU-MACOF and one CFU-ACOF clone, respectively. Similarly, the three tri-potent clones were derived from one quinti-potent CFU-MACOF and two quadri-potent CFU-ACOF clones, while the two bi-potential clones arose from a CFU-COF and a CFU-AOF clone respectively.

4.4. **Discussion**

Due to the >1:3 clonogenic frequency of HUCPVC populations in culture, we succeeded in isolating definitive single-cell-derived (SCD) clonal populations by seeding cells at sub-single-cell densities. By assaying the frequency and lineage differentiation capacity of these SCD clones, we quantified the proportion of self-renewing restricted progenitors to multi-potential stem cells within the overall mesenchymal population in culture.

These data, which illustrate daughter SCD clones being derived from equal or greater potential SCD parents, is the first reported evidence of self-renewing multi-
potent mesenchymal cells. One striking observation when the lineages are observed individually is the absence of the adipogenic lineage in the bi-potential clones that possessed either an osteogenic or chondrogenic capacity. The former theoretically arises at twice the frequency of the latter, and is supported by our previously published data in which we observed a 1:1 ratio of uncommitted to committed CFU-O progenitors in HUCPVC culture\textsuperscript{46}. The tri-potential clones illustrated that, when differentially expressed with osteogenic potential, the chondrogenic and adipogenic capacities were exclusive of each other. When these two lineages were expressed together, it was only in the quadri- and quinti-potential clones. Interestingly, the myogenic lineage was only expressed in the quinti-potential clone, suggesting that it is the first lineage forfeited when a multi-potential MSC differentiates.

Figure 4-7. Hierarchy of MSC differentiation.

These data elucidate a hierarchical mechanism of MSC differentiation (Figure 4-7) in which a self-renewing multi-potent MSC (CFU-MACOF) gives rise to more
restricted self-renewing progenitors that gradually lose differentiation potential until a state of complete restriction to the fibroblast is reached. The sum total of all these progenitors provide the $1: [2.6 \pm 1.4]$ clonogenic frequency observed in HUCPVC culture.

Based solely on in vitro evidence, hierarchical structures of MSC differentiation have been previously proposed (Figure 4-8). Aubin\textsuperscript{105} postulated that MSCs give rise to either a CFU-OC or CFU-OA bi-potential progenitor and that CFU-A and CFU-C are differentially forfeited to result in the osteogenic capacity required for bone formation. Similarly, Muraglia and colleagues\textsuperscript{106} concluded that bone represents the default in the mesenchymal pathway. However, both groups failed to include the myogenic and fibroblast lineages in their in vitro studies and, as our results demonstrate, it is this latter lineage that is the default in the mesenchymal hierarchy.

\begin{center}
\textbf{Figure 4-8.} Aubin\textsuperscript{105} postulated the hierarchical differentiation of an MSC into a terminally differentiated osteoblast through a series of OA or OC bi-potential progenitors (a), while Muraglia \textit{et al.}\textsuperscript{106} suggested that a tri-potential OCA sequentially differentiates into OC and O progenitors respectively (b).
\end{center}

Indeed, the primary responsibility of mesenchymal HUCPVCs \textit{in situ} is to maintain their niche’s stromal tissue by differentiating into fibroblasts that function to generate fibrous extracellular matrix. As a result, resident MSCs must follow a differentiation pathway in which all of the other differentiation lineages are by-passed; accordingly, for maintenance of the niche there must be a sequence of signaling events that prevent differentiation of the MSC and its more restricted progenitors into any phenotype other than fibroblastic. Similarly, we argue that, in the BM or other organs,
MSCs are responsible for maintaining their niche by giving rise to daughter cells that preferentially differentiate, not into cells of the bone, but rather cells of the stroma: fibroblasts that produce the stromal extracellular matrix. However, when these cells are removed from their natural environment, and provided with a specific set of cues \textit{in vitro}, their lineage capacity becomes evident.

**Conclusion**

Here we have shown that a multi-potent mesenchymal cell population is composed of a variety of progenitors with differential capacities. In a process of sequential restriction through the muscle, adipose, cartilage and bone lineages, a self-renewing multi-potent mesenchymal \textit{stem cell} gives rise to more restricted self-renewing progeny, until a state of complete restriction to the terminally differentiated fibroblast is reached.
5. CHAPTER 5: Discussion
This body of work has set out to answer one simple question: does a mesenchymal stem cell exist? To fully address this question the challenge was to show that a single multipotent mesenchymal tissue-derived cell could produce a daughter cell with identical capacity. The data illustrated herein definitively demonstrates this property \textit{in vitro}. This information along with the methods provided here will enable interrogation of MSC self-renewal in serially transplanted living recipients, as has been definitively demonstrated in the HSC system.

\textbf{5.1. HUCPVCs coordinate repair of multiple connective tissues in a long-bone defect model}

Given the growing body of literature supporting the perivascular provenance of MPCs two specific perivascular regions have been implicated as possible repositories of these cells, the sub-endothelial space and the perivascular compartment. In this work, cells were specifically isolated from the perivascular fraction of human umbilical cords. It was found here that these HUCPVCs spontaneously elaborated bone matrix \textit{in vitro}, and that this frequency of bone nodule formation doubled upon induction with osteogenic supplements. These data illustrated that the HUCPVC progenitor population is heterogeneous, as it contains at least three distinct populations: committed and uncommitted CFU-Os, and a population of restricted CFU-F progenitors.

The majority of MSC literature has relied on \textit{in vitro} analysis to determine the multipotential nature of putative MPCs, and very few studies have analyzed multiple mesenchymal lineages of putative MPCs \textit{in vivo}. The few multilineage analyses performed inoculated MPCs into diffusion chambers that were implanted either subcutaneously (s.c.)\textsuperscript{226,227} or intraperitoneally (IP)\textsuperscript{245}. This \textit{in vivo} diffusion chamber model is artificial as the MPCs, sequestered in a chamber, were only exposed to one of the three ‘niche’ conditions, paracrine and/or autocrine signaling. There was no cell-cell contact, nor cell-matrix interaction with the host. By implanting the diffusion chambers into an ectopic
site, s.c. or IP, the cells were not exposed to the normal signaling mechanisms of an MPC microenvironment. Accordingly, although bone and cartilage tissues did form within the chambers, this possibly did not reflect the normal behavior of these cells \textit{in vivo}.

![Figure 5-1. Stem cells within their microenvironment. Schematic diagram of the major types and spatial orientations of cells that make up the hair follicle (a), and the interactive signaling pathways that mediate hair follicle stem cell proliferation (b). The exact spatial relationships of haematopoietic and mesenchymal cellular components in the bone marrow microenvironment (c) are not well defined. (Adjusted composite from Moore & Lemischka)\textsuperscript{246}.](image)

The s.c. microenvironment has a distinct signaling system that maintains homeostasis of the dermal layers (Figure 5-1a,b). Several active signaling molecular mechanisms identified in this microenvironment control bulge stem cell fate within this homeostatic system. It has not been determined what effects these extrinsic signals nor others in the sub-dermal environment have on MPC fate, and as a result, the observations of bone and cartilage production in MPC-inoculated diffusion chambers placed s.c. may be an artifact of the model. Similarly, when implanted in the IP microenvironment, the cells are constantly immersed in body fluid. In a recent study investigating the effects of a bone-like mineral film on osteogenesis of BM MPCs, the authors used simulated body fluid (SBF) to simulate \textit{in vivo} conditions\textsuperscript{247}. Surprisingly, they found less ALP expression by MPCs on ‘bone-like mineral’ (BLM) coated surfaces than uncoated surfaces. In this case, BLM inhibited osteogenesis while some spontaneous ALP
was upregulated due to the presence of SBF. Thus, as SBF causes abnormal paracrine signaling of MPCs to differentiate down the osteogenic pathway, it is possible that this may be an artifact of the model. Accordingly, implantation of cells into the IP space is a less than ideal model to analyze MPC function.

In order to determine whether \textit{in vitro} bone elaboration by HUCPVCs was simply an artifact of culture, here, MPCs were transplanted into a prototypical mesenchymal environment. Transplantation directly into the marrow compartment (Figure 5-1c) enabled assessment of the direct effects of HUCPVCs on tissue repair. In this environment the cells were exposed to all elements of the ‘niche’: para'autocrine signaling, cell-cell contact, and cell-matrix interactions. Injecting the cells directly through the knee damaged the cartilage, bone and marrow tissue, and provided an ideal model to observe MPC contribution to the repair and reestablishment of homeostasis of these tissues. It has been shown herein that HUCPVCs contributed to multiple mesenchymal tissues including bone, cartilage, adipose and fibrous tissue \textit{in vivo}. By flushing the marrow of femurs after 2, 4 and 6 weeks, eGFP\textsuperscript{+} cells were found present in culture, demonstrating that the HUCPVCs survived up to 6 weeks \textit{in vivo}. Transplanted femurs displayed significantly more repair to bone, cartilage and stromal fractions compared to sham controls. Histological analyses showed complete HUCPVC-induced repair to the distal end of the femur at 2 weeks, while complete autologous repair was only achieved after 4 weeks. Micro CT data illustrated significantly more bone mineral density (BMD) at all time points, providing evidence that HUCPVCs caused more bone matrix elaboration compared to controls. This was further confirmed by the observation of human cells laying down new matrix in trabecular clefts. Putative human osteoblasts appeared to be secreting human osteocalcin causing mineralization of the underlying osteoid. It was further observed that newly formed human cartilage, as determined by human-specific collagen II staining, was distal to the new mouse cartilage. The human cells contributed to repair by producing chondrocytes that laid down human collagen II. Given the evidence that murine BM-derived MPCs\textsuperscript{248} cycle more rapidly than human BM-derived MPCs\textsuperscript{97} it is
likely that the mouse progenitors were able to migrate, proliferate and generate chondrogenic progenitors more quickly than the differentiating HUCPVCs. These data, combined with the observation that the majority of new bone and cartilage tissue was of host rather than human origin, suggest that in addition to directly contributing to repair, the HUCPVCs recruited and induced mouse mesenchymal progenitors to regenerate the damaged tissue. Rather than simply enlodging in the marrow, the HUCPVCs engrafted in this mesenchymal compartment, becoming functionally resident by directing autologous repair, as well as producing progeny that directly contributed to tissue restoration. During this reestablishment, the likely first step was rapid formation of the stromal tissue and recapitulation of the HSC microenvironment (Figure 5-1c). During repair, newly formed microvessels would facilitate formation of other tissue types including trabecular bone and cartilage; as this microvascular network would not only contribute oxygen and nutrients, but also a progenitor cell source. This progenitor source represents the multipotential perivascular cells previously described (see 1.3.3.6), that are observed as MPCs in culture. These MPCs could thus either infiltrate damaged areas by direct migration from nearby microvasculature, or possibly infiltrate along with the de novo vessel formation. As a result, these cells could migrate to areas requiring formation of new bone, cartilage and/or fat. The mechanism of this action is still unclear, although analyses of close time points (i.e. every 24 hours after transplantation) in this intrafemoral model may facilitate clarification of these tissue formation steps.

The in vivo work described herein is an example of the phenomenon where in vitro multipotential transplanted HUCPVCs not only contributed to tissue healing and matrix elaboration themselves, but also recruited the resident mouse progenitors to rapidly repair the damaged tissues. Accordingly, due to the observation that the majority of repaired tissue was of host rather than donor origin, it is possible that rather than displaying unrestricted self renewal and differentiation, only a few asymmetric cell divisions of the transplanted MPCs were initiated to produce the more restricted tissue-specific progenitors available for regenerating the damaged tissue. Further analysis by transplantation of
restricted progenitors, such as a COF restricted progenitor population, would provide further insight into the ability of these cells to contribute to tissue healing. When homeostasis of the mesenchymal compartment was disturbed by injury, rather than simply maintaining their microenvironment, the MPCs become required to reestablish homeostasis by repairing the damaged mesenchymal tissues. This phenomenon was observed in vivo, as, rather than clonally exhausting the supply of MPCs, the human cells possibly recruited the resident mouse and transplanted committed human CFU-C/O/F progenitors to rapidly repair the damaged tissues. In order to interrogate this possibility, it would be necessary to isolate the transplanted human cells, and assay their ability to regenerate the same tissues when transplanted into a secondary recipient. This would provide evidence that coordinated cellular response of progenitors in situ is the responsibility of the MPCs. It is proposed here that the scale of this MPC differentiation to repopulate the depleted progenitor population is relative to the level of disturbance that has been caused to the homeostasis of the tissue compartment.

The perivascular environment appears to be the primary source of mesenchymal progenitors in many organs including bone marrow. While occupying their putative ‘niche,’ these perivascular cells remain quiescent; but in most organs they will give rise to daughter cells that preferentially differentiate into the specific tissue type requiring replenishment. Accordingly, these cells will by-pass all other differentiation lineages as a result of a sequence of signaling events that prevent differentiation of the multipotent MPC into other non-required cell lineages. Thus, the primary responsibility of HUCPVCs in situ is to maintain the umbilical cord’s stromal tissue by differentiating into the myofibroblasts that elaborate the Wharton’s Jelly extracellular matrix. However, when the homeostasis of this, or any other, perivascular environment is disturbed during growth or by injury, the MPCs are required to produce not only the synthetically active myofibroblasts, but other tissue types such as the smooth muscle cells of the vasculature, as well as precursors of bone and cartilage. Accordingly, when these resident MPCs are removed from their natural environment and provided
with the specific inductive signals, it is possible to delineate their lineage capacity
_in vitro._

5.2. **HUCPVC-derived progenitors maintained in extended culture are multipotential**

Having determined multipotential capacity of HUCPVCs to differentiate into bone, cartilage and fibrous tissue _in vivo_, the _in vitro_ properties of these cells were interrogated. When isolated in culture, HUCPVCs maintained a myofibroblastic morphology, and formed CFU-F colonies at a frequency of 1:300 cells. This frequency is two to three orders of magnitude higher than that observed in human BM (1:10^4-10^5)\textsuperscript{97,192}. By seeding incremental dilutions of 300 cells per well, an exponential relationship between the number of cells seeded and the CFU-F frequency was observed. By seeding 1,200 cells, \(\sim 6\) colonies formed (CFU-F=1:200) rather than the expected 4; and by seeding 2,400 cells, \(\sim 14\) colonies were observed (CFU-F=1:170) rather than the expected 8. This does not conform to observations by Castro-Malaspina et al.\textsuperscript{78} who showed a linear relationship between cells seeded and number of CFU-F. However, it is clear from this result that some 'niche-mediated' signaling mechanism, most likely paracrine signaling, was potentiating colony formation. In order to further dissect this mechanism, each 'niche condition' should be investigated separately. To determine the possible effects of paracrine signaling, either conditioned media from HUCPVCs, or a trans-well assay in which a population of HUCPVCs could signal serial dilutions of physically separated cells could be used. To further determine the effect of cell-cell interactions, irradiated (non-proliferating) eGFP-labeled HUCPVCs could be seeded with serial dilutions of unlabeled HUCPVCs to observe the effects on CFU-F frequency. Finally, as collagen I is the major constituent of the umbilical cord ECM, in order to determine the effect of cell-matrix interactions, plates coated with varying amounts of collagen could be used to determine any potentiating effects on HUCPVC CFU-F formation. These experiments would provide a deeper understanding of the _in vitro_ 'niche' effects on HUCPVC populations.
It was further determined that HUCPVC populations doubled at ~20 hours when cultured with 15% FBS, and this proliferation decreased to 42 and 47 hours (not significantly different) with 5% and 2% FBS respectively. Although the effect of 15% FBS on CFU-F frequency was not assayed, it is possible that the higher proliferation rate was a function of a higher CFU-F frequency, as a larger number of CFU-F progenitors would increase the overall proliferation rate. It is important to note that a higher proliferation rate does not necessarily suggest a larger pool of multipotential MPCs. Although the lack of prospective isolation has made it impossible to assay the proliferation rate of the multilineage MPC population, based on evidence in the HSC\textsuperscript{249} and satellite stem cell\textsuperscript{124} systems, it is likely that the multipotential MPCs cycle more slowly than the fibroblast-restricted progenitors. This would be most evident in large colonies where, after having initiated the colony, the MPCs then become slow-cycling or quiescent, and allow the more restricted progenitors propagate the colony fibroblast population. This phenomenon has yet to be shown, although the experiments outlined below could begin to address these questions.

As the specific effects of serum on MPC populations is still unclear\textsuperscript{240}, the ability of HUCPVCs to become as ‘low-serum dependent’ as possible was assayed, such that at low serum levels, HUCPVC proliferation became independent of serum concentration, and the confounding effects of serum were limited. At low serum levels of 5% or 2%, the frequency of CFU-F formation remained stable (not significantly different) in culture at ~1:3 from P2 to P10. This is an important finding, as it illustrates that a significant pool of progenitors was maintained in extended culture. As discussed below (see 5.3), this high clonogenic frequency enabled isolation of definitive single cell-derived clonal populations.

As well, determination of this frequency could enable interrogation of the phenomena associated with CFU-F formation. Using a live-cell imaging system, 3 cells could be seeded in a well, and time-lapse images of each cell’s development could be captured. This strategy would definitively determine whether limiting dilution truly produces single cell-derived colonies, and if so,
whether there were any observable differences between the cell that produced the colony compared to the remaining two that did not. Importantly, it would be possible to further track how daughter cells are produced within this colony, and whether the original ‘colony-initiating-cell’ (CIC) continues to proliferate or becomes quiescent after one or more doublings. By then re-isolating the original CIC along with a few of its immediate progeny as well as distant progeny (either by laser micro-dissection or some novel cell-specific isolation strategy), it would be interesting to observe whether/how they contributed to the formation of new colonies, and if so, what similarities or differences occurred during the formation of each colony. Accordingly, a map or model of colony formation could be created, such that one could predict where the progenitor/stem cells reside within a given colony. As well, by using a strategy such as BrdU labeling of developing colonies at 1, 2 and 4 cells, significant insight could be gained into how the early colony-generating cells are maintained and behave to produce the remainder of the colony. Using such a strategy, one could possibly even begin to interrogate the “immortal strand hypothesis” by analyzing the fate of parent and daughter cells’ DNA.

Having determined the general kinetics of cultured HUCPVC CFU-F frequency, the ability of HUCPVCs to differentiate into the five mesenchymal lineages: muscle, adipose, cartilage, bone and fibrous tissue was analyzed. As discussed above, the HUCPVC population contained CFU-O progenitors that, either spontaneously or under induction, elaborated bone matrix in vitro. In repeating this work, it was further determined by RT-PCR that osteogenic genes Runx2, osteopontin and osteocalcin were upregulated. Collagen I upregulation was also observed, although this gene was also upregulated in uninduced HUCPVC populations indicating that CFU-F progenitors were producing functional fibroblasts that were actively producing collagen I in culture.

By further analyzing three-dimensional pellet cultures of HUCPVCs, elaboration of collagen II (by immunostaining) and glycosaminoglycans (by Alcian blue staining) was determined, as well as upregulation of chondrogenic genes Sox9, collagen II and aggrecan. These pellets demonstrated a fibro-
cartilage-like morphology, possibly expressing collagen I not present in the well-organized tissue observed in normal hyaline cartilage. Nevertheless, these results demonstrated chondrogenic differentiation of HUCPVCs in culture, suggesting that further refinement and optimization of the differentiation conditions may result in morphologically functional and useful tissue for cartilage-related therapies.

The adipogenic differentiation capacity of HUCPVCs was further determined. After 3 weeks of induction, HUCPVCs produced lipid vacuole-containing adipocytes that stained with oil red O, and upregulated lipoprotein lipase as analyzed by RT-PCR. While the PPAR-γ inducer, Rosiglitazone, was utilized, regrettably upregulation of PPAR-γ2 in the differentiated cells was not determined. Nevertheless, as with chondrogenic differentiation, HUCPVCs readily produced adipocytes in culture, and it is possible that this induction may be optimized to produce significant numbers of clinically useful adipocytes for future clinical applications.

Finally, the myogenic potential of HUCPVCs was determined. As these cells already maintain a myofibroblastic phenotype in culture, rigorous analysis to determine myogenic differentiation was necessary. In addition to determining the morphological expression of HUCPVC-derived multi-nucleated myotubes in culture, protein expression of early (MyoD) and late (fast skeletal muscle light chain (FSMLC)) markers by monoclonal antibody labeling was analyzed; as well as upregulation of five skeletal muscle-related genes by RT-PCR: MyoD, Myf5, desmin, myosin heavy chain (MHC), and FSMLC. This careful analysis determined that HUCPVCs, when grown to confluence, and induced with myogenic factors, hydrocortisone, Dex and horse serum, formed multinucleated myotubes in culture.

These data illustrate that bulk HUCPVC cultures maintain multipotential populations as they differentiate and elaborate multiple mesenchymal tissues both in vitro and in vivo. Nevertheless, these data did not provide an understanding of whether distinct populations of unilineage progenitors gave rise to the five mesenchymal phenotypes analyzed, or whether multipotential cells
were responsible for these observations.

5.3. HUCPVCs are a source of mesenchymal stem cells (MSCs)

In order to determine the stem cell nature of HUCPVC populations, it was imperative to isolate definitive single-cell-derived (SCD) clones. One other study succeeded in isolating definitive human SCD clones by FACS sorting single adherent BM cells into 96-well plates. In this study, SCD clones were analyzed for osteogenic and adipogenic capacity, although the authors failed to analyze individual clones for more than a single lineage. As well, the authors did not assay the self-renewal potential of the SCD clones to produce daughter cells with identical properties.

Here, initial cloning attempts utilized limiting dilution of 300 cells/well in 6-well plates and traditional cloning rings to isolate CFU-Fs. This method only resulted in a 22% yield, while a similar method of removing all cells around a CFU-F and allowing the colony to grow before passaging improved this yield to 60%. Other methods were used in an attempt to improve this frequency, including plating in agarose for single colony 'picking,' as well as single cell deposition by FACS. Neither strategy was successful. Nevertheless, as previously discussed, limiting dilution does not guarantee single-cell isolation, and accordingly, a more rigorous single-cell isolation method was developed.

It has been shown herein that HUCPVC populations maintain a high clonogenic frequency in culture of ~1:3. Accordingly, a dilution of 1 cell/50 µl was used to seed 1 cell/well in 35 96-well plates (3,360 wells). Based on a 1:3 clonogenic frequency, formation of 1120 colonies were expected, but only 145 developed, a frequency of 1:23. As previously discussed, incremental cell seeding densities resulted in an exponential increase of the number of CFU-Fs formed, possibly due to paracrine signaling systems. Accordingly, conditioned media (CM) was assessed for the ability to increase colony-forming efficiency; but, when seeded at a dilution of 1 cell/well with or without CM, no significant difference was observed between the number of colonies formed in each
condition. These data suggest that paracrine signaling does not potentiate colony formation, although to further study this outcome more rigorous analysis using limiting dilution (3 cells/well) should be performed. Having determined that each of the 1cell/well dilution-derived colonies had a probability of single cell derivation [P(SCD)] = 0.5; a more rigorous approach of 1 cell/2 wells [P(SCD)=0.75] in 1,921 wells (expecting 320 colonies) was used, forming only 68 colonies. In order to use a statistically significant probability of single cell isolation, a dilution of 1cell/5 wells was used [P(SCD)=0.96] in 6,720 wells. 32 of 45 colonies produced by this method survived expansion to the $10^6$ cells required for lineage analysis.

Using the same rigorous isolation method, of the 32 ‘parental’ clones, only 11 were able to produce daughter clonal populations with a P(SCD)=0.9999.

Having determined the P(SCD)=0.96 of the 32 parental clones, their SCD clonal purity was assayed. As they were produced from mixed gender suspensions of male and female cells, each clone was analyzed by FISH to determine absolute presence/absence of the Y-chromosome. 20 clones consisted of only female cells, while 11 were definitively male. One clone expressed a 60% Y-chromosome\(^{+}\) signal suggesting that it was of mixed origin. The observation of a mixed population being derived from such a rigorous SCD method suggests that other cloning methods, especially limiting dilution, are inefficient at producing SCD clones, and that ‘clones’ generated by these less rigorous methods are most likely of mixed origin. As, between 22 and 54 cells were analyzed in each clone by FISH, each was further statistically analyzed for clonal purity. It was determined that the probability of single cell derivation of each colony varied from $10^{-7}$ and $10^{-17}$ (i.e. $10^{-7}>[P(SCD)]>10^{-17}$); accordingly, the clones were considered to be definitively SCD.

The observation of a ~2:1 ratio of female to male parental clones is unexplained. The probability of this occurrence was 0.25 (the probability of a gender difference of 2 cells = $(1-k)^x$ where $k=0.5$ and $x=2$). This is not a significant difference, and it is likely that this ratio occurred due to chance, as when the 11 daughter clones were analyzed by FISH, the ratio was 5F:6M. This data suggests that upon extended rigorous selection of clonal populations in
culture, there was no selective difference between male and female cells.

All 32 parental and 11 daughter clones were analyzed for the ability to differentiate into the five mesenchymal lineages: muscle, adipose, cartilage, bone and fibrous tissue. Of the 32 parental clones, it was determined that three were quintipotential, as they differentiated into all five lineages (CFU-MACOF). Importantly, all three produced daughter clones that were CFU-MACOF, CFU-ACOF and CFU-COF respectively. This data, illustrating that a SCD CFU-MACOF parent produced a SCD CFU-MACOF daughter is definitive evidence of the two properties of stem cells: multilineage differentiation and self-renewal. Accordingly, it can be concluded that a mesenchymal tissue-derived self-renewing population of multipotential stem cells exists in culture. It is important to note that this observation is currently restricted to in vitro properties of these cells, and their in vivo self-renewal ability remains to be interrogated.

5.4. **HUCPV-MSCs produce restricted self-renewing progeny according to a deterministic hierarchy**

In addition to self-renewing to produce identical daughter cells, CFU-MACOF parental clones also produced daughters with diminished capacity. Re-cloning of one of these CFU-MACOF parents produced a CFU-ACOF daughter, illustrating forfeiture of the myogenic lineage. It is not clear whether the loss of this myogenic capacity was due to a spontaneously-produced CFU-ACOF cell being selected from a heterogeneous MACOF-produced parental population; or whether a CFU-MACOF cell was selected, and lost its self-renewal capacity some time during the ~20 doublings before it was assessed for lineage potential.

Due to the observation that after ~20 doublings, a self-renewing CFU-MACOF parent produced a CFU-MACOF daughter that was assayed at ~40 doublings, it has been shown that these quintipotential cells maintained their multipotential capacity in extended culture. Accordingly, it is suggested here that it was selection of a CFU-MACOF-derived CFU-ACOF cell rather than the extinguishment of a CFU-MACOF population that produced the CFU-ACOF daughter population observed. It is possible that the original MACOF cell
generated a population of progeny (the colony) with differential capacity (Figure 5-2), and accordingly, as the population expanded, the relative proportion of CFU-MACOF cells decreased. While the data does not currently bear this out, it is possible that the proportion of CFU-ACOF cells was higher, making the probability of isolating a SCD CFU-ACOF clone higher than that of isolating a CFU-MACOF clone. Similarly, CFU-COF cells were possibly more numerous than CFU-MACOF and CFU-ACOF cells, and accordingly could be more easily isolated, as observed by emergence of the COF daughter produced by the third CFU-MACOF parent.

Figure 5-2. Proposed model of MPC colony formation. The cells on the left side represent the middle of the colony and those on the right represent the outer edge. The MACOF’s produce more restricted progeny that occupy the space radially in relation to their differentiation capacity. (Darker shading indicates cells with greater potential, and lighter shading indicates cells that are more restricted).

When analyzed for self-renewal properties, the 4 ACOF parental clones only produced one daughter clone that also maintained ACOF potential, and accordingly self-renewed in culture. Of the 6 tripotential parental clones, only 3 (2 CFU-COF and 1 CFU-AOF) produced daughter clones. The CFU-COF parents produced a CFU-COF and a CFU-CF daughter, while the CFU-AOF parent self-renewed, producing a CFU-AOF daughter. This is an interesting finding, as there did not appear to be any crossover between lineages, i.e. a CFU-COF parent did not produce progeny with adipogenic potential. This data suggests strict
restriction of clones that are not able to cross beyond their lineage boundaries.

Of the 6 (4 CFU-OF and 2 CFU-CF) bi-potential parents, only 2 CFU-OF parents produced daughters that were CFU-OF and CFU-F only; while, of the 13 CFU-F-restricted parents, only 2 produced daughters that were also restricted to CFU-F. These data suggest that due to extended culture, CFU-F-restricted unipotential progenitor populations became extinguished more quickly than progenitors with capacity for more than one lineage, demonstrating that as the cells become more restricted, their self-renewal capacity was also diminished. As it appears that CFU-F progenitors are the terminal precursors in the progenitor pool, they are the default lineage that can safely become extinguished, because if needed, the higher order progenitors can continue to replenish their supply. As well, it was observed that similarly to the tripotential parents, bi- and unipotential parents did not give rise to progeny with any capacity other than that already inherent, providing further evidence for strict lineage restriction. Furthermore, the observation of only CFU-OF parents and not CFU-CF parents giving rise to progeny suggests that CFU-OF progenitors may be more robust, while CFU-CFs are possibly transient. Further analysis is required to examine this possibility.

As has been shown in the HSC system, these data have provided a hierarchy illustrating that MSCs self-renew and differentiate, producing a cascade of more restricted progeny with diminished self-renewal capacity that continue to differentiate until a terminally non-self-renewing cell is produced (Figure 5-3d). During generation of a colony, a self-renewing quintipotential MSC first forfeits its myogenic potential producing a quadripotential CFU-ACOF progenitor. A cell fate decision step then causes this CFU-ACOF precursor to either forfeit the adipogenic or chondrogenic lineage providing two possible tripotential progeny, a CFU-COF or CFU-AOF. The CFU-COF makes a similar decision to either forfeit its chondrogenic or osteogenic capacity giving rise to two possible progenitors, a CFU-OF or CFU-CF; while the CFU-AOF has no such choice, and can only forfeit its adipogenic potential to produce a CFU-OF progenitor. This restriction of two different tripotential precursors giving rise to the same CFU-OF descendant by two alternate pathways suggests that there may be a relative enrichment of
CFU-OF progenitors, at least in HUCPVC cultures. This phenomenon was observed, as there appeared to be two distinct, committed and uncommitted osteogenic populations within bulk HUCPVC cultures (see 2.3.3.3). Whether the committed osteogenic cells are the product of one of these pathways, or a transient descendant of both, remains to be elucidated. Nevertheless, the final two possible bipotential progenitors forfeit their respective osteogenic or chondrogenic capacities, finally producing a unipotential CFU-F. Like the CFU-OF progenitors, this final precursor population arises from two different pathways, and is the default lineage observed in MSC cultures.

Figure 5-3. Comparison of theoretical (a,b) and observed (c,d) MSC differentiation hierarchies. Caplan104 (a), and Aubin105 (b) postulated theoretical models of MSC differentiation, while Muraglia et al.106 (c) experimentally analyzed limiting dilution-derived CFU-F colonies for their multilineage potential to produce a hierarchy of MSC differentiation. These models illustrate how each respective author described the putative MSC producing theoretical (dashed circles and arrows) or observed (non-dashed circles and arrows) tri-, bi- or unipotential lineage-restricted progeny. These differ from the hierarchy described herein (d), which has shown definitive evidence of a cascade of sequentially more restricted progenitors producing CFU-F as their terminal lineage.

These findings are contrary to those postulated by Caplan104 and Aubin105.
as well as those observed by Muraglia et al.\textsuperscript{106} (Figure 5-3 a,b,c). Caplan’s proposed model\textsuperscript{104} described the differentiation of MSCs to produce an array of unipotential progenitors that are uniquely responsible for producing specific mesenchymal tissues. He further described adipogenesis having a distinct lineage from that of bone, cartilage, stroma and muscle. Aubin\textsuperscript{105} subsequently also postulated the steps of MSC differentiation, although she additionally suggested the possible existence of transitionary ‘bipotential’ progenitors. These osteo-adipo and osteo-chondro precursors purportedly produced bone as their default lineage, although Aubin suggested that due to their bipotential heritage, committed osteocalcin-expressing osteoprogenitors could “transdifferentiate” into adipose or cartilage. She further delineated muscle and fibroblastic lineages as distinct from bone, cartilage and adipose. Already, confusion began to appear in the MSC literature, as the exact lineage potential of MSCs was still undetermined; as well, the presence of ‘transitionary’ bipotential cells had yet to be shown. Addressing the first issue, emerging evidence\textsuperscript{114,242,251} at the time strongly indicated that bone, cartilage and adipose were the definitive terminal lineages of MSCs (see further discussion in 1.3.2).

Utilizing these three lineages, Muraglia et al.\textsuperscript{106} were the first to attempt to address the confusion surrounding MSC differentiation models by dissecting the differentiation potential of ‘clonal’ populations. As previously discussed (see 1.3.1), they used the definitive cloning assay of the time, limiting dilution, to isolate purported ‘clones,’ although they failed to determine the clonal purity of these ‘clones.’ They demonstrated that certain ‘clones’ exhibited tri- and bipotential capacity, and accordingly, this was the first evidence (although not definitive) of transitionary intermediate progenitors maintaining the ability to differentiate into more than a single lineage. Based on their evidence, they further suggested that bone is the terminal lineage of MSC differentiation, and in the absence of other specific lineage-inducing signals, MSCs will produce bone as their default phenotype.

In contrast to these three models, here it has been definitively shown that transitionary progenitors do exist along the differentiation pathway from MSC to
end mesenchymal phenotype. Importantly, it has been illustrated herein that all of these progenitors self-renew in culture and produce more restricted progeny according to a deterministic hierarchy. These restricted progenitors maintain strict lineage boundaries, and did not ‘transdifferentiate’ into any lineage other than to which they were already committed. Finally, in contrast to all previous work on MSCs, all five mesenchymal lineages were assayed herein, and a hierarchy of sequential forfeiture of myogenic, adipogenic, chondrogenic and osteogenic lineages, led to formation of the default fibroblastic lineage. Importantly, the default fibroblastic lineage observed here does not delineate all the other fibroblastic lineage potentials before complete termination of this pathway. For example, as with fibrous stroma, tendon and ligament are organized fibrous tissues. While the specific pathways leading to differentiation of these two specialized fibrous tissues have not been analyzed here, based on the hierarchy illustrated, it is possible that their precursors are more restricted than CFU-OFs, but less restricted than CFU-Fs. Rigorous analysis of clonal CFU-F populations with possible differential capacity to give rise to other specialized fibrous tissues will reveal further complexity to the hierarchy illustrated herein.

By assaying five mesenchymal lineages, the data illustrated here has demonstrated that the greater the potential a cell has for differentiation, the rarer it is within the mesenchymal compartment. The hierarchy produced provides insight into the development and relationships between the mesenchymal connective tissues. It has been observed herein that as the muscle lineage is the first to be forfeit in culture, this follows mesenchymal development in which the paraxial mesoderm gives rise to initially identical somites that specify into three regions. The dermamyotome and myotome give rise to skeletal muscles of the back, body wall and limbs, while the sclerotome produces the cartilage cells of the vertebrae and ribs that subsequently calcify and form bone. This follows the hierarchy in which initial divergence of the myogenic lineage is succeeded by forfeiture of the adipogenic lineage leaving a tri-potential CFU-FOC progenitor that gives rise to the cartilage, bone and marrow stroma. Sequential loss of the chondrogenic and osteogenic lineages inevitably produce the default fibroblastic
lineage observed in adult marrow stroma, the stromal tissue of the other organs of the body and many solid tumors.\textsuperscript{148,252}

To date, elucidating the biology of MSCs has been challenging because of the low frequency of MSCs within the heterogeneous BM population. By using a more homogeneous source of primary mesenchymal cells, HUCPVCs, it has been possible to dissect how an MSC differentiates, without the confounding effects of the variety of other stem cells, progenitors and terminally differentiated cells present in marrow. Here, it has been shown that populations of HUCPVCs that include multipotent MSCs coordinate tissue repair in response to injury. Clonal multi-potent parents give rise to a clonal multi-potent descendants, satisfying the two properties of stem cells that are required for maintenance of a mesenchymal environment: self-renewal and differentiation. The ability of these self-renewing progenitors to differentiate along multiple pathways becomes more restricted with the loss of the myogenic, adipogenic, chondrogenic, osteogenic and fibroblastic progenitor lineages respectively, illustrating the hierarchical mechanism of MSC fate decisions.

\subsection*{Future applications of HUCPVCs}

Described herein is a population of multipotential \textit{mesenchymal stem cells} that produce multiple mesenchymal tissues \textit{in vivo}, and self-renew and differentiate according to a deterministic hierarchy \textit{in vitro}. Due to the high clonogenic frequency within HUCPVC populations, it was possible to assay the self-renewal properties of these cells by deriving definitive SCD populations in culture that could be interrogated for \textit{in vitro} multipotential capacity. Importantly, while these data only illustrate the \textit{in vitro} self-renewal capacity of MSCs, the novel methods described herein make possible assaying clonal self-renewal of MSCs \textit{in vivo} that has hitherto been refractory to experimental evidence.

In order to assay MSC self-renewal properties \textit{in vivo}, HUCPVCs should first be permanently labeled with eGFP at a low moiety of infection so as to limit the number of genomic insertions. Clonal eGFP-labeled MSC populations can then be rigorously isolated by the methods described herein. Once expanded to
~10^6 cells, a small aliquot of these clones can be harvested for analysis of clonal purity by Southern blotting. Ideally, one would select clonal populations with only single insertions, although multiple insertion-clones could be analyzed by digestion with two or more restriction enzymes. Having determined the clonal purity of the populations, each clone can be individually implanted into multiple mesenchymal tissue sites such as the bone marrow and tibialis anterior muscle of NOD/scid mice. After 2 weeks, the mice can then be assayed for repair of their respective tissues to determine the multipotential capacity of each clone. In order to determine self-renewal, a parallel cohort of mice should be transplanted with the same set of clones (i.e. each clone is transplanted into 2 mice). After a given period of time (2 weeks for short-term repopulation analysis, or 12-16 weeks for long-term repopulation analysis), the transplanted tissues can be harvested and both sorted and unsorted eGFP cells transplanted into the same mesenchymal tissues of a new cohort of mice. After 2 weeks, these secondary recipients should be analyzed for repair of their respective mesenchymal tissues, and compared to the repair observed in the primary cohort. If any clonal populations produced the same repair in both primary and secondary mice, this would be the first definitive evidence of self-renewal in vivo, and thus, the stem cell nature of MSCs would be confirmed. Assuming this evidence can be shown, the next question would be what number of cells are required for repair, and whether fewer cells from clones with greater differentiation potential repair tissues at the same rate as a larger number of cells from more restricted clones. Having determined the capacity of each clone in the experiment described above, the cells can then be transplanted at limiting dilutions to observe what numbers of cells repair what variety of tissues. In this case, if an ACOF clone is observed producing large amounts of bone, but limited cartilage and adipose, it would then begin to delineate the frequencies of more restricted progenitors as compared to less restricted ones.

A significant finding of the work described herein is the default fibroblast stromal tissue generation capacity of HUCPVCs. Accordingly, it would be important to assay how this stromal tissue can be used to support other
differentiated cell types. Several investigators have tried co-culturing BM-derived MPCs with HSCs in order to facilitate ex vivo expansion of the HSC population\textsuperscript{143,251,253,254}, as well as co-infusion of both cell types in order to improve engraftment of HSCs \textit{in vivo}\textsuperscript{48,202} with varying results. As it has recently been shown that osteogenic cells regulate HSC self-renewal and differentiation in a ‘niche’ dependent manner\textsuperscript{24,255,256}, it is possible that the osteogenic differentiation capacity of HUCPVCs may facilitate the formation of an \textit{in vitro} HSC ‘niche.’ As well, tissue engineering of liver and kidney require management of a large stromal population to support the ex vivo expansion of functional parenchymal cells. It would be useful to assay whether HUCPVCs can support hepatocytes \textit{in vitro}, first in two-dimensional cultures, and if successful, in three-dimensional (3-D) cultures. Given their perivascular provenance, it is possible that, in combination with endothelial cells, HUCPVCs could also be used to create the smooth muscle layer of de novo vasculature for such 3-D tissue engineering applications. While these applications are a long way from being realized, HUCPVCs can continue to be used to address more fundamental questions.

For example, another important application of HUCPVCs would be to interrogate the exact geographic provenance of the MSCs isolated and analyzed herein. As previously mentioned (see 1.3.3.6), it is believed that MPCs are located in the perivascular compartment. Using thin (~50-200\(\mu\)m) cross sections of umbilical cords or vessels that are extracted from the cords prior to digestion, these sections could be cultured under osteogenic conditions \textit{in vitro}. In the event that osteogenic calcification occurs within these sections, this would be evidence of the location of osteogenic precursors. It is proposed here that if this calcification were to occur, it would either be in the perivascular space or in the sub-endothelial region of the vessels. Accordingly, one could use micro-dissection to excise these very specific regions for further analysis of MSC properties.

It has also been shown herein that HUCPVCs can be relatively easily transduced with eGFP using a lentiviral system. This transduction strategy can
be further developed to insert functional mesenchymal genes such as Runx2 or Sox9 to improve osteogenic or chondrogenic differentiation respectively. These strategies have been predominantly used for gene therapy applications to replace/regenerate defective tissues; although, they have been shown to be dangerous due to the tumor-forming properties of the mutated cells\textsuperscript{257}. As HUCPVCs can be relatively easily cloned in culture, post-transduced cells could be isolated to form clonally-pure populations. Each population could then be screened for tumorigenic capacity, and only populations that neither form tumors, nor silence the inserted gene, may be used safely for gene therapy applications.

Other applications for the use of HUCPVCs have been described above (see 5.2). Since only 3 cells need to be seeded to derive a CFU-F colony, HUCPVCs can be used to interrogate the mechanics of colony formation. As well, it is possible investigate the effects of different ‘niche’ conditions on colony formation. These data would provide significant insight into the conditions governing the fate of MSCs and their progeny.

5.6. Conclusions

From the work represented here, it can be concluded that the human perivascular compartment contains a population of multi-potent mesenchymal stem cells that clonally self-renew and differentiate according to a deterministic hierarchy in culture, and that upon injury, reestablish the homeostatic balance of their microenvironment.
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Appendix A

Lentiviral Transfection Protocol

Day 1:
• Passage 293T cells and seed in T75(s) with 12 ml DMEM (10% FBS) so that they are 60-70% confluent on day 3.

Day 3:
• Make up transfection mix:
  o For every T75 to be transfected, place:
    ▪ 15µg of GFP vector DNA
    ▪ 10µg of GagPol expressing plasmid
    ▪ 10µg of Rev expressing plasmid
    ▪ 10µg of Tat expressing plasmid
    ▪ 5µg of Vsv-G expressing plasmid
    ▪ and make up the remainder with dH2O so that the total volume is 450µL.
  o Add 50µL of 2.5M CaCl2 (for a total volume of 500µL per T75) and mix gently by pipetting up and down.
• Add above DNA mixture dropwise into 500µL (per flask) of 2X HeBS buffer using a P1000 Gilson pipettor with barrier tips. To ensure proper mixing, use a 1ml automatic pipettor to make bubbles in the HeBS while the DNA is being added.
• Vortex for 5 seconds.
• Incubate at room temperature for 20min. An evenly distributed precipitate should form.
• Remove old media from 293Ts. Add 9ml DMEM (10% FBS) per flask.
• Add transfection mix dropwise onto 293T media (1ml/flask). Mix gently.
• Incubate at 37°C overnight.

Day 4:
• Gently remove media and wash once with PBS.
• Discard media and PBS directly into bleach in the hood.
• Add 7.5ml DMEM (10% FBS) and incubate at 37°C for 3 more days.

Day 7:
• Collect supernatant off the cells.
• If cells are loose, spin at 700rpm for 5min to remove cells and debris.
• Pass supernatant through a 0.45 micron filter.
• Supernatant can be stored at -80°C.
• Concentrate vector by ultracentrifugation @25,000 rpm for 2 hours.
• Pour off supernatant and resuspend EACH pellet in 100ml DMEM.
• Allow pellets to dissolve overnight at 4°C.

Day 8:

• Transduce target cells in a 35mm dish with a total volume of 0.5ml viral supernatant for 5 hours
• Should run a titration of viral concentrations to determine optimal transduction concentration.
• After 5 hours add a further 1.5ml DMEM (10%FBS).
• Incubate for two days at 37°C.

Day 10:

• Remove supernatant from transduced cells and wash three times with PBS
• Cells are now safe to remove from virus quarantine area.
• Cells should be observed over next few days for presence of eGFP.
Appendix B

FISH protocol

FISH using VYSIS probes

REAGENTS

• DAPI II: Vysis 32-804831
• Formamide: Fisher #BP227500
• NP-40: Vysis #32-804818
• 20xSSC: Vysis #32-804850
• Probes (make sure at all times to minimize light exposure)

SOLUTIONS NEEDED

20xSSC
2xSSC (pH to 7.0!)
0.4xSSC/ 0.3% NP-40 (pH 7.0-7.5)
2xSSC/ 0.1% NP-40 (pH 7.0-7.5)

Denaturant solution: 49 ml Formamide, 7 ml 20xSSC, 14 ml dH₂O, pH to 7.0-8.0, store at 4°C

Ethanol Wash Solutions: 70%, 85%, 100%

PROTOCOL

• Prepare metaphase spreads on slides as outlined in a separate protocol. Don’t stain with Giemsa. Store slides covered with sealwrap at room temperature.

PRE-PREPARATION

• Incubate slides for 30 min in 2xSSC (pH 7.0) prewarmed to 37°C in a coplin jar (always, always, always do 4 slides at a time in the coplin jar, if you only have 2 slides, add another 2 blank slides)
• Dehydrate sequentially in 70%, 85% and 100 % ethanol series, 2 min each. Air dry.

PROBE PREPARATION

• 7 µl buffer (comes together with Vysis probes)
• 1 µl dH₂O
• 1 µl of each probe, for example CEP6 SpectrumGreen probe and CEP17 SpectrumOrange probe
• Centrifuge 1-3 seconds
• Vortex
• Recentrifuge
• Heat for 5 min. at 73 °C in a water bath to denature (N.B. Always set water bath 2˚C higher and measure temperature inside coplin jar)
• Use immediately (or keep for a short while longer at 73 °C if required)

SLIDE PREPARATION
• Place denaturant solution (70% formamide/ 2xSSC) in 73 °C water bath inside coplin jar (make sure temperature inside is correct, it may take 30 min to reach correct temperature)
• Denature slide for 5 min. – Immerse no more than four slides at a time
• Dehydrate in 70%, 85% and 100% ethanol for 2 min. each. Airdry.
• Apply 10 µl denatured probe and cover with a cover glass. Mark hybridizing area on the slide using a diamond scribe. Seal carefully with rubber cement.
• Place slides in a prewarmed humidified box (wrapped in metal foil to protect against light) and incubate overnight at 42˚C (you may use a hybridization oven).

RAPID WASH PROCEDURE
• Place 0.4xSSC/ 0.3% NP-40 in a 73°C water bath
• Remove cover glass and immediately place into wash tank with 0.4xSSC/ 0.3% NP-40
• Leave all slides in coplin jar for 2 min.
• Place slides in 2xSSC/ 0.1% NP-40 at room temperature 1 min.
• Airdry slides in darkness
• Apply 20 µl of DAPI solution (Vysis product) to the target area and put on cover glass (make sure it covers hybridized area).
• Examine slides on a fluorescence microscope