A Thesis

entitled

Testing for Osteogenic Potential of Human Mesenchymal Stem Cells

by

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Biologic and clinical interest in human mesenchymal stem cells (hMSCs) has risen dramatically over the last two decades. In orthopaedics, there is a great interest in developing therapies to improve bone healing by using a patient’s own hMSCs to form new bone. hMSCs can be isolated from different skeletal sites; however they may differ in their capability to form new bone \textit{in vivo}.

Therefore, it is important to develop methodology which will allow for prediction of hMSCs’ osteoblastic potential. This methodology includes testing of isolated hMSCs for the expression of a unique set of bone formation biomarkers prior to their surgical application. To identify the best skeletal site for hMSC isolation and their bone formation properties, we have tested bone specimens derived from patients undergoing orthopaedic surgeries at the UTMC. These studies were performed under institutional IRB protocol and Informed Consent from participating patients. Bone marrow specimens were isolated from iliac
crest bone marrow aspirates, fatty marrow, cartilage, and cortical and trabecular bone from distal femur and proximal tibia. After expansion, the phenotype of hMSC isolates were tested by assessing a *bona fide* marker of osteoblastic phenotype, enzymatic activity of alkaline phosphatase, and correlating these findings to the relative expression of bone-associated genes and candidate *ex vivo* bone biomarkers. In our limited sample size, we observed differences amongst five cultures of primary cells from different sources when tested for alkaline phosphatase activity and gene expression. Our results suggest that hMSCs within bone marrow aspirates may already possess a potential to differentiate into an osteoblasts, in contrast to hMSCs from other tissues. When comparing biomarkers for bone formation, hMSCs derived from marrow aspirates demonstrate an overall greater change in gene expression after osteogenic induction than hMSCs derived from other sites. Further, extracellular matrix-associated genes in marrow aspirate samples at day 0 such as, decorin and carboxypeptidase E, demonstrate a predictive correlation to hMSC’s osteogenic induction ability. Taken together, these preliminary results demonstrate the iliac crest marrow may be the best site to obtain hMSCs and testing for these candidate biomarkers may provide a clinician with insight into the osteogenic potential of their hMSCs to aid in surgical planning.
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Chapter 1

Testing for Osteogenic Potential of Human Mesenchymal Stem Cells

1.1 Introduction

Throughout life, bone continually undergoes a process called bone remodeling. During bone remodeling there is turnover and regular repair of bone tissue with support of newly formed osteoblasts. These osteoblasts arise from bone marrow stromal cells, also known as mesenchymal stem cells (MSCs) that predominately reside in the bone marrow stroma [1].

MSCs are multipotent stem cells that are able to differentiate into a variety of mesoderm-type cells, including adipocytes, chondrocytes, and osteocytes [2-3]. The International Society for Cellular Therapy has proposed three minimal criteria to identify MSCs: 1) the adherence to plastic, 2) the specific surface antigen expression (positivity for CD105, CD73, CD90 and the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II and 3) the multipotent capacity to differentiate into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions [4]. As unique
progenitors, MSCs isolates have been shown to differ from each other with respect to their osteogenic potential, but the cell properties responsible for these observations are not completely understood [5].

In recent years, surgeons have sought new strategies to optimize bone repair. One of these alternatives has been the clinical use of human MSCs (hMSCs). In orthopaedics, there is a great interest in developing therapies to improve bone healing by using a patient’s own hMSCs to form new bone [6]. Osteogenesis imperfecta, the nonunion of fractures and avascular necrosis of the femoral head remain some of the most challenging and clinically prevalent dilemmas in orthopaedic surgery [7]. In nearly every orthopaedic surgery, the “gold standard” for bone grafting is traditionally autologous bone harvested from the iliac crest. However, significant donor site pain and morbidity is associated with this procedure, and autologous bone cannot be used to treat avascular necrosis [8-10]. As a result, the use of autologous bone marrow aspirate for bone grafting has been advocated as a means to provide an osteogenic cell source.

Through surgical insertion of a large trochar needle into the iliac crest, iliac crest bone marrow can be collected, then concentrated and injected into the affected site. As this procedure has had variable success rates [11-13] and the major factors that affect successful bone regeneration are still unknown, it makes it very difficult to predict the outcome of these procedures. Hence, the safety, feasibility and efficiency of hMSC transplantation for clinical use are currently
the object of studies, both clinically and through \textit{in vitro} studies. As such, in order to use hMSCs in therapy-based treatment, identification of an \textit{ex vivo} cellular phenotype, also known as biomarkers, correlated to osteogenic potential to form bone \textit{in vivo} is critical to the efficacy of these treatments.

Clinically, it would be preferred to expand these hMSCs \textit{in vitro} prior to injection to obtain a consistent number of cells available for both reconstruction and repair of bone and cartilage. Even so, bone marrow aspirations must be re-injected during the same procedure according to the Food and Drug Administration regulations. As a result, an individual’s hMSCs only can be studied following the completion of the procedure. By studying marrow aspirates, it has been observed that initial plating contains a high concentration of hematopoietic stem cells with macrophages, endothelial cells and lymphocytes in the early BM monolayer [14, 15]. After exchange of growth media, only fibroblast-like spindle-shaped cells proliferate and form colonies. Since hMSCs adhere to the plastic, they are the primary cell type to remain in culture and can generate single-cell-derived colonies that can be expanded to obtain high numbers of cells for clinical use [16, 17]. However, it has long been established that these populations are not homogeneous preparations of stem cells. The identification of “optimal” conditions and methods for \textit{in vitro} cell culture should be investigated. Therefore, a focus of this study is to examine hMSCs isolated from a variety of skeletal tissue sites to determine if these stem cells behave differently as a result of culture technique and cell source.
Perhaps most important for clinical application is an understanding of the molecular and cellular changes that are associated with osteogenesis and bone formation. Bone formation is a complex process that involves numerous steps and interaction between the several genes [18-20]. Osteoblasts and their progenitors are responsible for bone formation, control of osteoclastic cell functions through the secretion of cytokines, and also support hematopoiesis by providing a supportive niche. Therefore, biomarkers known to be relevant for bone formation and the ability to form mineralized matrix in vivo have been used to indicate osteoblastic phenotype of cells ex vivo [19, 20]. However, these biomarkers, such as the production of alkaline phosphatase, type 1 collagen, osteopontin, bone sialoprotein and osteocalcin have not been predictive of the in vivo bone-forming capacity of hMSCs [19, 21]. Recent studies have identified genes believed to be associated with bone formation that are not within the traditional set of bone-formation biomarkers. Studies also have shown a regulatory function of genes involved with differentiation to osteogenesis and adipogenesis such as PPAR-γ [22].

Due to their heterogeneous nature, hMSCs may differ in their capability to form new bone in vivo not only between patients, but also between different harvest sites for the same patient. Furthermore, donor age and disease stage can also influence hMSC yield, proliferation rate, and differentiation potential [23, 24]. Consequently, it is of great importance to identify a set of biomarkers that are better at predicting the in vivo bone-formation capacity of hMSCs. Moreover,
their use for gene and cell therapy of skeletal diseases requires the long-lasting engraftment of hMSCs endowed with a residual proliferation potential sufficient to sustain the low, but continuous, bone turnover in adulthood [25]. The maintenance of their stem properties and the possibility to reprogram their commitment is therefore a field of primary interest given their potential use in regenerative medicine.

Thus, it is important to develop methodology which will allow for prediction of hMSCs osteoblastic potential at the time of a patient’s procedure. This methodology includes testing of isolated hMSCs for the expression of a unique set of bone formation biomarkers, prior to their surgical application. Through investigation of these genes, in vivo potential of bone formation can be predicted; and, therefore, potentially predict the ultimate patient outcome. To identify the best skeletal site for hMSC isolation and their bone formation properties, we have tested hMSCs derived from patients undergoing orthopaedic surgeries at the University of Toledo Medical Center (UTMC). Using these cells, we observed the relationships of hMSCs’ ability to proliferate and differentiate into osteoblasts and their correlation to the ex vivo biomarkers of bone formation. These data reveal striking differences between hMSC populations with regard to growth rate, endogenous levels of ALP expression, and the level of bone-specific and candidate biomarker gene expression following exposure to osteogenic media. As these differences were evident even between samples from the same donor, we suspect that the donor harvest site may be a determining
factor in hMSCs’ ability to differentiate towards pre-osteoblastic cells. As a result, careful genetic analysis of hMSC donor populations is necessary before administering cells to patients for therapeutic treatments.

1.2 Materials and Methods

CELL CULTURE

Bone Marrow Aspirate Cell Culture

Bone marrow derived hMSCs were harvested from the iliac crest of adult donors who underwent bone marrow collection for a related procedure after informed consent was obtained (Figure 1-1). An aliquot of whole bone marrow aspirate, usually less than one milliliter (ml) in volume, was transferred to a microcentrifuge tube. Cells were resuspended and 10µl was transferred to another microcentrifuge tube for counting. Ten microliters of Zapoglobin (Beckman Coulter, Miami, FL, USA), and 80µl of MEM-α Medium (Lonza, Versviers, Belgium) containing 15% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin was added to this tube, mixed and placed onto a hemacytometer for counting. The remaining marrow aspirate was transferred to a 30mm² or 60mm² culture plate (Becton Dickinson, Franklin Lakes, NJ, USA), dependent on the volume of bone marrow. A maximum volume of 0.5ml marrow/media suspension was plated onto 30mm² plate. For each aspiration, cell counts were attempted. The cell number per 1 ml was obtained by
multiplying the count by 100,000 (5-fold dilution in medium, 2-fold dilution in Zapoglobin, x 10,000 for the haemacytometer). Plates were placed into an incubator and maintained at 37°C with an atmosphere of 5% CO₂. After approximately five days, the non-adherent cells were removed and media was exchanged every 3-4 days. When they reached approximately 60% confluence, they were detached, pooled and re-plated for a further 3-5 passages.

*Tissue Derived Cell Culture*

Tissue-derived hMSCs were isolated directly from discarded pieces of bone and tissue (Figure 1-2). These tissues were obtained from the operating rooms of UTMC after being removed from patients in accordance with the standard-of-care treatment. Tissue was brought to the laboratory and processed within one hour of the harvest. For processing, tissue was observed and categorized according to the type of tissue (Table 1.1). Cartilage and fat pieces were harvested with a sterile scalpel blade, while bone pieces were harvested using a micro-drill that created fine bone pieces. These pieces of tissue were suspended in MEM-α Medium (Invitrogen, Carlsbad, CA, USA) containing 15% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin on 30mm² or 60mm² cell culture plates. Plates were placed into an incubator and maintained at 37°C with an atmosphere of 5% CO₂. After two to three days, plates were observed for cell growth. Once several colonies were observed, media was exchanged. When they reached approximately 60% confluency, they were detached, pooled and re-
plated for typically 2-5 passages. Tissue suspensions were maintained for 10-14 days before they were considered an unsuccessful isolation due to no observable colony formation.

EX VIVO CELL DIFFERENTIATION STUDIES

Alkaline Phosphatase (ALP) and Calcium Binding Assays

MSCs were plated at a density of 150 cells/well in 96-well plates (469 cells/cm²) in osteogenic medium consisting of MEM-α Medium (Invitrogen, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) supplemented with 10 mM β-glycerophosphate (Sigma Chemical Co., St. Louis, MO, USA), 50μg/mL L-ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA), 10nM dexamethasone (Sigma Chemical Co., St. Louis, MO, USA). The media was changed every four days. Cells were grown in the first three columns of the 96-well plate. By doing so, eight replicates were available for the control values, osteogenic-treated values, and values for cells without ALP substrate. The control cells were grown in standard growth media, MEM-α Medium (Lonza, Versviers, Belgium) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Cells were assayed on days 1, 3, 6, 9 and 12. Alkaline phosphatase assays were completed by preparing an ALP Buffer and ALP Substrate. ALP buffer was a 1:3 dilution of 1.5M ALP Buffer (Sigma Chemical Co., St. Louis, MO, USA) and sterile water. Phosphatase Substrate was
made by adding one 40mg Phosphatase Substrate capsule (Sigma Chemical Co., St. Louis, MO, USA) into 10ml of sterile water. Initially, each well was washed with 250μl of 0.1M pH 7.4 HEPES. After careful aspiration, 75μl of ALP Buffer was added to each well. Seventy-five microliters of Phosphatase Substrate solution was then added to sixteen of the twenty-four cell containing wells and also to eight wells with no cells to serve as the experimental blank. Sterile water was added to the remaining eight wells containing cells. The entire plate was then placed into the spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) the plate was read at 0, 5, 15 and 30 minutes at 405nm.

The calcium content in the cell lysates were measured according to a previously published method using Sigma Diagnostic Kit # 587-A, which measures the amount of purple-colored calcium-OCPC (o-cresolphthalein complexone) complex formed as a result of binding between OCPC and free calcium generated by osteoblasts. After thirty days of continuous treatment with osteogenic media or control media, cells were incubated at room temperature for four hours with 1.485 mL acetic acid (0.1%) in each well of the six-well plate in order to extract calcium from mineralized constructs. Twenty microliters was then added to a 96-well plate. Equal amounts (150 μL) of calcium binding reagent (o-cresolphthalein complexone, 0.24% 8-hydroxyquinoline, and surfactant) and calcium buffer reagent (500 mM 2-amino-2-methyl-1,3-propanediol) provided from the kit were added to each well in the plate and was incubated with CaCl₂ and sterile water at room temperature for 10 min. The
standards in concentrations ranging from 5 μg/mL to 100 μm/mL were prepared using CaCl₂. The absorbance of each well was measured using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) at 570 nm. The calcium content from each sample was normalized to the total cell number at each time point, \( \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{Standard}}} \times ([\text{standard}]) \).

**MTT Assay for Cell Proliferation**

MSCs were plated in 96-well plates at 150 cells/well and incubated at 37°C in 5% CO₂ incubator. One hundred and fifty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St. Louis, MO, USA) was added to each well at 5.0 mg/ml final concentration. MSCs were incubated in the MTT solution for a uniform time for each MSCs primary cell line. Formazan products were dissolved with Dimethylsulfoxide (Sigma) and the optical density was measured using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) at 570 nm and 650 nm. Cell growth was calculated using a MTT Standard Curve by a performing a serial dilution of the same MSCs. Eight replicates were used for each treatment.

**RNA Isolation and real-time quantitative polymerase chain reaction (qPCR)**

MSCs were plated at a density of ~25,000 cells per 100mm² culture dish and maintained in standard growth media or osteogenic supplemented media. At days 1, 3, 6, 9, 12, Trizol (Invitrogen, Carlsbad, CA, USA) was added to the
osteogenic plate and total RNA was isolated as described by the manufacturer. For the MSCs maintained in standard growth media, Trizol RNA isolation was performed at near confluence.

The cDNA was synthesized from RNA samples using the Verso cDNA Kit (Thermo Scientific, Waltham, MA, USA) as described by manufacturer’s instructions. qPCR was performed using Power SYBR Green PCR Master Mix Kits (Applied Biosystems, Foster City, CA, USA). The amount of cDNA used for each qPCR was 1.0µg. The amplification reactions were performed using the following primers: COL121A: forward, 5’-ACTCAGTGTCCTCCCTTGACAGAT-3’; reverse, 5’-GAGGTGACCATTGGACACAGAA-3’; CPE: forward, 5’-CATCTCCGTGGAAGGAATAGAC-3’; reverse, 5’-CTGGAGCTGAGGCTGTAAGTTT-3’; DCN: forward, 5’-GCCGAGAAGTTCTGATGAC-3’; reverse, 5’-TCAGAACACTGGACCACCTCG-3’; LOXL4: forward, 5’-CCAGCTTCTGTCTGGAGGAC-3’; reverse, 5’-AATGTCATGCCCAGGTGTGTG-3’; LUM: forward, 5’-GTGCCTCCTGGAATCAAGTATC-3’; reverse, 5’-GTGCCTCCTGGAATCAAGTATC-3’; MFAP5: forward, 5’-AGGTAAATTTCTCATCCCAGCA-3’; reverse, 5’-AGGTAAATTTCTCATCCCAGCA-3’; COL1A: forward, 5’-CAAGCTTCTGGACACATGGG-3’; reverse, 5’-CAAGCTTCTGGACACATGGG-3’; RUNX2: forward, 5’-GGGTCTTGGCTGATGTACCAGT-3’; reverse, 5’-GGGTCTTGGCTGATGTACCAGT-3’; CTTGACCATAACCGTCTTCACA-3’;
GT CATCAAGCTTCTGTCTGTGC-3'; Osteocalcin: forward, 5'-CAGGCGCTACCTGTATCAATG-3'; reverse, 5'-TCAGCCAACTCGTCACAGTC-3'; DLX5: forward, 5'-CGAGTCTTCAGCTACCGATTCT-3'; reverse, 5'-TACTGTTAGGGGTAGAGGAGTTT-3'; and 18s was used to normalize each qPCR amplification. Reactions were carried out using a StepOne Plus qPCR Machine (Applied Biosystems, Foster City, CA, USA).

1.3 Results

MSC Harvest and Preparation

A total of fifteen tissue samples were collected from donors with an average age of 60.6 years (range, 33-93). Eight of the tissue donors were female, six were male, and one was unknown. When comparing donor medical conditions and ability to proliferate MSCs in vitro, there was no correlation found between body mass index, diabetes mellitus or a history of smoking. There were no significant differences between age and gender and successful expansion. Amongst harvest sites, the iliac crest bone was the only tissue source that did not provide successful hMSC expansion in culture, but only one tissue sample was available for testing (Table 1.3).

Within the bone marrow aspirates, a total of seven bone marrow samples also were collected with an average age of 49.1 years (range, 24-64). Within this group four of the marrow donors were female, three were male. Five of the concentrated marrow samples were taken from the iliac crest and one sample
was harvested from the distal femoral. One marrow sample was the product of reaming during intramedullary nailing of the femur. In contrast to the other marrow aspirates, this sample was not centrifuged and concentrated in the operating room. Amongst all harvest sites, the iliac crest bone marrow was the only site to proliferate in culture. Expansion was unsuccessful when less than 350μl of marrow aspirate was available for plating (Table 1.2).

hMSCs were plated from a variety of tissue sources and performed heterogenous hMSC preparation by using a scalpel and/or drill to harvest tissue for plating. Regardless, the primary cultures showed a distinct population of spindle-shaped or flat hMSCs in the plate and no morphological differences were observed between these varying preparation methods. As cultures proliferated and were passaged, a morphological difference between tissue sources was observed. Cells harvested from cartilage and yellow bone sources were observed to maintain a spindle shape (Type I Cells) and grow rapidly, while cells harvested from red bone typically grew at a slightly slower rate and were more broad and transparent under 100x magnification (Type II Cells). This spindle and broad shape also was observed simultaneously in several cultures, demonstrating a heterogenous cell culture. In most cultures, the broad shaped hMSCs were the dominant cell type by the fourth passage (Figure 1-5E).

Assessment of Growth within Five Representative Cultures

Through evaluation of a MTT assay, the growth profile of each primary
cell line was determined (Figure 1-6). BM-006 MR demonstrated the least amount of growth over the twelve day culture, whereas MA-3 exhibited significantly greater levels of proliferation than all other cell lines. BM-006 CT and MA-4 demonstrated very similar growth profiles over the twelve day time period. Overall, each hMSC culture displayed similar growth rates until day 6. After day 6, cells begin to proliferate at varying rates, suggesting that harvest site may impact putative hMSC proliferation since marrow aspirates were observed to dramatically expand. BM-003 YB was not available for analysis.

**Differentiation Potential Analysis**

Of successfully expanded cultures, five representative cultures were analyzed for their osteogenic potential. Since ALP expression is upregulated during the early phase of osteoblast differentiation, levels of this enzyme should reflect the number of committed osteogenic cells within the culture population. hMSCs from BM-003 YB, BM-006 CT, BM-006 MR, MA-3 and MA-4 were cultured in osteogenic medium and tested for ALP activity. Figures 1-(7-11) shows the ALP activity from a twelve-day culture. BM-003 YB, BM-006 and MA-3 were observed to have the most significant difference in ALP activity after osteogenic treatment (Figure 1-7, 1-8, 1-10). Despite being from the same donor, BM-006 MR did not show a significant difference in ALP activity after the twelve day treatment with osteogenic treatment versus control cells (Figure 1-9). MA-3 showed a less significant difference in ALP ALP activity after osteogenic
treatment versus the control cells (Figure 1-10). We did not observe any difference in ALP activity in the osteogenic treated cells versus the control cells in MA-4 (Figure 1-11). However, when comparing overall ALP activity, MA-3, MA-4 and BM-006 MR displayed the highest expressions of ALP (Figure 1-12). This overall change in ALP expression from day 0 to day 12 was used to correlate osteogenic induction with gene analysis data.

Mineralization

Calcification assays also were performed in these cells (Figure 1-13). BM-003 YB showed the greatest mineralization after thirty days of osteogenic treatment, followed by MA-4, BM-006 MR and finally MA-3. There was no difference observed in calcification after thirty days for BM-006 CT.

Gene Expression Analysis

To identify the molecular phenotype predictive of osteogenesis, we compared differences in gene expression of hMSCs at day 0 and day 9 treated with control MEM-α medium and then to gene expressions of hMSCs at day 12 treated with osteogenic medium. By using this technique, it was possible to evaluate changes in gene expression that occurred simply due to culture growth or as a result of osteogenic induction.

Correlation of initial gene expression to ALP Activity
First, the gene expression of candidate genes from each primary cell line at day 0 was correlated to the increase in ALP activity (Table 1.4). After normalization to the expression of COL1A1 which was almost equal in all cell lines, the resulting fold-change was evaluated for correlation. Interestingly, the r-values from the correlation test for genes CPE, DCN, LOXL4 and PPAR-γ showed the greatest correlation (r-values > 0.75) to an increase in ALP activity while there was little correlation with genes known to be associated with bone formation. Low expression of CPE and PPAR-γ at day 0 strongly (r-values > 0.90) correlated to the largest increase in ALP activity after twelve days of osteo-induction. Whereas canonical genes associated with bone formation demonstrated little correlation (r-values < 0.50) to an increase in ALP activity. However, high correlation amongst relative fold-change was observed after separate analysis of tissue-derived hMSCs and marrow-aspirate-derived hMSCs (Table 1.5). This observation indicated that although these hMSCs were harvested from different patients, the tissue source of hMSCs may have the greatest influence on genetic phenotype.

**Gene expression of marrow aspirations**

To look more closely at the correlation of gene expressions of candidate biomarkers in MA-3 and MA-4 cells, fold changes were normalized to BM-003 YB cells (Table 1.5). BM-003 YB was selected to normalize due to its low ALP activity and minimal changes in gene expression. Following normalization, MA-
3 and MA-4 clearly showed similar decreased expression of DCN, LOXL4 and LUM. On the other hand, MA-3 and MA-4 displayed similar increased expression of RUNX2 and CPE. When comparing BM-006 CT and BM-006 MR, these hMSCs were derived from the same patient, but displayed markedly different levels of gene expression. DCN was increased in both BM-006 CT and MR, but were almost two-fold apart at 4.73 and 2.87, respectively. LOXL4 was the only gene that was found to have increased expression in BM-006 MR, but deceased expression in CT. PPAR-γ2 and CPE displayed a similar decrease and increase in expression, respectively, in both BM-006 CT and MR. Unlike the marrow aspirates, tissue-derived hMSCs had opposite expression of LUM and RUNX2. LUM showed decreased expression in BM-006 CT and MR, while increased expression was observed in MA-3 and MA-4. Conversely, RUNX2 was down regulated in CT and MR, while MA-3 and MA-4 had similarly increased expression. Overall, these data show that MA-3 and MA-4 have a more similar genetic phenotype than BM-003 YB, BM-006 CT and MR.

Factors of gene expression

Lastly, to draw direct comparisons between cell lines and changes in gene expression resulting from osteogenic induction or standard growth, gene fold changes were calculated and compared (Figure 1-14). The expression difference of each candidate gene for standard growth was the difference between day 0 of growth and day 9 of growth in control MEM-α medium (Figure 1-14A). The
expression difference of each candidate gene as a result of osteo-induction was determined through the difference of day 0 of growth with control media and day 12 of growth with osteogenic growth medium (Figure 1-14B). BM-003 YB candidate gene expression displayed minimal overall change in most genes. For BM-003 YB, COL12A1, LUM, COL1A, DLX5 and osteocalcin significantly (>5 fold) were downregulated after osteo-induction. DLX5 and osteocalcin expression changed from -12.5 to -33.6 or a 21.53 decrease in DLX5 expression and a 38.47 decrease, from -2.8 to -41.27, in osteocalcin. COL12A1, LUM and COL1A were downregulated after osteo-induction by 10.68 fold, 7.77 fold, and 5.62 fold, respectively. Also in BM-003 YB, PPAR-γ was upregulated as a result of osteo-induction changing from -13.75 to +1.10, an upregulation of 14.85 as a result of osteo-induction. The greatest changes observed in BM-006 CT were a seventeen fold increase of LOXL4 and a 35.20 fold increase of PPAR-γ as a result of osteo-induction and 9.39, 44.24 and 51.79 fold decreases of COL12A, DLX5 and COL1A, respectively. In BM-006 MR, the greatest changes in gene expression were a 6.7 upregulation of PPAR-γ2, a 6.75 downregulation of COL1A, a 10.39 upregulation of DLX5 and a 20.60 upregulation of PPAR-γ. In MA-3, CPE was upregulated 29.27 fold as a result of osteo-induction. DCN, PPAR-γ and PPAR-γ2 also were upregulated 15.58, 10.22 and 52.47 fold, respectively. In MA-3, the only significantly downregulated gene was LOXL4 which decreased 10.3 fold in expression. Four genes in MA-r, CPE, DCN, PPAR-γ and PPAR-γ2, were upregulated by 29.27 fold, 15.58 fold, 10.22 fold and 52.47 fold, respectively.
Similarly, MA-4 displayed a significant downregulation of LOXL4 by 45.13 fold and upregulations of CPE, DCN and PPAR-γ by 31.55 fold, 16.56 fold, and 9.6 fold, respectively.

**Correlation of gene expression changes**

Most of the changes in gene expression were similar amongst the primary cell lines. To compare these changes, a table was created to correlate fold changes as a result of time, a result of osteo-induction or both (Table 1.6). The values within the table are the numerical fold changes of each bar expressed in Figure 1-14A and 1-14B. Each value was colored to illustrate correlative changes in expression between fold changes from day 0 to day 9 in untreated, control cells and fold changes as a result of a twelve day treatment with osteogenic induction. Values were colored white if there was no observed correlation between time and osteo-induction. If gene expression increased similarly in both untreated and osteo-induced cells, the value was colored yellow. If gene expression increased in only the osteo-induced cells, the value was colored green. If gene expression increased in untreated cells as a result of time, but increased significantly more with osteo-treatment, the value was colored blue. If expression decreased, apart from of being untreated or osteo-treated, the value was colored red.
1.4 Discussion

Advancement of knowledge is critical to the development of new orthopaedic procedures. Over the past couple of decades, many insights into MSC biology and their regenerative potential has been revealed, and now is of great interest to the orthopaedic community. Although the culture of hMSCs has been well-studied, reports are rare regarding the osteogenic potential of hMSCs derived from different tissue sources and what the best method is to harvest hMSCs from patient samples. In this study, we harvested hMSCs from fifteen donors and tested five tissue samples from four different donors. We analyzed the cellular growth, and studied the osteogenic differentiative potential and genetic expression of several candidate genes in order to understand which tissue source might be considered optimal to isolate and expand for clinical orthopaedic applications.

We defined hMSCs by their adherence to plastic culture surface during isolation, morphology and their ability to be induced towards an osteoblastic phenotype as determined by ALP activity. hMSCs have numerous characteristic properties that have been well-defined by a number of investigators [1-4], but since we did not perform a cell sorting experiment to determine the specific surface antigen expression, there may be a heterogenous population of cells present in the culture. Our simple isolation technique may permit a high variability of cells in culture, but it also may be a more realistic representation of
the patient’s ability for osteogenesis since these cells are grown directly from donor tissue. In addition, the differentiation of MSCs largely is driven by signals from culture conditions or the microenvironment in vivo, particularly the microenvironment of rapidly developing or injured tissues [26]. In most cases the signals that drive differentiation in vivo remain indeterminate, and therefore, cannot be replicated ex vivo. In our study we did our best to mimic this local in vivo microenvironment by culturing cells with whole pieces of intact tissue in the culture media. By leaving the marrow aspirates and tissue-derived cultures untouched for several days post-plating to best simulate this microenvironment, we obtained successful expansion. We were able to successfully harvest seven different tissue types from our fifteen donors (Table 1). Most often, cartilage derived hMSCs (CT) universally were observed to expand from the majority of patients, regardless of age or disease state. Yellow bone marrow tissue samples (YB) also had an overall high success of expansion within our donor population, suggesting that the presence of fat may be beneficial to hMSC culture. Dissimilar to other studies [27-29], hMSCs derived from the bone periosteum often did not expand in culture, or expanded as cells with an older morphology. One observation to note during culture of all hMSCs is that these hMSCs often presented two morphologically distinct cells: Type I cells that are spindle shaped and grow rapidly, and Type II cells that are broad and grow slowly [17, 30, 31]. Moreover, in our experiments we observed that after several passages, more Type II cells were present within the culture. Also observed were cells with
intermediate morphologies. Other authors also demonstrated that samples of hMSCs obtained from iliac crest aspirates varied widely in their culture expansion [29, 32]. Likewise, similar variations in both morphology (Figure 1-5) and in growth profiles were observed (Figure 1-6).

To examine the possibility that cellular differentiation varies amongst these heterogeneous populations, we measured levels of ALP enzyme activity in each donor tissue sample for a twelve day period. For comparison, we cultured cells in control media and also in osteogenic supplemented media. Since ALP expression is upregulated during the early phase of osteoblast differentiation, its expression serves as an indicator of osteogenesis. When ALP activity was monitored over the twelve day period, significant differences in ALP activity were evident between the donor samples. Starting from day 0, MA-3 and MA-4 ALP activity dramatically increased by 9.39 and 20.39 μUnits, respectively, while BM-003 YB ALP activity minimally increased 0.68 μUnits. Measuring ALP activity in BM-006 CT and MR, despite being from the same patient, ALP activity increased by 2.79 and 7.12 μUnits, respectively. These variations could be due to several factors. BM-003 YB was derived from a 93 year old woman and although there was a significant increase in ALP activity after osteo-treatment as compared to the control culture, the overall change in ALP activity was considerably lower than the other tested cell lines. This observation can likely be contributed to her relatively old age. It has been previously shown that there are age-dependant differences in healing and differentiative potential with older
MSCs, which may lack the cytokines and stimulatory factors important for osteoprogenitor expansion and differentiation [33-35]. The different ALP activity in the two samples derived from BM-006 may be due to fact that the CT culture was derived from cartilage, whereas the MR culture was derived from red bone. Since CT is from a connective tissue other than bone, its response to osteostimulation may be endogenously low. MA-3 and MA-4 expressed considerably more ALP activity than the other hMSC cultures, but the differences between control and osteo-treated cultures were relatively small. This may indicate marrow aspirates are more differentiated, and therefore, readily induced over time without osteo-treatment since osteo-treatment was not necessary for these cell lines to illustrate an increase in ALP activity.

To further confirm the different nature of these hMSC populations, we measured the induction levels of several candidate bone biomarkers as well as bone-specific genes by qPCR. By comparing gene expression at day 0 with the ALP activity of each cell line, this study determined how the expression of genes at day 0 correlated to a future increase in ALP activity (Table 1.4). Two candidate genes initially described by Larsen et al., decorin (DCN) and lysyl oxidase-like 4 (LOXL4), show a strong correlation in all cell lines to expression levels predictive of an osteoblastic phenotype. The only other genes with an r-value greater than 0.75 not proposed by Larsen et al. were carboxypeptidase-E (CPE), and peroxisome proliferator-activated receptor gamma (PPAR-γ), a well-established gene that plays an important role in the control of adipocyte development as well
as glucose and fatty acid metabolism. More recently, PPAR-γ also has been shown to be involved in regulatory pathways of osteoblast differentiation from marrow mesenchymal stem cells [22]. This may explain why initially low expression of PPAR-γ correlates to future expression of a pre-osteoblastic phenotype. CPE encodes a carboxypeptidase that cleaves C-terminal amino acid residues and is involved in the biosynthesis of peptide hormones and neurotransmitters, including insulin. Since CPE and PPAR-γ are both involved in glucose metabolism, it is possible that their low expressions at day 0 may be predictive of hMSC’s ability to differentiate towards an osteoblastic phenotype.

Looking more closely at these other candidate bone biomarkers from the day 0 evaluation, it was discerned that several of these biomarkers play roles within the extracellular matrix. DCN is a protein enriched within connective tissue, that binds to type I collagen fibrils, and plays a role in matrix assembly. DCN was found to be a candidate gene for in vivo bone growth, and also contributory to bone loss of DCN knockout mice [16, 30]. LOXL4, a gene also described by Larsen et al., is important in collagen assembly by osteoblastic cells due to their role in cross-linking of collagen molecules as well as elastins [31, 32]. It is valuable to note, that correlations to ALP activity may have been much higher if not all cell lines were included in the correlation analysis. hMSCs derived from marrow aspirates have similar expression levels and often do not correspond to the expression levels of tissue-derived hMSCs.
In order to draw additional information from gene expression levels, we normalized candidate biomarkers to 18S and then to BM-003 YB expression since these cells had very low ALP activity. Using this method, it was possible to observe whether or not the candidate biomarkers were similarly expressed in tissue samples and how expression in MA-3 and MA-4 compared since these samples expressed dramatically more ALP activity. Here, we show that DCN, LOXL4, Lumican (LUM) and PPAR-γ2 have low levels of expression in MA-3 and MA-4. Like the other candidate genes, LUM is an extracellular matrix related gene. LUM belongs to the small leucine-rich proteoglycan family and plays an essential role in regulation of collagen fibril formation in bone matrix [33, 34].

Also in MA-3 and MA-4, RUNX2 and CPE are approximately expressed three and four fold higher than BM-003 YB, respectively. Interestingly, when looking at expressions of these genes in BM-006 CT and MR, DCN, LUM, and RUNX2 were oppositely regulated. The RUNX2 gene encodes a protein that is essential for osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression [35]. Of these, CPE is the only putative biomarker that displayed similar expression in all cell lines.

To provide a complete analysis of the changes in expression associated with osteo-induction or simply with culture growth over time, expression levels were normalized to 18S and analyzed (Figure 1-14, Table 1.6). All candidate genes were compared with canonical bone-related genes. Candidates included,
collagen XII alpha1 (COL12A1), a homotrimer found in association with type I collagen with an association that is thought to modify the interactions between collagen I fibrils and the surrounding matrix, CPE, DCN, LOXL4, LUM, and microfibrillar associated protein 5 (MFAP5), Canonical genes associated with bone formation were COL1A, distal-less homeobox 5 (DLX5), a protein that plays an important role in skeletal development and binds to the homeodomain-response elements in the RUNX2 gene distal promoter [35,36], osteocalcin, a bone-derived protein that also plays a role in glucose metabolism, PPAR-γ and PPAR-γ2. By color-coding Table 1-6, it is apparent that there are numerous relationships between these genes. Central to determining if a bone biomarker is predictive of hMSC’s ability to differentiate towards an osteoblastic phenotype is to observe gene changes after osteo-treatment. Expression of CPE, DCN, PPAR-γ dramatically increases in MA-3 and MA-4. Since these two cell lines had markedly greater ALP activity, it suggests these genes may be involved in differentiation to pre-osteoblastic cells. LOXL4 also is considerably down regulated after osteo-treatment, indicating that downregulation of LOXL4 also may be necessary for osteoblast differentiation. Interestingly, canonical osteoblastic markers, like COL1A, RUNX2 and osteocalcin were not correlated to an increase in ALP activity. This finding also was present in the study by Larsen et al., and was suggested that these in vivo osteoblastic markers lack the sensitivity to determine bone-formation capacity in vitro. These data, suggested that the aforementioned extracellular-matrix related proteins may provide a
more predictive molecular signature for bone formation. More testing of these markers is necessary, both ex vivo and in vivo, to determine if these genes can serve as biomarkers and do in fact influence osteogenesis in vivo similarly to ALP activity observed ex vivo. Moreover, the molecular signature described here may only represent a few of the genes involved in the complex osteoblastic phenotype with several others yet to be identified. The ability to prospectively test an hMSC sample for a set of biomarkers remains to be determined. A set of biomarkers will be pivotal to quality and successful application of hMSCs for bone regeneration. Taken together, the preliminary results of this study demonstrate that the iliac crest marrow may be the best site to obtain hMSCs and testing for these candidate biomarkers may provide a clinician with insight into the osteogenic potential of their hMSCs to aid in surgical planning.
1.5 Conclusions

- Four candidate genes, decorin, carboxypeptidase-E, lysyl oxidase-like 4 and peroxisome proliferator-activated receptor gamma show a strong correlation in expression levels predictive to an osteoblastic phenotype.
- The source of hMSCs directly affects the ability to differentiate into pre-osteoblastic cells.
- hMSCs from a geriatric patient displayed low variation of gene expression and low alkaline phosphatase activity suggesting that with age hMSCs may lose their differentiation capacity.
- hMSCs obtained from marrow aspirates already may possess the ability to differentiate into pre-osteoblastic cells.
- These results agree with the clinical practice to inject a patient’s own hMSCs obtained from iliac crest bone marrow aspirations into a non-healing fracture to induce new bone formation at the fracture site.


1.6 Tables and Figures (In order of appearance)

Table 1.1: Tissue Classification Key

<table>
<thead>
<tr>
<th>Label</th>
<th>Tissue Description</th>
</tr>
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<tbody>
<tr>
<td>MA</td>
<td>Marrow Aspirate</td>
</tr>
<tr>
<td>MR</td>
<td>Red Marrow Bone</td>
</tr>
<tr>
<td>YB</td>
<td>Yellow Marrow Bone</td>
</tr>
<tr>
<td>CT</td>
<td>Cartilaginous Tissue</td>
</tr>
<tr>
<td>AD</td>
<td>Adipose Tissue</td>
</tr>
<tr>
<td>PR</td>
<td>Periosteal Tissue</td>
</tr>
<tr>
<td>WB</td>
<td>White, Bony-like Tissue</td>
</tr>
<tr>
<td>TFB</td>
<td>Fat Beneath Tumor</td>
</tr>
<tr>
<td>CTN</td>
<td>Newer Cartilage</td>
</tr>
<tr>
<td>CTO</td>
<td>Older Cartilage</td>
</tr>
<tr>
<td>PRN</td>
<td>Newer Periosteal Tissue</td>
</tr>
<tr>
<td>PRO</td>
<td>Older Periosteal Tissue</td>
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</tbody>
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Table 1.2: Bone Marrow Aspirate Donor Data

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<th>Age</th>
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<th>Harvest Site</th>
<th>Total Marrow Volume</th>
<th>Initial Cell Count</th>
<th>Successful Expansion</th>
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<td>MA-1</td>
<td>64</td>
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<td>N/A</td>
<td>N/A</td>
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<tr>
<td>MA-2</td>
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<td>F N/A</td>
<td>Iliac Crest</td>
<td>140ul</td>
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<tr>
<td>MA-3</td>
<td>59</td>
<td>F N/A</td>
<td>Iliac Crest</td>
<td>1000ul</td>
<td>2.8x10⁷</td>
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</tr>
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<td>MA-4</td>
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<td>1000ul</td>
<td>7x10⁷</td>
<td>Yes</td>
</tr>
<tr>
<td>MA-5</td>
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<td>F N/A</td>
<td>Iliac Crest</td>
<td>N/A</td>
<td>1.2x10⁸</td>
<td>Yes (terminated due to fungus)</td>
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<td>MA-6</td>
<td>38</td>
<td>M Smoker</td>
<td>Femoral Canal</td>
<td>10ml (unconc.)</td>
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<tr>
<td>MA-7</td>
<td>24</td>
<td>F N/A</td>
<td>Distal Femur</td>
<td>350ul</td>
<td>3x10⁷</td>
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Table 1.3: Tissue Donor Data

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<tr>
<th></th>
<th>Age</th>
<th>Gender</th>
<th>Related Medical Conditions</th>
<th>Harvest Site</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Successful Expansion (plates)</th>
<th>BMI*</th>
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<td>BM-001</td>
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<td>68</td>
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<td>93</td>
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<td>Thrombocytopenia, Venous Insufficiency</td>
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<td>149.9</td>
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<td>22.7</td>
</tr>
<tr>
<td>BM-004</td>
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<td>F</td>
<td>Hypertension, Smoker until 2005</td>
<td>Iliac Crest</td>
<td>67.6</td>
<td>154.9</td>
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<td>N/A</td>
<td>N/A</td>
<td>Knee</td>
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<td>N/A</td>
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<td>N/A</td>
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<tr>
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<td>Epilepsy</td>
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<td>185.42</td>
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<td>29.4</td>
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<tr>
<td>BM-007</td>
<td>69</td>
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<td>117.93</td>
<td>172.5</td>
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<td>39.6</td>
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<tr>
<td>BM-008</td>
<td>75</td>
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<td>Type 2 Diabetes Mellitus</td>
<td>Knee</td>
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<td>154.9</td>
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<td>24.6</td>
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<tr>
<td>BM-009</td>
<td>56</td>
<td>M</td>
<td>Type 2 Diabetes Mellitus, Hypertension, Type 2 Diabetes Mellitus</td>
<td>Knee</td>
<td>112.5</td>
<td>175.3</td>
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<td>36.4</td>
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<tr>
<td>BM-010</td>
<td>50</td>
<td>F</td>
<td>Degenerative joint disease, Hypertension, Diabetes Mellitus, Hypothyroidism bilateral knee developmental orthopaedic disease</td>
<td>Knee</td>
<td>99.8</td>
<td>167.5</td>
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<td>BM-011</td>
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<td>N/A</td>
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<tr>
<td>BM-012</td>
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<td>Degenerative joint disease, Hypertension, Diabetes Mellitus, Hypothyroidism bilateral knee developmental orthopaedic disease</td>
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<td>175</td>
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<td>BM-013</td>
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<td>73</td>
<td>150</td>
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<td>BM-014</td>
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<td>Syncope</td>
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<td>127</td>
<td>157.5</td>
<td>Yes (7/7)</td>
<td>51.2</td>
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Figure 1-1: On the oblique obturator view, the tip of the trochar needle was located at the midpoint between the inner and outer wall of the iliac crest and advanced. The depth of the needle advancing into the iliac crest was about 4 to 5 centimeters.
Figure 1-2: Tissue Samples were obtained directly from the operating rooms of UTMC. Each specimen was categorized as a specific tissue type and fragments of tissue were harvested in culture plates within 1 hour of being removed from the patient. A) Cartilage Harvested from piece of distal femur B) Harvest of “CT” cells C) Harvest of “MR” cells D) Harvest of “YB” cells E) Harvest of “MA”
Figure 1-3: Images A-E illustrate various points during hMSC development. White arrows point to putative hMSCs. Image A is six days after “YB” tissue harvest and shows very early hMSCs beginning to appear on the culture plate (100x). Image B is eighteen days after plating “YB” and twelve days after Image A was taken (100x). Image C was taken thirteen days after plating a different “YB” sample and shows hMSCs growing from bone pieces with visible fat droplets in the media (40x). Image D shows a “MR” culture six days after harvest with hematopoietic stem cells and hMSCs in the medium (100x). Image E shows an initial hMSC developing amongst millions of hematopoietic stems from a bone marrow aspirate (100x). Image F is of MA-3 and shows hMSCs arises from a cluster of cells(100x).
Figure 1-4: Following tissue harvest, hMSCs begin to migrate out of the tissue pieces and adhere to the culture plate. Images A-F illustrate the growth pattern of hMSCs in a cartilage culture from a 63 year old male. A) Eight days after initial plating hMSCs have migrated out of a piece of cartilage and moving outward (100x). B) Ten days after initial plating, cells have become dense around the cartilage border and confluent surrounding the cartilage (100x). C) At 40x magnification ten days after plating, hMSCs clearly become decreasingly confluent farther from the cartilage piece. D) Thirteen days after plating, hMSCs have become completely confluent surrounding the cartilage piece in the microscope field at 40x magnification. E) The cartilage piece has been removed on day 13, demonstrating that the hMSCs arise from the edge of the cartilage and move outward. F) At 40x magnification on day 13, a distinct border of the cartilage piece is visible.
Figure 1-5: Experimental cultures following nine days of growth in control medium. All cultures were initially plated with a density of 4,325 cells/well in a six-well plate. Image A) BM-003 YB. B) BM-006 CT. C) BM-006 MR. D) MA-3. E) MA-4.
Figure 1-6: Growth profiles of each primary cell line as determined by MTT Assay. Absorption values are the average of eight replicates. Cell number was determined by creating a standard curve with known cell number. BM-003 YB was not available for analysis.
Figure 1-7: Alkaline phosphatase activity in differentiating BM-003 YB Cells.
Figure 1-8: Alkaline phosphatase activity in differentiating BM-006 CT Cells.
Figure 1-9: Alkaline phosphatase activity in differentiating BM-006 MR Cells.
Figure 1-10: Alkaline phosphatase activity in differentiating MA-3.
Figure 1-11: Alkaline phosphatase activity in differentiating MA-4.
Figure 1-12: Alkaline phosphatase activity in primary cell lines at day 0 (black) and day 12 (grey) following induction of osteoblast differentiation.
Figure 1-13: Mineralization in all cell lines after 30 consecutive days of osteogenic treatment.
TABLE 1.4: A) Relative expression fold-change of candidate biomarker genes examined by real-time quantitative PCR at day 0. B) Relative expression fold-change of canonical genes associated with bone formation. Relative changes in gene expression were assessed for correlation to pre-osteoblastic phenotype using ALP activity and normalized to Col1A1 expression which was nearly identical in all cell lines.

<table>
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<tr>
<th></th>
<th>DLX5</th>
<th>OC</th>
<th>PPAR-γ</th>
<th>PPAR-γ2</th>
<th>RunX2</th>
<th>ALP Activity [µUnits/min]</th>
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<tr>
<td>BM-003 YB</td>
<td>0.71</td>
<td>0.70</td>
<td>0.52</td>
<td>0.56</td>
<td>0.55</td>
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<td>0.53</td>
<td>0.54</td>
<td>0.49</td>
<td>2.79</td>
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<td>0.51</td>
<td>0.56</td>
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<td>-0.44</td>
<td>-0.90</td>
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<table>
<thead>
<tr>
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<th>DCN</th>
<th>LoxL4</th>
<th>LUM</th>
<th>MFAP5</th>
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<td>BM-003 YB</td>
<td>0.77</td>
<td>0.57</td>
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<td>0.69</td>
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<td>0.76</td>
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<td>BM-006 MR</td>
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<td>MA-4</td>
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<td>-0.74</td>
<td>-0.30</td>
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Table 1.5: Gene expression of candidate biomarkers in MA-3 and MA-4 cells (high ALP activity) normalized to BM-003 YB cells (low ALP activity). Red indicates decreased expression, and green indicates increased expression.

<table>
<thead>
<tr>
<th></th>
<th>BM-003 YB</th>
<th>BM-006 CT</th>
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<td>1.84</td>
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Figure 1-14: Fold differences of tested genes for each cell line. Each bar represents the positive or negative fold-change of gene expression as compared to initial gene expression in A) uninduced control hMSCs and B) osteo-induced hMSCs.
Table 1.6: Comparison of gene expression as a result of time versus osteogenic treatment. The values for untreated cells represent the fold-change from day 0 to day 9 of growth under control conditions. Osteo-induced values represent the fold change in expression in osteogenic induced cells.

<table>
<thead>
<tr>
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<th>Osteo-induced cells</th>
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</table>

Key
- No correlation in expression
- Expression changes in a time-dependent manner
- Expression changes in an induction-dependent manner
- Expression changes in a time-and-induction-dependent manner
- Expression decrease
References


14. Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad


formation in diffusion-chambers by subcultured cells derived from the periosteum, Bone 1990;11:181–188.


