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Characterization of Stromal Cells Derived from Bone Marrow, Dermis, and Myocardium

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ABSTRACT

Stromal cells originate from a variety of tissues and may have possible therapeutic applications in tissue repair in myocardial infarcts. This project investigated the relationships between human bone marrow-derived stromal cells (BM/MSC), dermis-derived stromal cells, and cardiac-derived stromal cells by *in vitro* characterization for surface markers, secretory profiles, differentiation capacity, and DNA methylation patterns. Cell characterization is important for cell therapies and it helps to better understand the functions of these cell types and the mechanism of actions.

Following the guideline of the International Society for Cell Therapy (ISCT) for Mesenchymal Stem/Stromal Cell (MSC) definition, we compared the expression of 7 minimal, and 11 additional surface markers for all these cell types. Most of these markers displayed identical expression for all cell types, while two markers stro-1 and CD146 showed high variability, related to the donors and passage numbers than to the cell strain. The secreted protein profile of all four cell types was also observed to be similar. Although differentiation tests indicated some differences between the strains, these assays are very sensitive to culture conditions and therefore have limitations. The DNA methylation footprint is characteristic and well conserved between different tissues, and is generally less sensitive to donor source and culture conditions. Here we demonstrate the application of this novel methylation footprint technique for cell characterization. In contrast with other methods, methylation analysis indicated that BM/MSC and DF are different from each other in two methylation markers. Cardiac stromal cells, both atrial and ventricular, have similar methylation patterns to DF, regarding the selected genes. Whether the differences between BM/MSC vs. DF and cardiac stromal cells at these methylation points reflect the solid tissue origin of these cells, or whether there are additional differences in the genome for methylation points between DF and cardiac stromal cells or between atrial and ventricular stromal cells are not known from this study. However, our study demonstrated that the methylation analysis could be a useful assay to delineate the relationship of stromal cells and fibroblasts as well as their relation in tissue origin. Future experiments will investigate the importance of altered methylation patterns with *in vivo* functions in myocardial infract repair.

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BACKGROUND

Cell Therapy for Myocardial Infarctions

An acute myocardial infarction (MI) occurs when there is a blockage of oxygen to the heart. This lack of oxygen quickly causes damage and necrosis of the surrounding heart tissue. Current treatments for this disease are coronary bypass surgery and a number of drugs such as statins, ACE inhibitors, and beta blockers. However, more novel techniques, such as cell therapy, are being developed to treat this disorder. Cell therapy is being researched to alleviate the after effects on damaged tissue from an acute myocardial infarction.

The theory behind cell based therapeutics is that by introducing cells into diseased tissue, they will have the ability to influence its healing and regeneration. Cell therapy is described as "a strategy aimed at replacing, repairing, or enhancing the biological function of a damaged tissue or system by means of autologous or allogeneic cells" (Bordignon et al., 1999). The cells used for therapy should have a great regenerative capacity much like stem cells, which can differentiate into multiple lineages. One of the first cell types investigated for use in cell transplantation was skeletal myoblasts (SkM) because of their structural resemblance to cardiac myocytes. The belief was that the SkM cells could integrate into the structure of the myocardium and improve cardiac function. However, *in vivo* data indicated only varying success for this therapy. This resulted possibly because of the lack of the cells' ability to fully integrate into the structural myocardium (Lyon and Harding, 2007).

There has also been a focus on two important groups of cells - embryonic and adult stem cells. The benefit of embryonic stem (ES) cells is in their totipotency. These

primitive cells, under optimal conditions, could differentiate into many cell types including cardiac myocytes. Certain animal models have already shown improved cardiac function after a myocardial infarction using ES cell transplants (Menard et al., 2005; Singla et al., 2006). Because of the multitude of ethical reasons, ES cell research in humans is still in its infancy and will have many obstacles to overcome before it will be advanced. Appealing replacements to ES cells are the adult stem (AS) cell populations that remain in the body post embryonic development. AS cells are found in less frequency, but still have the ability to be self-renewing and multipotent. One population of AS cells that are currently being researched is mesenchymal stem/stromal cells (MSC). MSCs are capable of differentiation into multiple lineages and are even believed to be immuno-tolerant (Pittenger et al., 1999; Ryan et al., 2005).

MSC transplantations are attractive because the cells secrete a number of factors that either help build and maintain the extracellular matrix or mitigate inflammation and the associated cell damage from an infarct. Furthermore, genetic modification of MSCs is also possible, leading to combined genetic and cell therapies. These modifications could target vasculogenesis, resistance to apoptosis, and an enhanced ability to recruit stem cells.

Cells for therapy, in general, to treat a myocardial infarct could come from three possible sources. The first would be an autologous cell transplant, where the cells would originate from the patient. An autologous transplant would bypass the harmful effects of graft versus host disease (GVHD), yet the difficulties of extracting cells from a patient, and then attempting to isolate and modify them *in vitro* are likely to remain significant obstacles. An allogeneic cell transplant is a transplantation of cells which are coming

from another human donor. This situation presents a beneficial scenario because cells could be ready as the patient needs the treatment, whereas an autologous treatment may take weeks to culture and modify the cells appropriately. However, the disadvantages of an allogeneic treatment would be the need to maintain the cells on hand as well as the potential for GVHD. Lastly, and probably the least likely, is a xenogeneic transplantation of cells coming from another species. This would present an increased probability of a negative immune reaction.

The choice of what cell to use is one of the most critical aspects of this type of therapy. Cells need to be multipotent, yet not in such a way that they could become tumorigenic. Cells also need to produce the right kind of proteins and soluble factors that would positively influence repair, yet not trigger the patient's immune system.

Bone Marrow as a Source of Adult Stem Cells

Bone marrow is responsible for generating the hematopoietic, endothelial, and stromal cell populations throughout the body. Hematopoietic cells are the progenitors that produce all cells of the blood system, including erythroid, lymphoid, and myeloid cells. Endothelial cells comprise the tissue responsible for the epithelium of blood vessels and endocardium of the heart. Stromal cells function in cooperation with the bone marrow and various other cell populations. They have a crucial role in maintaining hematopoiesis by supporting the hematopoietic environment; they comprise the connective tissue of several organs, support the parenchymal cells of an organ, and consist of progenitor cells that have a capacity to differentiate into many different cell lineages.

As a part of their function to support neighboring environments stromal cells *in vivo* have been reported to differentiate into macrophages, adipocytes, osteogenic cells, and reticular cells (Deans and Moseley, 2000). The *in vivo* relationship of these cells greatly depends on the developmental stage of the bone marrow and the current state of the hematopoietic environment (Deans and Moseley, 2000). It has been discovered, however, that the plasticity of these cells depends largely on a single cell population in the larger heterogeneous one (Pittenger et al., 1999). This population, named mesenchymal stem/stromal cells (MSCs), is believed to proliferate undifferentiated, as well as differentiate into the aforementioned lineages. This fraction of the larger stromal cell population plays a critical role in the development and maintenance of the bone marrow, and therefore could play a similar support role in other tissues. The mesengenic process, beginning with the MSC, is detailed in Figure 1.

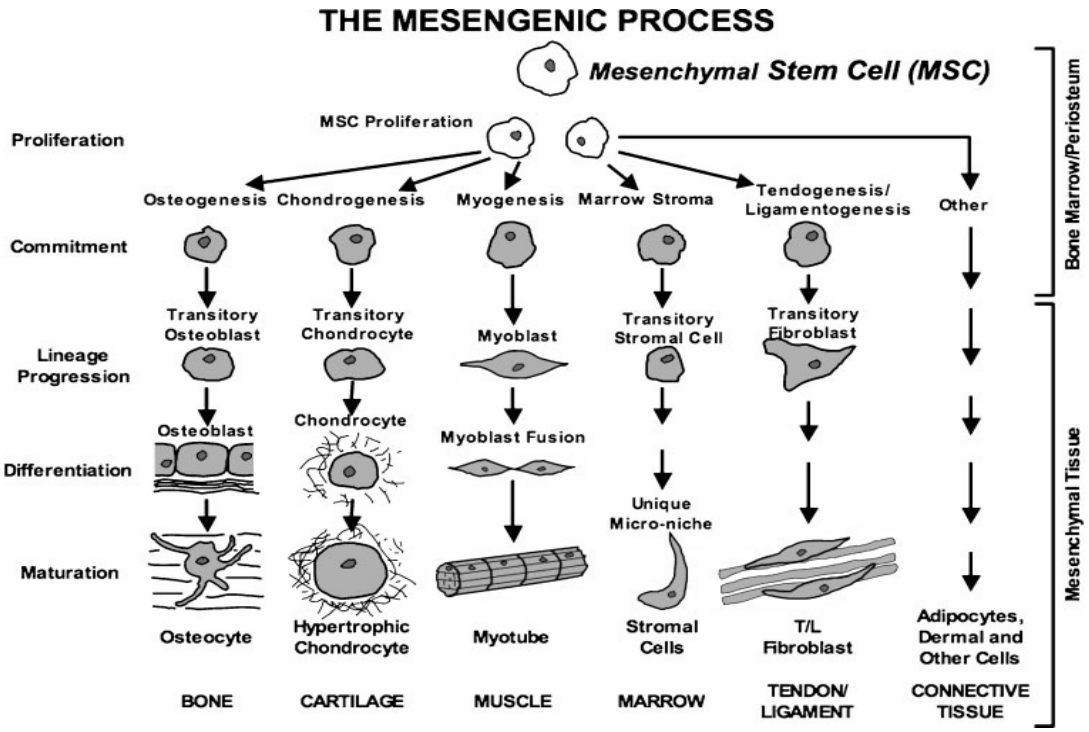

Figure 1. The Mesengenic Process. Figure from Dennis JE, Caplan AI (2006) Mesenchymal Stem Cells as Trophic Mediators. *Journal of Cellular Biochemistry* **98**: 1076-1084.

Mesenchymal Stem/Stromal Cells

Depending on the publication, the terms “stem cell” and “stromal cell” have been used interchangeably in naming mesenchymal stem/stromal cells (MSC). MSCs were first defined as a part of the heterogeneous bone marrow population without a separate designation, and were initially thought to only be responsible for supporting hematopoiesis (Friedenstein et al., 1976). This is due to the fact that MSCs *in vivo* represent only a minute fraction, approximately 0.0001%, of the nucleated cell population in the bone marrow (Barry, 2003). In 1991, Simmons and Torok-Storb used a novel antibody (stro-1) to define a population of stromal cell precursors that are not hematopoietic progenitors (Simmons and Torok-Storb, 1991). However, the use of stro-1 as a primary marker of MSCs was not widely accepted. A stromal cell progenitor, also considered a mesenchymal progenitor, holds great interest in terms of therapy because of the wide range of tissues that it influences.

MSCs however, have been difficult to define through a traditional and absolute set of characteristics. The International Society for Cellular Therapy (ISCT) published a position statement to begin the official definition of an MSC. These characteristics include three minimal criteria: the ability to propagate as an attached monolayer *in vitro*; positive expression of CD105, CD73, and CD90, and negative expression of CD45, CD34, CD14 (or CD11b), CD79α (or CD19), and HLA-DR; and *in vitro* differentiation into adipocytes, osteoblasts, and chondrocytes (Dominici et al., 2006). Each of these criterions taken separately is not considered conclusive characterization to qualify a cell as an MSC.

MSCs have been isolated in many places from the body, but most notably from the bone marrow. Aspirates, usually from the pelvis or the iliac crest, are exposed to a gradient where the mononuclear cells are isolated and selected for by plastic adherence. MSCs can also be isolated from a variety of places such as liver, lung, spleen (Anker et al., 2003), cord blood (Liu and Hwang, 2005), adiposus tissue (Im et al., 2005), synovial tissue (Giurea et al., 2006), dental pulp (Yang et al., 2007). However, it is difficult to establish whether all of these populations derived from different tissues are similar, even though they uphold the three minimal ISCT MSC criteria.

Although their differentiation capabilities were believed to be their primary mechanism in repair, later studies suggested that it is their ability to secrete soluble factors and alter the tissue microenvironment that represents their primary mechanism (Phinney and Prockop, 2007). MSCs use these soluble factors to mediate wound healing through different roles including recruitment of stem cells, supporting angiogenesis and in intervening with apoptosis. MSCs, by secreting SDF-1, interact with the surface receptor CXCR4 to mobilize and recruit stem cells as well enhance the survival and growth of cells expressing the CXCR4 receptor (Zhang et al., 2007). The release of growth factors such as VEGF has been shown to mediate angiogenesis and vasculogenesis by regulating blood vessel wall assembly (Ball et al., 2007). MSCs also secrete proteins involved in extracellular matrix metabolism (for example MMP and TIMP family proteins) which is necessary for angiogenesis. MSCs can express a number of important cytokine and chemokine receptors, such as CCR7, CCR9, CXCR5, and CXCR6 that assist in leukocyte trafficking and cell compartmentalization (Honczarenko et al., 2006). Cell compartmentalization has been shown to be important in maintaining

the framework for micro-environmental niches (such as the niche for regulation of hematopoiesis *in vivo*) as well as for responding to stress situations in the body (Gupta et al., 1998).

Suppression of inflammation and immunomodulation are other key mechanisms by which MSCs can be useful for therapy. MSCs are reported to inhibit or limit inflammation by suppressing the production of inflammatory cytokines such as TNF-α, IL-6, and IL-8 (Guo et al., 2007). They also are reported to suppress T cell proliferation via the secretion of cytokines such as IL-10 and TGF-β (Di Nicola et al., 2002). MSCs may also be able to halt the maturation of dendritic cells leading to an immunologic tolerant state (Zhang et al., 2004). Immunomodulation is important primarily for two reasons: for immune tolerance of cells during therapy and in changing the immune reaction evoked by the disease. Both of these characteristics are essential for cell therapy in myocardial infarct repair to be successful without raising a harmful immune response in the patient.

Considering the roles that MSCs can play, they have become a prime target for therapeutic testing. Their current and proposed uses for regeneration include a wide range of conditions. MSCs have already been found to be useful in bone diseases such as osteogenesis imperfecta where the bone makes insufficient amounts of type I collagen. Researchers used culture-expanded MSCs after chemotherapy and a fresh bone marrow transplant to enhance the effectiveness and longevity of the growth rate of new bone (Horwitz et al., 2002). There have also been promising trials in animal models using both allogeneic and xenogeneic transplants. These include cerebral ischemia, pulmonary

fibrosis, autoimmune encephalomyelitis, nephropathy, engraftment for reduction of tumors, lung injuries, and myocardial infarctions (Chamberlain et al., 2007).

Reports in the literature suggest that even though unmodified MSCs have promise, they are more effective when modified. In cell therapy for myocardial infarcts, Akt-modified MSCs have shown enhanced activity when compared to unmodified MSCs in ventricular remodeling and in restoring cardiac function (Mangi et al., 2003). Akt1 is a serine threonine kinase that has been shown to be a powerful survival signal in many cellular systems (Datta et al., 1999). Up-regulating the CXCR4 receptor on MSCs using an adenoviral vector to over express the CXCR4 gene has also been shown to promote improved angiogenesis over normal MSCs (Zhang et al., 2007). These modifications are believed to stimulate better recruiting and engraftment abilities, enabling MSCs to survive in areas where conditions are hypoxic and scarring has begun.

Fibroblasts

MSCs, although distinct, have been shown to have a close relationship to other cell types *in vitro*, namely fibroblasts (Covas et al., 2008). The relationship, however, between MSCs and fibroblast-like cells has not been well characterized in the literature. In this MQP project, dermal fibroblasts (DF), or dermis-derived stromal cells, were used for comparison with MSCs to analyze *in vitro* similarities and differences. These fibroblasts originate from the lowest layer of skin consisting mostly of connective tissue, and are usually defined as mesenchymal-derived spindle-shaped cells that produce the major interstitial collagens - types I, II, III, and IV (Covas et al., 2008).

Fibroblast cells are important components of many types of tissue. They are responsible for upholding the extracellular matrix by providing the connective

nonfunctional frame for the tissue. These cells mainly function through cell-to-cell signaling and utilize secretory molecules to assemble the extracellular matrix, properties that are very similar to MSCs. Fibroblasts, like MSCs, can also be derived from a variety of tissues.

Similarities based on gene expression have been reported between different types of fibroblasts and between fibroblasts and MSCs. The connection has been found in several genes, such as VIM (intermediate filament protein family; responsible for the structural feature of cells), LGALS1 (galectin protein family; involved in cell-cell and cell-matrix interactions), MMP2 (matrix metalloproteinase family; involved in breaking down the extracellular matrix), TAGLN2 (early marker of differentiated smooth muscle), ANXA2 (annexin protein family; implicated in cell growth regulation as well as signal transduction pathways) (Covas et al., 2008). According to the study, this indicates a close biological relationship between these cells, supporting morphological as well as functional data.

Since MSCs and fibroblasts have a number of similar gene expression characteristics as well as both potentially being able to influence the tissue framework during wound healing, it is important to gain a better understanding between these two cell types. Also, given that fibroblasts are similar to MSCs on a genetic and morphological level *in vitro*, it is reasonable to suspect that they too may be candidates for cell based therapies.

Cardiac Stromal Cells

The third cell type that is of particular interest in this study is cardiac-derived stromal cells. These cells have become an interest because of the important role they play

in the adult myocardium during typical development and in diseased states. The myocardium of an adult human is comprised of a large number of cells: cardiac myocytes, non-myocytes and the extracellular matrix by which they are encompassed (Baudino et al., 2006). Myocytes are the most abundant individual cell type because they are responsible for composing the muscle that pumps blood into and out of the heart.

However, the non-myocytic cells in the myocardium are also critical for normal functioning. These cells include mast cells, endothelial cells, and stromal cells (Baudino et al., 2006). Stromal cells of the heart are becoming more significant because of their known connection with the extracellular matrix. Recent literature has also suggested that the role of these cells may be even broader than just matrix remodeling as they could have a sentinel role in mediating mechanical, electrical, and chemical signals for the "global myocardial response" (Baudino et al., 2006).

Due to their role in extracellular matrix synthesis, cardiac stromal cells are targets because of wound healing. Typically, injury to the myocardium induces hemostasis, infiltration of immune cells, phagocytosis of necrotic cells and cellular debris, repopulation of inflammatory repair cells within the zone of injury by chemotaxis, followed by increased proliferation and subsequent matrix remodeling (Brown et al., 2005). This makes cardiac stromal cells heavily involved and highly important in the stable and functional regeneration of the myocardium following an infarct, and it is therefore important that they become well characterized.

Cardiovascular Cell Biology: The Genzyme Lab

The research aim for the Cardiovascular Cell Biology Laboratory at Genzyme Corporation (Framingham, MA) is to address, through cell therapy, myocardial infarcts.

Infarcts pose a major threat to the survival of the heart because the lack of oxygen forces the tissue to become necrotic or apoptotic. Even with appropriate medical care, those who suffer from a myocardial infarct may still die. The hope is that MSC treatment may improve the function of the infarcted heart.

As stated above, MSCs possess certain characteristics and biological behaviors that make them attractive for use in myocardial repair following MI. This includes their hypothesized ability not to provoke an immunogenic response, their ability to secrete soluble factors, and their reported potential to differentiate into several tissue types. To analyze whether MSCs, and in particular bone marrow-derived stromal cells (BM/MSC), would be a good candidate for cell therapy, this lab has undertaken many experiments. A variety of techniques have been used to isolate cells needed for research, as well as to characterize them to maintain positive identification through several passages. More in-depth studies have used animal models to analyze the effects *in vivo*.

Even though MSCs are currently a main target cell for therapy, the group has and will continue to look for additional candidates to reach the goal of improving heart function following an infarct. Previous studies have looked at skeletal muscle derived myoblasts for therapy because of their structural and functional similarities to cardiomyocytes. Future studies will explore the possible use of other cells that may be better suited for the task. This may consist of using adult cardiac stem cells, or MSCs that have been modified. Modifications would aim at increasing the soluble factors present in the infarcted area, and increasing the longevity of the transplanted cells.

PROJECT PURPOSE

Stromal cells are known to originate from a variety of tissues, and may have possible therapeutic applications in tissue repair. Although stromal cells grow as an attached monolayer *in vitro* under similar culture conditions as MSCs, and have a high secretory capacity, there is not a clear single set of characteristics that can definitively identify these cells based on propagation *ex vivo*. Thus it is uncertain whether these cells have a collective identity as one specific cell type or whether their tissue origin determines cell identity.

In order to better understand the stromal connection to MSCs the *in vitro* relationship of bone marrow-derived stromal cells, dermal fibroblasts, and cardiac-derived stromal cells, were studied. These cells were characterized through cell surface markers, specific secretory profile in culture medium, stimulation with molecules in culture, differentiation capacity, and DNA methylation patterns. The importance of finding an efficient and stable way of characterizing cells is to better explain the functional differences observed for similar cell types of different origin (stromal cells or fibroblasts) and for the same cell type exposed to different conditions (such as diseases or injuries).

METHODS

Isolation and Expansion of Human Cells

Bone marrow-derived MSCs were obtained from consenting healthy human adult donors from a commercial source. Fresh bone marrow was diluted with EDTA and isolated through a Ficoll density gradient by centrifuging the cells at 2000rpm for 20 minutes. The mononuclear fraction was removed and lysed with red blood cell lysis buffer. After a 3 minute incubation with lysis buffer, remaining intact cells were centrifuged at 450 x *g* for 10-12 minutes and were plated on plastic surface at a seeding density of approximately 6.0 x 10^5 cells/cm^2. The media was replaced the next day to remove all non-adhering cells, and regular media exchanges took place every other day thereafter. BM/MSCs were maintained in MSC culture medium: DMEM (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 µg/mL Gentamicin (Invitrogen), and 1µg/mL Amphotericin B (Invitrogen). Cells were then subcultured every 7-10 days by washing with PBS, incubating with 0.25% Trypsin (Gibco, Invitrogen), diluted with the defined culture medium, centrifuged at 1200rpm for 5 minutes, and re-plated at a seeding density of 5,000 cells/cm^2.

Dermal fibroblasts were purchased from a commercial source. Cells were plated according to the manufacturer, and then maintained in the same culture medium used for BM/MSCs. Atrial and ventricle cardiac cells were isolated from human heart tissue after the tissue was minced and digested with collagen and trypsin. The tissue was obtained from consenting adults that had undergone coronary bypass surgery. Both cardiac cell types were maintained in the same media used for culturing BM/MSCs. Pre-adipocytes and osteoblasts were purchased from a commercial source. Cells were received growing

in flask and were maintained according the manufacturers' instructions. All characterization and experimental protocols were performed on cells in passages 3 through 5.

Flow Cytometry

Analysis of cell surface antigen expression on all cell types was performed on freshly harvested cells using the panel of fluorochrome-labeled monoclonal antibodies found in Table 1 in the Results. Cells were incubated with each antibody at the concentration, temperature and time suggested by the manufacturer's instructions. Non-specific fluorescence was determined by incubating with the appropriate isotype matched monoclonal antibody. Data were analyzed by collecting 10,000 events on a Becton Dickinson FACSCalibur system (BD Biosciences) using Cell-Quest Pro software.

Protein Secretion Measurement

The Human Cytokine LINCO*plex* Kit (Linco Research, Millipore) was used in combination with the Luminex 200 Total System (Luminex Corporation) to measure the secreted cytokines and chemokines in the culture media after conditioning with exponentially growing cells for 48 hours. The 21-plex kit was conducted in accordance with the manufacturer's protocol. Cell supernatant was collected from exponentially growing cultures in passage 4, and frozen prior to use in the multiplex kit. Briefly, samples were thawed and assayed without dilutions. The 96-well plate including the background, standards, controls and samples were incubated with a solution with beads containing the cytokines investigated, which can be found in Table 3 in the Results. After an overnight incubation, the plate was incubated with a cocktail of detection antibodies

and then a streptavidin-phycoerythrin solution. The plate was then run on a Luminex instrument.

The cell supernatant was also sent for SearchLight service testing (Pierce Biotechnology, Inc.) to quantitate growth factors present in the culture media after conditioning with exponentially growing cells for 48 hours.

Adipogenic Differentiation

Cells in passage 4 were grown to a confluent density in 24 well plates, and incubated with adipogenic media (see below), with a media change every 2-3 days per manufacturers' instructions. Adipogenic media consisted of 90% low glucose DMEM, 10% FBS, 1 μM Dexamethasone, 0.5 mM IBMX, 10 μg/mL Insulin, 100 μM Indomethacin, and 50 μg/mL Gentamicin (Chemicon International) as suggested by the kits' supplier. After 3-4 weeks of culture, cells were stained with Oil Red O solution to confirm the presence of lipid droplets.

For the quantitative analysis of differentiation, photographs were taken, with a Nikon camera, of five randomly selected fields of the culture dish. These pictures were subsequently analyzed using MetaMorph software program.

Chondrogenic Differentiation

Cells in passage 4 were harvested and re-suspended at a concentration of 1.25 x 10^6 cells/mL in 1X CLC ACG Media containing 95.5% high glucose DMEM with L-glutamine and HEPES buffer (Gibco, Invitrogen), 2.0% FBS, 0.4% 250X Cholesterol Lipid Concentrate (Invitrogen), 2.0% Insulin-Transferrin-Selenium-X (Invitrogen), 5 ng/mL TGF-β2 (R&D Systems), and 100 μg/mL Ascorbate (Wako Chemicals). After re-

suspension, 200 µL of cells were added to each well of a 96-well V-bottomed plate (Matrix Technologies) using a multi-channel pipette. The plate was centrifuged for five minutes at 500 x *g* and incubated for 24 hours. After the centrifugation, cells were re-suspended from the pellet through gentle aspiration and release of media into the well. Complete media changes occurred every 2-3 days. After four weeks, pellets were removed, placed in cassettes, fixed with 10% Neutral Buffered Formalin, micro-sectioned and were stained by histology for Toluidine Blue and Safranin-O. Typically three pellets (randomly selected) were used per slide for sectioning and staining.

Osteogenic Differentiation

Cells in passage 4 were grown to a confluent density in 24 well plates and incubated with osteogenic media (see below) with media changes every 2-3 days per manufacturers' instructions. Prior to growth in the well, plates were pre-coated with vitronectin and fibronectin. Osteogenic media consisted of 87% low glucose DMEM supplemented with L-glutamine, 10% FBS, 0.1 µM Dexamethasone, 0.2 mM Ascorbic Acid2-Phosphate Solution, 10 mM Glycerol 2-Phosphate Solution, and 50 µg/mL Gentamicin (Chemicon International) as suggested by the kits' supplier. After 14-17 days of culture, cells were stained with Alizarin Red Solution to confirm the presence of calcium deposits.

Quantitative analysis and photographing of differentiation were preformed similar to the adipogenic assay.

DNA Methylation Analysis

Cells were harvested and frozen following normal culturing methods. Genomic DNA was extracted from frozen cell pellets through the use of a DNeasy kit. The genomic DNA was sent to Genzyme Biosurgery (Genzyme Corp, Cambridge, MA) for DNA methylation analysis. Briefly, genomic DNA was bisulfite treated by a commercially available kit following the suggested protocol. A PCR assay was performed on the bisulfite modified DNA samples, using the same forward and reverse primers in combination with two different probes for each gene. These probes were designed according to the methylation sites, assuming that the DNA at the methylated cytosine sites is not sensitive to bisulfite treatment, but unmethylated cytosines will be converted to uracils (Figure 10 in Results), according to the QAMA method (Zeschnigk et al., 2004). The cycle times (Ct) were determined for both probes (Ct1 = methylated probe Ct, and Ct2 = unmethylated probe Ct) by amplification with the ABI 7500 Fast real-time PCR system. The ΔCt value (difference between the Ct values of the two probes) were calculated and converted to a percent using the formula: percent methylation (%M) = (1/R+1) x 100; where $R = (1+E)^{\Delta Ct}$, and E = PCR efficiency, and ΔCt = Ct1 - Ct2 (Lehmann et al., 2001). The PCR efficiency was assumed to be 1.0 (100% efficient) for all assays.

RESULTS

1. Cellular Morphology

Dermis-derived fibroblasts (DF) were obtained from commercial sources while bone marrow-derived mesenchymal stromal cells (BM/MSC) were isolated from healthy donors using the techniques mentioned in the Methods section. Cardiac cells from the atrium and ventricle were isolated from donors with a chronic illness and had undergone coronary bypass surgery. The cultivation of all four cell types was under the same media and culture conditions as defined by the International Society for Cellular Therapy (ISCT) and shown in the Methods section. The cells displayed similar cellular morphology as shown in Figure 2. DFs (Fig. 2A), atrial cells (Fig. 2C), and ventricle cells (Fig. 2D) exhibited typical fibroblastic morphology such as having a large spindle shaped body with branched cytoplasm and a circular shaped nucleus. BM/MSCs (Fig. 2B) also present characteristic fibroblast growth, aligned in parallel clusters, when the culture is more confluent and the cells are not allowed to expand and proliferate over large areas. The figures also illustrate healthy cells unstressed by culture conditions, age (passage number), and proliferative capacity as determined by strong adherence to the surface of the culture vessel and by the apparent interaction between neighboring cells. It was not the aim or purpose of the research to characterize the cultivation techniques of these particular cells, but a brief synopsis of cellular morphology and growth characteristics is necessary to understanding the resemblance between the cell types on a single cell level.

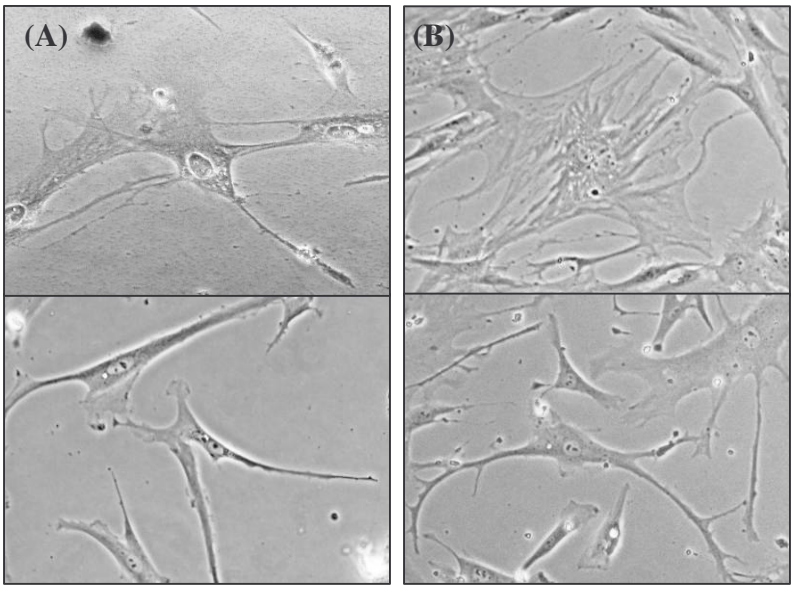

Figure 2. Cellular Morphology. Typical light microscope view (40X) of the four different cell types used in this project for research allowed to adhere to culture plates: ***(A)*** Dermal fibroblast (DF), ***(B)*** Bone marrow-derived mesenchymal stromal cells (BM/MSC), ***(C)*** Cardiac cell isolated from the atrium, and ***(D)*** Cardiac cell isolated from the ventricle. All cells are in passage 4. Photos were taken on day five of their respective passage, where each type was cultured with the seeding density of 5000cells/cm^2.

2. Surface Marker Characterization

The definition of an MSC has been organized by the ISCT, as described in the Background. Briefly, the selected minimal markers are positive surface expression of CD105, CD90, and CD73 and no surface expression of CD45, CD34, CD19, and CD14. The combination of these surface markers were chosen because they distinguish between type and function of cells (Dominici et al., 2006). The lack of expression of the four listed surface antigens is critical as they represent the cell types most likely found to be contaminating an MSC culture isolated from bone marrow (blood cells, macrophages, and B-cells). The best example of this is CD45 and CD34 expression which distinguishes hematopoietic cells from non-hematopoietic cells of the bone marrow (Dominici et al., 2006).

Due to the non-specific morphology and surface markers of the minimal criteria we further extended the analysis for functional differences. The use of additional markers followed to investigate whether these cell strains - BM/MSCs, DFs, and cardiac cells - could be distinguished based on a larger panel of markers. Some of the markers used, such as positive expression of CD44 and CD29, have been consistently used as additional MSC criteria markers (Falanga et al., 2007; Jo et al., 2007; Hou et al., 2007). In terms of functional characterization, the MHC proteins were analyzed to see if the cells would raise an immune response. Other examples include CD117 and CXCR4, which designate cell types that hold characteristics that are stem-like and smooth muscle actin expression, which distinguishes muscle cells that have become differentiated. All of the surface markers investigated is identified in Table 1, along with their cellular expression and known function.

Surface Antigen Characteristics			
	Ag	**Cellular Expression**	**Function**
ISCT MSC Positive Markers	***CD105***	Endothelial cells, activated monocytes and macrophages, BM cell subsets	Binds TGF-β
	CD90	Endothelial cells, smooth muscle cells	*Unknown*
	CD73	B- and T-cell subsets	Ecto-5'-nucelotidase
ISCT MSC Negative Markers	***CD45***	Hematopoietic cells	Pan leukocyte marker
	CD34	Hematopoietic and endothelial progenitor cells	Ligand for CD62L (L-selectin)
	CD19	B-cells	Co-receptor for B cells
	CD14	Monocytes, macrophages	Receptor for complex of lipopolysaccharide and lipopolysaccharide binding protein (LBP)
Additional Surface Markers	***CD133***	Stem/progenitor cells	Endothelial progenitor cell (EPC) marker
	CD117	Hematopoietic progenitors	Stem-cell factor (SCF) receptor
	CD44	Leukocytes, erythrocytes	Mediates adhesion of leukocytes
	CD31	Monocytes, platelets, granulocytes, T-cell subsets, endothelial cells	Adhesion molecule
	CD29	Leukocytes	Integrin B1 subunit, associates with CD49a in VLA-1 integrin
	MHCI	Nucleated cells and leukocytes	Immunogenic purposes
	MHCII	Antigen presenting cells (APCs)	Immunogenic purposes
	SM-α	Smooth muscle cells	Marker of smooth muscle differentiation
	CXCR4	Immature CD34+ hematopoietic stem cells	Binding to SDF-1; Cofactor for fusion and entry of T-cell line
	CD146	Endothelium	Pericyte marker, potential adhesion molecule
	STRO-1	Bone marrow stromal cell and erythroid precursors	*Unknown*

Table 1. Surface Antigen Characteristics. Cellular expression and the primary function of the surface marker antigens used to characterize the different cell types.

Each of the four cell types - BM/MSCs, DFs, atrial and ventricle cardiac cells - were analyzed for their surface marker expression for both the minimal and additional markers. For the comparison and analysis of surface marker expression the presented data were based on one donor in the fourth passage after cultures were grown to approximately 70% confluency. Flow cytometry, however, was performed on three BM/MSC donors, two DF donors, and a single donor for each of the cardiac cells, on passages three through five. Multiple trials found stable expression between the donors and through the passages tested for most of the markers (data not shown).

2.1 Homogenous Expression

Homogenous expression of cell surface markers refers to all cells, in a given cultured population, having or not having that particular surface antigen. Typically homogenous negative or positive expression was observed for all four cell types. A positive result was defined as having surface antigen expression greater than 95% above the isotype control. Conversely, a negative result was defined as having positive expression less than 2% above the isotype control. The representative profiles for positive expression (Fig. 3A) and negative expression (Fig. 3B) through flow cytometry are shown in Figure 3. This quality is important because primary cultures are not homogenous. All cells identified as positive or negative in the analysis have the same or similar corresponding profile. The summary of surface marker expression on the four different cell types is shown in Table 2.

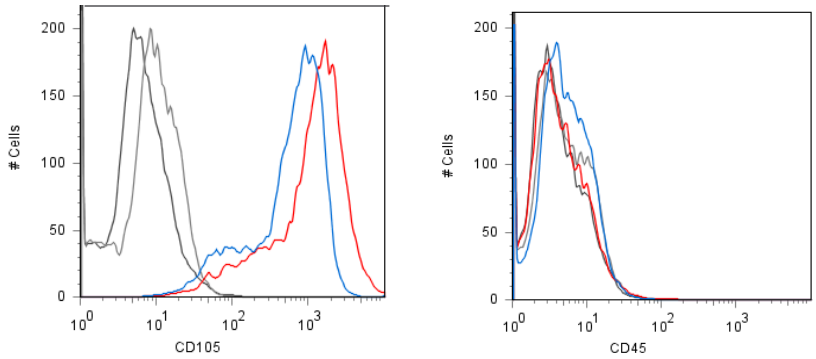

Figure 3. Representative FACS Profiles. Positive flow cytometry profile ***(A)*** and negative flow cytometry profile ***(B)*** for a particular antibody for primary staining (red and blue line) and isotype controls (dark and light gray lines).

According to the minimal markers, BM/MSCs were found to have positive expression for CD105, CD90, and CD73, and negative expression for CD45, CD34, CD19, and CD14. DFs, atrial and ventricle cells were observed to have identical expression concerning these markers as well. This indicated that these cell types upheld the surface marker component of the minimal criteria, according to the ISCT, to be classified as an MSC. BM/MSCs were also found to positively express CD44, CD29, and MHCI, but lacked expression of CD133, CD117, CD31, MHCII, CXCR4, and, α-SM actin. Interestingly, DFs and both types of cardiac cells had identical expression as MSCs regarding these additional markers as well.

	Surface Antigen	Bone Marrow MSC	Dermal Fibroblasts	Atrial Cardiac Cells	Ventricle Cardiac Cells
ISCT Positive Markers	CD105	+	+	+	+
	CD90	+	+	+	+
	CD73	+	+	+	+
ISCT Negative Markers	CD45	-	-	-	-
	CD34	-	-	-	-
	CD19	-	-	-	-
	CD14	-	-	-	-
Additional Surface Markers Investigated	CD133	-	-	-	-
	CD117	-	-	-	-
	CD44	+	+	+	+
	CD31	-	-	-	-
	CD29	+	+	+	+
	MHCI	+	+	+	+
	MHCII	-	-	-	-
	α-SM Actin	-	-	-	-
	CXCR4	-	-	-	-
	CD146	~~~~	~~~~	~~~~	~~~~
	STRO-1	~~~~	~~~~	~~~~	~~~~
	+	Indicates phenotype is Positive (≥ 95%+)			
	-	Indicates phenotype is Negative (≤ 2%+)			
	~~	Indicates Variable Expression			

Table 2. Surface Antigen Expression. Overall surface marker expression by specific cell type. A '+' sign correlates to 95% of the overall cell population expressing the surface marker. A '-' sign correlates to less than or equal to 2% of the overall cell population expressing the surface marker.

2.2 Variable Expression

Two markers, CD146 and stro-1, were differentially expressed between the investigated cell types. Stro-1 is a surface antigen that can be found on precursors of a number of stromal type cells, while CD146 is a perivascular marker (Shi and Gronthos, 2003; Sacchetti et al., 2007). Expression through passages and donors of BM/MSCs was highly variable. Some donors had very little expression of stro-1 and as passage number increases, this expression was observed to fade. The fluctuation between different donors and passages may be due to culture dependent conditions or other unknown factors. The

variability seen through donors and passages was also demonstrated for DFs and atrial and ventricle cardiac cells. These results can be found in the bottom of Table 2.

One of our intentions was the isolation and *in vitro* comparison of stro-1^+ and stro-1^- cells. To this end, we performed sorting of stro-1 positivity by two methods- FACS and magnetic beads. The frequency of the stro-1+ cells was approximately 5-10% of the overall cell population in early passage two and three cell cultures, confirming what has been reported in the literature. The follow-up on the sorted cells and whether they retain their positive expression, however, could not be completed due to technical difficulties.

3. Analysis of Proteins Secreted into the Culture Media

According to published literature, one of the main mechanisms by which MSCs can mediate their positive effects is by secreting proteins. This led to the hypothesis that MSCs utilize paracrine and autocrine signaling. These signaling mechanisms could modulate inflammation and matrix remodeling as well as recruit other stem cells. In this MQP study, the secretion of a number of different factors - cytokines, chemokines, growth factors, and extracellular matrix proteins involved in metabolism - were analyzed through culturing the cells in normal conditions and assaying the supernatant. Cytokines and chemokines are critical in both the innate and adaptive immune response. The inflammation associated with myocardial infarction could cause cardiac myocyte apoptosis mediated by anoikis. By blocking extracellular matrix degradation through the secretion of factors such as TIMPs, MSCs could prevent this collateral cell damage. Growth factors and extracellular matrix proteins are also important in angiogenesis and

vasculogenesis. The assay techniques utilized were a multiplex cytokine kit measured on a Luminex instrument and a specially selected panel of secreted factors using a contract assay services (SearchLight) to measure other soluble factors. The different secreted and soluble factors investigated are noted in Table 3.

Culture supernatant was collected 48 hours after the medium was conditioned with exponentially growing cells. Typically cultures were 60% confluent when conditioning began and 80% confluent when supernatant was collected and the cells were harvested and counted. The measurements for both assays were given in ng/ml and were further normalized to cell number and hours conditioned. For easier comparison and viewing, the absolute level of DFs was taken as a baseline reference point (1 relative unit). BM/MSCs and both cardiac cell types were compared to and expressed as a fraction of DFs. The absolute level is the average value of four different trials on each cell type. The relative protein expression as normalized to DFs is visualized in Figure 4 for the Luminex results, and Figure 5 for the SearchLight results (discussed in the next subsection).

	Protein Secretion Characteristics	
Cytokine	**Primary Source(s)**	**Activity**
G-CSF*	Fibroblasts and monocytes	Stimulates neutrophil development and differentiation
GM-CSF*	Macrophages, T-cells	Stimulates growth and differentiation of myelomonocytic lineage cells
IFNγ*	T-cells, NK cells	Macrophage activation; Increased expression of MHC molecules; Suppresses Th2 cells
IL-1α*	Macrophages, endothelial cells	T-cell activation; Macrophage activation
IL-1β*	Macrophages, endothelial cells	T-cell activation; Macrophage activation
IL-2*	T-cells	T-cell proliferation
IL-4*	T-cells, mast cells	B-cell activation; Induces differentiation into Th2 cells
IL-5*	T-cells, mast cells	Eosinophil growth and differentiation
IL-6*	T-cells, macrophages, endothelial cells	T- and B-cell growth and differentiation; Acute phase protein production
IL-7*	Non T-cells	Growth of pre B-cells and pre T-cells
IL-10**	T-cells, macrophages	Suppresses of macrophage functions
IL-12p70*	Macrophages, dendritic cells	Activates NK cells; Induces CD4 T-cell differentiation into Th1-like cells
IL-13*	T-cells	B-cell growth and differentiation; Inhibits macrophage inflammatory cytokine production
IL-15*	Many non T-cells	Stimulates growth of intestinal epithelium, T cells, and NK cells; Enhances CD8 memory T cell survival
IL-17*	CD4 memory cells	Induces cytokine production by epithelia, endothelia, and fibroblasts
TNFα*	Macrophages, T-cells, NK cells	Local inflammation; Endothelial activation
TGFβ1*	Chrondrocytes, monocytes, T-cells	Inhibits cell growth; Anti-inflammatory; Induces switch to IgA production
Chemokine	**Primary Source(s)**	**Activity**
Eotaxin*	T-cells, macrophages, smooth muscle, fibroblasts	Recruits eosinophils, usually for an allergic response
IL-8*	Macrophages, epithelial cells	Recruits neutrophils during inflammation
IP-10*	Monocytes, endothelial cells, fibroblasts	Activates T-cells; Inhibition of BM colony formation and angiogenesis
MCP-1*	Fibroblasts, mononuclear cells, smooth muscle cells	Recruits cells of the immune system during inflammation
MIP-1α*	Macrophages	Activates granulocytes; Induces the release of other pro-inflammatory cytokines
Rantes**	Monocyte/macrophage, T-cell	Recruits leukocytes; Activates NK cells
SDF-1β**	Endothelial cells, stromal fibroblasts, osteoblasts	Recruits EPCs from BM for angiogenesis; Activates and recruits lymphocytes
Growth Factor	**Primary Source(s)**	**Activity**
EGF**	Submaxillary gland, Brunners gland	Promotes proliferation of mesenchymal, glial and epithelial cells
FGF basic**	Many cells associated with ECM	Promotes proliferation of many cells; Inhibits some stem cells; Induces mesoderm to form in early embryos
HGF**	Mesenchymal cells	Regulates cell growth, cell motility, and morphogenesis
PDGF-BB**	Platelets, endothelial cells, placenta	Promotes proliferation of connective tissue, glial and smooth muscle cells
PLGF**	Placental cells, endothelial cells	Molecular marker for inflammation; Type of vascular endothelial growth factor
VEGF**	T-cells, monocytes, keratinocytes	Mediates increased vascular permeability; Induces angiogenesis, vasculogenesis, and endothelial cell growth; Promotes cell migration; Inhibits apoptosis
ECM Protein	**Primary Source(s)**	**Activity**
MMP-2**	Epidermal keratinocyte, T-cells, mast cells, eosinophil	Involved in the breakdown of the ECM in normal physiological processes; Degrades type IV collagen
MMP-9**	T-cells, mast cells, eosinophil	Involved in the breakdown of the ECM in normal physiological processes; Degrades type IV/V collagen; Involved in IL-8 induced mobilization of hematopoietic progenitor cells from BM
TIMP-1**	Astrocytes, T-cells	Inhibitor of MMP family; Promotes cell proliferation in a wide range of types; May also have an anti-apoptotic function
TIMP-2**	Endothelial cells	Inhibitor of MMP family; Suppresses proliferation of endothelial cells; Has a possible angiogenesis role

Table 3. Protein Secretion Characteristics. Primary sources and known activity of the cytokines, chemokines, growth factors, and extracellular matrix (ECM) proteins analyzed through the Luminex instrument and the SearchLight Multiplex Assay. Chemokines are shown using their common name. Proteins marked * were measured with the Luminex, proteins marked ** were measured through SearchLight.

3.1 Cytokine & Chemokine Analysis

All four cell types - BM/MSCs, DFs, and both cardiac derived cells - showed undetectable levels of expression in eight different cytokines: IL-2, IL-4, IL-5, IL-7, IL-12p70, IL-13, MIP-1α, and TNF-α (data not shown). All of these interleukins are primarily cytokines produced by T-cells, or activate the differentiation and proliferation of T-cells. MIP-1α and TNF-α are important secreted factors that respond to inflammation.

BM/MSCs showed slightly greater expression, a 3 to 4 fold increase, of IFN-γ and IL-6, while the levels for GM-CSF, IL-1β, IL-15, IL-8, and IL-10 were not more than 1 fold increase than DFs, as seen in Figure 4. Atrial expression, however, showed much greater relative expression in IL-6, but it was the numbers for G-CSF and IL-8, roughly 30 fold increases, that dominated the secretion profile. Ventricle cells showed a similar expression to atrial cells with elevated IL-6 and IL-8 levels. The considerable increase in both cardiac cell types could be due to their origin in a heart that had previously experienced a chronic disease.

Three cytokines, IL-1α, IL-17, and IP-10, were not detectable in DFs, and therefore no relative expression profile was generated. The other cell types, BM/MSCs and both CSCs, showed moderate secretion levels for these cytokines (below 100 ng/ 1000 cells).

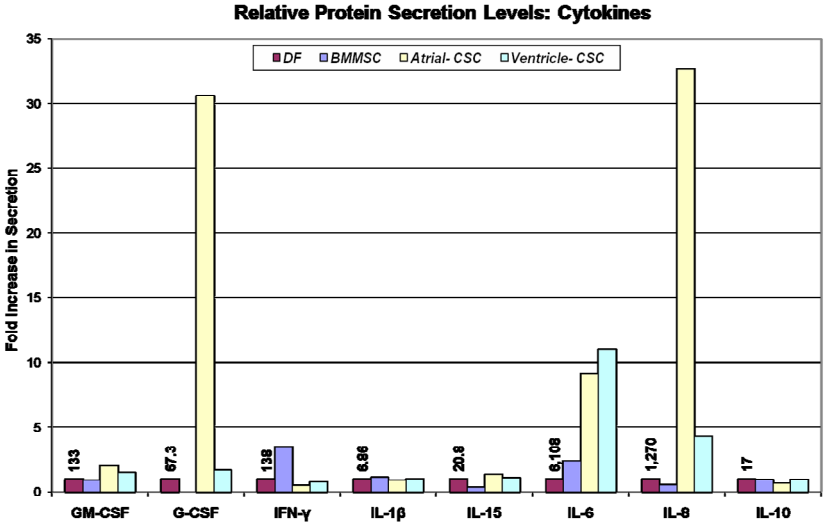

Figure 4. Relative Protein Secretion. This figure illustrates the relative level of protein being expressed in the conditioned medium, as normalized to DFs, and measured by the Luminex assay. The normalization was done by taking the average expression level and dividing the MSC, atrial or ventricle value by the DF value. The *y*-axis is the fold increase in secretion; the *x*-axis gives the secreted factor. For each factor, DFs are measured as 1, the baseline value. The numerical value above the bar representing DFs is the absolute average value of each particular protein in ng per 1,000 cells.

3.2 Analysis of Growth Factors & Proteins Involved in ECM Metabolism

For the majority of the proteins investigated, levels were relatively similar. Comparing BM/MSC secretion to DF HGF, PLGF, and VEGF levels were significantly

higher in BM/MSCs. From these factors, VEGF is similarly high in both atrial and ventricular cardiac cells. Conversely, PLGF secretion is much lower in both types of cardiac stromal cells. The secretion of this factor is smaller even when compared to DF levels. The two different cardiac cell types show differences between each other in the secretion of HGF and MMP-2.

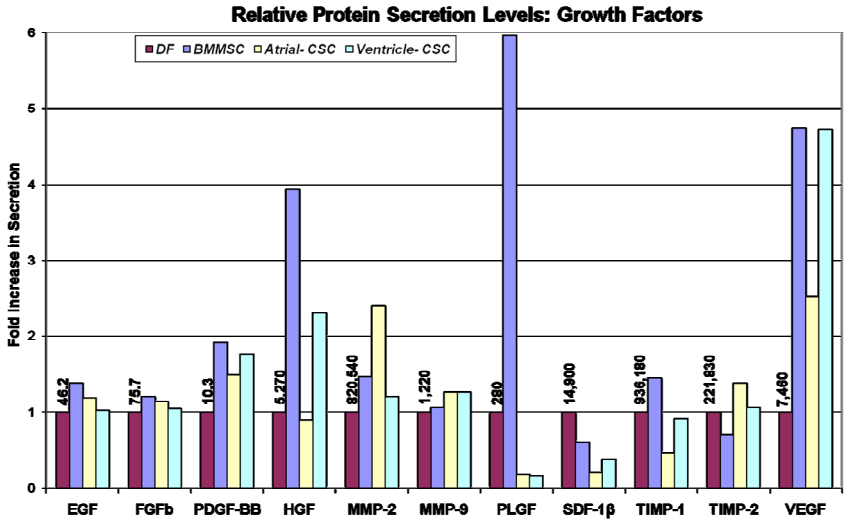

Figure 5 Relative Protein Secretion. This figure illustrates the relative level of protein being expressed by the SearchLight assay. The same normalization was preformed as in Figure 4. The *y*-axis is the fold increase in expression; the *x*-axis gives the secreted factor. For each factor, DFs are measured as 1, the baseline value. The numerical value above the bar representing DFs is the absolute average value of each particular factor in ng/1,000 cells.

The cardiac cells also showed extensive increases in expression in MCP-1, and more notably, in RANTES (data not shown). Both cardiac cells showed approximately 20-fold increases in the expression of MCP-1 over both DF and BM/MSCs. MCP-1 is up-regulated in sites of injury or infection and stimulates the recruitment of many inflammatory molecules. Cardiac cells also showed a substantial increase in RANTES, as both had over 100-fold increases over DFs and BM/MSCs. RANTES is a chemokine that also regulates inflammatory processes by recruiting T cells. The vast expression in these two cardiac cell types is most likely attributed to the fact that they were isolated, as mentioned before, from tissue that had or was currently going through an injury.

4. Stimulation with Molecules Involved in Inflammation

The cell types were also observed for their response to two critical cytokines, IFN-γ and TNF-α, involved in innate and adaptive immunity via *in vitro* applications. IFN-γ, a regulator of the innate immune reaction, up-regulates co-stimulatory molecules in macrophages and dendritic cells and is also responsible for a higher presence of antigen presenting cells. The importance of investigating the reaction to IFN-γ was to study whether exposure to these conditions (pro-inflammatory) would induce a change in their immune character. The immune characteristics of a cell become especially significant when evaluating the use of allogeneic cells. TNF-α is an important check-point cytokine in the immune response to myocardial diseases. It is released in early phase myocardial injuries triggering monocytes to differentiate into macrophages. Although our original intention was the side by side comparison of BM/MSCs, DFs and cardiac stromal cells, this work was primarily focused on the comparison of BM/MSC and DFs.

4.1 IFN-γ Stimulation

Since cell therapy treatments would likely use allogeneic cells, it is important to know first if they could become antigen presenting *in vitro*. This may be an indicator of whether it might activate an immune response in the patient. In order to assess whether a cell may become antigen presenting, the effect of IFN-γ in up-regulating co-stimulatory molecules (CD40, CD80, and CD86) and MHC II was observed. IFN-γ was added at a concentration of 200ng/mL to expanding BM/MSC, DF, atrial and ventricular cardiac cultures; these cultures were compared to those that did not receive the treatment. After an incubation period of three days in either normal conditions or in the presence of IFN-γ,

cells were harvested, counted, and analyzed for the presence of the co-stimulatory molecules and MHCII by flow cytometry. The outcome was the same for all three BM/MSC donors, both DF donors, and both cardiac stromal cell donors: IFN-γ did not up-regulate surface marker expression of co-stimulatory molecules CD40, CD80, and CD86, but could, however, up-regulate MHCII surface antigen expression as shown in Table 4. Cell counts between cultures with IFN-γ and without IFN-γ stimulation showed no significant differences (data not shown).

Cell Type	Treatment	CD40	CD80	CD86	MHCII
BM/MSC	+ IFN-γ	-	-	-	+
	- IFN-γ	-	-	-	-
DF	+ IFN-γ	-	-	-	+
	- IFN-γ	-	-	-	-
Atrial	+ IFN-γ	-	-	-	+
	- IFN-γ	-	-	-	-
Ventricle	+ IFN-γ	-	-	-	+
	- IFN-γ	-	-	-	-

Table 4. Summary of IFN-gamma Results. Flow cytometry was used to analyze the surface antigen expression of CD40, CD80, CD86, and MHCII for BM/MSC, DF, atrial and ventricle cardiac stromal cells cultured with and without IFN-gamma Antibodies were incubated with cells harvested from expanding passage 4 cultures using the time, concentration, and temperature suggested by the manufacturer's instructions. Cultures of each cell type were harvested and stained for negative controls. '+' indicates positive surface expression of the specific surface antigen, '-' indicates no expression specific surface antigen.

Further experiments between BM/MSCs and DFs to observe their responsiveness to IFN-γ were analyzed while studying protein secretion levels and PGE_2 concentrations between stimulated and non-stimulated cultures (using the same three day incubation period). The reason for investigating the change in cytokine and growth factor secretion is to observe whether IFN-γ influences the cell to secrete more pro-inflammatory proteins or modulate the secretion of these proteins. BM/MSC and DF supernatants were assayed for protein secretion levels between cultures treated with and without IFN-γ. As shown in Table 5A, cytokines such as IP-10 and IL-1α were secreted at much higher levels (greater than a 60-fold increase) in cultures stimulated with IFN-γ. IL-15, G-CSF, MIP-1α, and IL-6 were secreted at moderately high levels (a 4- to 12-fold increase) between

the same cultures. All other cytokines measured showed either little or no change. Other than the difference between the amounts of IP-10, there were many similarities between BM/MSC and DF cultures. Although no cultures, BM/MSC or DF, exhibited significant down-regulation of any cytokines, IL-8 was the only protein that consistently had lower levels when stimulated with IFN-γ.

A:	MSC (str.1)	MSC (str.2)	DF (str.1)	DF (str.2)
IP-10	1020.65	2407.71	6300.00	1935.34
IL-1A	71.55	69.15	61.65	74.30
IL-15	11.35	5.20	3.67	4.50
G-CSF	6.17	6.17	4.20	10.50
MIP-1A	10.55	4.88	4.88	0.00
IL-6	4.44	3.21	4.22	4.00
IL-7	3.02	2.81	4.62	2.18
IL-1β	2.35	1.34	2.13	2.11
GM-CSF	2.11	1.79	1.33	1.21
EOTOX	1.57	0.60	0.07	1.23
IL-8	0.24	0.23	0.46	0.25
IL-10	0.39	1.00	0.00	2.56

B:	MSC (str.1)	MSC (str.2)	DF (str.1)	DF (str.2)
MCP-1	48.2	33.4	7.8	3.6
RANTES	18.1	28.3	106.0	13.3
EGF	1.45	1.11	1.00	0.88
FGFB	0.99	1.67	1.17	2.06
MMP-2	1.08	0.98	1.18	1.22
MMP-9	1.25	2.07	1.16	2.11
TIMP-1	1.00	1.42	1.38	2.21
TIMP-2	1.13	0.87	1.27	1.13
PLGF	0.90	1.09	2.08	1.54
PDGF-BB	1.31	1.23	1.00	0.92
SDF-1B	0.34	0.98	0.66	1.03
VEGF	1.06	0.80	0.60	0.80
TGF-B1	1.46	1.39	2.57	1.10
HGF	1.70	0.80	1.75	0.28

Table 5: Relative Levels of Secretion in Response to IFN-gamma. Protein secretion levels of BM/MSC and DF, 2 donors each (labeled as either strain 1 or strain 2), were measured by a 21-plex Luminex kit **(A)** for cytokines and the SearchLight assay **(B)** for growth factors. Measurements were obtained in pg/mL and normalized for cell count. Measurements for cultures with IFN-gamma were divided by the corresponding culture without IFN-gamma to obtain the relative secretion ratio.

Using the second assay method there were only significant changes in secretion for MCP-1 and RANTES (greater than 10-fold increase), as shown in Table 5B. All other soluble factors measured showed little or no change. Interestingly, the amounts of MCP-1 and RANTES, although higher in stimulated cultures, showed dramatically different amounts between BM/MSC and DF. This may point to differences in how these cells modulate the effect of IFN-γ.

Prostaglandins are lipid mediators involved in suppressing T cell proliferation and differentiation. Specifically, we studied the secretion of prostaglandin E_2 (PGE_2) in the supernatants of BM/MSC and DF cultures. After the three day incubation with IFN-γ, the supernatant was extracted and measured on a competitive enzyme immunoassay for the amount of PGE_2 content. As shown in Figure 6, both BM/MSC donors showed up-

regulation of PGE_2 in the culture supernatant following stimulation with IFN-γ. This differs with the result from the supernatant collected from both strains of DF, as the PGE_2 content was slightly higher after incubation. Whether this difference indicates an important change in the IFN-γ signaling pathway between the two cell types is unknown, however, as we did not further investigate this question.

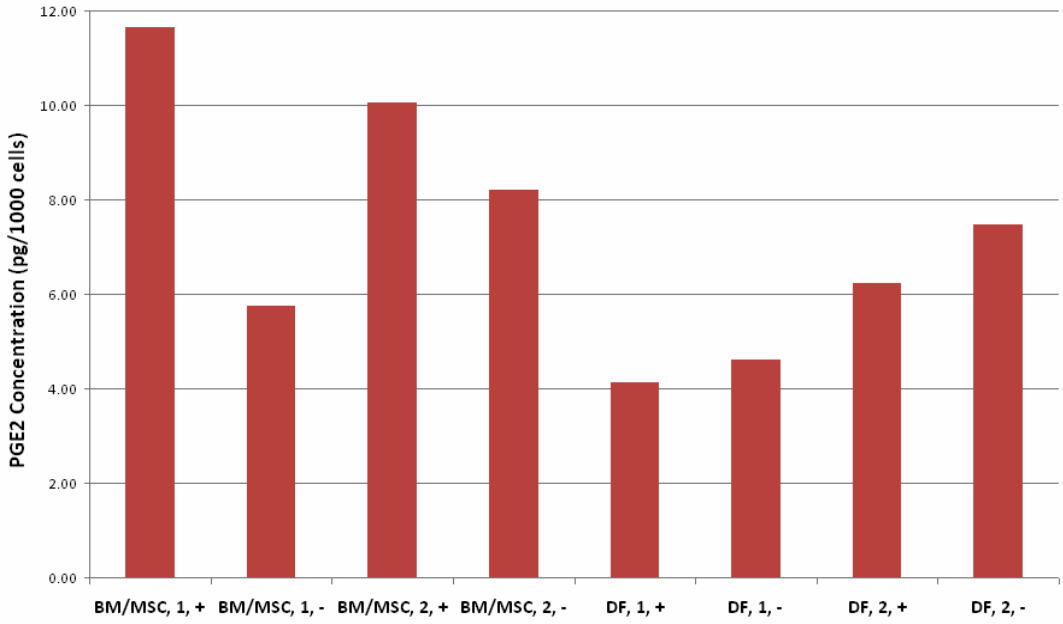

Figure 6: IFN-gamma Induced PGE_2 Concentrations. Culture supernatant of Passage 4 proliferating BM/MSC and DF cultures were analyzed for their PGE_2 concentrations with a commercial ELISA kit. Two donors of BM/MSC and DF were used, recorded as either a 1 or 2, along with whether the culture was stimulated with IFN-gamma (+) or without IFN-gamma (-).

4.2 TNF-α Stimulation

TNF-α is a cytokine that represents an important step in inflammation during the critical time period after a myocardial injury. It is critical in the beginning phase of inflammation as it triggers and regulates a wide variety of immune cells. Since a cell therapy treatment would target the early stage of inflammation to prevent scarring and fibrosis, it would be important to study how transplanted cells would react to the cytokines prevalent in the damaged environment. To study this aspect of wound healing,

varying concentrations of TNF-α (0, 10, and 50ng/mL) were added to expanding BM/MSC and DF cultures in a time-course experiment. Culture supernatant was harvested for measurement of secreted proteins with a cardiovascular 21-plex kit at two time points - day one and two following incubation with the molecule.

Although there were no significant findings with most of the cytokines measured, four cytokines did show an increase in both DF and BM/MSC cultures: IL-6, IL-8, MCP-

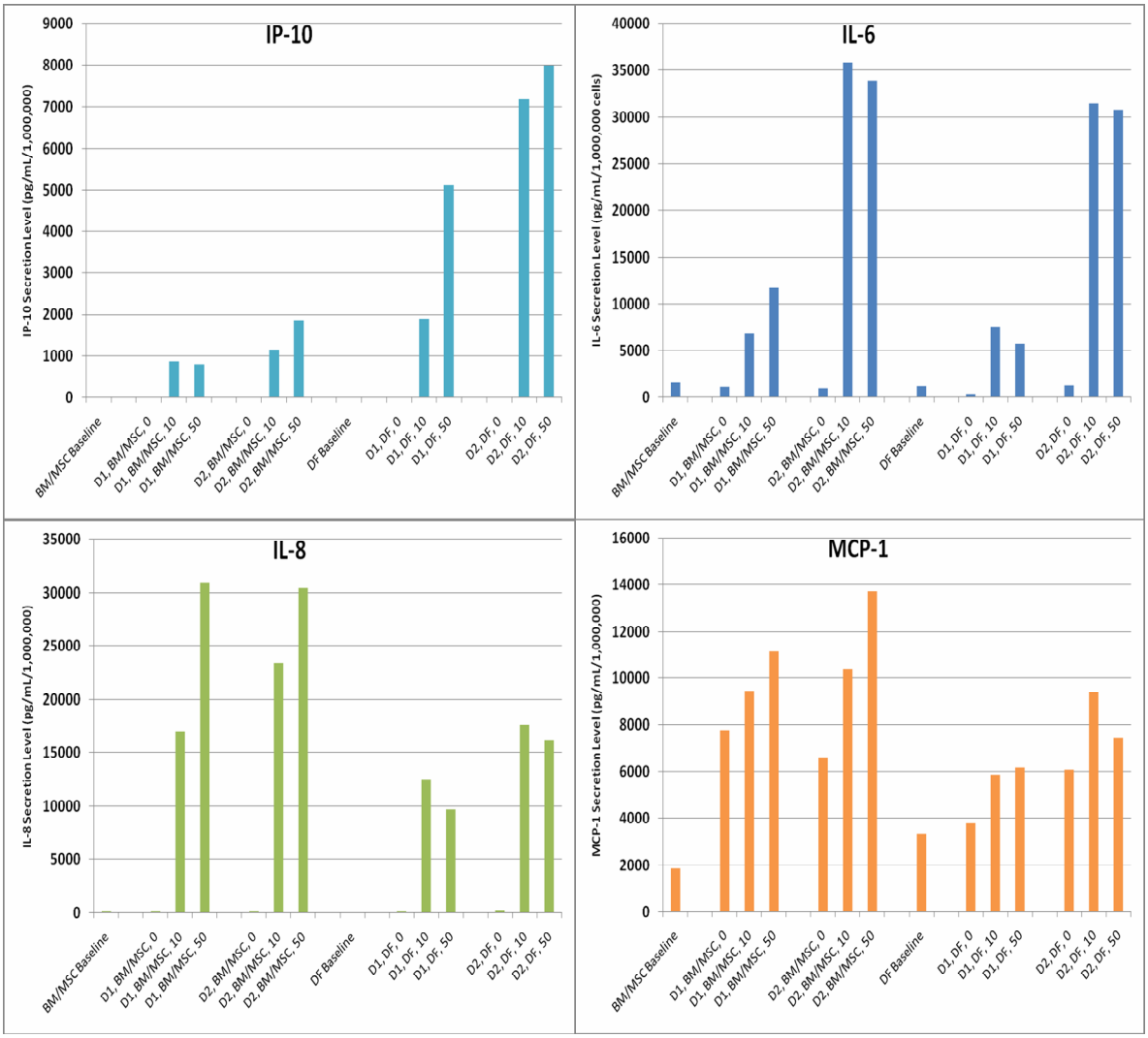

Figure 7. Affect of TNF-alpha on Cytokine Secretion Levels. Cytokine secretion levels of BM/MSC and DF for IL-6, IL-8, IP-10, and MCP-1 were measured by a cardiovascular 21-plex kit. Measurements were obtained in pg/mL and normalized for cell count. The *x-axis* of all graphs show the baseline BM/MSC (BM/MSC Baseline) measurement followed by day 1 (D1) cultures that were incubated with either 0 ng/mL (0), 10 ng/mL (10), or 50 ng/mL (50) TNF-alpha followed by day 2 (D2) measurements with the same nomenclature. BM/MSC measurements are followed by DF measurements using the same naming scheme. The *y-axis* represents the measurement of secreted proteins in the supernatant by pg/mL/1,000 cells.

1, and IP-10, as shown in Figure 7. The secretion of these cytokines was observed to be dose dependent. IL-8 and MCP-1 reached their maximum secretion levels on the first day, while IL-6 and IP-10 levels showed further increases on day 2 in both cell types. This could suggest IP-10 and IL-6 secretion is directly regulated by TNF-α. Alternatively, IL-8 and MCP-1 secretion may be regulated by mechanisms other than TNF-α. Cultures were also harvested for cell count, however, data showed no significant changes between cultures that were incubated with or without the molecules (data not shown).

5. Differentiation Capability

According to the ISCT, MSCs must be able to differentiate into at least two of three defined cell lineages: bone, fat and cartilage. Proliferating passage four cells were harvested and plated into three different culture conditions, as detailed in the Methods. The osteogenesis assay was performed over the duration of 14 days, the adipogenesis assay over 21 days, and the chondrogenesis assay over a period of 28 days. All assays were performed on BM/MSCs, DFs, and both cardiac cells as well as positive controls for each assay. The positive controls included pre-adipocytes for the adipogenic assay, osteoblasts for the osteogenic assay, and chondrocytes for the chondrogenic assay. The adipogenesis assay used Oil Red O solution to visualize the production of lipid vacuoles. The osteogenesis assay used Alizarin Red solution to visualize the presence of calcium deposits, a main component in bone formation. The chondrogenesis assay used Safranin-O and Toluidine Blue, both of which are used as histological stains for cartilage.

5.1 Differentiation Analysis

BM/MSCs clearly differentiated into adipocytes as seen by the bright red spots, in Figure 8A and 8E, corresponding to lipid droplet formation. The lipid droplet formation in the BM/MSC culture was very well visible before the staining was applied. This result was also homogenous throughout the entire plate. DFs and both cardiac cells did not show any signs of lipid formation. In fact, many cells were lost during this assay for these cell types. This may because of the fragility of the differentiated cells in culture, the inability to tolerate the differentiation conditions, or the particularly high confluency rate. The pre-adipocyte control culture homogenously differentiated into adipocytes with a large amount of lipid droplet formation.

All cell types appeared to produce some level of calcium as seen by the reddish staining of the cells as shown in Figures 8F-8J. BM/MSCs were observed to have clusters of differentiated cells producing the calcium deposit. This contrasted to the more homogenous presence of the Alizarin stain throughout the DF and cardiac cell cultures. The control osteoblasts culture produced the most homogenous culture where it seemed all cells stained for calcium deposits.

The Toluidine Blue and Safranin-O staining used to detect cartilage and the characteristic lacuna formation of the cell pellet during the assay was not observed in any of the cell types. The figures for the Safranin-O stain are represented in Figure 8K-8O. They show partial deep red staining, but not homogenous or prevalent enough to definitively comment on the positive nature of these cells. It may be possible that these cells are still in the differentiation phase.

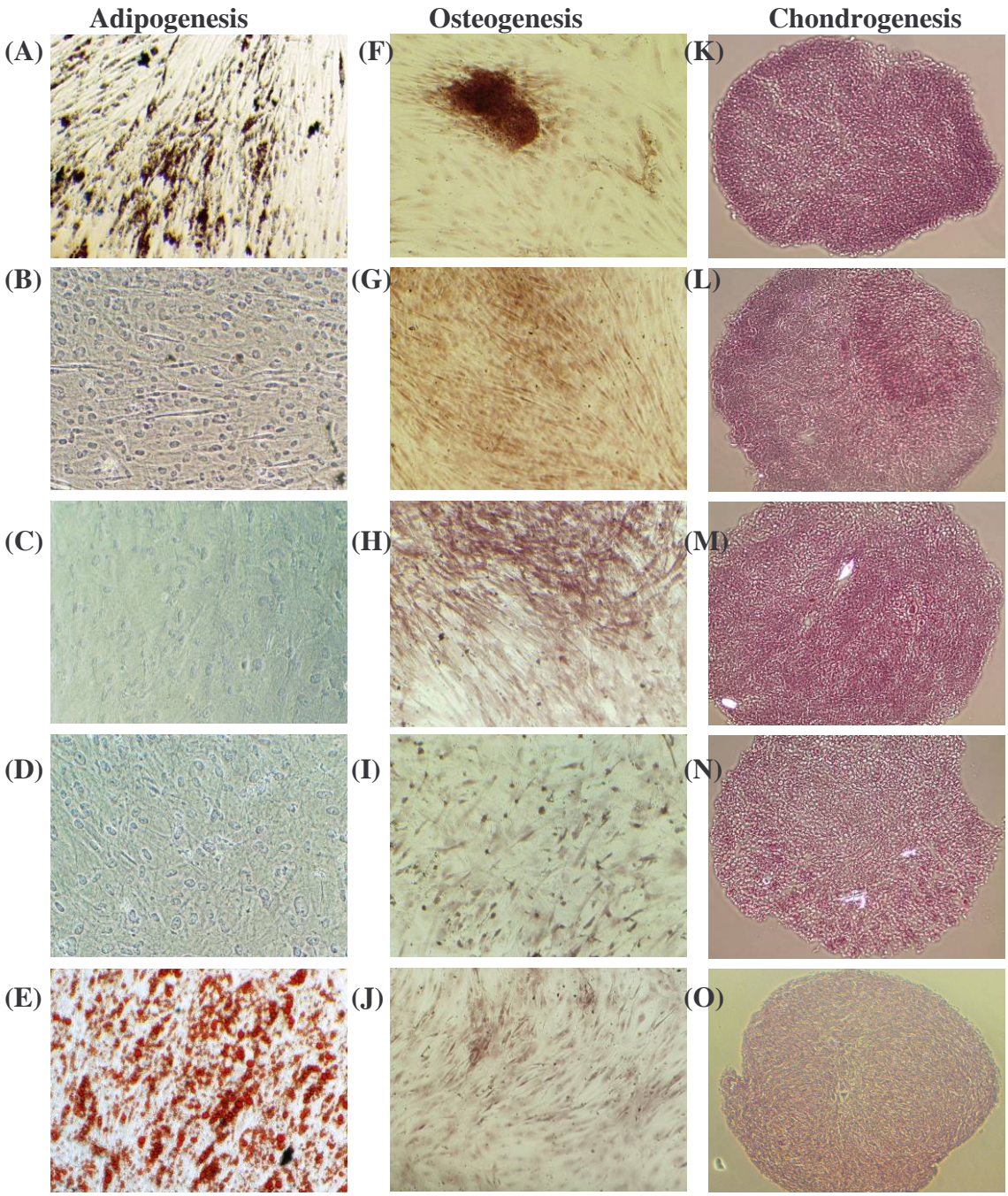

Figure 8. Differentiation Assay. Adipogenic pictures, ***(A)-(E)***, were taken with 20X magnification on a light microscope. Osteogenic pictures, ***(F)-(J)*** were taken at 10X and Chondrogenic ***(K)-(O)*** pictures were taken at 40X. Cell types are BM/MSCs (***A, F, K***), DFs (***B, G, L***), atrial cells (***C, H, M***), ventricle cells (***D, I, N***), pre-adipocytes (***E***), osteoblasts (***J***), and chondrocytes (***O***).

5.2 Quantification of Cell Differentiation using MetaMorph

Quantitative analysis was applied on the results of the adipogenic and osteogenic assays because of the difficulty in being able to clearly observe the amount of differentiation in the cultures. In order to quantitate the results, five random photographs were taken of the adipogenic and osteogenic cultures to assess the degree of differentiation. Using the MetaMorph software program, a color threshold was selected by defining a specific color which correlated to positive differentiation, as determined by the positive controls. The amount of pixels of this defined color was measured as a percent of the field in the randomly selected photograph. The color threshold settings were then applied to the corresponding random photographs in order to compare the percent differentiation in BM/MSCs, DFs, and both cardiac cell types. Figure 9 represents the data obtained from this quantification.

In the pre-adipocyte culture, lipid vacuoles as stained by Oil Red O, occupied on average approximately 45-55% of the field as illustrated in Figure 9A. This was compared to approximately 20% of the BM/MSC field, and 0% of the field for DFs or either of the cardiac cells. Calcium produced by osteoblasts and stained by Alizarin Red, occupied approximately 25-30% of the field as represented in Figure 9B. BM/MSCs had approximately 20-25% of the field stained, followed by DFs and atrial cells with approximately 10-15% and lastly ventricle cells with 8%.

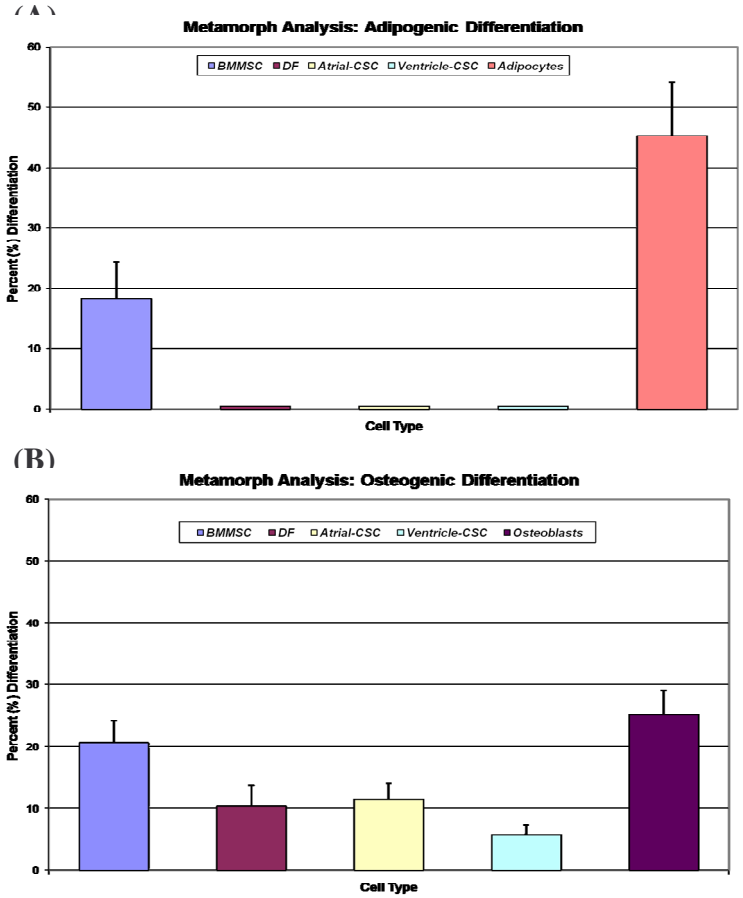

Figure 9. Quantitative Differentiation Analysis. This figure represents the analysis of ***(A)*** Adipogenic differentiation and ***(B)*** Osteogenic differentiation by the Metamorph computer program. The *y*-axis represents the percent of the area that contains the selected pigment that corresponded to differentiation. Each analysis was preformed on five random pictures for each cell type, with the error bar showing the standard deviation between the samples.

6. DNA Methylation

Unlike the previous assays, a more consistent way to characterize the cell types was desired that would show both reproducibility and a clear distinction between the cells. To address this point, DNA methylation patterns were analyzed to look for more stable characteristics that were less sensitive to a number of factors such as donor, passage number, and culture conditions. The cause of the variety of cell types in the body is differential gene expression, which in turn is partially the result of DNA methylation. Methylation is specific to different cell types and even though it can be subject to changes in the promoter (and subsequently transcription), is likely to be more stable.

The use of this technique for cell identification purposes was demonstrated by Baron et al., 2006, and Rapko et al., 2007. In these papers, genetic markers were used to distinguish chondrocytes from synovial fibroblasts, and MSCs from articular chondrocytes. We applied this method to see whether the same epigenetic markers could distinguish BM/MSCs from DFs and cardiac-derived stromal cells. This DNA methylation characterization assay was based on eight methylation points in the genome. These markers were chosen because of their ability to discriminate chondrocytes from synovial fibroblasts and MSCs from articular chondrocytes in a genome-wide discovery experiment (Baron et al., 2006; Rapko et al., 2007).

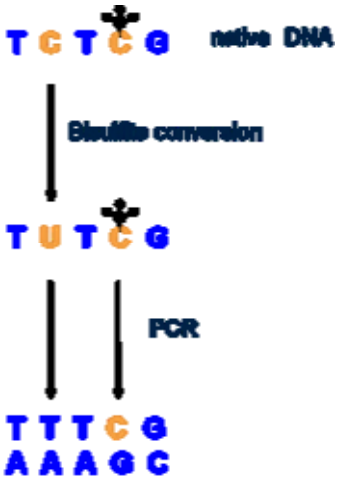

Figure 10. Bisulfite Conversion. This figure, used with permission from S. Rapko, illustrates the mechanism of sodium bisulfite on genomic DNA.

For this purpose, genomic DNA was isolated from exponentially growing BM/MSCs, DFs, atrial and ventricle cells, cultured to passage four. Methylation was detected through QAMA real-time PCR. Bisulfite conversion causes the un-methylated cytosine residues in the DNA to convert to uracil and, following PCR, convert to thymine (methylation of cytosine bases only occurs if it is followed by a guanine basis, known as CpG sites), this conversion is diagramed in Figure 10. Methylated cytosine residues, on the other hand, remain protected and do not become converted.

The percent methylation of the selected markers is shown in Figure 11. Atrial and ventricular cardiac stromal cells were quantified together as cardiac stromal cells in the figure because of their high similarity to one another. By previous experiments, 80%

methylation or above, as compared to the controls, was considered over-methylation while below 30% methylation was considered under-methylation. BM/MSCs showed under-methylation in markers *GENZ2* and *GENZ7* and over-methylation in the markers for *GENZ1, GENZ3, GENZ8,* and *GENZ6*. In comparison, DFs showed some similarities in the methylation patterns, but differed greatly in *GENZ6, GENZ7,* and *GENZ1*. Interestingly, the cardiac-derived stromal cells showed an identical expression pattern to DFs in all eight markers tested. *GENZ7* and *GENZ6* were observed to give the highest amount of differences between BM/MSCs and DFs.

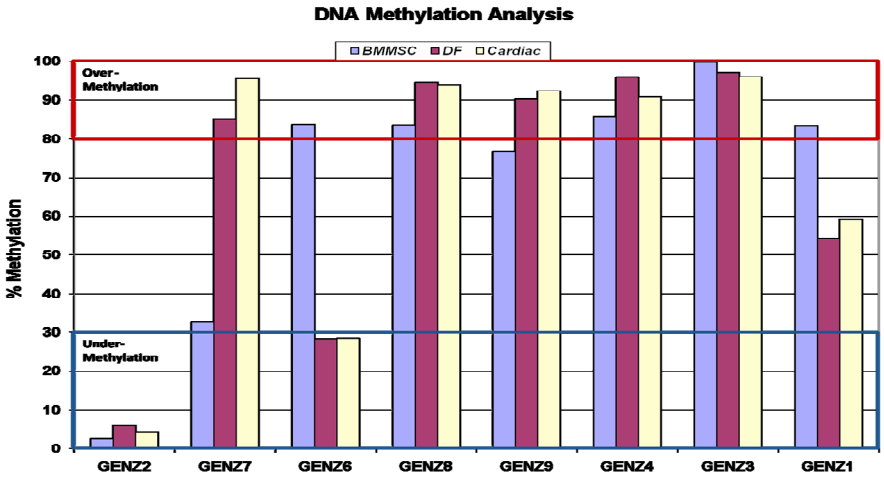

Figure 11. Mean DNA Methylation Patterns. These values were obtained by correlating the C/t value of methylated and unmethylated fluorescing probes to a standard curve. These are the mean values of at least 2 donors.

DISCUSSION

Stromal cell progenitors, and in particular mesenchymal stem/stromal cells (MSC), are hypothesized to be candidates for cell therapy applications because of functional roles they may play during wound healing. MSCs, however, originate from a variety of tissues, and their relation to one another is unknown. This MQP applied a number of assays to observe whether different origins of stromal cells, such as bone marrow-derived (BM/MSC), dermis-derived (DF), and cardiac-derived stromal cells, could be distinguished *in vitro*. We used side by side comparison characterization studies including surface antigens, secreted proteins, DNA methylation, and cell differentiation capability to address this question.

The *in vitro* growth characteristics of all cell types investigated were similar as each showed characteristic fibroblastic morphology. We also found that all cell types expressed the minimal surface markers according to the ISCT standards. Differences were observed in the differentiation tests as BM/MSC differentiated into two lineages, while the other cell types showed the capability for differentiation into only one. However, these tests did not seem to be consistent and are difficult to reproduce as they rely more on culture conditions than cell strain. This was seen in previous experiments in the lab which showed DFs could in fact differentiate into adipocytes.

We further extended the characterization to 11 additional surface markers. By these markers, however, the four cell types were still undistinguishable. We tried to follow the variable expression of one particular marker, stro-1, because it is believed to be a marker for pericytes and stromal progenitors (Shi and Gronthos, 2003). These

experiments, however, were unsuccessful in culturing a homogenous stro-1^+ population, and the data was not conclusive.

All cell types were observed to have similar secretion profiles for many cytokines and growth factors, including a high secretory capacity for SDF-1β, VEGF, and HGF. This indicates the cells were able to secrete proteins important for angiogenesis as well as stem cell recruitment, migration and proliferation (Zhou et al., 2007). They also secreted matrix metallo-proteases, such as MMP and TIMP proteins, involved in extracellular matrix metabolism. Finally, these cells secreted pro-inflammatory cytokines IL-6, IL-8, and MCP-1. The *in vivo* effect of the cells producing these cytokines is unknown; however we would hypothesize that this would be a negative effect.

Even under challenging conditions, the cells responded similarly by up-regulating the MHCII surface antigen, but not up-regulating co-stimulatory molecules in the presence of IFN-γ. We would expect that these cells would respond similarly under *in vivo* conditions. A follow-up on the regulatory pathway of these cells' particular response to IFN-γ should be further investigated. Additional studies into the differences in PGE_2 secretion (data limited to BM/MSC and DF) could help solve whether these results are characteristic to cell type or variable depending on the culture conditions.

Since the previously described assays showed high similarity for all cell types, we continued to explore additional markers to distinguish and characterize the cells. DNA methylation was found to be a new approach for these purposes. Based on preliminary work preformed by Genzyme Biosurgery, we used eight methylation markers to screen for differences between the cells. Cardiac derived stromal cells and DFs were both observed to have the same methylation patterns in the selected genetic markers, but were

found to be different than BM/MSCs. It is unknown whether these particular differences in gene expression come from the solid tissue origin of the cells, or if they represent a different stage of maturation. Further improvement of the methylation markers, including exploring more genes, could give new information about the origin and development stages of stromal cells. Along these lines, characterization of disease related cells, such as the origin of the cardiac stromal cells, might show differences that could eventually be targeted for therapy.

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