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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

MASTER OF SCIENCE IN DENTISTRY

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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.Sc.D. degree and for this assignment.

STUDENT SIGNATURE: _____

Annapurna Bondalapati B.D.S. Date

DEDICATION

To my loving husband, adorable kids and wonderful family for all of your love and support throughout my education. Thank you for standing by me in my endeavors.

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Abstract

BMP2 ENHANCES OSTEOGENESIS OF HUMAN GINGIVAL STEM CELLS IN A PEPTIDE-BASED HYDROGEL

DEGREE DATE: JUNE 14, 2017 Annapurna Bondalapati, B.D.S.

COLLEGE OF DENTAL MEDICINE NOVA SOUTHEASTERN UNIVERSITY
Directed by: Dr. Uma Devi Kandalam, Assistant Professor, Department of Pediatric Dentistry, NSU College of Dental Medicine.

Purpose: The aim of the study was to investigate effect of the growth factor, Bone Morphogenetic Protein 2 (BMP2), on the differentiation of Human gingiva derived stem cells (HGMSCs) in a self-assembled three-dimensional (3D) peptide hydrogel. The study has two parts; part 1 comprised of optimizing the dose of BMP2 and Part II was investigating the effective delivery method of BMP2 in enhancing the osteogenic differentiation of HGMSCs

Methods: Human gingiva derived mesenchymal stem cells (HGMSCs) cultured in the peptide hydrogel (3-D cultures) were treated with 50, 100 and 200 ng/ml BMP2. The cells in the osteogenic differentiation of HGMSCs in the peptide gel was evaluated at one week. The expressions of osteogenic marker genes Alkaline-Phosphatase (ALP), Runt related transcription factor 2 (RUNX2), Collagen Type I (COL1) were measured using quantitative PCR. The results were compared with monolayer cells treated with BMP2 (2-D culture). Furthermore, to evaluate the effective delivery method, cells were encapsulated in Puramtrix and BMP2 was administered either in culture medium or encapsulated in Puramatrix. The cells were treated for one week and early osteogenic markers genes were measured. ANOVA was used to evaluate the results and $P < .05$ was considered statistically significant.

Results: The results of the study demonstrated a dose dependent upregulation (*P*<.05) of the genes including ALP, RUNX2 and COL1 at all concentrations (50,100 and 200ng/ml). There was significant up regulation of gene expression in 200ng/ml compared to 50ng/ml (*P*<.05). BMP2 treatment accelerated the mineral deposition in HGMSCs. Overall results of our study demonstrated that the application of osteoconductive agent rhBMP2, stimulated the osteogenic differentiation regardless of the delivery method that was used in this study. However, BMP2 entrapped in Puramatrix™ showed significantly high ALP expression at 100ng/ml compared to 200ng/ml concentration

Conclusions: The Puramatrix™ hydrogel in combination with BMP2 supported osteogenic differentiation of HGMSCs. This novel tissue engineered cell-scaffold system with growth factor has potential for the regeneration of bone in craniofacial defects.

Key words: Gingiva derived stem cells, Peptide hydrogel, Bone morphogenetic Protein -2 Osteogenic differentiation, Craniofacial defect

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Abbreviations:

BMMSCs	Bone marrow derived mesenchymal stem cells
CNCC	Cranial neural crest cells
DMEM	Dulbecco's modified eagle medium
HGMSCs	Human gingival derived mesenchymal stem cells
MSCs	Mesenchymal stem cells
NC	Neural Crest
OM	Osteogenic Medium
rhBMP-2	Recombinant human bone morphogenetic protein 2
ALP	Alkaline Phosphatase
COL 1	Collagen Type 1
RUNX 2	Runt related Transcription Factor 2

Chapter 1 INTRODUCTION

1.1 Craniofacial Defects

Large size craniofacial defects in children arise from many etiologies including open skull trauma, infection, congenital anomalies and tumor. Reconstruction of these large size defects poses a reconstructive challenge because of children's unique physiology, developing anatomy, and dynamic growth. Over three-quarters of all craniofacial defects observed in the US per year are cleft palates[1, 2] (Figure 1). The complications of the cleft palate include wound dehiscence, residual lip and/or nose deformity, feeding difficulties, speech abnormalities, dentofacial anomalies and psychosocial problems [3] [4] .

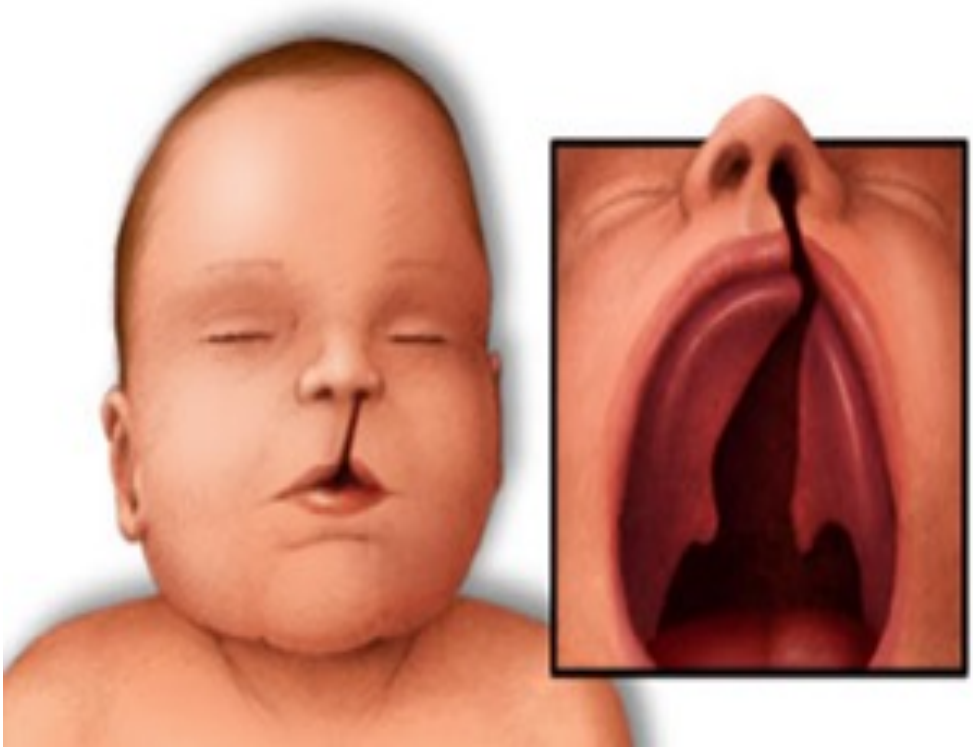


Figure 1: Cleft Palate

Current treatment modalities for management critical size palatal defects are limited. Autologous bone grafting for reconstruction of craniofacial bone is considered the gold

standard in pediatric care. The sources of autograft are cancellous bone derived from the iliac crest, cortical bone from symphysis of mandible, and cortico-cancellous bone from the rib (Figure 2). The gold standard autograft has the ability to reincorporate into the skull (osseointegration), lower risk of material rejection, and ability to allow growth of the skull. Nevertheless, with pediatric patients, additional considerations must account for the high incidence of bone resorption, the immature osseous skeleton, limited availability of bone tissue and the harvest is often associated with undesirable side effects associated with donor site morbidity and repeated surgeries. Allografts, on the other hand are accompanied with infections. Recent developments in stem cell based tissue engineering approaches offer an alternative solution.

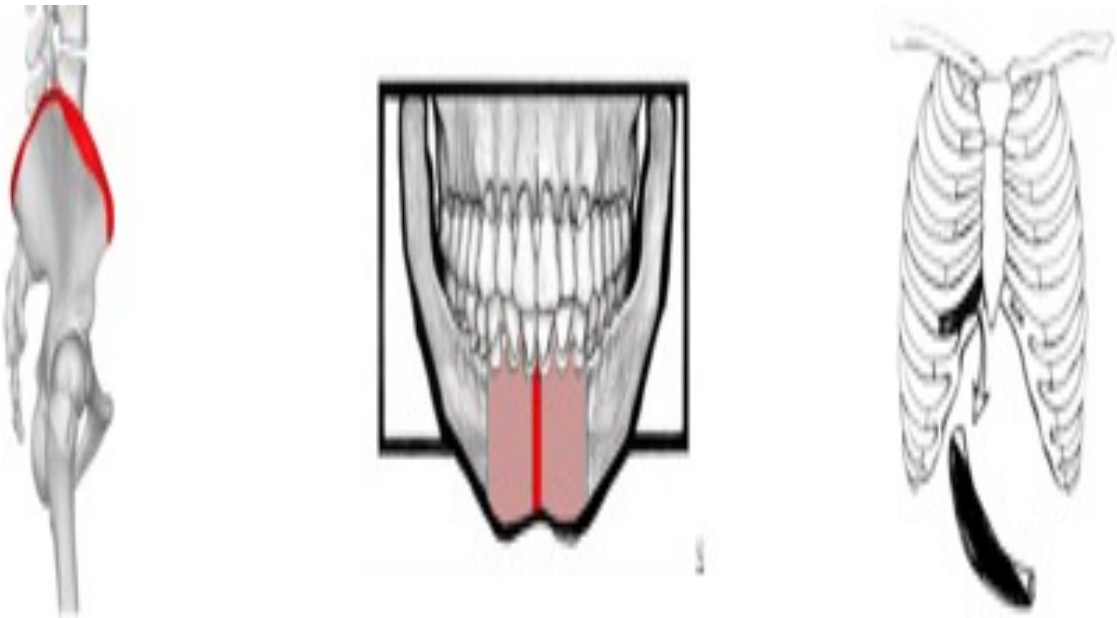


Figure 2: Sources of Autografts

1.2 Tissue Engineering

Tissue engineering approaches represent a promising alternative that would serve to facilitate bone regeneration even in large craniofacial skeletal defects. Engineering bone requires the combination of osteogenic cells, osteoconductive scaffolds, osteoinductive growth factors. (Figure 3)

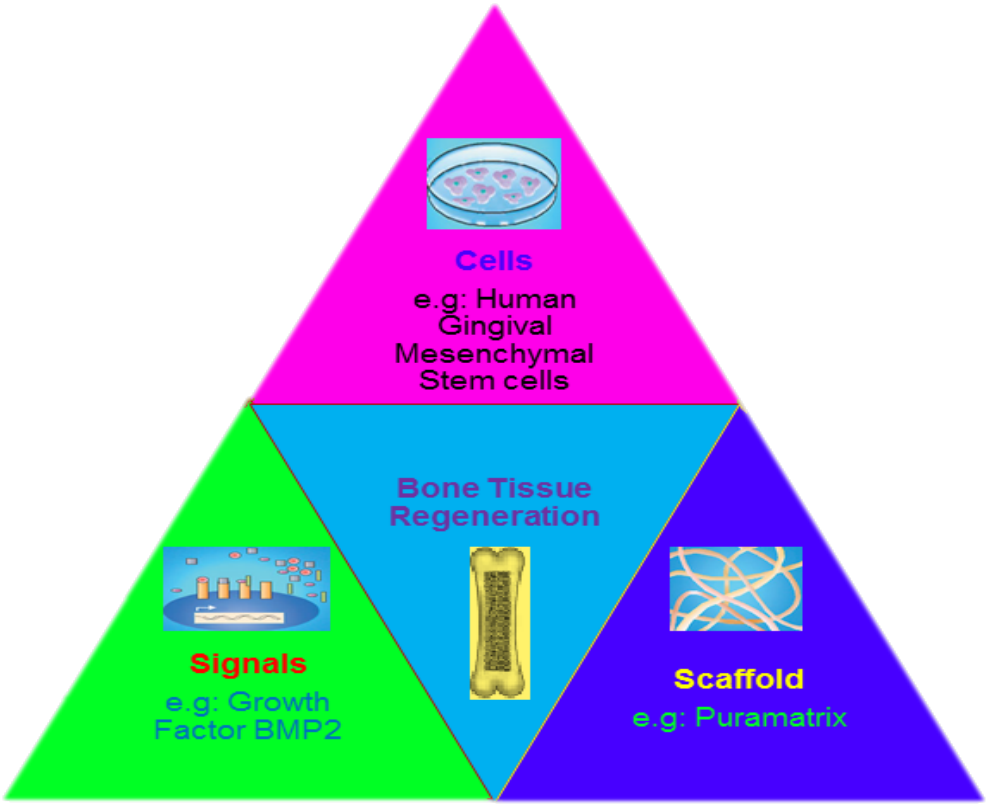


Figure 3: Essential Components of Tissue Engineering

1.3 Mesenchymal Stem Cells

Mesenchymal stem cell based approaches are promising alternatives to facilitate bone regeneration in critical size defects. Mesenchymal stem cells (MSCs) as potential source for bone tissue regeneration has been explored in recent years [5, 6]. MSCs are unspecialized cells which reside in adult tissues. They are highly proliferative with the intrinsic ability of self-renewal. MSCs have multipotent differentiation capacity and they are capable to differentiate into multiple cell types including osteocytes, chondrocytes, adipocytes, tenocytes and myoblasts [7-9]. (Figure 4)

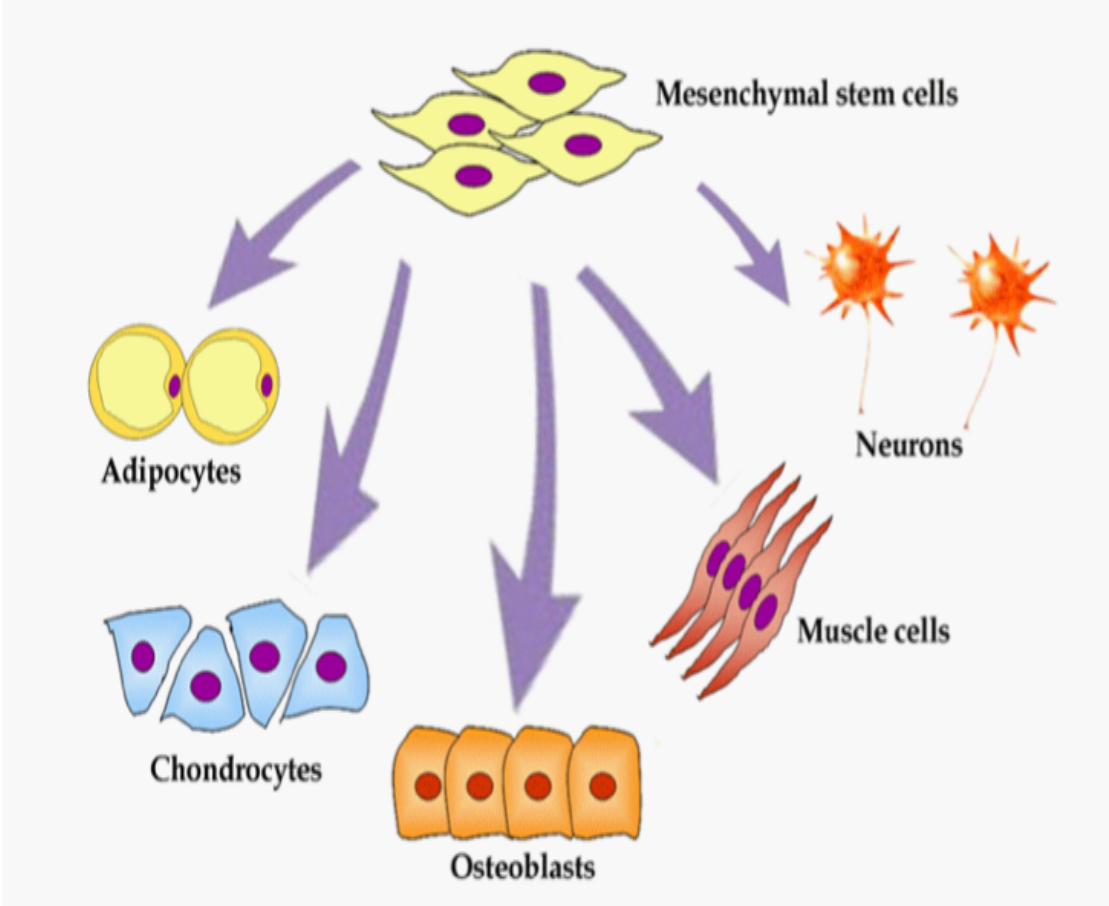


Figure 4: Differentiation potential of Mesenchymal Stem Cells. MSCs can differentiate in a large variety of human tissues including osteogenic, chondrogenic, adipogenic and neuronal lineages. Recently, it was also demonstrated the presence of human MSC like cells in adult skeletal muscle.

MSCs have high immunomodulatory capacity and inhibit the Major Histocompatibility Complex (MHC)-mismatched lymphocyte response (immunogenicity). MSCs have been shown to express MHC class I and other immune related molecules as VCAM-1 and LFA-3 [10-12].

MSCs can be obtained from a wide spectrum of adult tissues such as bone marrow, umbilical cord and orofacial tissues such as gingiva. Bone marrow has been a major source for the isolation of MSCs as bone marrow derived mesenchymal stem cells (BMMSCs) have been proven through clinical trials that they are an effective treatment for the osseous defects [13]. However, BMMSCs are highly variable with limited self-renewal and differentiation capacity. Furthermore, bone marrow aspiration is known to be an invasive and painful procedure, and considered as a complicated procedure for general practitioners. In recent years, studies have explored the isolation of MSCs from other tissue sources, including adipose tissue, umbilical cord, umbilical cord blood and stem cells from orofacial region [14].

1.4 Orofacial Stem Cells:

Recently, isolation of stem cell populations from orofacial region has been gaining attention. Orofacial stem cells are originated from neural crest cells that can be differentiated into cartilage and bone to form craniofacial skeleton. While autologous BMMSCs obtained from distant part of the body far from craniofacial region are the traditional stem cell source for the repair and regeneration of long bones [15-17]; the reconstruction of bone in the craniofacial region showed partial success as the long bones are originated from mesoderm and the bones

in the craniofacial region are derived from the neural crest (ectodermal origin). This suggests that neural crest associated cells might be a superior cell source for the reconstruction of bone in the orofacial region as compared to BMMSCs obtained from long bones [18]. Moreover, orofacial mesenchymal stem cells (OMSCs) are readily accessible from the oral cavity, can be easily expanded, highly proliferative and have ability to differentiate into osteogenic, odontogenic, adipogenic and neurogenic precursor cells. (Figure 5)

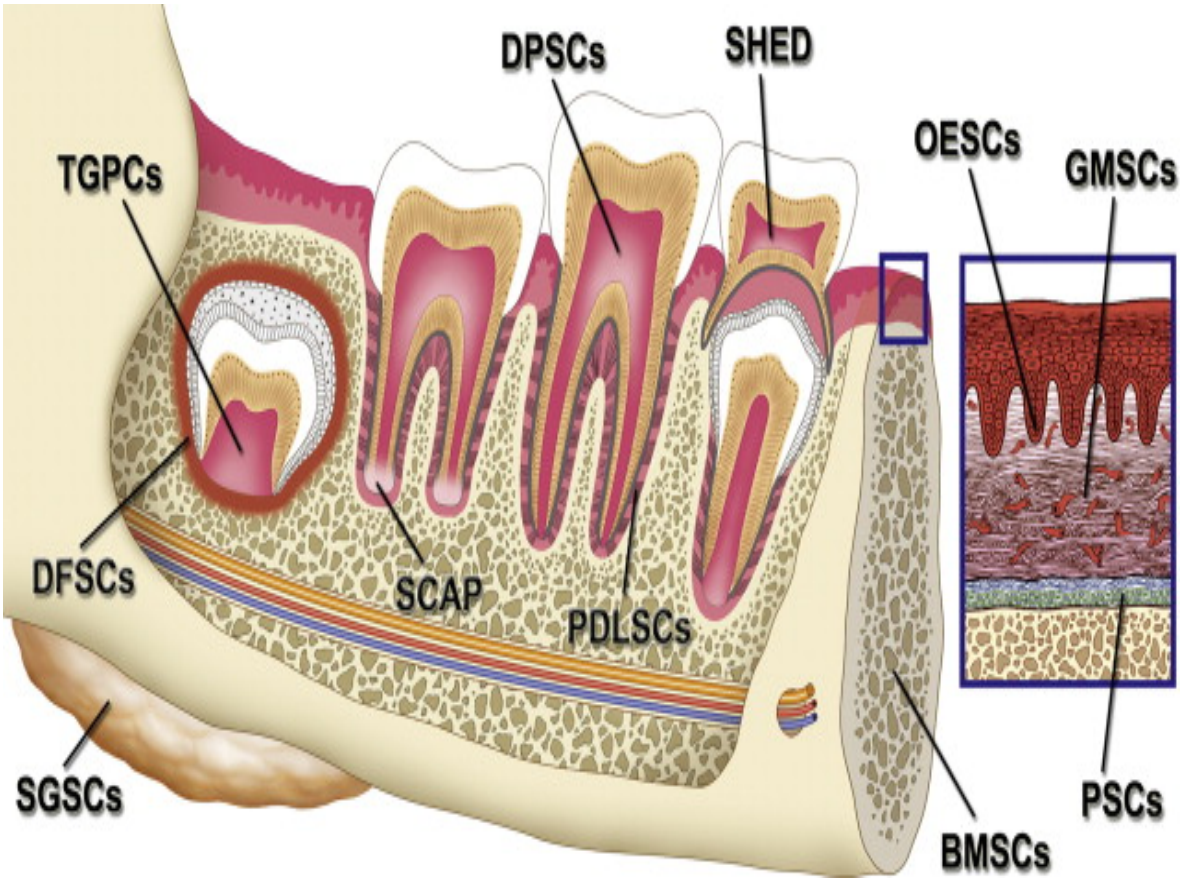


Figure 5: Sources of adult stem cells in the oral and maxillofacial region. BMSCs: bone marrow-derived MSCs from orofacial bone; DPSCs: dental pulp stem cells; SHED: stem cells from human exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFSCs: dental follicle stem cells; TGPCs: tooth germ progenitor cells; SCAP: stem cells from the apical papilla; OESCs: oral epithelial progenitor/stem cells; GMSCs: gingiva-derived MSCs, PSCs: periosteum-derived stem cells; SGSCs: salivary gland-derived stem cells (Reference: Stem Cells in Dentistry – Part I: Stem cell sources (Hiroshi Egusa, DDS, PhD, Wataru Sonoyama, DDS, PhD, Masahiro Nishimura, DDS, PhD, Ikiru Atsuta, DDS, PhD, Kentaro Akiyama, DDS, PhD).

Orofacial/dental tissues are specialized tissues that do not undergo continuous remodeling unlike the bone marrow stem cells [18]. As these stem cells are derived from the neural crest region, which is originated from ectoderm, the dental mesenchyme is often termed as 'ectomesenchyme' due to its earlier interaction with the neural crest. Thus, orofacial stem cells may possess different characteristics similar to those of neural crest cells [19, 20]. Studies suggested that dental tissue derived stem cells are more appealing for craniofacial application due to their increased commitment to differentiate into craniofacial tissues when compared to non-dental derived stem cells. MSCs derived from orofacial tissue possess multi differentiation ability and have the capacity to give at least three distinguishable lineages of cells including osteo/odontogenic, adipogenic and neurogenic.

1.5 Human Gingival Mesenchymal Stem Cells:

Human gingiva is a well-known tissue enriched with adult mesenchymal stem cells, 90% of which originated from cranial neural crest cells (CNCC) [19, 20]. The ectomesenchymal origin of these cell types exhibit characteristics akin to those of neural crest cells. The neural crest derived origin makes Human Gingival Mesenchymal Stem Cells (HGMSCs) interesting candidates for their use in craniofacial bone tissue engineering. GMSCs have shown stem cells properties and immunomodulatory abilities as those of BMMSCs. Usually the gingiva overlying the alveolar ridges and retro molar region is frequently resected during general dental treatments and can often be obtained as a discarded biological sample. GMSCs proliferate faster than BMMSCs, display a stable morphology and do not lose their MSC characteristics with extended

passaging [21] and differentiate into various lineages, when cultured in appropriate inductive media [22-26]. The distinctive feature of GMSCs is that, they display potent immunosuppressive and anti- inflammatory functions. Recently, the mechanisms of the immunomodulatory effect of human GMSCs on the innate immune cells have been investigated [20].

MSCs derived from human gingiva (HGMSCs) can readily be obtained with technique minimally invasive methods and can maintained/preserved under standard laboratory conditions (Figure 6). Being derived from neural crest, HGMSCs offer distinctive advantages for craniofacial bone-tissue repair and regeneration.

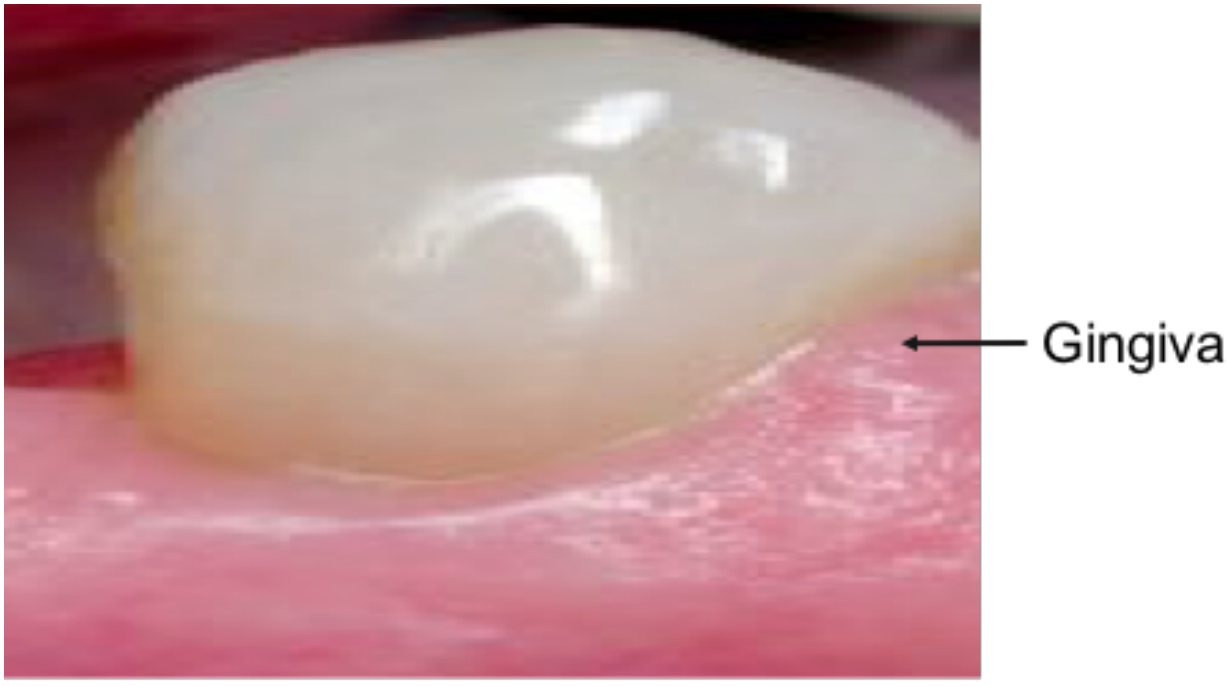


Figure 6: Human Gingival Tissue

1.6 Scaffold

A right combination of cells-scaffold and growth factors is essential for regeneration. Hydrogel scaffolds are able to mimic natural extracellular matrix

of many tissues and are able to form solid constructs that permit homogenous distribution of the cell. Hydrogels offer the convenience of incorporating growth factors and cells prior to injection into the *in vivo* site to enable gel formation. Injectable hydrogels present a novel approach of local delivery of stem cells in tissue engineering applications, enabling surgeons to transplant cells in a minimally invasive way. They are naturally biocompatible, as they do not cause an immune response or inflammatory reaction. These gels are degraded by hydrolysis, action of enzymes and/or dissolution. The use of injectable hydrogels has been tested for bone tissue engineering. Injectable hydrogels are novel strategy for local delivery of stem cells as they can fill irregular shapes and voids in the bone defects to and maximizes cell adhesion and interaction enhancing bone regeneration [27].

Self-assembled short peptide scaffolds are new class of hydrogels, with implantable or injectable mode of delivery of the cells as well as growth factors. The nanofiber structures of these peptides (<10nm in diameter) are several times thinner than the cells, which permits them to surround the cells in a manner similar to the natural extracellular matrix.

Self- assembling peptides have ability to form stable hydrogels and have been used *in-vivo* animal studies for repairing bony defects. In particular, liquid hydrogel can fill the three dimensional (3D) irregular defects in the craniofacial region and assists in enhanced healing without forming a scar. Furthermore, these 3D scaffolds mimic the defect while being rigid enough to support cells and flexible to blend into host tissue. 3D scaffolds increase cell proliferation, migration and viability compared to preformed 2D scaffolds. Our study intends

to use a 3D peptide based hydrogel biomaterial, Puramatrix™, with over 99% water content that can self- assemble into 3D interweaving nanofibres

1.7 Puramatrix™

Puramatrix™ is a chemically defined hydrogel, devoid of any proteins of animal origin and has ability to carry, deliver the cells on to the defect site (Figure 7). It can mimic natural extracellular matrix and present a novel approach for delivery of stem cells with ease. Additionally, it acts as a dynamic liquid support to carry living cells, drugs and growth factors and have the ability to deliver cells at the defect site without inflammatory reaction with minimal immune response and reduce scar formation[28, 29]. Nanostructured biomaterials are gaining popularity in regenerative medicine because they mimic natural extracellular matrix in a nano scale. Physical and biological parameters of this scaffold can be modified due to its synthetic nature. Bioactive modifications can be made, which makes it versatile in terms of cell adhesion while increasing its stability. PuraMatrix™ hydrogel is capable of both ionic and hydrophobic interactions. These interactions trigger spontaneous self-assembly enabling cell encapsulation and filling in both *in vitro* and *in vivo* applications. We intend to utilize this property to encapsulate HGMSCs within the PuraMatrix™ for the purpose of site-specific delivery of cells and growth factors.

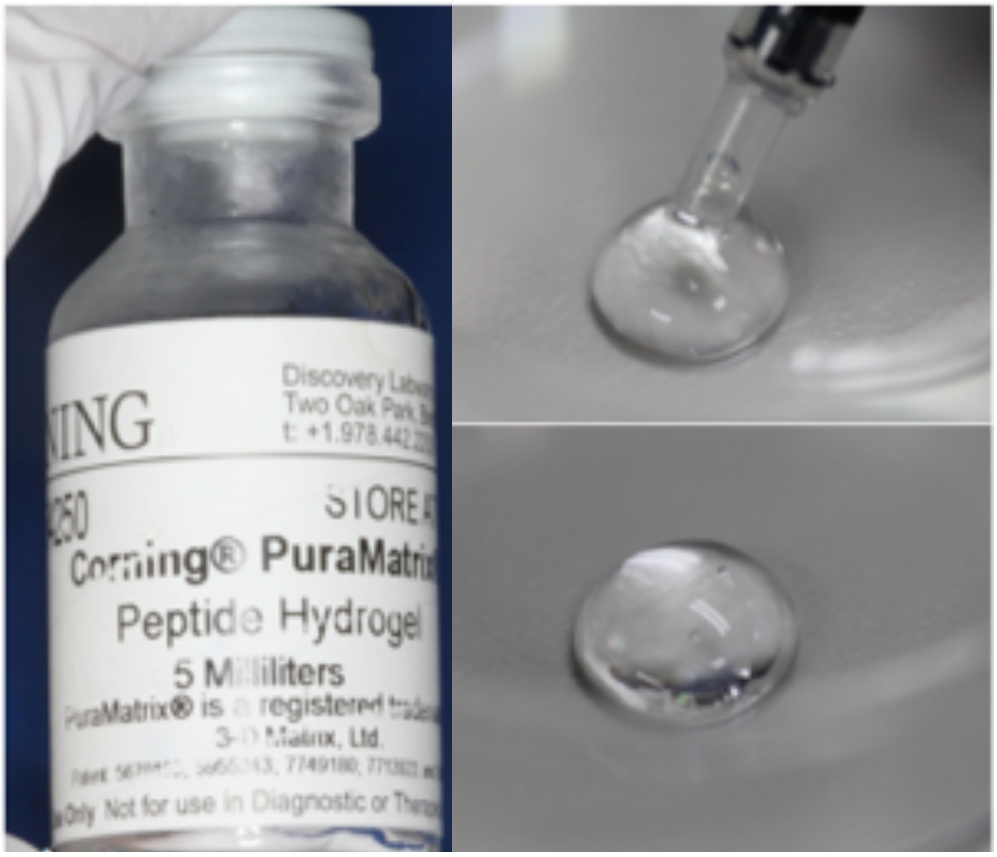


Figure 7: Puramatrix™ – Self assembled peptide scaffold (Figure showing its injectability)

Characteristics	Puramatrix Synthetic ECM	Natural ECM	Synthetic Scaffold	Puramatrix Advantages
Composition	Patented 16 mer peptide in 0.5-1.0% w/v	Collagen, Fibronectin, Cadaver tissue, Basement membranes	PLA,PLGA,carbon fiber, calcium phosphate	Animal-free, reproducible cell culture and cell signaling.
Fiber size	7-10nm diameter	5-10nm diameter	10,000-100,000nm looks 2D relative to cell	Approximates in vivo ECM nano –scale
Pore size	50-200 nm	50 – 400 nm	20,000 – $1*10^6$ nm	Encapsulates like ECM
Water content	99.5 – 99.9%	80 - 97%	60 – 80%	Better hydration and nutrient diffusion
Mechanical Strength	Low to mid, cells can migrate within it	Low to mid	Mid to High	More rapid ingrowth, breakdown

Table 1: Characteristics of Puramatrix

1.8 Growth Factor

In bone tissue engineering, in concert with osteoprogenitor cells and scaffolds, a plethora of growth factors enhance osteogenesis. Growth factors are particularly interesting because of their ability to target specific cellular receptors and actively trigger various cellular signaling processes [30]. Major players in the skeletal tissue engineering are members of the TGFβ superfamily, notably the members of bone morphogenetic protein superfamily (BMPs).

1.9 Bone morphogenetic Proteins

Bone morphogenetic proteins are a family of osteoinductive proteins that promote differentiation of mesenchymal cells into osteoblasts and promote neovascularization. Among the 15 identified BMPs, BMP-2 and BMP-7 (i.e., osteogenic protein-1) are now commercially available and have been

investigated as an alternative to bone auto grafting in a variety of clinical situations, including spinal fusions, internal fixation of fractures, treatment of bone defects, and reconstruction of maxillofacial conditions.

Bone morphogenetic protein-2 (BMP-2) is currently the one of the only Food and Drug Administration (FDA)-approved osteoinductive growth factor used as a bone graft substitute. BMP2 successfully induced osteoblastic differentiation of mesenchymal stem cells [31]. While BMP2, conventionally used in large quantities (as high as 10 μg/mL), in *invivo* applications, have adverse effects such as enhanced bone formation in undesired site, inflammation and respiratory distress [32]. Furthermore, I*n vitro* studies report using wide range of doses of BMP2 (100-1000ng/ml). However, optimal dose of BMP2 may differ with the type of stem cells and the scaffolds employed. Our previous studies demonstrated that concentration range from 50ng/ml to 200ng/ml BMP2 could induce osteogenic differentiation of HGMSCs [33] in monolayer cultures. In this project, we intend to establish optimal dose of BMP2 in HGMSCs encapsulated in Puramatrix^{TM} hydrogel.

Another major limitation of clinical BMP-2 treatments is the rapid diffusion from the implant site, causing a significant decrease in local concentration, as well as rapid loss of bioactivity in the soluble form. Soluble or free BMP-2 quickly loses bioactivity due to proteolytic degradation and denaturing caused by physiologic conditions (i.e. pH, temperature, salt concentration). Therefore, recent efforts have focused on encapsulating the BMP molecule to protect it from degradation or enhancing the binding affinity of BMPs to the carrier material [34]. Matrix based systems for growth factor delivery for regenerating

the tissue provides controlled release and stabilizes the loaded signaling molecule. However, limited studies have established on the mode of the delivery of growth factor and their effect on osteogenic differentiation on mesenchymal stem cells. In this study, we intend to test and compare two types of delivery methods to establish the suitable method of delivery that can enhance osteogenic differentiation of HGMSCs encapsulated in Puramatrix^{TM}.

1.10 Innovation*:*

The current surgical protocol for repair of critical size defects in the craniofacial region require the gold standard autografts. Cell based tissue engineering aims to replace or facilitate the repair and regeneration of damaged tissue by applying the combinations of biomaterials, cells, and bioactive molecules (growth factors). Growth factors play a crucial role in harnessing and controlling tissue regeneration. A primary goal of growth factor delivery for bone tissue engineering is to accelerate healing and enhance bone formation. Additionally, the dose of BMP2 remains crucial in bone tissue engineering applications. Currently supra-physiological dose has been used which is resulting in adverse effect. It is essential to optimize the dose. Another aspect of the use of growth factors in tissue engineering is controlled delivery. The conventional methods of administering BMP2 in the culture medium might be homogeneous, however, in this method only small amount reaches to the cells related to biological signaling pathways. In contrast, the availability of growth factor in direct contact with cells might be an efficient way of delivering. The effect of delivery mode on osteogenic differentiation of HGMSCs have never been tested. Hence in this study we intend to investigate the dose and delivery strategy of BMP2 on

HGMSCs encapsulated in the Puramatrix™, a new biomaterial that serves as synthetic extracellular matrix. Overall goal of this study was to assess the effect of BMP2 on osteogenic differentiation of PuraMatrix™ encapsulated HGMSCs.

1.11 Objectives

The long-term goal of this project is to develop a 3D injectable scaffold for the purpose of bone regeneration in patients with cleft lip and palate defects using HGMSCs and minimal dose of BMP2.

The objective of this study was to assess the effect of BMP2 on osteogenic differentiation of PuraMatrix™ encapsulated HGMSCs. This study has two specific aims

1.12 Specific aims

Specific aim 1a) is to assess optimal dose of BMP-2 on osteogenic differentiation of PM encapsulated HGMSCs.

Different doses (50,100 and 200ng/ml) of BMP2 will be administered to HGMSCs in monolayer cultures (2-D) as well as Puramatrix™ encapsulated HGMSCs (3-D) cultures for one week and the osteogenic maker gene expressions were measured.

Specific aim 1b) is to examine and compare BMP2 delivery strategies that enhance the osteogenic differentiation of HGMSCs

The two types of delivery methods include

1. HGMSCs stimulation with soluble BMP2: The HGMSCs will be encapsulated in Puramatrix™ and BMP2 will be supplemented with osteogenic medium at regular intervals for one week and osteogenic

differentiation will be investigated by measuring osteogenic markers gene expression.

2. HGMSCs stimulation with adsorbable method: HGMSCs loaded with BMP2 will be encapsulated in Puramatrix^{TM} will be induced with osteogenic medium for one week and the osteogenic differentiation will be examined by measuring the osteogenic marker gene profiles.

This cell-scaffold -growth factor composite will have potentially therapeutic benefit for the repair of bony defects.

1.13 Hypothesis

In this study, we would like to test these two hypothesis

1. The optimal concentration of BMP2 that can trigger the osteogenic differentiation
2. Whether Puramatrix encapsulated BMP2 can enhance osteogenic differentiation of HGMSCs, if so whether the mode of delivery method influences on the osteogenic differentiation of HGMSCs.

Null Hypothesis:

1. Mode of BMP2 delivery does not influence the osteogenic differentiation of HGMSCs

2: Concentration of BMP-2 has no effect on osteogenic differentiation of PM encapsulated HGMSCs.

Alternative Hypothesis:

1. Mode of BMP2 delivery may influence the osteogenic differentiation of HGMSCs

2: Concentration of BMP-2 has an impact on osteogenic differentiation of PM encapsulated HGCMSCs.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Commercially available PuraMatrix^{TM} hydrogel BD Biosciences, San Jose, CA) was used for the study. Mesenchymal stem cell medium was obtained from Sciencell (Carlsbad, CA). Commercially available rhBMP2 (R&D systems, Minneapolis, MN) was used. All other necessary chemicals and lab supplies were obtained from Sigma (St. Louis, MO) and VWR international (Atlanta, GA), respectively.

2.2 Overall Study Design:

Human gingival tissue was obtained upon approval of Institutional Review Board. HGMSCs were isolated using enzymatic digestion method. Cells isolated from the tissue were cultured and expanded under standard culture conditions. Cells at 70-80% confluency were induced with osteogenic supplements and osteogenic differentiation of HGMSCs was determined by gene expression of various osteogenic differentiation marker genes. Mineral deposition of osteogenically induced cells was confirmed by Alizarin Red and Von Kossa staining techniques. Cells were encapsulated in 3D PuraMatrix^{TM} scaffold and then supplied with CM in one group and OM in other group. Cells were encapsulated in PuraMatrix^{TM} in different cell concentrations ($1x10^4$, $2x10^4$ $4x10^4$ and $8x10^4$ cells/ml). Cell proliferation was assessed by WST assay. Cells were encapsulated in PuraMatrix^{TM} with different concentrations of BMP2 (50ng/ml, 100ng/ml, 200ng/ml) for 1 week. Cells grown in osteogenic medium served as a control. Osteogenesis was determined by gene expression studies, mineralization studies and ALP assay.

Specific Aim 1a:

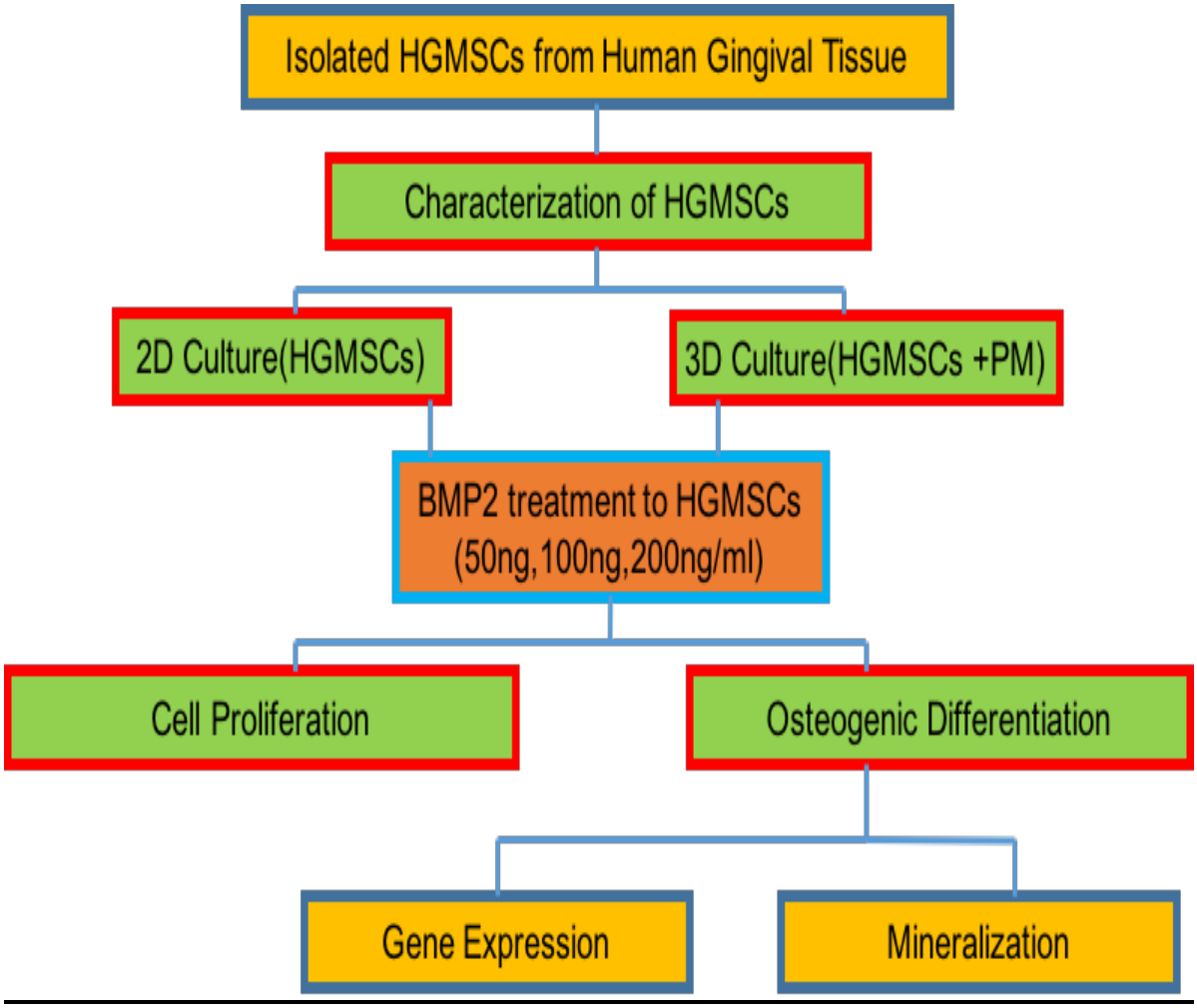


Figure 8: Specific Aim 1a: To assess optimal dose of BMP2 on osteogenic differentiation of Puramatrix™ encapsulated HGMSCs

Specific Aim 1b:

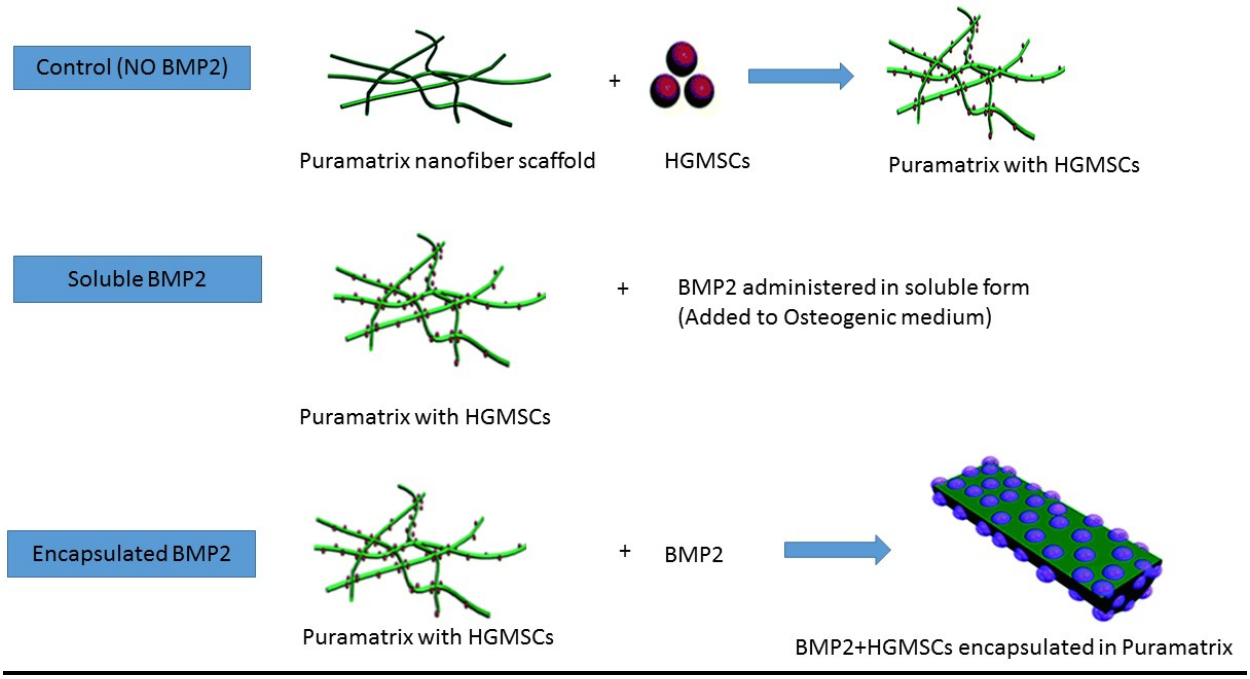


Figure 9: Specific Aim 1b: Examine and compare BMP2 delivery strategies that enhance osteogenic differentiation of HGMSCs

2.3 Isolation of gingival stem cells and Cell culture

Gingival tissue was obtained upon approval of Institutional Review Board. Mesenchymal stem cells were isolated from the gingival tissue using standard procedures.

2.3.1 Enzymatic Digestion

Briefly, the gingival tissue was minced thoroughly to make to smaller tissue samples and were digested enzymatically using 1mg/ml collagenase and 0.2% dispase for 15 minutes. The first cell suspension was discarded to avoid the interference of epithelial cells. The tissue samples were further digested with 1mg/ml collagenase and 0.2% dispase and the cell suspension was pooled. The cell suspensions were centrifuged and the cell pellet were plated in tissue

culture flask and grown under standard culture conditions, in a humidified incubator at 37°C and 5% CO_2.

2.4 Cell Culture:

The cells were cultured in growth medium (DMEM, 10% FBS and 1% antibiotics) at 37°C and 5% CO_2. To ensure uniform cell population first two passage cells were kept further expansion and cells from third or fourth passage were used for all studies.

2.5 Characterization of HGMSCs surface markers by flow cytometry method

Monolayer cells ($1x10^6$ per group) were used to detect the surface markers using Miltenyi Kit per manufacturer's instructions using a flow cytometer FACAria IIIu (BD Biosciences, San Jose, CA). The specific markers positive for mesenchymal stem CD73, CD90, CD105, and negative for CD34 were measured at the facilities at University of Miami using a fluorescent activated cell sorter FACAria IIIu (BD Biosciences, San Jose, CA) with adjusted florescence compensation setting. Negative samples were used to set up the thresholds of quadrant markers

2.6 HGMSCs encapsulation and culture in Puramatrix™ (cell–gel constructs 3D culture)

The cells (HGMSCs) from 3rd or 4th passage was used for all of experiments. Based on our pilot studies we have chosen to use 0.5% Puramatrix™ for all the experiments. The encapsulation method was followed per manufacturer's instructions. Briefly, the cells in 10% sucrose solution were mixed in 250µL of Puramatrix solution at 1:1 ratio and the cells suspended in the gel (2×10^6

cells/gel) were slowly released into the growth medium. After 30 minutes of the incubation at 37^{o}C the growth medium was replaced to and cells were fed with new growth medium and gelation was examined under microscope. The cell-gel constructs were finally incubated at 37^{o}C, 5% CO_2.The cell-gel constructs were further incubated under standard culture conditions and cell morphology was monitored under phase contrast microscope on daily basis.

2.7 BMP2 Treatment:

After two days of the encapsulation, the culture medium was replaced by osteogenic medium (culture medium + 50µg/ml Ascorbic acid, β-Glycerophosphate Dexamethasone). Cell-scaffold inserts placed in 12 well plate provided with osteogenic medium. The medium was replenished twice a week. The cell-gel construct supplemented with osteogenic medium considered as control group. The experimental groups were supplemented with increasing concentrations of BMP2 (50ng/ml, 100ng/ml and 200ng/ml). Experiments were performed to measure alkaline phosphatase enzyme activity, osteogenic marker gene expression and mineralization. All experiments were repeated five times. Detailed experimental procedure is given below.

2.8 Cell Proliferation Assay (WST Assay)

Two types of assays were planned in this experiment. To determine the effect of BMP2 on HGMSCs proliferation, and to examine whether Puramatrix ^{TM} could support the cell proliferation.

2.8.1 BMP2 treatment to HGMSCs

The cells seeded in 96 well plate (10,000 cells/ per well) were allowed to attach overnight and subsequently induced with various concentrations of

BMP2(50,100 and 200ng/ml) and grown for 1,2 and 3 days. The cell proliferation WST assay was performed after designated time points.

2.8.2 HGMSCs in Puramatrix Gel

Cells incubated for designated period (1, 2 and 3 days), were WST-1 reagent was added to the cells cultured in growth medium to 1:10 final concentration. The assay was conducted at 24, 48 and 72 hours' time intervals and the absorbance was measured using micro-plate reader.

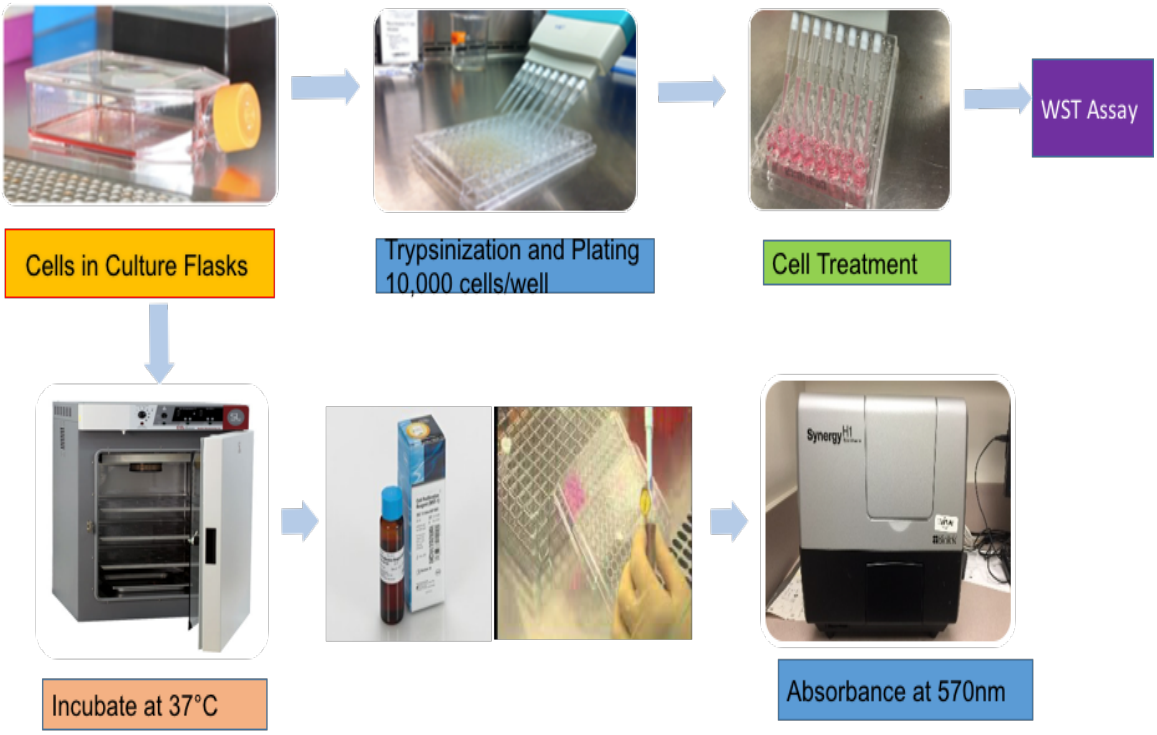


Figure 10: WST Assay

2.9 Gene expression by quantitative PCR

Briefly, the cells were released from the gel by mechanical disruption and RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) method per manufacturer's instruction. RNA was quantified and cDNA was measured using to standard protocols. Osteogenic marker genes ALP, Collagen type I and Runt-related transcription factor 2 (RUNX2) expression were assessed after

one week using Quantitative PCR (Step–One plus Applied Biosystems, Foster City, CA). Expression levels were determined by using 2-ΔΔCt methods

2.9.1 Osteogenic marker gene expression of HGMSCs treated with BMP2:

The effect of BMP2 on HGMSCs monolayer cultures and cells embedded in Puramatrix was examined for their osteogenic differentiation. The cells at 70 to 80% confluency was replaced with osteogenic differentiation medium (DMEM,10% FBS, antibiotic and antimicrotic reagent, 50µg/ml ascorbic acid, β-glycerophosphate and dexamethasone induced with different concentrations of BMP2 (50,100 and 200µg/ml). The cells without BMP2 were designated as control. The gene expression of osteogenic markers genes was measured using quantitative PCR method.

2.9.2 Osteogenic markers gene expressions of HGMSCs embedded in Puramatrix treated with BMP2

From the data that was obtained from cell viability experiments, the cell density to embed in the Puramatrix^{TM} was determined. The cells at 2 x 10^6 cells were mixed in 250µL of Puramatrix^{TM} solution and the cell-scaffold insert was placed in the each well of a 12-well culture plate and allowed to solidify. After two days, the culture medium will be replaced by osteogenic medium (control group). The experimental groups were supplemented with increasing concentrations of BMP2 (50ng/ml, 100ng/ml and 200ng/ml). The medium was replenished twice a week for both control group and experimental group.

2.10 Gene expression studies of Puramatrix™ encapsulated HGMSCs with BMP2

In order to determine the osteogenic differentiation of Puramatrix™ encapsulated BMP2, different concentrations of BMP2 (50, 100, 200ng/ml) were loaded along with HGMSCs on to Puramatrix™ and cells were differentiated in osteogenic medium. The cells with osteogenic medium with out BMP2 were considered as control

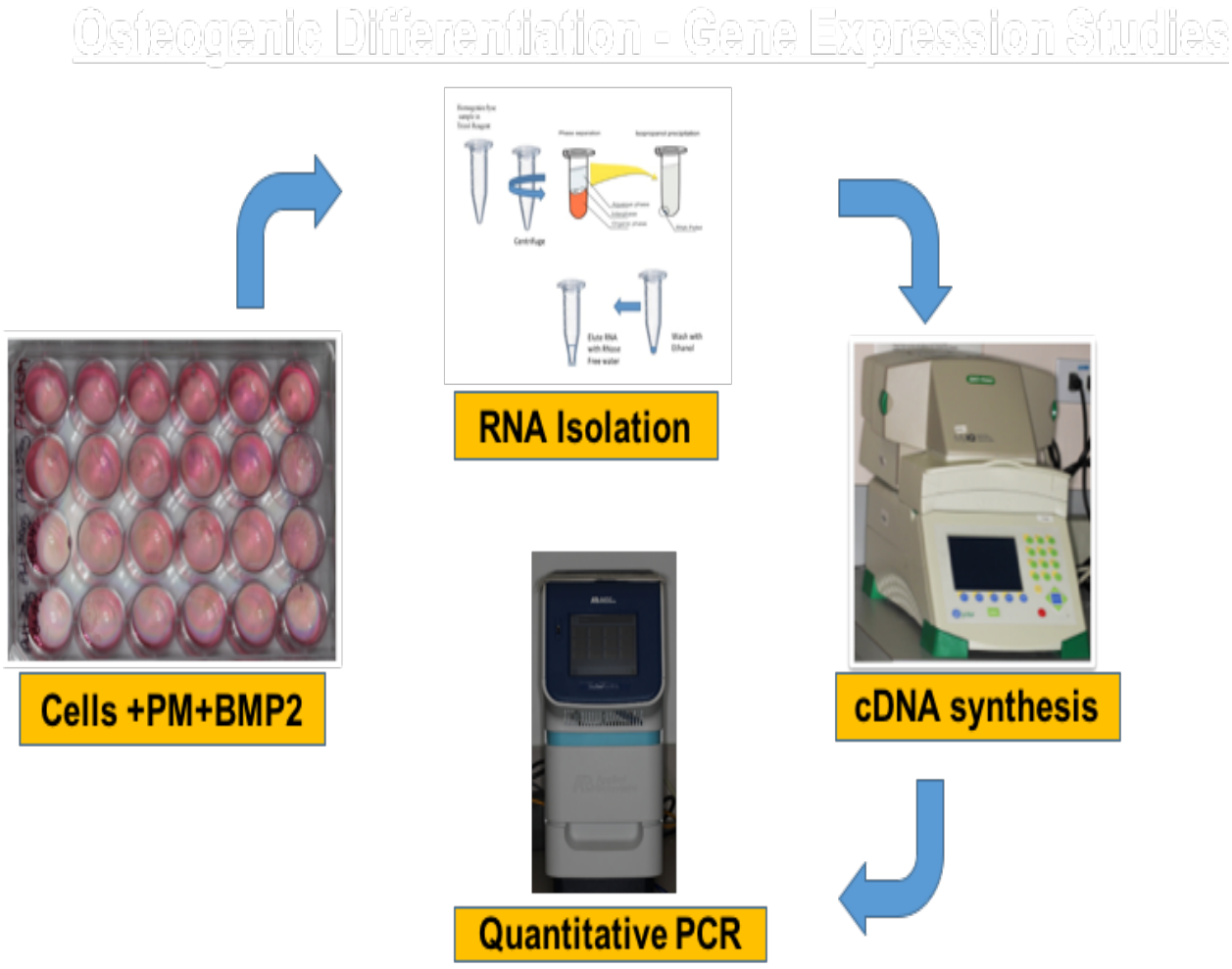


Figure 11: Gene Expression studies

2.11 Mineralization

Cells cultured in growth medium and osteogenic medium were grown for 3 to 4 weeks and *in vitro* mineralization was conducted. The detailed experimental design is given below.

2.11.1 Alizarin Red Staining

Determination of calcium deposits were detected to the constructs grown at 21-day time. The cells were stained with 2% filtered Alizarin Red stain (Sigma-Aldrich, St Louis, MO) for ten minutes to detect the calcium deposits. Calcium deposits appeared as orange color stain in the section.

2.11.2 Von Kossa Staining

To determine the presence of phosphate based mineral, cells stained by applying 2% silver nitrate (Sigma-Aldrich, St Louis, MO) solution for 1 hour under bright light. The reaction was stopped by adding the developing solution, viz. 1% sodium thiosulphate (Sigma-Aldrich, St Louis, MO) for 1 min and observed under phase contrast microscope (Olympus, XI 50)

2.12 Statistical analysis

Following the guidelines from Cohen [31], for a power of 0.8, and alpha of 0.05 and for an independent test of two means a samples from no less than 6 donors will be employed. Thus, total 6 biological replicates and 2 technical replicates for the sample obtained from each donor. To evaluate differences between or among groups, analysis of variance (ANOVA) was performed. A P-value < 0.05 is selected for significance of the statistical tests.

CHAPTER 3 RESULTS:

3.1 Isolation and culture of human gingival stem cells:

The cells isolated from the tissue were seeded at a density of 2 x 10^4 cells/cm^2 and fed with growth medium (DMEM supplemented with 10% FBS and 1% antibiotics). The cells reached 70 to 80% confluency after 7 to 8 days' post seeding. The cell population was homogenous; cells were tightly adhered with spindle-shaped cells (Figure 12)

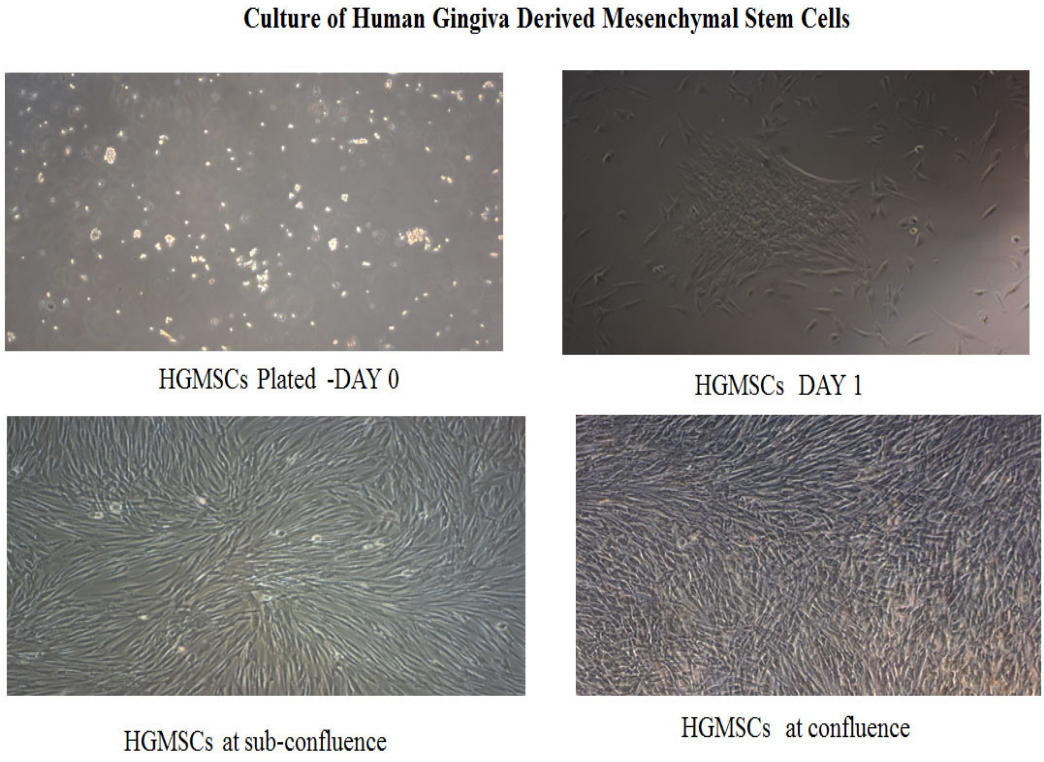


Figure 12: Showing gingival stem cells isolated from human gingival tissue

3.2 Flow cytometry analysis

The flow cytometry results confirmed positive for CD 73, CD 90, CD105 (all above 90%) and negative for hematopoietic stem cell marker CD 34 (Figure 13)

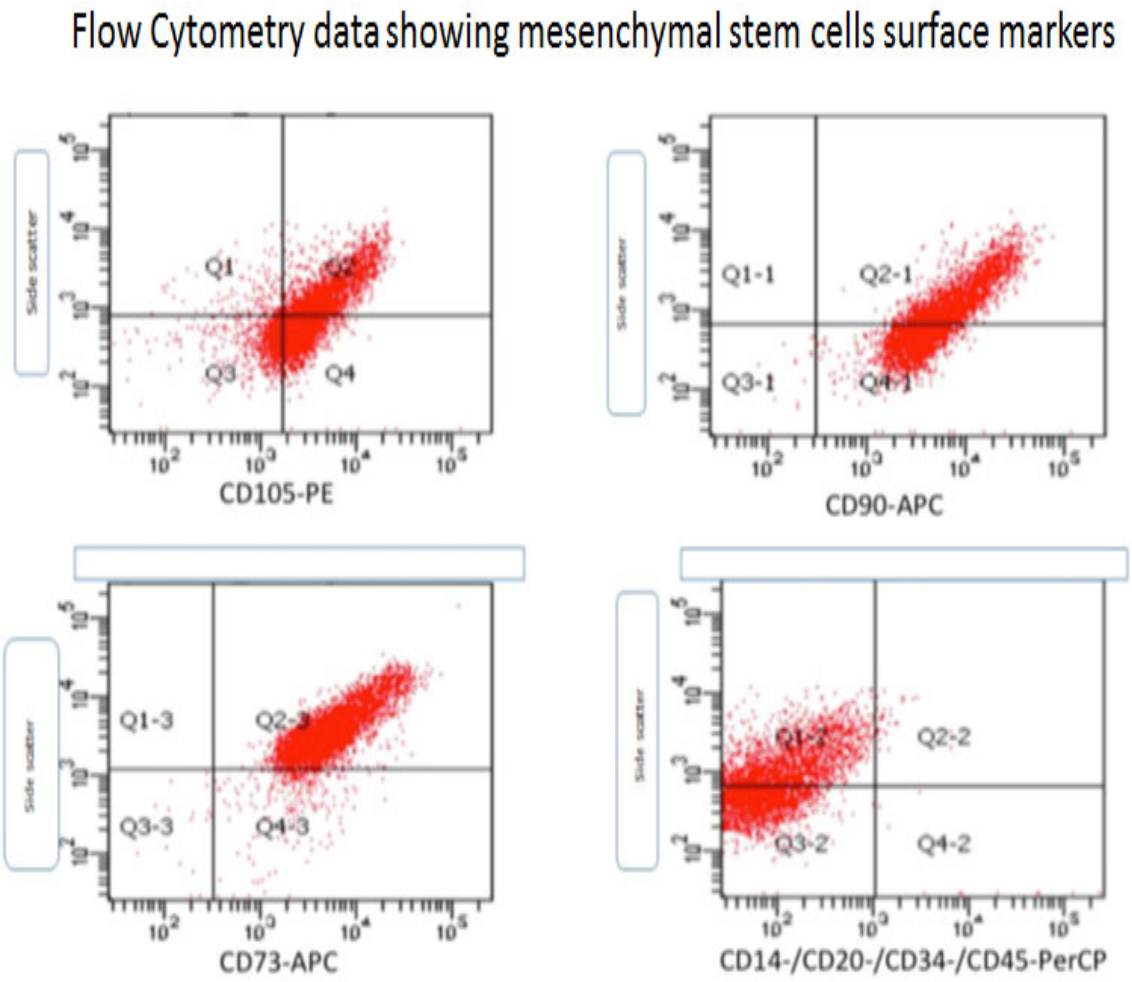


Figure 13: Flow Cytometry data showing surface markers of mesenchymal stem cells. The cells showed over 90% positive to CD 73, CD 90, CD 105 and negative to CD 34

3.3 Cell Growth in PuraMatrix™ (cell–gel constructs 3D model)

For all our studies cells from passage 3 or 4 were used. Cell morphology and growth characteristics were monitored sequentially under light microscope. Under phase contrast view, HGMSCs seeded on to the PuraMatrix™ nano

scaffolds showed spherical structures at Day 0. Cell growth was observed from day1. On Day 5, the cells attained their original spindle shaped. Morphological Observation of cells encapsulated in Puramatrix showed interconnections between the cells. (Figure 14).

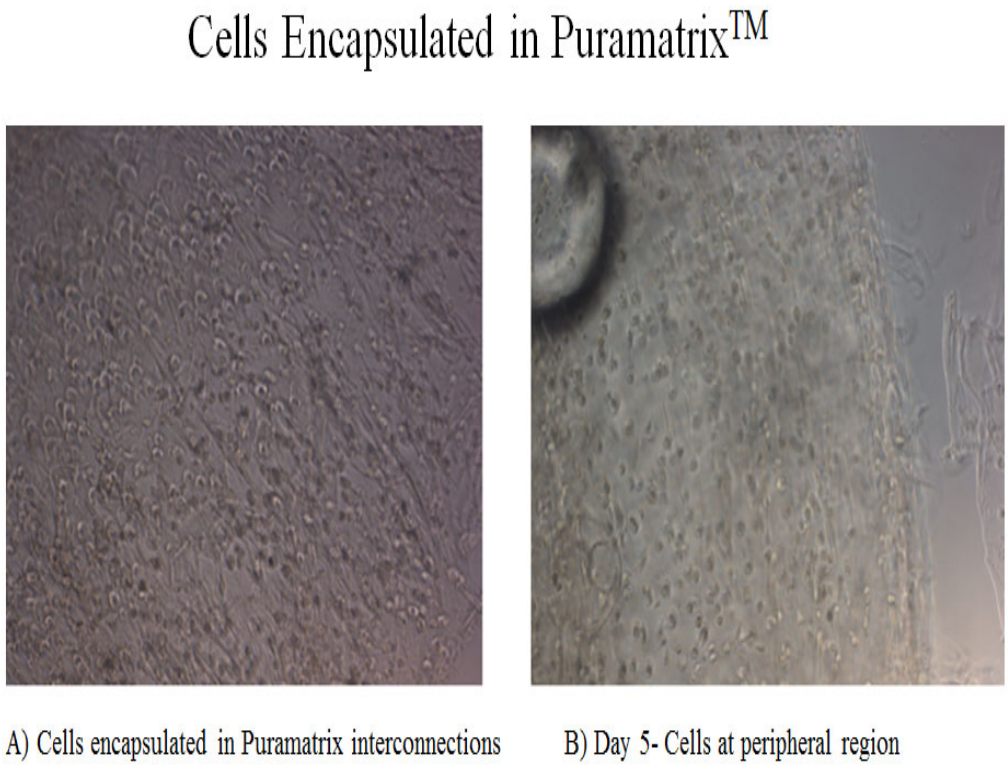


Figure 14: Human gingival stem cells encapsulated in Puramatrix™. The cell morphology showed spindle shaped cells

3.4.1 Proliferation of HGMSCs induced in BMP2

WST assay is a quantitative assay to measure the cell proliferation. Based on our pilot studies, the BMP2 doses were determined. The cell viability has not decreased at any concentration that we selected. There was a significant increase in the cell number was observed at all concentration from day 2. There was significant increase in the cell number at all concentrations on day 2 and

day 3 (Figure 15). Thus we determined that the doses we used will not inhibit the cell proliferation.

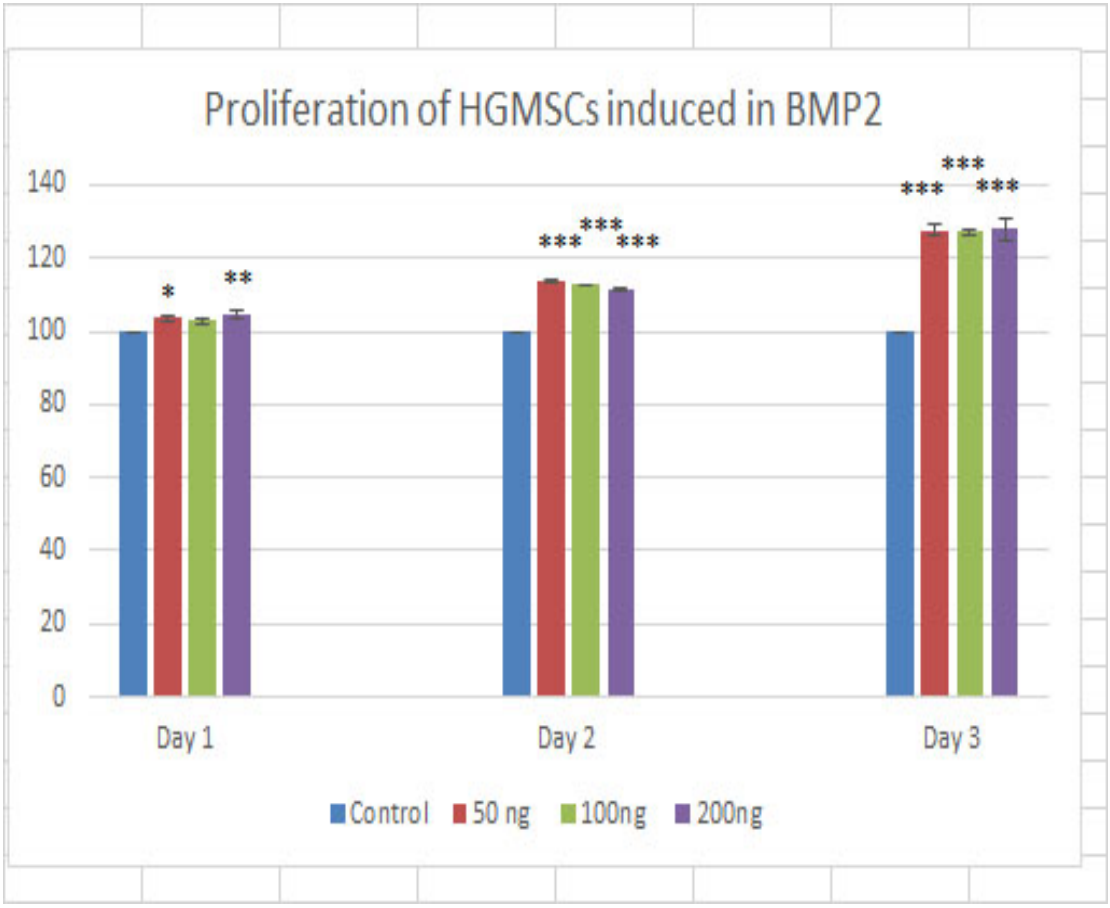


Figure 15: Cell Proliferation in BMP2

3.4.2 HGMSCs encapsulated in Puramatrix™: Cell Proliferation

The cells seeded at 0.1 million, 1 million, 0.3 million and 3 million cells per ml. The cell proliferation was observed at 1, 2 and 3 days. Our results revealed that there was slight increase in the cell number within the group from day 1 to day 3. There was significant increase in cell proliferation at the density of 300,000 on day 3 (Figure 16).

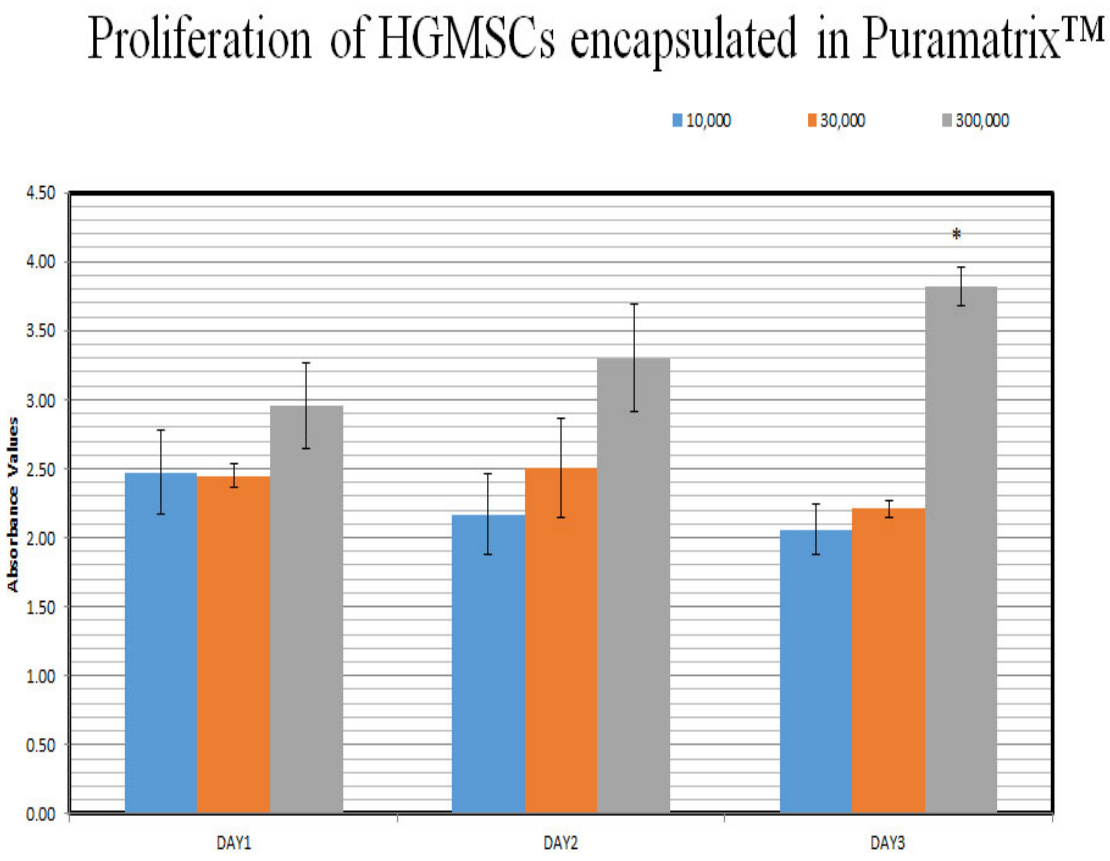


Figure 16: Cell Proliferation in Puramatrix

3.5 Gene expression studies:

Gene expression of various genes have been investigated at day 7. The effect of BMP2 was compared to monolayer cultures (2- D) cultures with the Puramatrix™ encapsulated HGMSCs (3-D cultures). The ALP gene expression upregulated by 40, 80, and 150% in 3-D cultures compared to 2-D cultures. Although there was significant increase in ALP gene expression compared to control group, there was no significant difference among the experimental group in 2-D cultures. On the other hand, the ALP gene expression has significantly increased ($P<0.05$) in cells induced with 200ng/ml BMP2 group compared to 50ng/ml in 3-D cultures (Figures 17, 18). Dose dependent upregulation has been observed in ALP, Col I and RUNX2. Nevertheless, the

expression of Collagen Type I and RUNX2 genes expressed similar manner in cells cultured in 2-D environment as well as 3-D cultures. (Figures 19, 20, 21, 22)

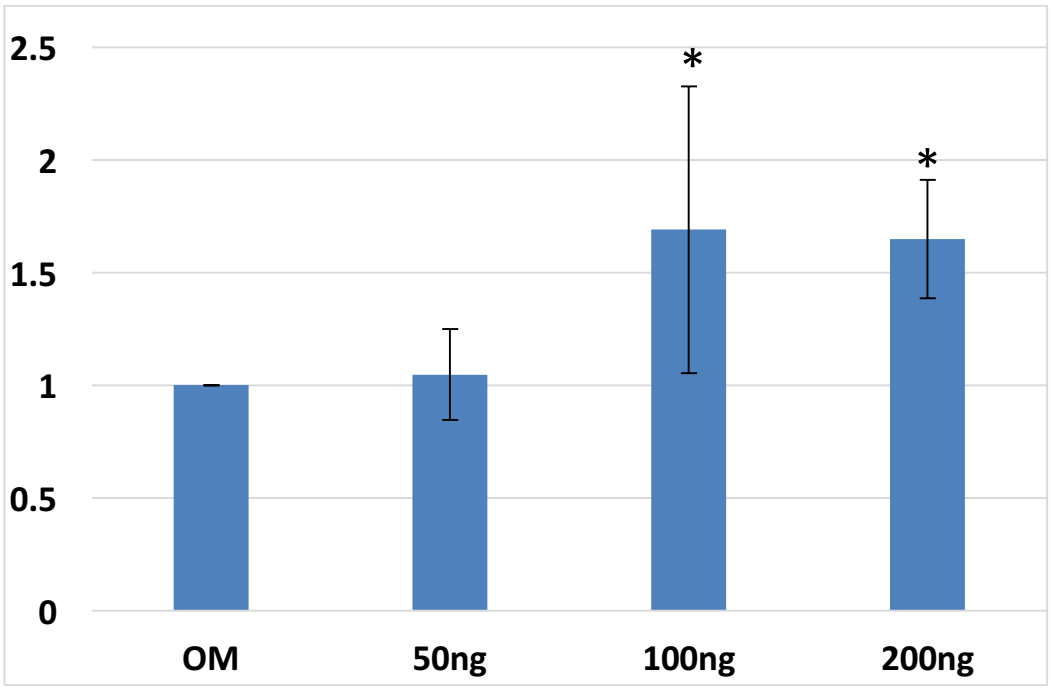


Figure 17: Relative Gene Expression of Alkaline Phosphatase– 2D Culture (1week)

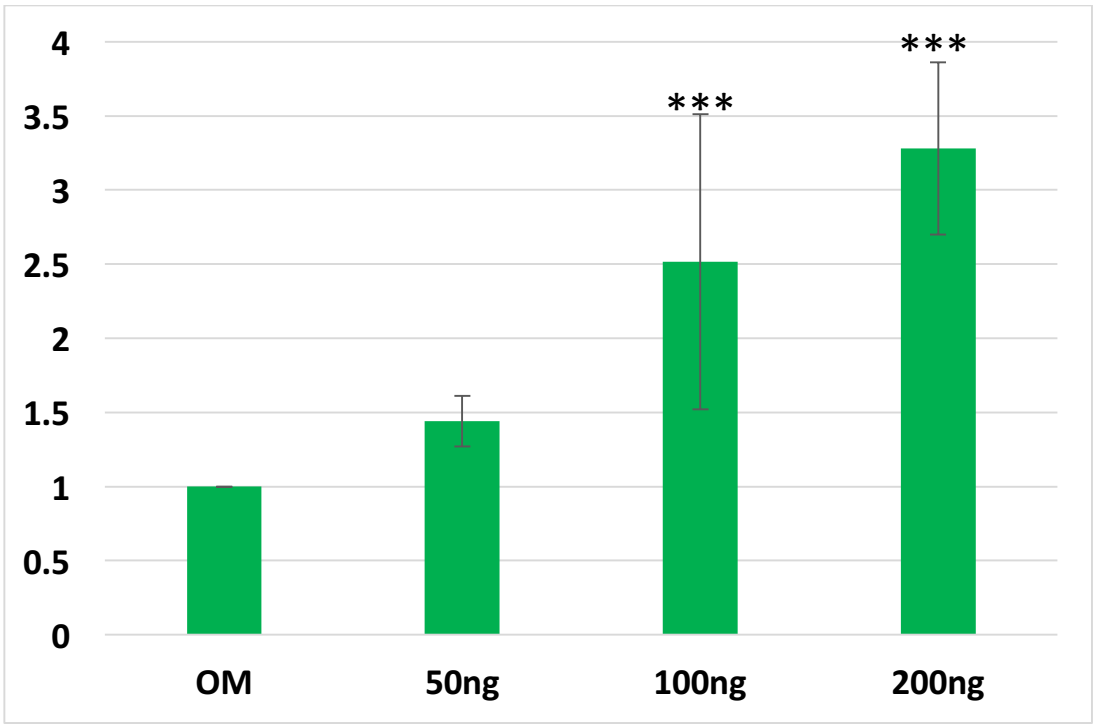


Figure 18: Relative Gene Expression of Alkaline Phosphatase- 3D Culture (1week)

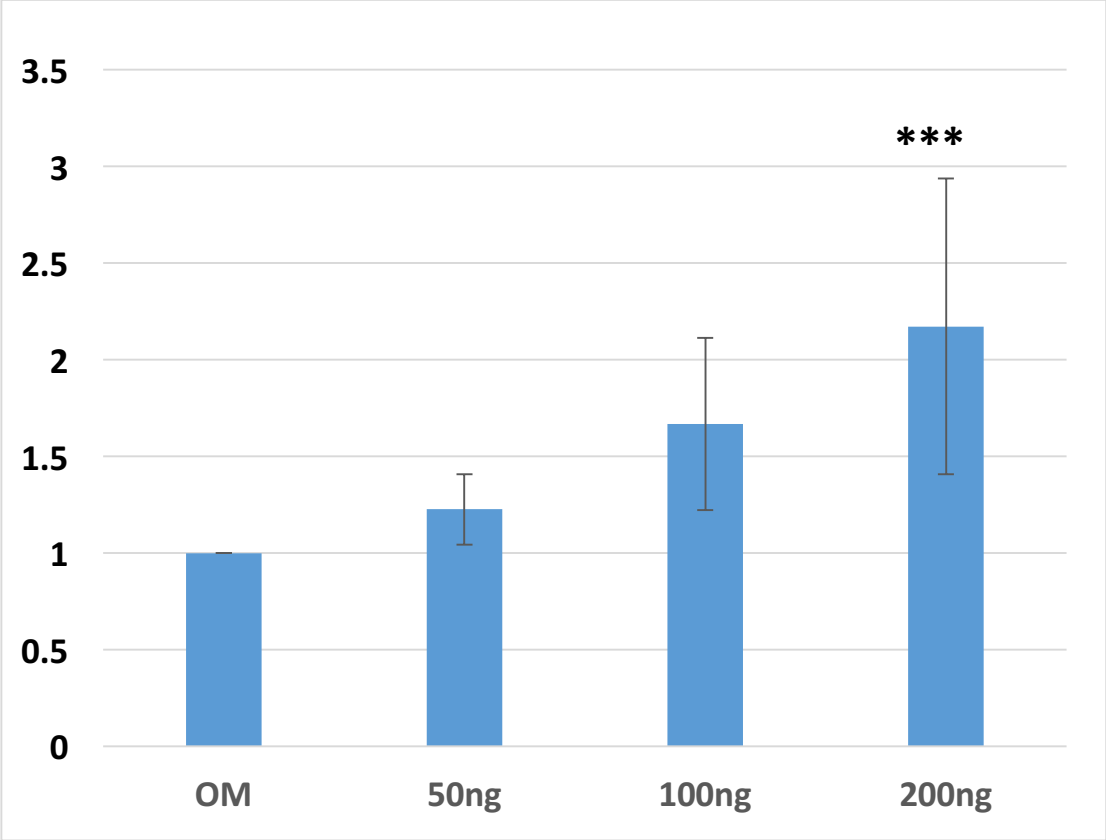


Figure 19: Relative gene Expression of Type 1 Collagen – 2D Culture (1week)

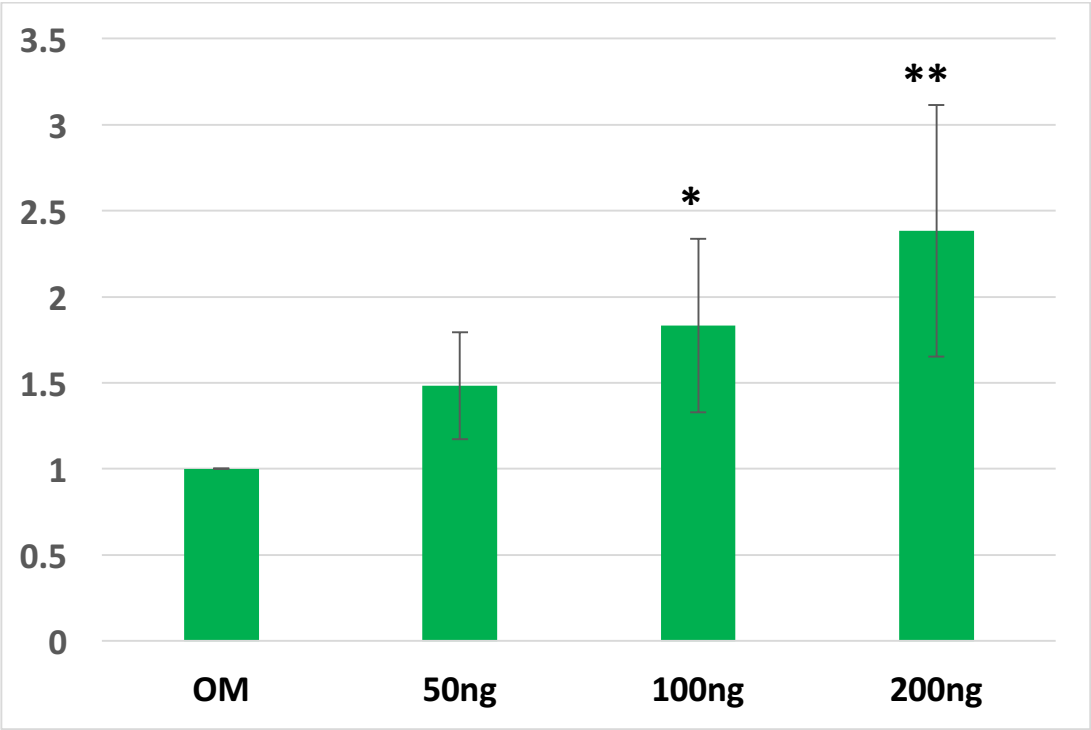


Figure 20: Relative Gene Expression of Type 1 Collagen – 3D Culture (1week)

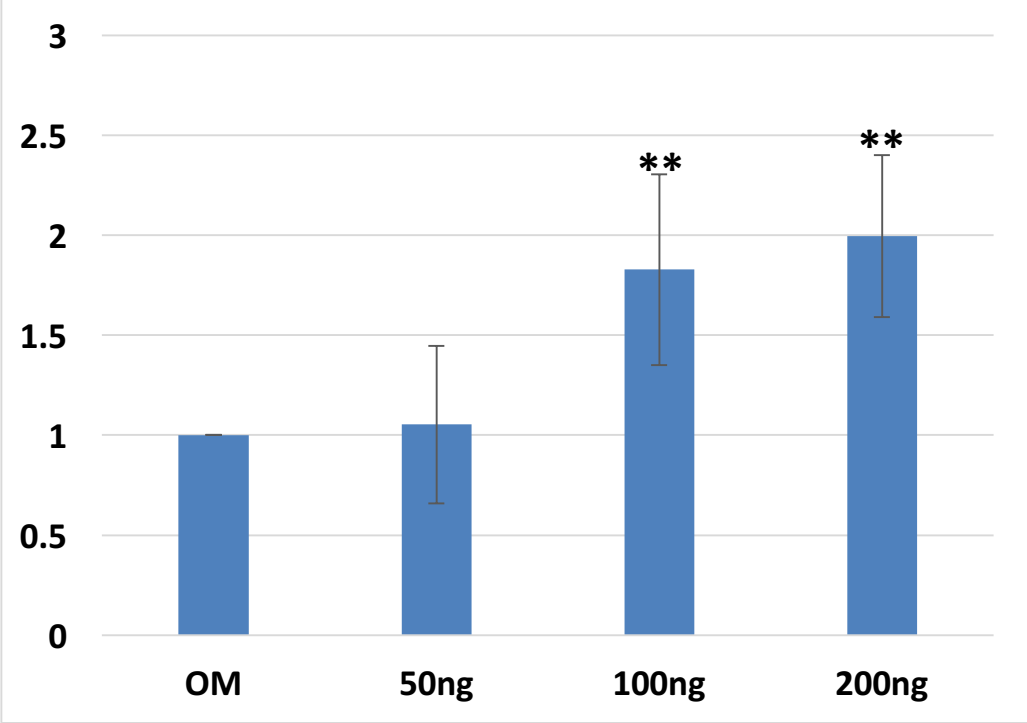


Figure 21: Relative Gene Expression of RunX2 – 2D Culture (1week)

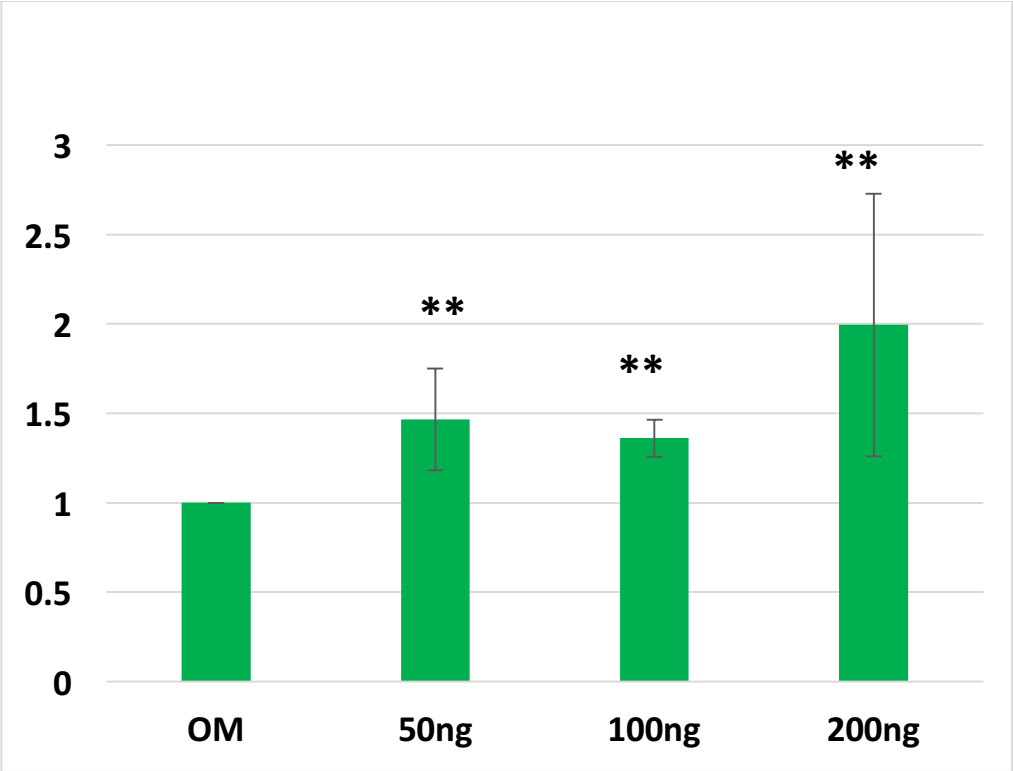


Figure 22: Relative Gene Expression of RUNX2 – 3D Culture (1week)

Specific Aim 1b: Different delivery strategies of BMP2 effect on HGMSCs osteogenic differentiation –

BMP2 induced osteogenic differentiation of HGMSCs: Two types of delivery methods were compared in this study (Figure 9). 1) The HGMSCS were encapsulated in Puramatrix™ and BMP2 was administered in the culture medium (soluble method). 2) The HGMSCs and BMP2 were encapsulated together in Puramatrix (adsorbable method)

The results of the study demonstrated that the application of osteoconductive agent rhBMP2, stimulated the osteogenic differentiation regardless of the delivery method that was used in this study. However, BMP2 entrapped in Puramatrix™ showed significantly high ALP expression at 100ng/ml compared to 200ng/ml concentration (Figure 23, 24, 25)

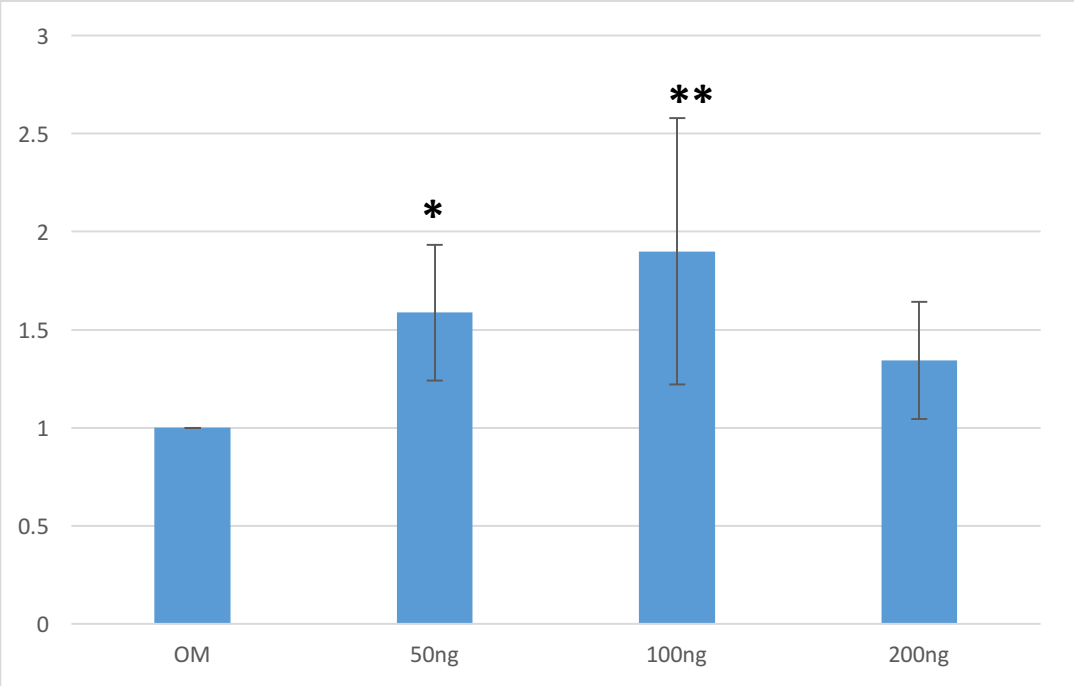


Figure 23: **Relative Gene Expression of ALP for the cells treated with or without osteogenic differentiation medium for one week**

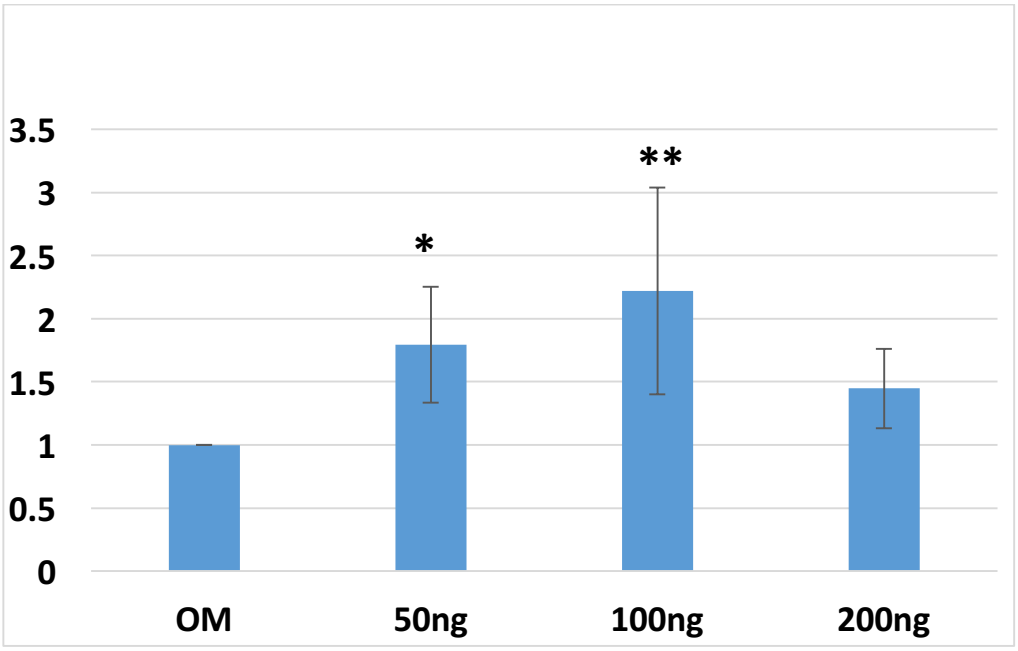


Figure 24: Relative Gene Expression of Type 1 Collagen for the cells treated with or without osteogenic differentiation medium for one week

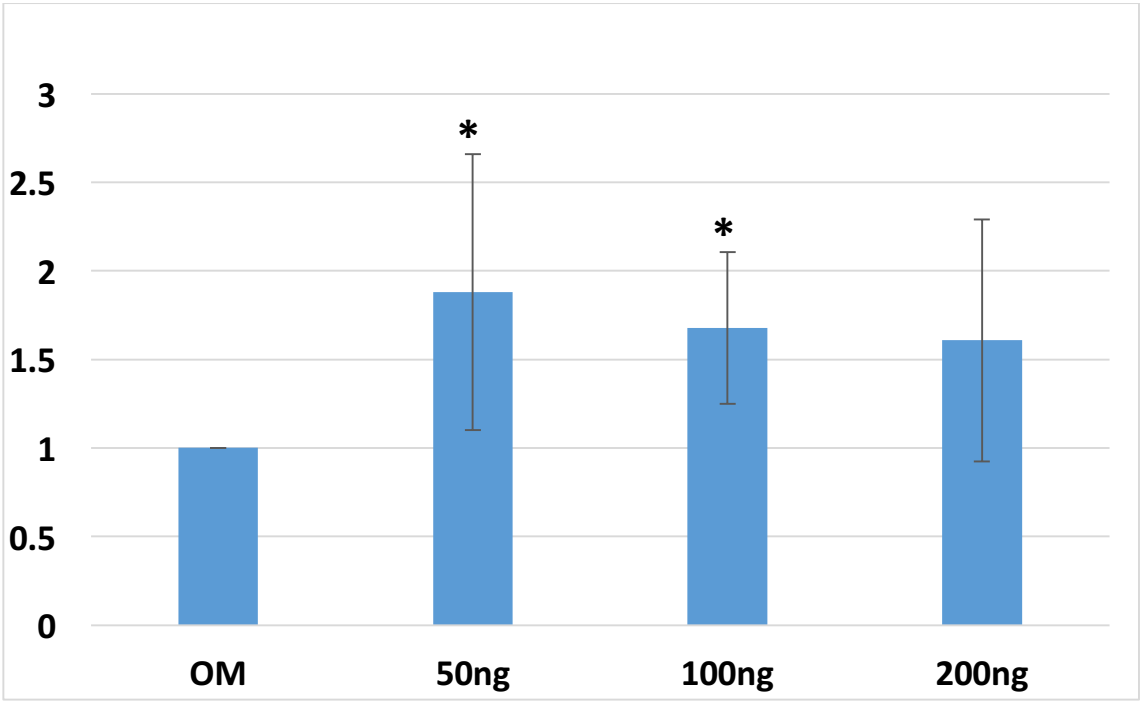


Figure 25: Relative Gene Expression of RUNX2 for the cells treated with or without osteogenic differentiation medium for one week

3.6 Mineralization:

Monolayer culture and 3D Culture of HGMSCs were induced with different concentrations of BMP2 supplemented with osteogenic medium. HGMSCs induced with OM showed enhanced mineral depositon after 35 days. In BMP2 induced cells within 3 weeks enhanced mineral deposition was observed. The cells showed positive for Alizarin red and Vonkossa stains. BMP2 enhanced the cell differentiation (Figure 26, 27)

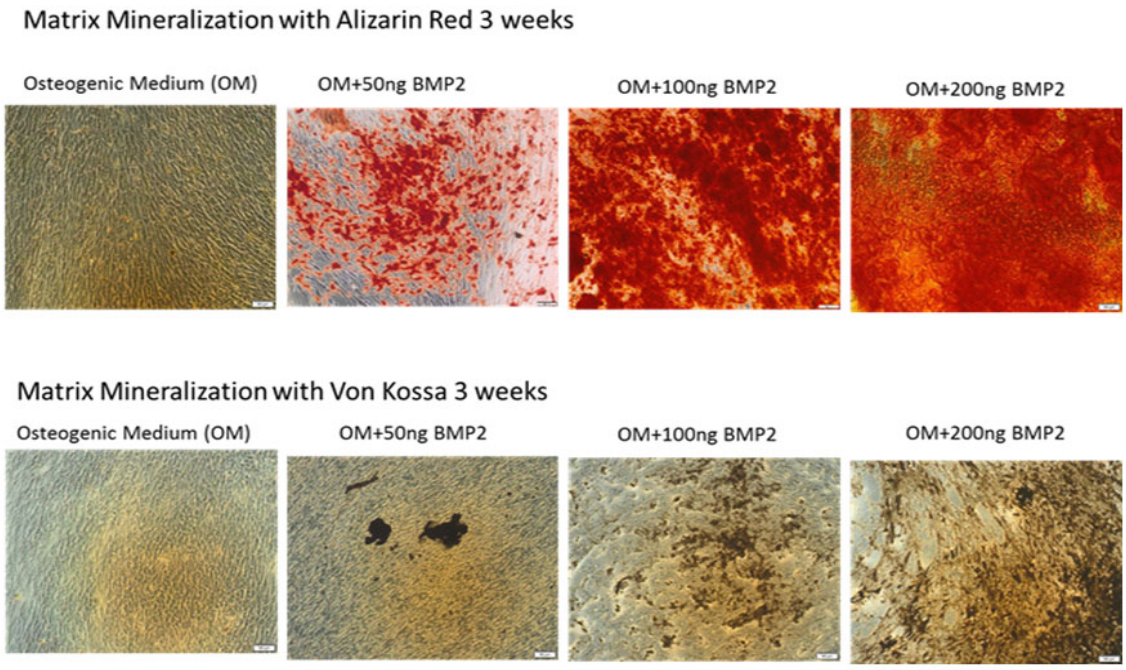


Figure 26: Mineralization 2D Culture – Alizarin Red and Von Kossa stain

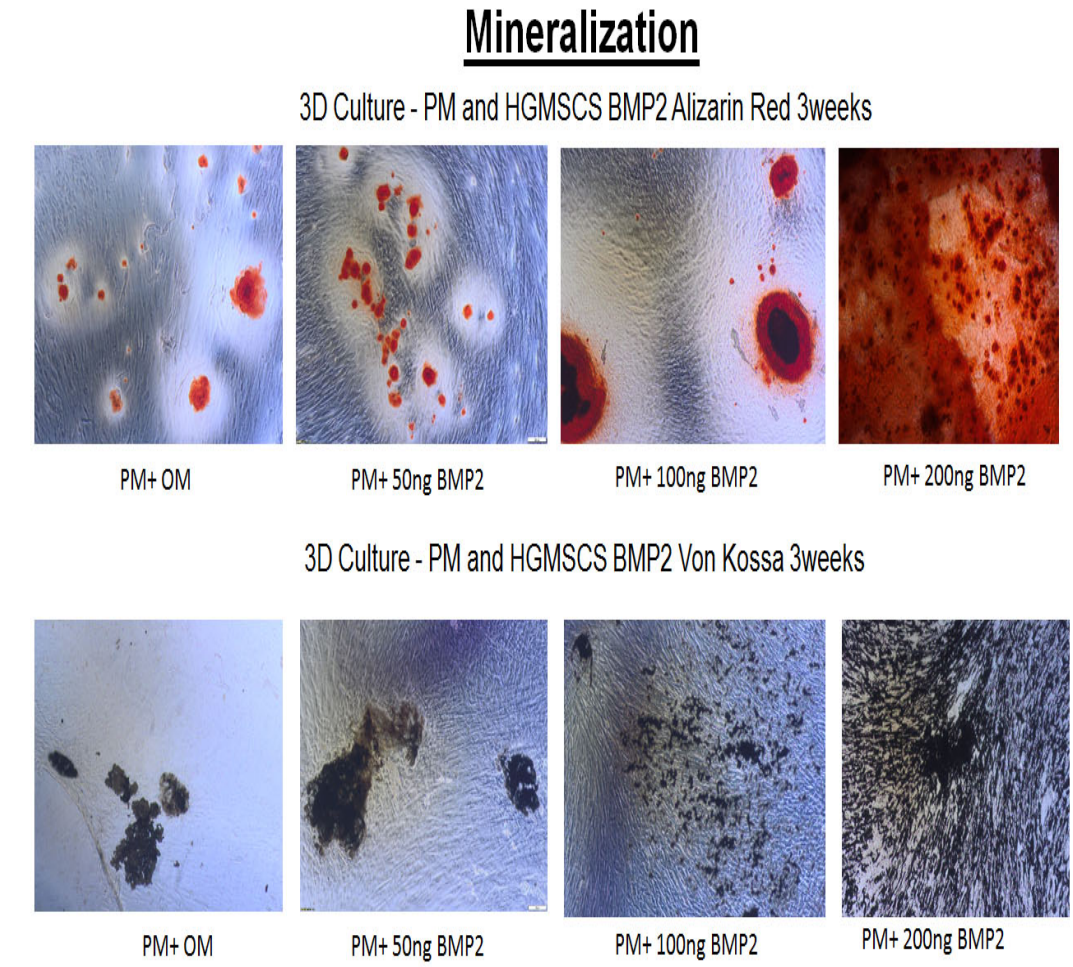


Figure 27: Mineralization 3D Culture - Alizarin red and Von Kossa Stain

CHAPTER 4 DISCUSSION AND CONCLUSIONS

4.1 Discussion:

Cell based bone tissue engineering approaches are governed by the successful use of stem cells, several signaling molecules and osteo-conductive scaffolds. Two main approaches are mainly utilized to develop engineered tissue;

1) utilizing the scaffold as extracellular matrix to support stem cell growth and migration and 2) scaffold-based delivery of signaling molecules (growth factors).

In this study a self-assembling hydrogel scaffold, Puramtrix^{TM}, was used to deliver stem cells. Our study demonstrated that Puramatrix^{TM} supported the cell growth at all concentrations tested providing adequate niche for the cell survival. The data from our study suggested that Puramatrix^{TM} can support the growth of $3x10^6$ per/ml cells without causing any toxic effect. Our results are in agreement with a previous study by Cavalacanti et al [35]. However, in our study, the cells up to 3 million survived during the entire experimental period where as in Cavalacanti et al's study; there was a decrease in cell number, that were encapsulated above 800,000 cell per ml. This difference in the percent survival could be due to the concentration of the gel and the type of the cells that was used. We further investigated the effect of growth factor on HGMSCs proliferation and differentiation.

Owing to the critical role of growth factors in controlling basic cellular functions, and their ability to directly elicit and orchestrate tissue regeneration, a wide range of growth factors has been tested for regeneration of bone. Bone morphogenetic proteins (BMPs) are frequently used growth factors, which play

essential role in skeletal development, bone formation and mesenchymal stem cell (MSC) differentiation [36]. The BMP signaling pathway plays many crucial roles in bone formation and is involved in multiple stages of the developmental process, including osteoblast differentiation, mesoderm patterning, bone formation, and craniofacial development [37]. They elicit new bone formation in ectopic and orthotopic sites. Of these most commonly used is BMP2. However, BMP2 is labile and expensive and the over dose of BMP2 might cause deleterious effects especially when used in pediatric population. Keeping in the view of its use in pediatric population, in this study two aspects have been proposed. 1) to establish the optimal dose of BMP2 2) whether the mode of delivery of BMP2 can influence the osteogenic potential of HGMSCs. Puramatrix^{TM}, a hydrogel scaffold was used to deliver stem cells and BMP2. The effect of BMP2 on HGMSCs proliferation was investigated on monolayer cultures. Although BMP2 is known to promote the differentiation of cells into the osteoblast lineage, none of the concentrations that were used in our study inhibited the cell proliferation. Our results are in agreement with previous reports [38, 39].

In our study, the early osteogenic markers genes ALP and Col Type I were upregulated when the cells were stimulated with BMP2. BMP2 in synergy with dexamethasone (a traditional osteogenic inducer) upregulated the osteogenic differentiation in human bone marrow stem cells [40]. In our study, the enhanced expressions various osteogenic markers of HGMSCs induced with BMP2 was also in the presence of Dexamethasone. Additionally, BMP2 enhanced the early osteogenic marker gene expression in both 2-D cultures as

well as 3-D cultures. There was a significant increase by 1.3, 1.5 and 2 folds in 50,100 and 200ng/ml concentrations respectively when HGMSCs cultured in Puramatrix^{TM} hydrogel (3-D). The enhancement is dose dependent manner and much higher comparative to 2 D cultures. Chen et al [41] conducted a detailed study on the effects of periodic heat shock on hMSCs and reported an upregulation of ALP activity. In our study, although we have not measured activity of ALP, our results showed ALP gene upregulation. RunX2 is the earliest transcription factor expressed during osteogenic differentiation. In the presence of BMP2, the expression of RUNX2 was found to be comparable in both 2 D cultures and in and 3 D cultures. It is interesting to note that the study by Chen et al. demonstrated the inhibition of RUNX2 expression when the cells were induced with heat shock. Nevertheless, the cells embedded in Puramatrix^{TM} during differentiation showed significant increase when compared to the undifferentiated cells. Although we found a significant increase in the expressions of RUNX2 and collagen gens compared to the osteogenic medium, the effect was similar in 2-D and 3-D cultures. It may be attributed to the fact that matrix stiffness and elasticity of the material could affect the stem cell differentiation [42] .

Mineralization is a hallmark for osteogenic differentiation. In our study, we identified profound increase in the mineralization process when cells treated with BMP2. The effect was significant in the cells treated with 100 and 200ng/ml. This data is in agreement with many other studies [43, 44]. Our study revealed that BMP2 induced osteogenic differentiation and hastened the mineralization process, particularly in the 200ng/ml dose but not the 100ng/ml

dose. This could be due to the potential for high bath-to-bath variability between the two samples, although the experiments were performed under similar conditions.

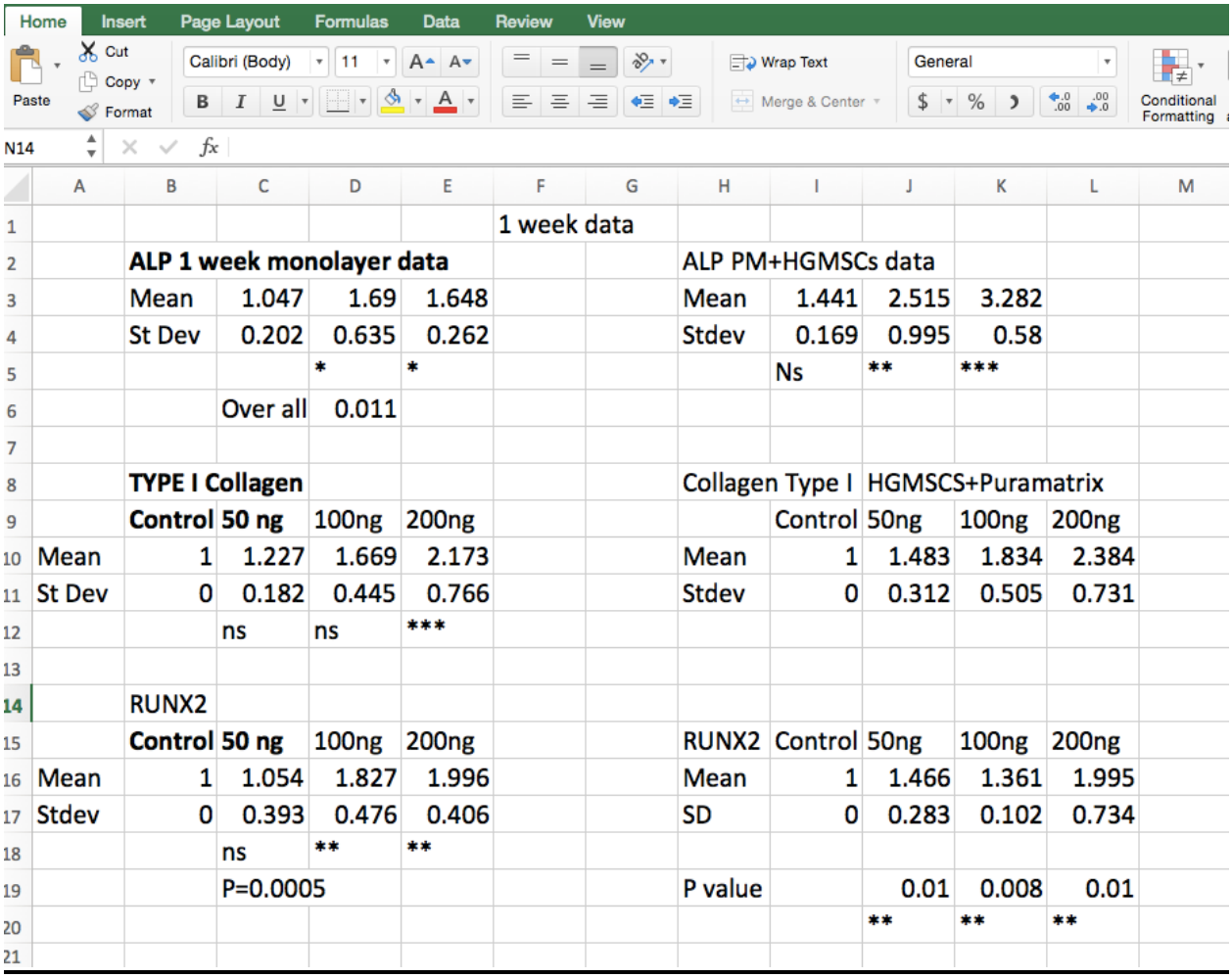
We further investigated the effect of delivery method whether the osteogenic differentiation pattern depends on the type of delivery of growth factor. Overall results of our study demonstrated that the application of osteoconductive agent rhBMP2, stimulated the osteogenic differentiation regardless of the delivery method that was used in this study. However, BMP2 entrapped in Puramatrix^{TM} showed higher ALP expression at 100ng/ml compared to 200ng/ml concentration. In the present study, in the solubilized method, the BMP2 was added in the culture medium to the Puramatrix^{TM} encapsulated HGMSCs on every other day which resulted in using high amount of BMP2 than the encapsulated method. It is interesting to note that the upregulation of gene expression of osteogenic markers was not significantly different between these two groups. Conventional methods of administering BMP2 in the culture medium might be homogeneous, however, in this method only small amount reaches to the cellular micro domains related to biological signaling pathways because of Brownian motion of BMP-2 released from a matrix utilizing a protein delivery system could efficiently bind to the receptor site [45-47].

4.2 Conclusions:

Mesenchymal stem cells are a potential stem cell based strategy for the repair of the craniofacial region. The mesenchymal stem cells derived from gingiva are a promising cell source as they originate from neural crest region, which is responsible for craniofacial development. This is the first comprehensive study

to investigate the effect of BMP2 in three different culture conditions. Our overall results indicated that BMP2 enhanced HGMSCs osteogenic differentiation ability in all culture conditions. However, the effect of BMP2 is significantly higher in the cells cultured in 3-D environment compared to 2-D cultures. Thus this study revealed that the combination of osteogenic gingival stem cells and osteoinductive BMP2 in an osteoconductive Puramatrix^{TM} hydrogel might be a promising alternative for craniofacial bone regeneration.

5. RAW DATA:



	A	B	C	D	E	F	G	H	I	J	K	L	M
1						1 week data							
2		**ALP 1 week monolayer data**						ALP PM+HGMSCs data					
3		Mean	1.047	1.69	1.648			Mean	1.441	2.515	3.282		
4		St Dev	0.202	0.635	0.262			Stdev	0.169	0.995	0.58		
5				*	*				Ns	**	***		
6			Over all	0.011									
7													
8		**TYPE I Collagen**						Collagen Type I		HGMSCS+Puramatrix			
9		**Control**	**50 ng**	100ng	200ng				Control	50ng	100ng	200ng	
10	Mean	1	1.227	1.669	2.173			Mean	1	1.483	1.834	2.384	
11	St Dev	0	0.182	0.445	0.766			Stdev	0	0.312	0.505	0.731	
12			ns	ns	***								
13													
14		RUNX2											
15		**Control**	**50 ng**	100ng	200ng			RUNX2	Control	50ng	100ng	200ng	
16	Mean	1	1.054	1.827	1.996			Mean	1	1.466	1.361	1.995	
17	Stdev	0	0.393	0.476	0.406			SD	0	0.283	0.102	0.734	
18			ns	**	**								
19			P=0.0005					P value		0.01	0.008	0.01	
20										**	**	**	
21													

Gene expression studies - Monolayer culture and HGMSCS with Puramatrix™

	D	E	F	G	H	I	J
1							
2							
3							
4			**3 D Culture with BMP2 encapsulated**				
5							
6	**ALP**	OM	50ng	100ng	200ng		
7	Ave	1	1.616	1.7	1.429		
8	St Dev	1	0.347	0.678	0.298		
9							
10							
11	**COLLAGEN**	OM	50ng	100ng	200ng		
12		1	1.794	2.22	1.448		
13	Stdev	0	0.461	0.817	0.313		
14			*	**			
15							
16	**RUNX2**	OM	50ng	100ng	200ng		
17							
18							
19	Mean	1	1.881	1.678	1.608		
20	St Dev	0	0.779	0.427	0.684		
21			*	*			

Gene Expression studies - 3D Culture with BMP2 encapsulated

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