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Gene expression profiles of cytokines during osteogenic differentiation of human gingiva derived mesenchymal stem cells

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Gene Expression Profiles of Cytokines During Osteogenic Differentiation of Human Gingiva Derived Mesenchymal Stem Cells

Reem Ahmad Almashat

A Thesis Presented to the Faculty of the College of Dental Medicine of Nova Southeastern University in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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By

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I certify that I am the sole author of this thesis, and that any assistance I received in its preparation has been fully acknowledged and disclosed in the thesis. I have cited any sources from which I used ideas, data, or words, and labeled as quotations any directly quoted phrases or passages, as well as providing proper documentation and citations. This thesis was prepared by me, specifically for the M.S. degree and for this assignment.

STUDENT SIGNATURE: _____

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DEDICATION

I dedicate this project to my country, Saudi Arabia, for believing in me and for sponsoring my education in the United States. I would also like to dedicate this work to my loving, supportive family and friends. To my parents, Maysa Qutob and Ahmad Almashat, who have raised me to be who I am today and have always been there for me throughout my life. Thank you for your endless love, prayers and support. To my loving sisters, Salma, Nada and Leena Almashat. Having you by my side from thousands of miles away kept me going forward. To my baby brother, Yazan, thank you for just being you.

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Last but not least, I wish to express my appreciation to all the individuals who volunteered their participation in this project, Dr. Anapurna Bondalapati, Ms. Shaileen Dokhte Kavir and research collaborators at University of Miami. Special thanks to Ms. Christine Manguno, for her help in the lab experiments and for taking great care of my cells.

ABSTRACT

Gene Expression Profiles of Cytokines During Osteogenic Differentiation of Human Gingiva Derived Mesenchymal Stem Cells
August 2015

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Background: Therapeutic management of bone loss in the craniofacial region as a consequence of trauma, surgery or congenital malformations presents a clinical challenge. Mesenchymal stem cells (MSCs), due to their inherent plasticity, are potential candidates for cell based therapies for the repair and reconstruction of craniofacial bone tissue. Guided differentiation of stem cells to osteogenic precursors is marked by spatio-temporally regulation of gene expression profiles including that of transcription factors, cytokines, extracellular matrix proteins, enzymes and several signaling pathways. Cytokines, produced by both immune and non-immune cells can influence both immuno-modulatory responses in the host and also affect cell physiology. Understanding the cytokine expression profiles will be of great advantage in developing methods for effective bone regeneration with minimal immunological insults either on the graft or on the host. **Objective:** The objective of the present study is to investigate the gene expression profiles of the various cytokines of HGMSCs in normal and osteogenic conditions. **Methodology:** HGMSCs were isolated from gingival tissues by standard enzymatic methods. HGMSCs were guided to osteogenic precursor cells and the

differentiation process was monitored by measuring stage specific expression of genes and proteins. Mineral nodule formation of osteogenic differentiation was analyzed by using Alizarin red and Von Kossa Staining methods. Gene expression profiles of various pro- and anti-inflammatory cytokine profiles of HGMSCs were investigated using quantitative real time PCR at 1, 2 and 3 weeks post-induction with osteogenic medium. **Results:** The osteogenic differentiation of HGMSCs was confirmed by alkaline phosphate enzyme activity assay, gene and protein expression studies of osteogenic markers. Mineral nodule formation was observed after 4 weeks of osteogenic induction. The results of cytokine profile expressions revealed that there was a significant upregulation in the expression of TGF-β at all-time points. The gene expression of IL-10 was more or less consistent with an overall increase of 40% over that of controls at all time points studied. We observed a significant decrease in the mRNA expression of IL-6 and IL-1β with respect to their control group ($P<0.05$) and the expression of IL-8 was upregulated significantly. **Conclusion**: There is an overall enhancement in the expression of anti-inflammatory cytokines IL-10 and TGF-β during the osteogenic differentiation of HGMSCs that indicates a potential shift of cytokines to dampen immune responses. The reduction of IL-6 and IL-1β expression is an added advantage to reduce the acute phase and inflammatory responses, favoring HGMSCs to be cells of choice for repair and regeneration of craniofacial bones. A beneficial combination of the cytokines expressed by HGMSCs during osteogenic differentiation to reduce acute phase and long term immune responses will facilitate the achievement of our long term goal.

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List of Abbreviation

ALP	Alkaline Phosphatase
BMMSCs	Bone Marrow Derived Mesenchymal Stem Cells
BMP	Bone Morphogenic Protein
CNCC	Cranial Neural Crest Cells
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
HGMSCs	Human Gingival Mesenchymal Stem Cells
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
MSCs	Mesenchymal Stem Cells
PBS	Phosphate Buffered Saline
TGF-β	Transforming Growth Factor- β
TNF-α	Tumor Necrosis Factor-α

CHAPTER 1: INTRODUCTION

1.1 Background

The craniofacial region is a complex structure composed of bone, cartilage and vascular network. Numerous conditions such as trauma, congenital malformation and progressively deforming skeletal diseases may cause damage to the craniofacial region. In the United States, more than 15 million fractures are being treated each year [1]. Approximately, 1,600,000 bone grafts are performed each year and 6% of those are in the craniofacial region [2]. Craniofacial fractures are common (58.5% of total fractures) in the pediatric population, the majority of which are critical size defects. Reconstruction of the critical size bony defects using autologous bone graft requires a huge quantity of bone, and is associated with the donor site morbidity [3,4]. Allografts, on the other hand are accompanied with infections and delayed bone formation [5,6]. The emergence of stem cell based tissue engineering strategies are recognized as promising approaches to regenerate biological tissue substitute for critical size bony defects [7,8]. Mesenchymal stem cells (MSCs) as a potential stem cell source for tissue engineering applications have been widely studied [6,9]. Their high proliferative nature, self-renewal potential, ability to differentiate into multiple cell lineages, and their immunoprevilage nature make them a valuable resource for the regeneration of tissue [10].

1.2 Cytokines:

In bone tissue regeneration, MSCs display a unique capability of homing into injury or inflammatory sites to facilitate the repair of damaged tissue [11,12]. This phenomenon is

mediated by several growth factors and cytokines. Cytokines are a group of small molecular weight proteins produced by a wide variety of cells and influence a plethora of other cellular behavior in an autocrine or paracrine manner [11,13]. Cytokines act through specific receptors and regulate maturation and growth of stem cell populations. Cytokines can potentiate or attenuate the effects of other cytokines and influence cell signaling behavior. In addition, they can influence immune responses and alter host-graft interactions. Stem cells alter their cytokine profiles during their differentiation and in the host-milieu. In their effort to avoid allorecognition, many stem cells alter the local environment by modulating cytokine profiles [11,12]. The immunosuppressive phenomenon of MSCs is modulated by suppressing the pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), Interleukin-8 (IL-8) and Interleukin-1β (IL-1β) and enhancing the anti-inflammatory cytokines including transforming growth factor- β (TGF-β) and Interleukin-10 (IL-10) [14,15].

1.2.1 Tumor Necrosis Factor-α:

TNF-α is the most potent osteoclastogenic cytokine produced during inflammation. It has been reported that a prolonged exposure to TNF-α leads to chronic inflammation and bone destruction [16]. The expression of TNF-α increases immediately after bone injury, it then returns to base line for a few days then again is increased in later phases of bone formation and modeling [17]. *In-vivo*, eradicating the signaling pathways of TNF-α has been shown to preclude endochondral ossification.

1.2.2 Interleukin-6:

Members of IL-6 family are pro-inflammatory cytokines that include IL-6, IL-11,

leukemia inhibitory factor, cardiostrophen and oncostatin M. IL-6 regulates the pleiotropic functions of tissues and cells and is crucial for bone metabolism [18,19]. IL-6 as an osteotrophic factor, stimulates bone resorption[18]. IL-6 plays a major role in bone resorption in several physiological and pathological conditions such as bone loss due to estrogen deficiency, and hypercalcimia found in the blood stream of hamster ovarian cells that have been transfected with the IL-6 gene [18]. IL-6 has an important role in osteogenesis of mesenchymal stem cells [20]. The activity of IL-6 is employed through a cell surface receptor that has two components: 1) a ligand-binding IL-6 receptor (IL- 6R) and 2) a non-ligand-binding but signal-transducing protein gp130 **(Figure 1-1).** Cytokines that use gp130 act directly on the osteoblasts/stromal cells. However, they do not act on osteoclast progenitors to induce osteoclast formation.

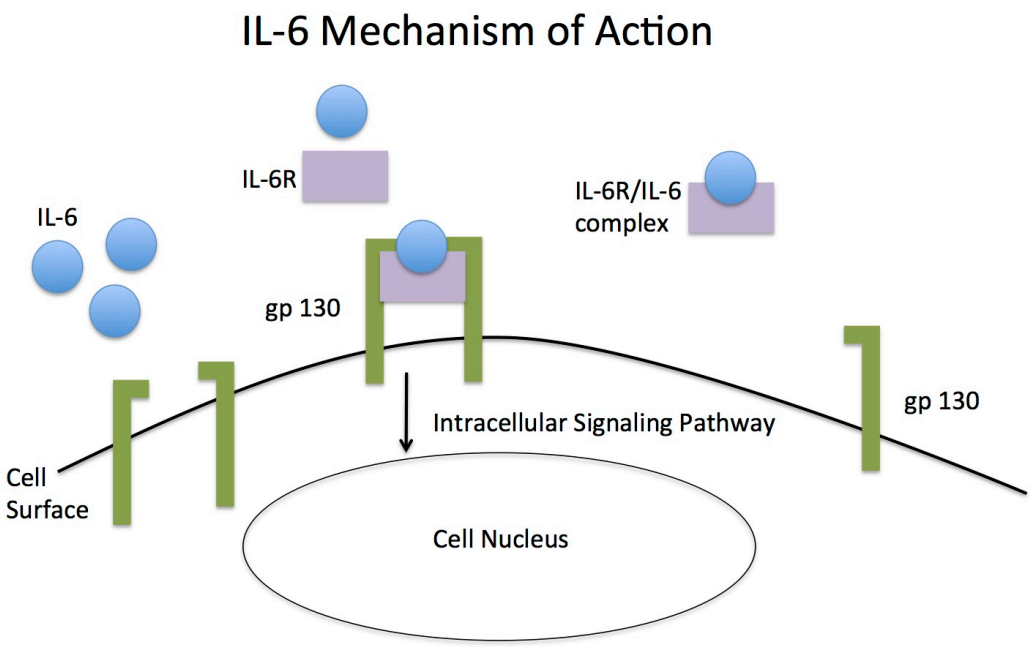

Figure 1-1. The mechanism of action of IL-6

1.2.3 Interleukin-1β

IL-1β, a proinflammatory cytokine, plays a main role in the events of endochondral bone formation by human bone marrow mesenchymal stromal cells (BMMSC). It also contributes in remodeling of the forming hypertrophic cartilage. Low doses of IL-1β (50 pg/mL) enhance colony-forming units- fibroblastic (CFU-f) and osteoblastic (CFU-o) in both number and size in the absence of further supplements [21]. In osteogenic culture, IL-1β enhances calcium deposition and BMP-2 expression by the activation of NF-ϰB and ERK signaling. IL-1β regulates both early and late events of the endochondral bone formation by BMMSC. TNF-α and IL-1β are considered the two master regulators of inflammation in fracture healing [21].

1.2.4 Interleukin-8:

IL-8 is a member of the alpha chemokine family of cytokines originally identified as a neutrophil chemo attractant. IL-8 is often associated with inflammation increased by oxidant stress [22,23].The expression of IL-8 and IL-1β is found to be high in the UCMSCs when compared to BMMSCs[24].

1.2.5 Interleukin-10:

IL-10 is an anti-inflammatory cytokine, that inhibits T-cell proliferation and other effector responses [25]. It works by blocking TGF-β synthesis, which acts as an endogenous suppressor of hematopoiesis in osteogenic cultures [26]. IL-10 plays a prominent role in tempering immunomediated inflammation including infection, allergy, and

autoimmunity. MSCs derived IL-10 can facilitate suppression of T-cell proliferation and infiltration. It has also been shown that IL-10 attenuates the severity of graft-vs-host disease after allogenic stem cells transplantation [25]. IL-10 has been reported as one of the most significant cytokines produced by the MSCs playing anti-inflammatory roles. The expression of IL-10 was significantly increased in cultures containing both BMMSCs and T cells than cultures of T cells alone [25].

1.2.6 Transforming Growth Factor-β:

TGF-β is a growth factor that promotes and maintains bone formation. In serum-free cultures, TGF-β was found to stimulate the formation of osteoblast-like cells and express bone specific markers including alkaline phosphatase (ALP) and oteocalcin [27]. TGF-β family signaling is involved in the maintenance of embryonic stem cells identity, maintenance of self-renewal, proliferation and pluripotenty in both mouse and human embryonic stem cells [28]. TGF-β1 induces Smad3-dependent nuclear accumulation of β-catenin in MSCs, which is required for the stimulation of MSCs proliferation. Members of the TGF-β family have also been implicated in directing decisions regarding the fate of MSCs. BMPs induce differentiation of mesenchymal stem cells into cells with chondroblast or osteoblast phenotypes *in-vitro*. In most cell types, TGF-β signaling additionally controls the expression of a plethora of homeostatic genes whose activity determines cell proliferation, extracellular matrix production, paracrine factor secretion, cell-cell contacts, immune function, and tissue repair [29].

1.3 Osteogenesis

Osteogenic differentiation is a spatio-temporally regulated event, involving stage-wise expression of genes that regulate a variety of growth factors and cytokines. Progression of stem cells to osteogenic precursor cells ensues in three distinct phases: 1) proliferation (Day 0- Day 4), 2) extracellular matrix maturation (Day 4 –Day14) and 3) mineralization (Day 14 to Day 21) [30,31]. In the proliferative level **(Figure 1-2),** MSCs are extremely mitotic; they exhibit high expression of histone proteins. During the extracellular matrix maturation phase, MSCs condense into compact nodules. Genes associated with extracellular matrix proteins such as collagen type I; fibronectin and TGF-β are expressed during this stage. Furthermore, many non-extracellular matrix proteins including ALP, osteopontin and osteocalcin are upregulated. During mineralization, the MSCs will differentiate into osteoblasts. These cells will secrete osteoid, a collagen-proteoglycan matrix, that binds to calcium salts to become calcified. Most importantly, osteogenic differentiation is determined by two transcription factors, RUNX2 and Osterix. RUNX2, the master regulator of osteogenesis, influences many extracellular matrix proteins such as collagen type I, osteopontin, bone sialoproteins and osteocalcin.

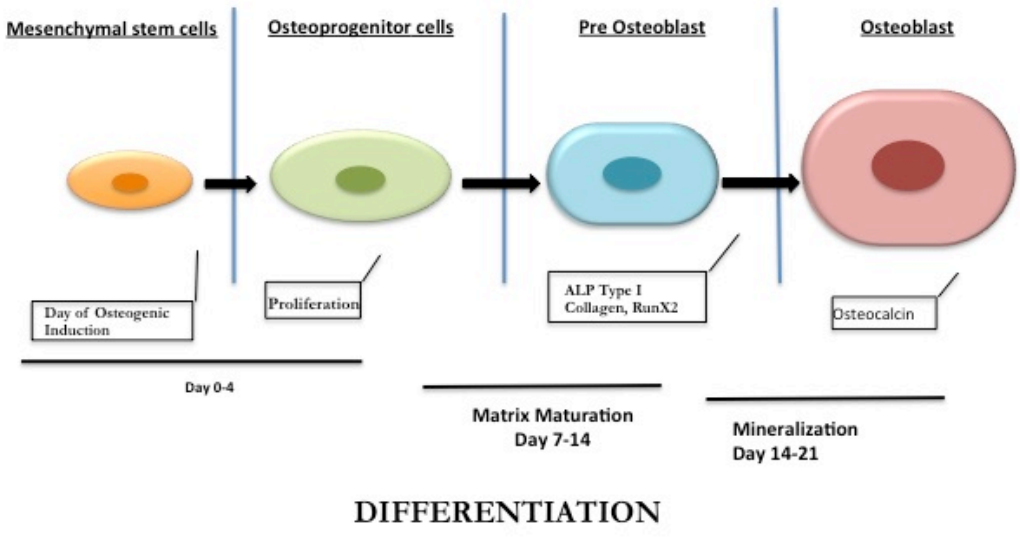

Figure 1-2. Phenotypic changes during osteogenesis

1.4 The aim of the study

Many studies have reported the cytokine levels of stem cells in basic culture conditions [32,33]. However, very limited studies have focused on stem cells undergoing osteogenic differentiation [34,35]. In this study, we propose to investigate the cytokine profiles of mesenchymal stem cells derived from human gingival tissue (HGMSCs) in normal and osteogenic conditions.

1.5 Human gingival mesenchymal stem cells (HGMSCs)

Human gingival connective tissue is a reservoir of mesenchymal stem cells. HGMSCs can be obtained from discarded tissues in the dental clinics with minimally invasive procedures. HGMSCs can be easily isolated, have a high proliferative capacity, display stable phenotype and maintain a normal karyotype [36]. The distinctive feature of HGMSCs is their potent immunomodulatory effects on multiple types of innate immunity cells especially macrophages, mast cells and dendritic cells [37]. Furthermore, 90% of which are originated from cranial neural crest cells (CNCC) [38]. CNCC play an important role in the formation of tooth and alveolar bone found in the craniofacial region [38,39].

1.6 Innovation:

Our long-term goal is to develop an immunocompatible model and regenerate the bony tissue at the site of the defect using a novel stem cell-based system. MSCs derived from human gingiva are highly proliferative and efficiently differentiate into osteoblasts. HGMSCs, while maintaining their initial phenotype after many passages, they do not express HLA-dr surface molecule making them immunoprevilaged and potentially an excellent option for allografting. They have been shown to be potent immumodulators and anti-inflammatory cells capable of improving healing in previous inflammatory diseases [36-38,40]. Understanding the cytokine expression profiles will be of great advantage in developing methods for effective bone regeneration with minimal immunological insults either on the graft or on the host. Many studies were reported on the cytokine profiles of normal culture conditions in various types of stem cells [13,33-35,41,42].

This is the first study to investigate the gene expression profiles of cytokines of HGMSCs during osteogenic differentiation. The information obtained from this study will provide in-depth insights into cytokine-regulation of osteogenesis and will help in optimizing a cell-system that favors immunocompatible.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials:

Dulbecco's Modified Eagle Medium (DMEM), Trypsin, Gene Expression Assay Mix, cDNA kit (Life Technology, Carlsbad, CA), Phosphate Buffered Saline (PBS), Penicillin/Streptomycin (Sigma; St. Louis MI) and Fetal Bovine Serum (FBS); (Atlanta Biologicals; Lawrenceville, GA) were used for the Study. The tissue culture flasks and other required lab supplies were obtained from VWR International Company (Atlanta, GA).

2.2 Overall study design:

The cells isolated from 6 healthy male/female donors were selected for the study. Isolation of HGMSCs from human gingival tissue was approved by the Institutional Review Board (IRB # 02071304) protocol at Nova Southeastern University. Each batch of cells was expanded until the cells reached confluence and subsequently were cryopreserved. All batches of cryopreserved HGMSCs were revived at the same time and cultured under standard culture conditions. Six individual experiments were conducted at similar experimental conditions. The cells were then induced with osteogenic medium and osteogenic differentiation was monitored at 1, 2, 3 and 4 weeks intervals. The cells cultured in complete medium (DMEM+ 10% FBS+ 1% antibiotic) served as the control group. The osteogenic potential of cells cultured in growth medium with osteogenic supplements obtained from each batch was examined for their osteogenic potential. Mineralization was confirmed after 4 weeks by mineral deposition assay using Alizarin Red and Von Kossa. The osteogenic marker genes ALP and osteopontin were measured

at each time point. The gene expressions of 6 different cytokine profiles (TNF-α, IL-8, IL-1β, IL-6, IL-10, and TGF-β) were measured using quantitative PCR at 3 different time points (1, 2 and 3 weeks) (Figure 2-1, 2-2)

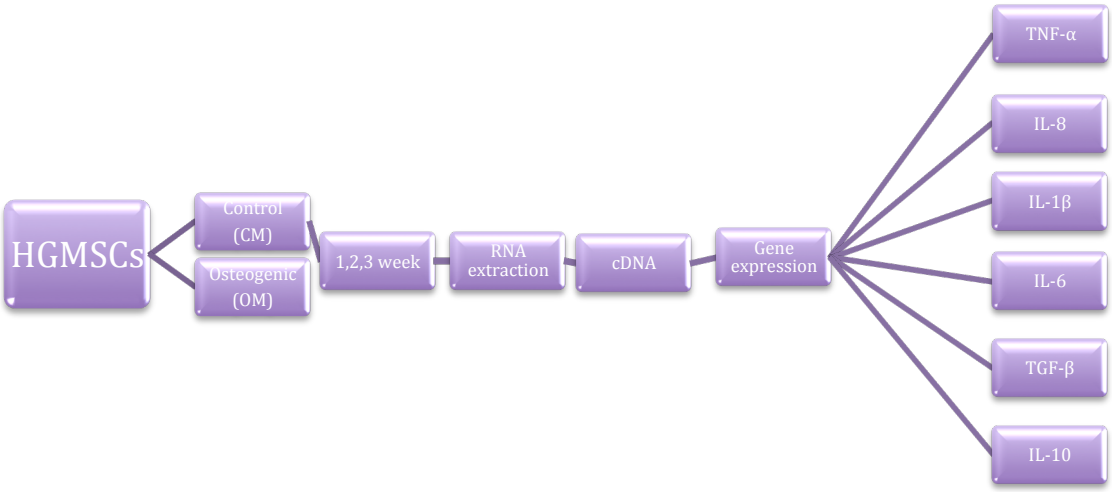

Figure 2-1. A flow chart showing the overall study design

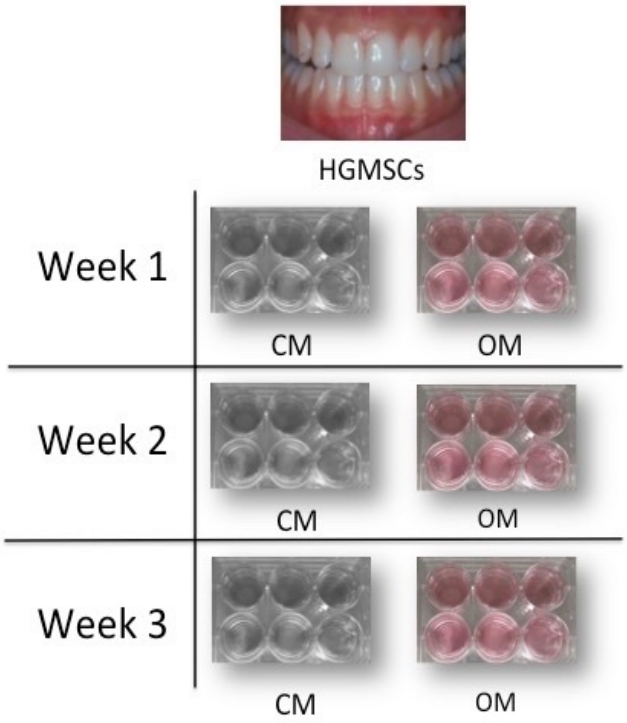

Figure 2-2. Study design. The figure shows HGMSCs treatment with both CM (complete medium) and OM (osteogenic medium) at 3 different time points (1, 2, and 3 weeks). CM was considered the control.

2.3 Sample size

Six individual experiments were conducted under similar experimental conditions. Cryopreserved cells obtained from 6 donors were randomly selected for the study. There were 2 groups: 1) Control group: cells cultured in complete medium 2) Experimental group: cells cultured in osteogenic medium. The cells were harvested at 1,2 and 3 weeks for the gene expression studies. The sample size was as follows: 6 biological samples X 3 time points X 6 genes X 2 culture conditions= 108 control and 108 osteogenically differentiated samples with a total of 216 samples (Table 1).

	Time points	Genes examined	Gene Sequences
Group I **Control**	**week 1** 6 samples CM OM **week 2** 6 samples CM OM **week 3** 6 samples CM OM	TNF-α	Hs00174128_m1
		IL-8	Hs00174103_m1
		IL-1β	Hs01555410_m1
Group 2 **Experimental**		IL-6	Hs00985639_m1
		TGF-β	Hs00234244_m1
		IL-10	Hs00175123_m1

Table 1: Sample size and distribution

2.4 Methods:

2.4.1 Isolation of mesenchymal stem cells from gingival tissue:

The gingival tissues obtained from NSU clinics were de-epithelialized and minced into tiny pieces (2x2 mm) and rinsed with DMEM supplemented with 10%, FBS (Life technology, Carlsbad, CA), 400 mmol/ml L-glutamine, 100 U/ml penicillin, 100 lg/ml streptomycin and 1% amphotericine [43]. HGMSCs were isolated from the gingival tissue using standard procedures [44]. Briefly, cells were digested enzymatically using 0.1% collagenase and 0.2% dispase for 30 minutes **(**Figure 2-3**).** The first cell suspension was discarded to avoid the interference of epithelial cells. The tissue samples were further treated with 0.1% collagenase and 0.2% dispase. Then the cell suspension was collected. The procedure was repeated twice and the cell suspensions were pooled. They were

centrifuged and the cell pellet was plated ($1x2x10^4$ cells cm^2) in a tissue culture flask and allowed to adhere to the flask for 48 hours in a humidified incubator at 37°C and 5% CO_2 (Figure 2-4). Each batch of cells was cultured under standard culture conditions. After reaching 70-80% confluency, they were cryopreserved.

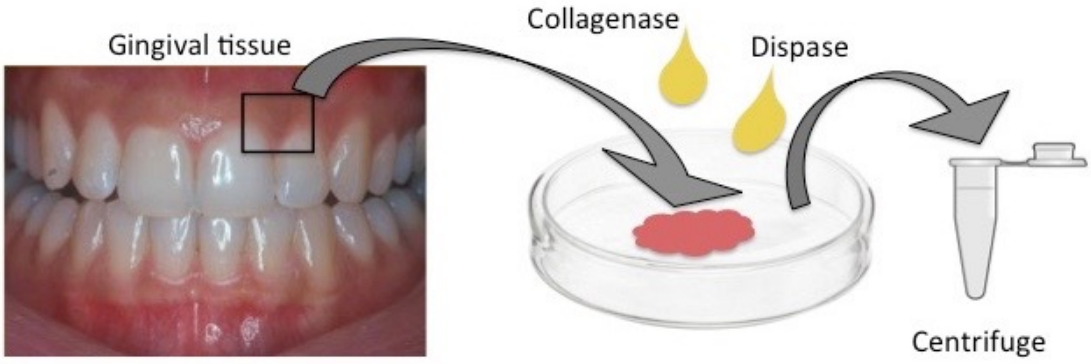

Figure 2-3. Isolation of MSCs from human gingiva

Figure 2-4. Cell culture incubator

2.4.2 Cell culture:

Cryopreserved cells were revived and resuspended in DMEM supplemented with 10% FBS and 1% antibiotics and seeded at $1x2x10^4$ cells cm^2 in a tissue culture flask and incubated at 37°C and 5% CO_2. After 48 hours, non-adherent cells were removed and cells were fed with culture medium for every 2-3 days. Cells cultured under specific culture conditions were subsequently sub-cultured at 90% confluence and expanded.

2.4.3 Characterization of HGMSCs by surface markers by flow-cytometry method:

Cells at the concentration of 1 $x10^6$ cells were used to measure the surface markers using Miltenyi Kit according to manufacturer's instruction. The specific markers positive for mesenchymal stem CD 73, CD 90 and CD 105 and negative for CD 34 were identified at the facilities of University of Miami using a florescent activated cell sorter FACAria (BD Biosciences, San Jose, CA) with an adjusted florescence compensation setting. Negative samples were used to set up the thresholds of quadrant markers.

2.4.4 *In-vitro* confirmation of osteogenic differentiation

For the differentiation assays, the cells grown at 70-80% confluency were induced with osteogenic supplements including, 50 μg/ml Ascorbic Acid, 10 mM β-Glycerophosphate and 100 nM Dexamethasone (Dex). Osteogenic differentiation assays were performed to confirm the presence of mineral nodules. In order to maintain consistency, the 4^{th} or 5^{th} passage cells were used in this study. In order to confirm osteogenic differentiation for every batch, cells obtained from each donor were induced with osteogenic differentiation

medium for four weeks. Mineralization was confirmed by both Alizarin Red and Von Kossa staining.

2.4.5 Alizarin Red staining:

After 4 weeks of treatment, cells with or without osteogenic supplements were washed with PBS and fixed in 10% buffered formalin solution for 10 minutes. After rinsing with distilled water, the cells were incubated in 1% Alizarin Red solution for 10-15 minutes followed by a gentle wash with distilled water.

2.4.6 Von Kossa staining:

Mineral deposition was investigated after week 4 for cells treated with and without osteogenic media. After a designated period of time, cells were rinsed with cold PBS and fixed in 10% formalin for 10 minutes at room temperature. The cells were then rinsed twice with distilled water and incubated in 2% silver nitrate and exposed to UV light for 30 minutes. The stain was washed and cells were exposed to bright light for 15 minutes, and dehydrated in 100% ethanol.

2.5 Gene expression studies:

2.5.1 RNA extraction

RNA was extracted from the cells using tri-reagent (Life technologies, Carlsbad, CA). Reverse transcription was performed to generate cDNA. About 2 ug of total RNA was reverse transcribed by using MMLv reverse transcriptase using cDNA synthesis kit as per manufacturer's instructions. The total RNA was used for cDNA synthesis by reverse transcriptase with a (Figure 2-5).

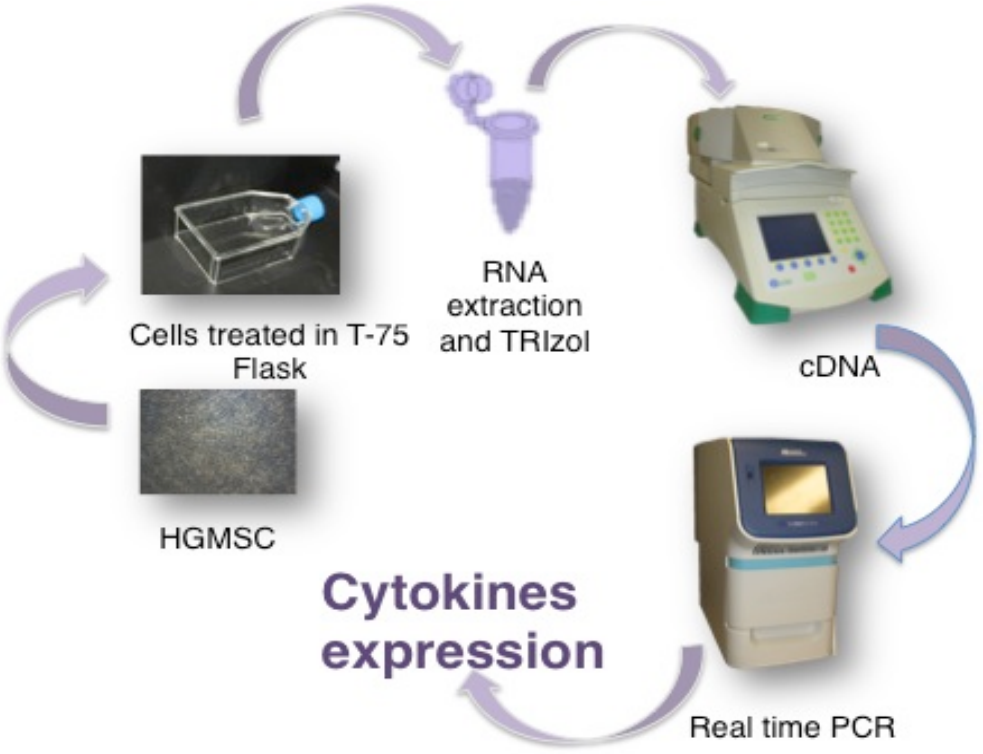

Figure 2-5. Experimental procedure showing gene expression study

2.5.2 Quantitative PCR and cDNA preparation:

The reverse transcriptase products were amplified with primers (Table 1) specific for TNF-α, IL-1β, IL-6, IL-8, IL-10, TGF-β and β-actin a housekeeping gene, using a gene expression assay kit (Life Technology, Carlsbad, CA). Real-time PCR was performed and monitored by using a StepOne Plus Real-Time PCR System (Life Technologies, Charlsbad, CA). cDNA was analyzed by commercially available primers and probes from Life Technologies (Carlsbad, CA) following the manufacturer's instructions.

2.6 Statistical analysis:

To evaluate differences between or among groups, paired t-test and analysis of variance (ANOVA) were performed. A P-value ≤ 0.05 was selected for significance. For all the data analysis, GraphPad Prism 5 software was used.

CHAPTER 3: RESULTS

3.1 Cellular morphology:

The cells with passages 4 and 5 were used for all the experiments. Cell suspension was seeded at a density of $1x10^6$ and cultured under standard culture conditions. The cells were tightly adhered after 36 hours after seeding and were widely spread showing fibroblast morphology. The cells reached 70-80% confluency after one week (Figure 3-1).

Cell Culture

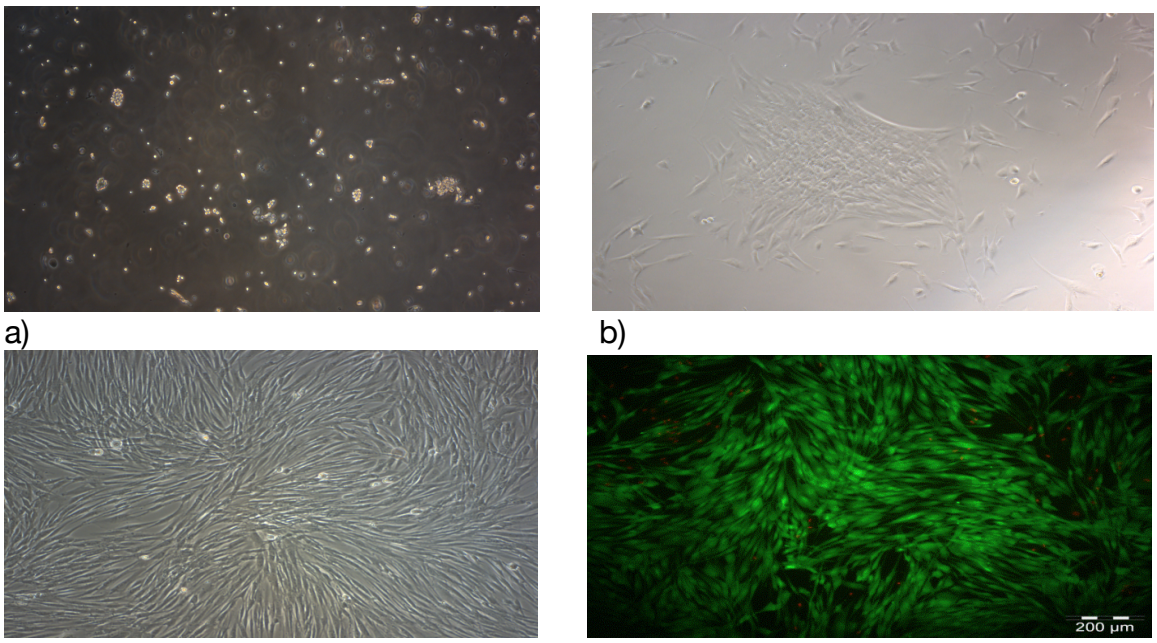

Figure 3-1. A microscopic view showing: **a)** HGMSCs plated in T75 flask at day 0. **b)** HGMSCs at day 1. **c)** Cells at sub-confluence. **d)** Cells stained with florescent showing viable cells (green cells). Red color indicates dead cells

3.2 Cell surface markers:

The flow cytometry data revealed that the cells were positive for mesenchymal stem cells markers CD73, CD90, CD105. They were negative for haemopoietic stem cells markers CD34. All batches of cells were examined for their surface markers (Figure 3-2).

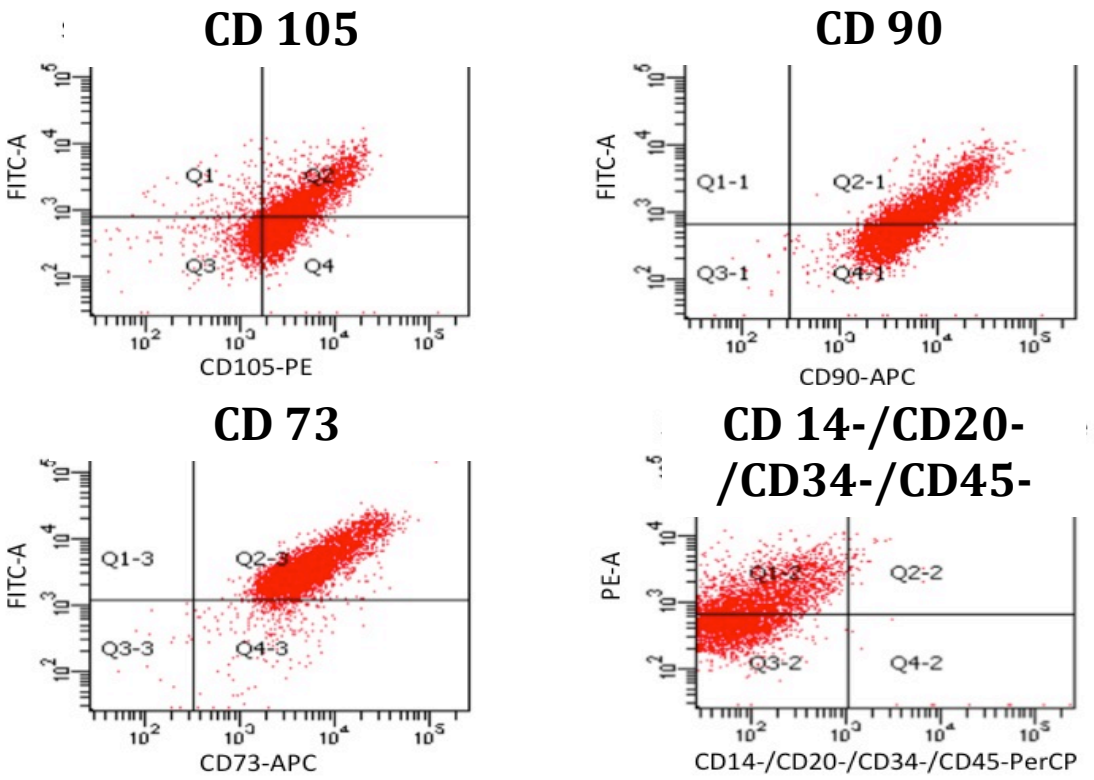

Figure 3-2. Flow cytometry showing HGMSCs surface markers

3.3 *In-vitro* assays to confirm osteogenic differentiation:

The cells obtained from each donor were examined for their osteogenic potential. Various assays were conducted including ALP activity, osteogenic marker gene expression and mineralization to determine osteogenic potential.

3.3.1 ALP activity:

The results of ALP activity indicated that there was a gradual increase in ALP activity at 7 and 14 days post induction. Over 48% increase at the 7th day followed by 71% increase at the 14th day was observed (Figure 3-3).

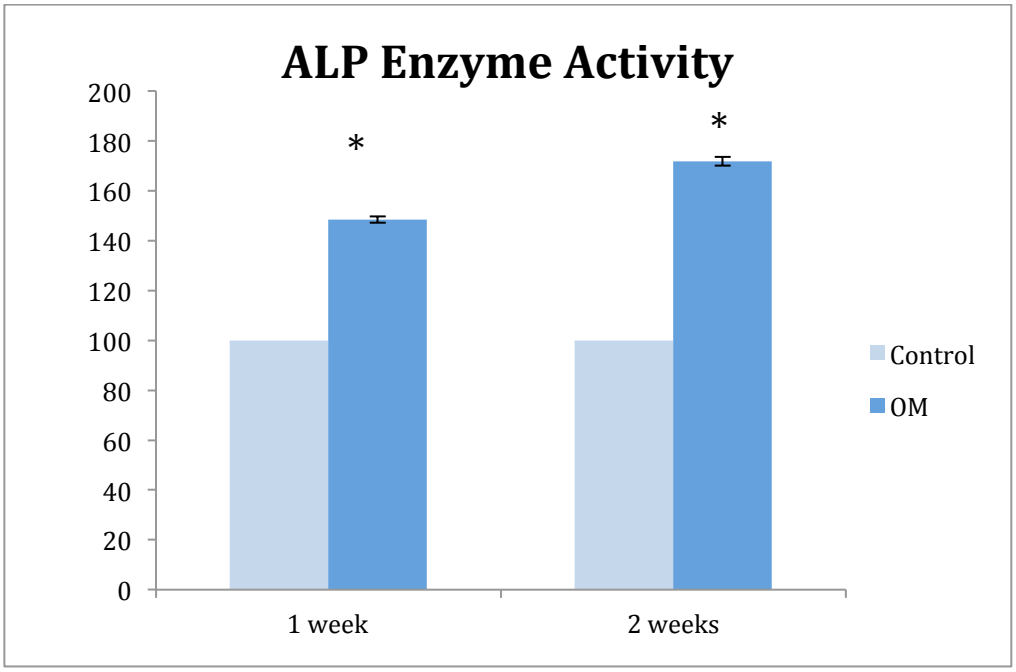

Figure 3-3. ALP enzyme activity, an early marker for osteogenic differentiation is increased at 1 week and 2 weeks

3.3.2 Osteogenic marker genes:

In order to confirm the differentiation, various osteogenic marker genes were investigated. The cells obtained from all batches showed expression of osteoblast specific genes such as ALP, Collagen type I and osteopontin. The results of RT-PCR

demonstrated that there was a significant upregulation of mRNA expression in all genes (Figure 3-4).

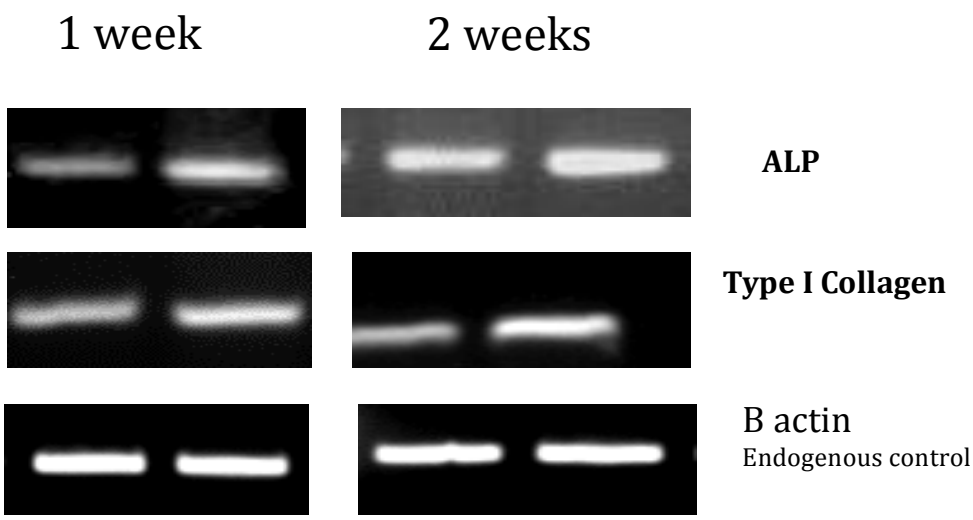

Figure 3-4. Gene expression of osteogenic markers

3.4 In-vitro mineralization experiments:

Mineralization is the hallmark for osteogenically differentiated cells. The cells induced with or without osteogenic supplements were monitored at weekly intervals to examine mineral nodule formation. Our results revealed that after 4 weeks of osteogenic induction, the cells showed mineral nodules (Figure 3-5).

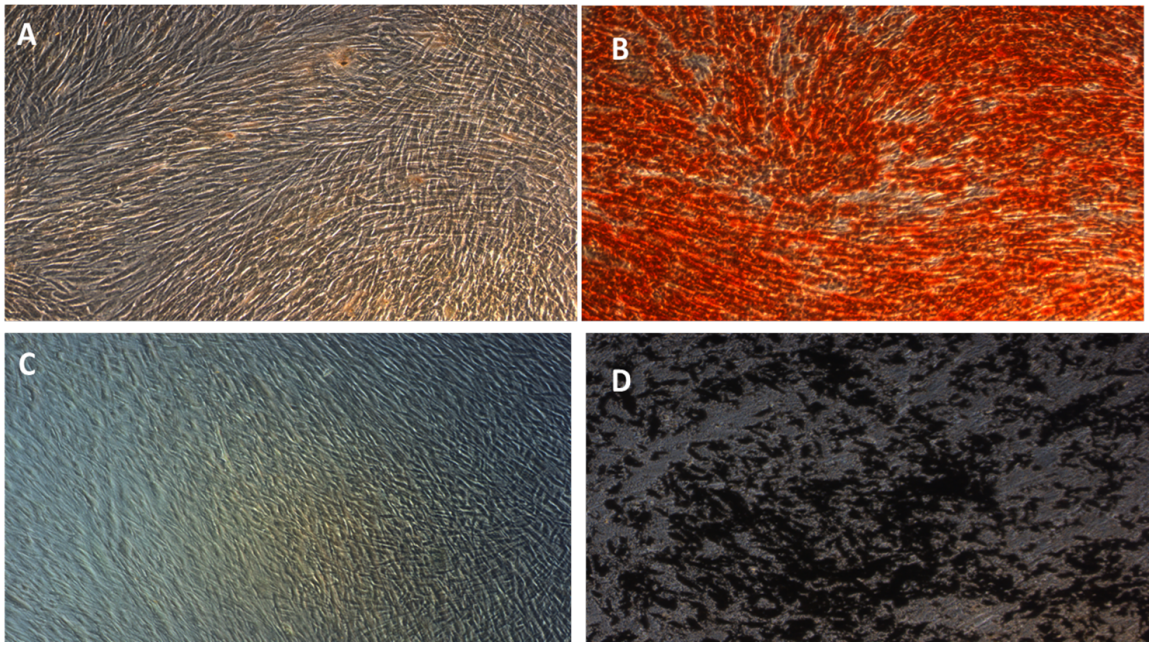

Figure 3-5. *In-vitro* mineralization: HGMSCs 4 weeks mineralization with Alizarin Red (A&B) and Von Kossa (C&D). A&C shows cells treated in CM, B&D shows cells treated in OM

3.5 mRNA expression profiles of cytokines:

To examine the changes in cytokine expression in undifferentiated and osteogenically differentiating cells, the cells cultured with or without osteogenic supplements were harvested at 7, 14 and 21 days' time intervals. A total of 6 cytokines were selected for the study. The mRNA expression was measured using quantitative PCR method at the designated time points. The results obtained from all six biological samples were analyzed in this study. The quantitative PCR experiments were conducted using 3 replicas from each sample. The average value of the experimental group (cells induced with osteogenic medium) was compared with the average of the control (cells devoid of osteogenic supplements). The relative gene expression was measured by using 2 δδ Ct method.

Gene expression profiles of the cytokines in this study revealed interesting findings. The overall results demonstrated that the gene expression levels of cytokines were altered during the differentiation of the MSCs. In our study, we measured the gene expressions of TGF-β, IL-10 (anti-inflammatory cytokines), IL-6, IL-8, TNF-α and IL-1β (pro-inflammatory cytokines). The results of our study demonstrated that the cytokines including TGF-β, IL-6, IL-8, IL-10 and IL-1β were expressed in undifferentiated and differentiated cells. However, it is interesting to note that TNF-α was expressed in undifferentiated cells obtained from two donors, but none of the differentiated cells expressed TNF-α at any time point. Our results revealed that the relative gene expression of IL-6 and IL-1β was significantly down regulated in Dex induced cells when compared to control (cells cultured without osteogenic supplements) in HGMSCs. Furthermore, there was a time dependent down regulation observed in the cells treated with osteogenic induction medium.

Our results revealed that there was an upregulation in the expression of TGF-β. While there was an 82% increase in the expression at day 7, a slight decrease was observed at day 14 and a gradual increase at day 21. There was an increase in the expression of IL-10 with a range from 39% to 41% at all-time points. There was significant decrease observed in the expression of IL-6 and IL-1β compared to the control group at all time points. It is interesting to note that the IL-6 expression was down regulated by 27% at week 2 when compared to 1 week cell cultures with a gradual increase at 3 week time point. The levels of IL-8 were significantly increased at 1 and 2 week time points (Figures 3-6 → 3-10)

IL-10

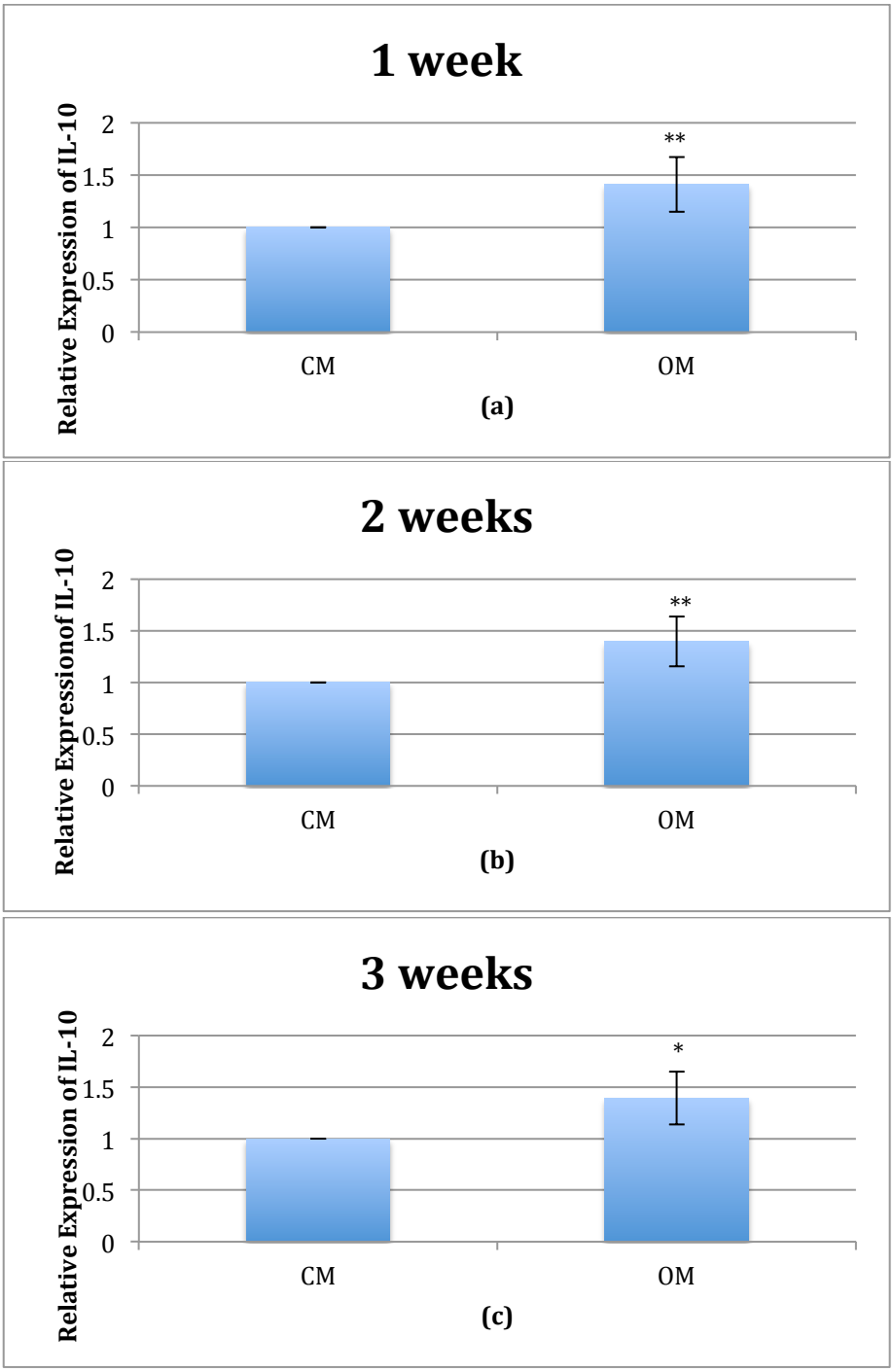

Figure 3-6. HGMSCs were induced with osteogenic medium (OM) for 1, 2 and 3 weeks and compared to control (cells grown in complete medium without osteogenic supplements). The figure shows the relative gene expression of IL-10. **a)** Cells cultured in complete medium with or without osteogenic supplements for 7 days. **b)** Cells cultured in complete medium with or without osteogenic supplements for 14 days. **c)** Cells cultured in complete medium with or without osteogenic supplements for 21 days.

TGF-β

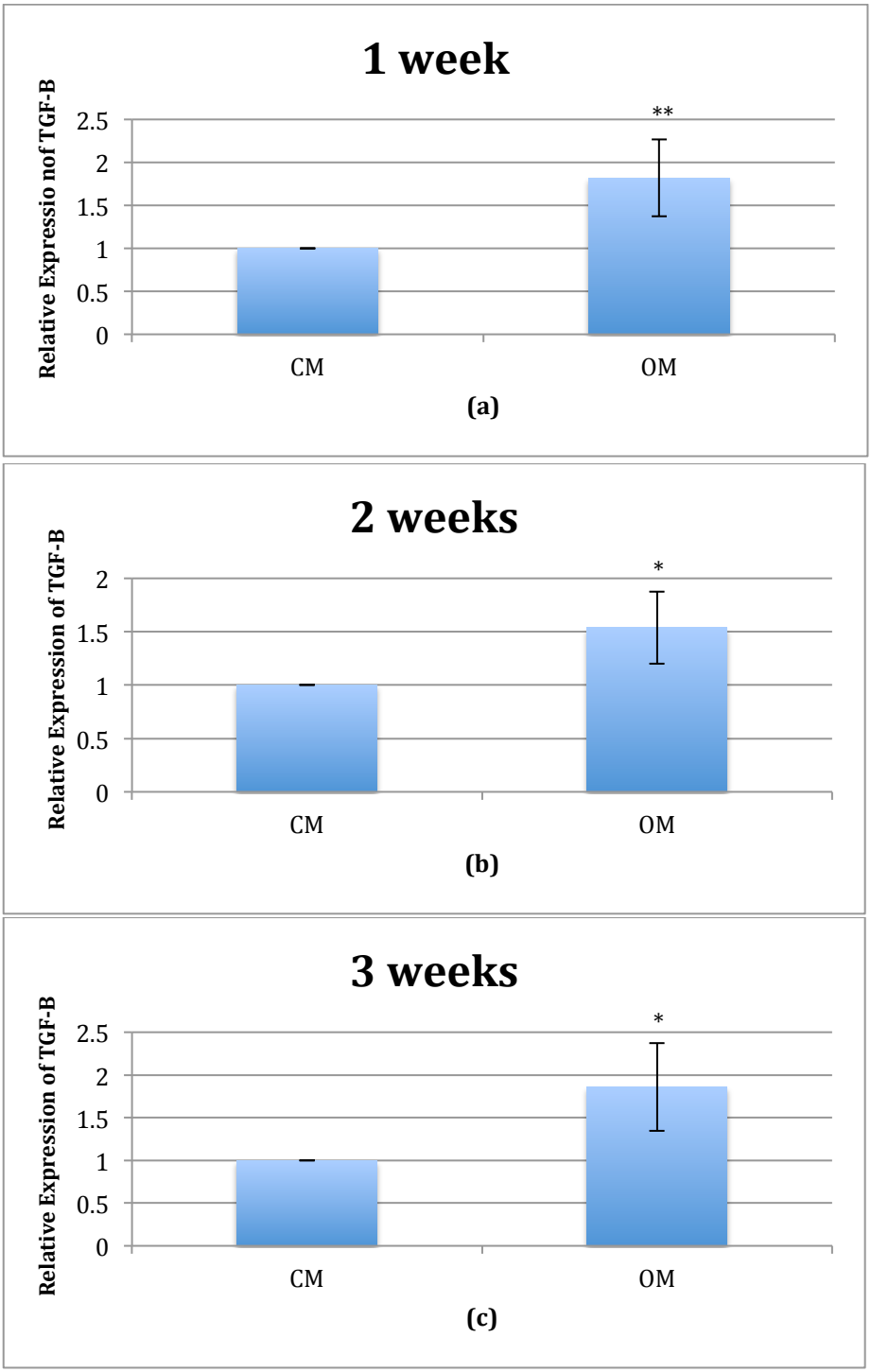

Figure 3-7. HGMSCs were induced with osteogenic medium (OM) for 1, 2 and 3 weeks and compared to control (cells grown in complete medium without osteogenic supplements). The figure shows the relative gene expression of **TGF-β**. **a)** Cells cultured in complete medium with or without osteogenic supplements for 7 days. **b)** Cells cultured in complete medium with or without osteogenic supplements for 14 days. **c)** Cells cultured in complete medium with or without osteogenic supplements for 21 days.

IL-1β

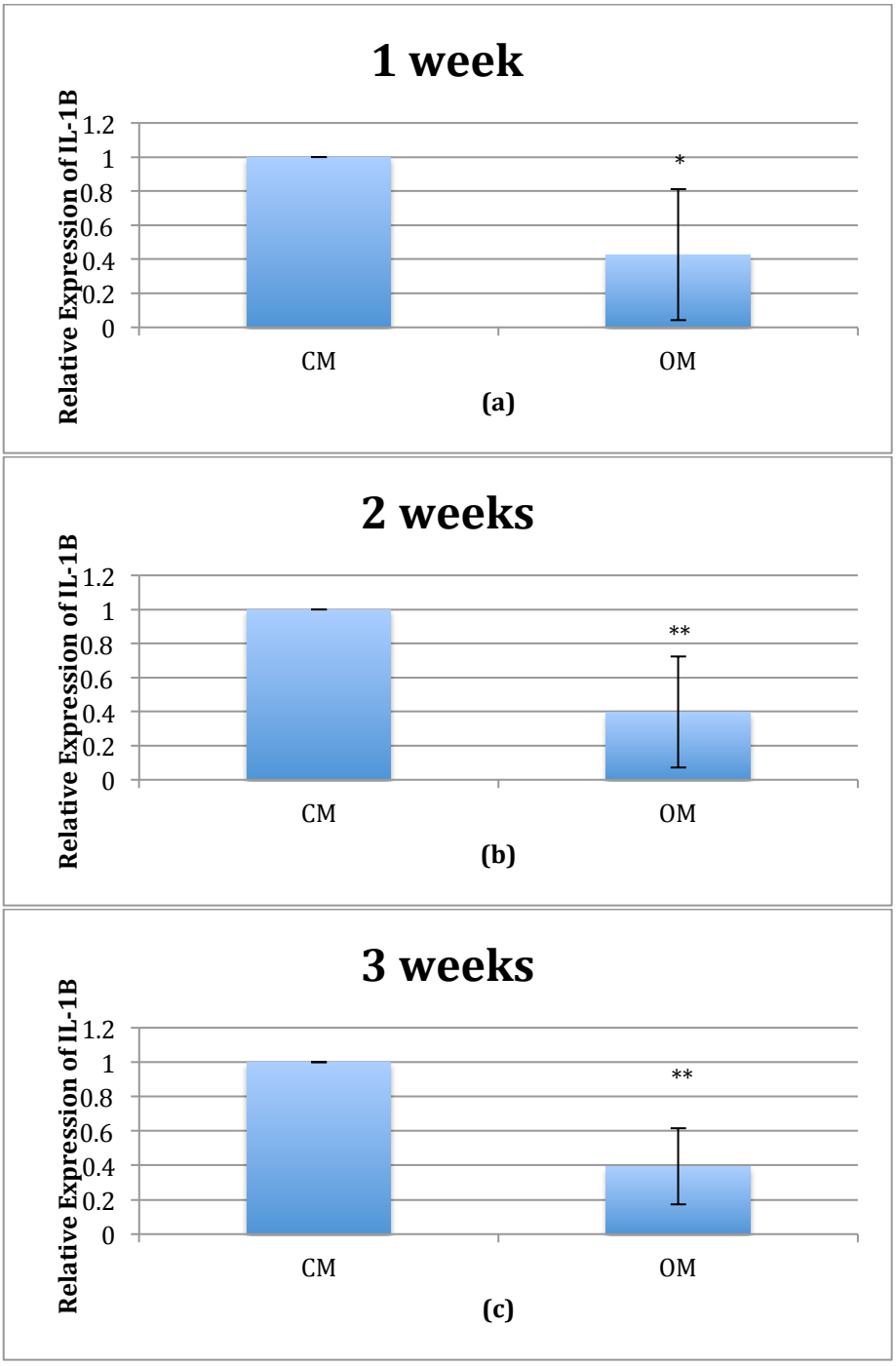

Figure 3-8. HGMSCs were induced with osteogenic medium (OM) for 1, 2 and 3 weeks and compared to control (cells grown in complete medium without osteogenic supplements). The figure shows the relative gene expression of **IL-1β**. **a)** Cells cultured in complete medium with or without osteogenic supplements for 7 days. **b)** Cells cultured in complete medium with or without osteogenic supplements for 14 days. **c)** Cells cultured in complete medium with or without osteogenic supplements for 21 days.

IL-6

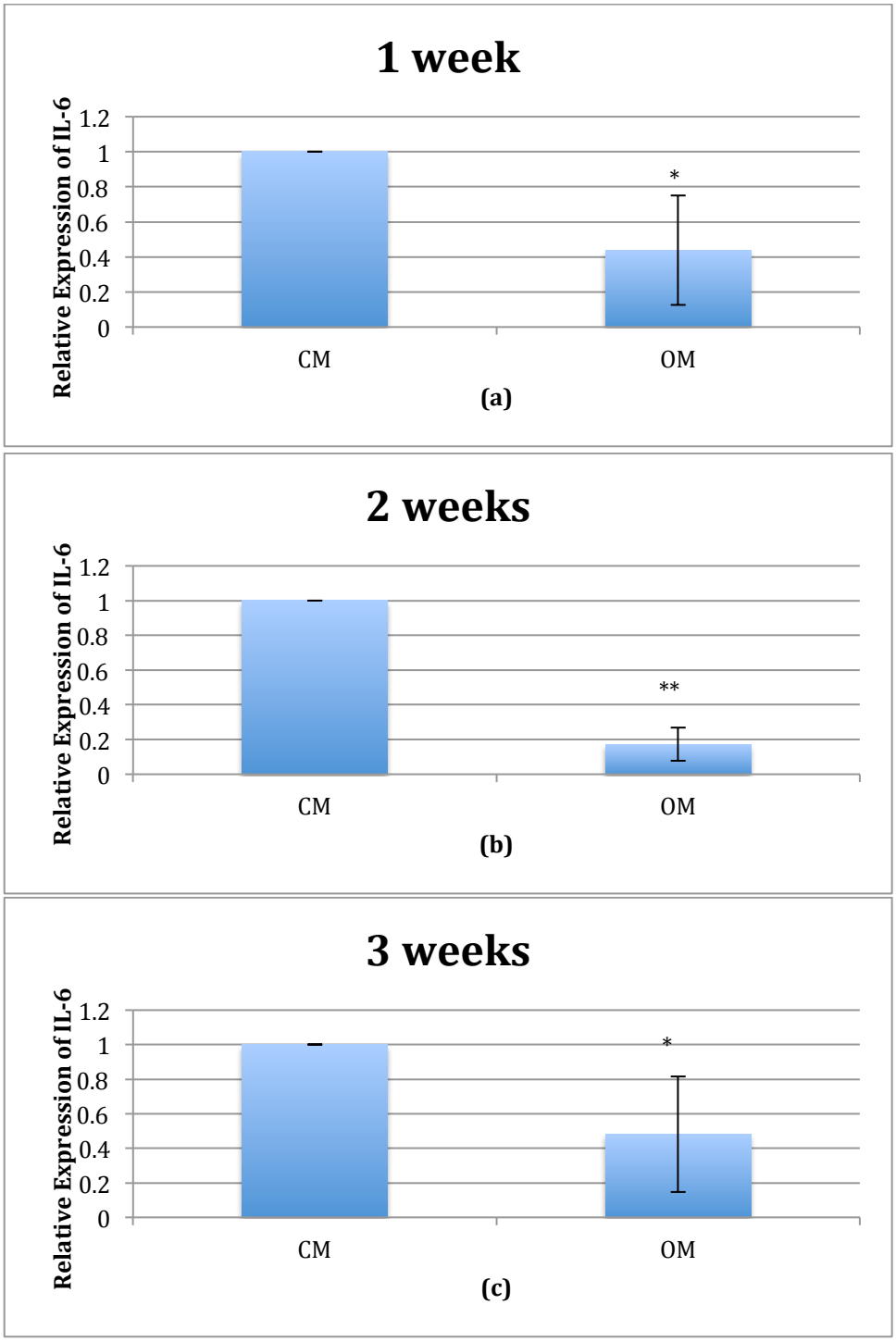

Figure 3-9. HGMSCs were induced with osteogenic medium (OM) for 1, 2 and 3 weeks and compared to control (cells grown in complete medium without osteogenic supplements). The figure shows the relative gene expression of **IL-6**. **a)** Cells cultured in complete medium with or without osteogenic supplements for 7 days. **b)** Cells cultured in complete medium with or without osteogenic supplements for 14 days. **c)** Cells cultured in complete medium with or without osteogenic supplements for 21 days.

IL-8

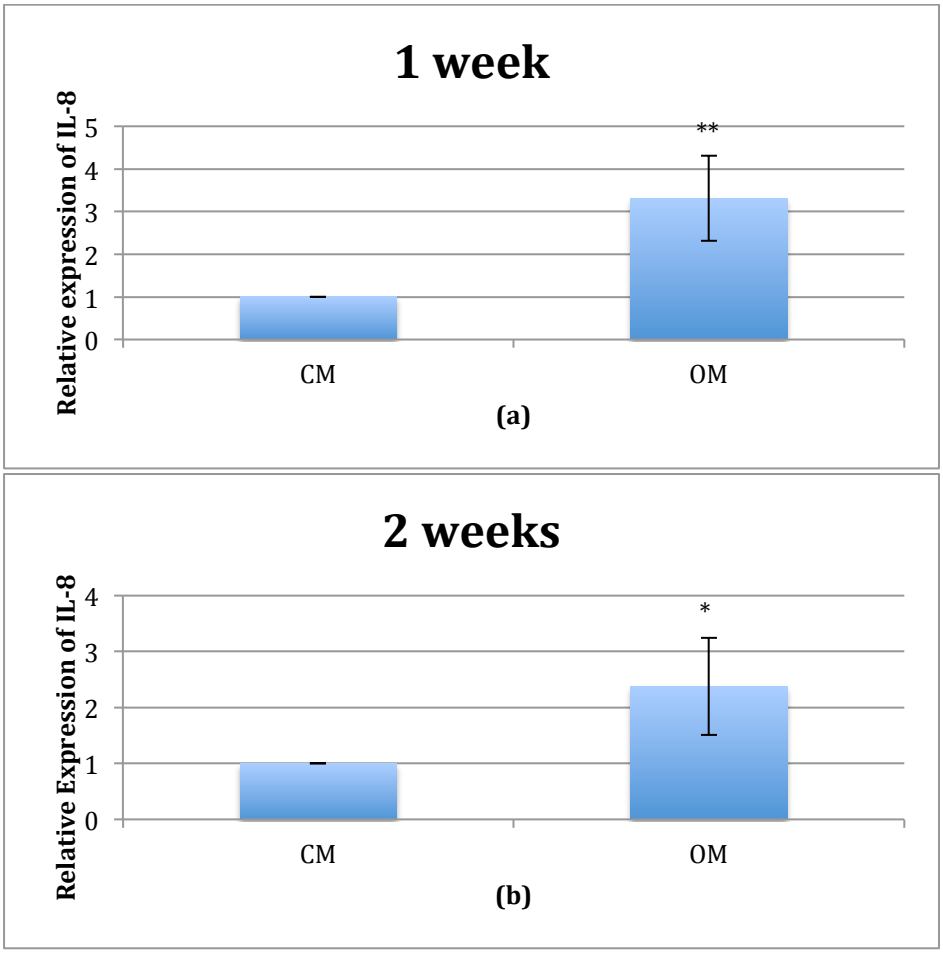

Figure 3-10. HGMSCs were induced with osteogenic medium (OM) for 1 and 2 weeks and compared to control (cells grown in complete medium without osteogenic supplements). The figure shows the relative gene expression of **IL-8**. **a)** Cells cultured in complete medium with or without osteogenic supplements for 7 days. **b)** Cells cultured in complete medium with or without osteogenic supplements for 14 days.

CHAPTER 4: DISCUSSION

MSCs, due to their inherent plasticity, are potential candidates for cell based therapies for the repair and reconstruction of the craniofacial bone tissue. According to minimal criteria of the International Society of Cellular Therapy (ISCT), in order to be considered as MSCs, they need to follow three important criteria. 1) Cells need to adhere to plastic surface and 2) they should express specific panel of surface markers and 3) differentiate into osteogenic, chondrogenic and adipogenic lineages under specific culture conditions. In parallel to their role as undifferentiated cell reserves, MSCs have immunomodulatory functions which are exerted by cytokines. Cytokines or growth factors are decision makers of cell fates toward survival versus death and interaction versus protection. In addition to their immuno-modulatory responses they regulate stem cell differentiation. The present study was focused on the elucidation of gene expression profiles of various cytokines and their role during osteogenic differentiation of HGMSCs.

In this study, we demonstrated that HGMSCs were positive to mesenchymal stem cell surface markers. The results of the study confirmed that HGMSCs can potentially differentiate to osteogenic lineage. The overall results demonstrated that the expression of cytokine levels altered during osteogenic differentiation. (Table 2) summarizes the *in-vitro* studies of the cytokines expression.

In our study, while undifferentiated and osteognically differentiating cells expressed TGF-β, the increase in the expression of TGF-β in osteogenically differentiated cells ranged from 50-80% at the designated time points. TGF-β is one of the most predominate

immunomodulatory cytokines produced and constitutively secreted by MSCs. TGF-β is involved in a cell-contact dependent inhibition of T-cell proliferation by MSCs [29].

IL-10 is the most commonly discussed cytokine in relation to its immunomodulatory effects on MSCs. However, according to previous reports, the secretion of IL-10 is quite contradictory. While few researchers reported the secretion of IL-10 by MSCs, others reported that MSCs secrete IL-10 only under specific conditions [11]. IL-10 inhibits the production of Th1 and proinflammatory cytokine TNF-α. MSCs produced high levels of IL-10 when they were co-cultured with the lymphocytes. In our study, the IL-10 levels were upregulated around 40% in osteogenically differentiated cells compared to undifferentiated cells at all-time points observed. Vlasselaer et al demonstrated an interesting finding that the cells induced with IL-10 inhibited the osteogenic activity in mouse BMMSC [26]. However, the IL-10 did not affect the ongoing synthesis of ALP and collagen during the first 9 days of the culture; nevertheless it maximally suppressed these proteins at day 15 –day 18 when the cells were induced with IL-10 from the day 1 culture.

TNF-α and IL-1β play an important role in bone metabolism and regulate the function of osteoblasts and osteoclasts. In our study, TNF-α gene expression was randomly observed in both undifferentiated and differentiated conditions. The expression of TNF-α was observed only in the undifferentiated cells obtained from two donors of the cells obtained from 6 donors. Osteogenically differentiating cells did not express TNF-α. The limited number of donors might be a limitation in the study, and it could be cell type dependent. In Kim et al studies, the protein expression of TNF-α was minimal in both

undifferentiated and differentiated cells of BMMSCs [45]. Furthermore, Sumansinghe et al reported that mechanical strain has minimal effect on the expression of TNF-α [46]. Taken together, during osteogenic conditions, mechanical stimulus might not influence the levels of TNF-α.

Our data demonstrated that IL-1β was expressed in undifferentiated and differentiated cells. There was a gradual down regulation in the expression level ($p=<0.05$) in the osteogenically differentiating cells compared to control at all-time points tested. Kim et al reported the IL-1β expression was insignificant in BMMSCs cultured in basal medium as well as differentiating medium [45]. In mechanically strained BMMSCs significantly low levels of IL-1β were observed at later point of time. They predicted that IL-1β might have expressed at the earlier time points in their study [46].

In our study, the expression of IL-8 increased in time dependent manner with maximal expression at week 2. Our results were in agreement with the study of Sumansinghe et al [46]. This group of researchers found an enhanced expression of IL-8 in the continuous presence of 100nM Dex. Furthermore, they showed that the mechanical strain enhanced the IL-8 expression significantly during the days 1-3 and 6-9 days relative to unstrained controls. While Dex dependent down regulation of IL-8 has been observed by Chaudhary et al in BMMSCs [47], Kim et al [45] studies demonstrated a significant increase in IL-8 expression in the same cells model when the cells were induced with Dex for 14 days. Taken together, it was predicted that the expression levels depend on the duration of Dex induction in the culture medium. It is interesting to note that our results demonstrated that

the IL-8 expression in undifferentiated cells was insignificant at week 3. On the other hand osteogenically differentiated cells exhibited high levels of IL-8 expression. From the above studies, we have predicted that Dex might be influencing the IL-8 expression.

Our results revealed that the cells undergoing osteogenic differentiation have significantly down regulated the expression of IL-6 in HGMSCs. It was also observed that the IL-6 expression was down regulated in time dependent manner. Cells incubated in the osteogenic supplements for two weeks inhibited IL-6 expression by 50 to 25% than cells incubated in normal culture conditions. Our results were in agreement with the results observed in bone marrow stem cells. Studies have shown that the expression of IL-6 was inhibited by bone marrow derived MSCs when treated with Dex in the culture medium [8]. These findings were also supported by another study reporting the reduction in the expression of IL-6 when MSCs were subjected to osteogenic differentiation [42,45]. In another study, IL-6 expression was observed to be down regulated after the first day of Dex in cell culture [46]. The same study indicated that IL-6 mechanical stimuli decrease the inhibition of IL-6 expression by dexamethasone. Irradiation of MSCs by cyclic strain enhances the release of IL-6 even with the presence of dexamethasone in cell culture [46]. It is interesting to note that Li et al have demonstrated that when BMMSCs and peripheral blood leucocytes undergo differentiation, there was a decrease in IL-6 level [48]. It suggests that IL-6 down regulation was differentiation dependent but not cell phenotype dependent. Their study showed a significant decrease in IL-6 mRNA expression in vascular endothelial growth factor (VEGF) induced endothelial cells compared with undifferentiated MSCs. The *in vivo* study of Li et al on cardiovascular tissue engineering

demonstrated that when allogenic stem cells were implanted in rat infraction model they improved cardiac function, but after the cells were myogenically differentiated, they lost the immunoprevilage property and there was cellular rejection [48]. All these studies collectively state that IL-6 might have immunosuppressive properties and modulation of IL-6 may improve the immunoprevilage property of MSCs.

Author	Year	Cell type	Culture condition	Cytokines
Kim et al	2005	Human Bone marrow MSC	Complete medium	• IL-6, IL-8, were expressed • IL-1β was not expressed
			Osteogenic medium	• IL-6 decreased expression • IL-8 was expressed • IL-1β was not expressed
Samanasunghe et al	2008	Human Bone marrow MSC	Complete medium	• TNF-α anf IL-1β had very low expression • IL-6 increased • IL-8 less expression
			Complete medium+ cyclic strain	• TNF-α and IL-1β had very low expression • IL-6 decreased • IL-8 increased expression or the first 3 days
			Osteogenic medium	• TNF-α and IL-1β had very low expression • IL-8 greatest expression
			Osteogenic medium+ cyclic strain	• TNF-α and IL-1β had very low expression • IL-6 increased • IL-8 decreased expression with 10% strain
Malaval et al	1998	Rat calvaria cells	Complete medium without Dex	• IL-6 expression observed
			Complete medium with Dex	• IL-6 expression decreased
Hwang et al	2009	Human bone marrow Cord blood Amnion and decidua of placenta	Complete medium	• IL-6 and IL_8 were expressed by all cell types • IL-6 was lower in cord blood
Park et al	2009	Human Bone marrow MSC	Complete medium	• IL-6 high expression • IL-8 moderate expression • TGF-β detected with no measurable value

Table 2: *In-vitro* studies of various authors on cytokines expression studies

CONCLUSION

There is an overall enhancement in the expression of anti-inflammatory cytokines IL-10 and TGF-β during the osteogenic differentiation of HGMSCs indicating a potential shift of cytokines to dampen immune responses. The reduction of IL-6 and IL-1β expression is an added advantage to reduce acute-phase and inflammatory responses, favoring HGMSCs to be the cells of choice for repair and regeneration of craniofacial bones. A beneficial combination of the cytokines expressed by HGMSCs during osteogenic differentiation to reduce acute phase and long term immune responses will facilitate the achievement of our long term goal.

APPENDICES

Gene Expression of IL-10

1 week		2 weeks		3 weeks	
CM	OM	CM	OM	CM	OM
1	1.90	1	1.01	1	1.288
1	1.26	1	1.41	1	1.66
1	1.26	1	1.64	1	1.37
1	1.26	1	1.52	1	1.7
1	1.15	1	1.58	1	1.22
1	1.54	1	1.22	1	1.13
1	1.51				
Mean	1.41	Mean	1.396667	Mean	1.394667
Sd	0.26	Stdev	0.240389	StDEV	0.256184
P value	0.0056	P	0.0099	P=	0.0103
	**	Stdev	**		*

Gene Expression of IL-6

1 week		2 weeks		3 weeks	
1	0.77	1	0.24	1	0.11
1	0.13	1	0.05	1	0.53
1	0.11	1	0.12	1	0.08
1	0.74	1	0.09	1	0.52
1	0.12	1	0.25	1	0.93
1	0.77	1	0.28	1	0.71
Mean	0.44				
SD	0.31	Mean	0.17	Mean	0.48
		SD	0.10	Stdev	0.34
P=	0.01				
	*	P	< 0.0001	P	0.01
			***		*

Gene Expression of IL-1

1 Week Data		2 Weeks 'Data		3 weeks' Data	
CM	OM	CM	OM	CM	OM
1	0.86	1	0.05	1	0.601
1	0.08	1	0.46	1	0.0589
1	0.59	1	0.95	1	0.456
1	0.14	1	0.12	1	0.285
1	0.85	1	0.18	1	0.307
1	0.04	1	0.67	1	0.659
		1	0.36		
Mean	0.43			Mean	0.39
SD	0.39	Mean	0.40	STDEV	0.22
P Value	0.0421 *	SD	0.33		
				P value	0.009 **
		P Value	0.0027		
		Significance	**		

Gene Expression of IL-8

1 WEEK		2 WEEKS	
CM	OM	CM	OM
1	7.68	1	6.25
1	4.60	1	12.44
1	4.07	1	9.24
1	9.38	1	3.50
1	7.56	1	4.49
Mean	6.66	1	2.55
SD	2.01	Mean	6.41
P value	0.02	SD	3.78
*		P	0.02 *

Gene Expression of TGF-β

1 week		2 weeks		3 weeks	
CM	OM	CM	OM	CM	OM
				1	1.563
1	1.34	1	1.62	1	1.39
1	2.18	1	1.30	1	2.7
1	1.23	1	1.39	1	1.7
1	2.03	1	2.09	1	1.94
1	1.8	1	1.29		
1	2.34				
Mean	1.82	Mean	1.5382	Mean	1.8586
SD	0.45	SD	0.34		0.5115
P=	0.0068	P=	0.0233	P=	0.0199
	**		*		*

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