DEVELOPMENT OF GELATIN MICROBUBBLE BASED PLGA SCAFFOLD FOR APPLICATION IN BONE TISSUE ENGINEERING

by

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ABSTRACT

DEVELOPMENT OF GELATIN MICROBUBBLE BASED PLGA SCAFFOLD

FOR POTENTIAL APPLICATION IN

BONE TISSUE ENGINEERING

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The University of Texas at Arlington, 2012

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The overall goal of the study was to develop a delivery system for a potential bone tissue engineering application focusing on fabrication of gelatin microbubble (MB) based PLGA scaffolds capable of recruiting Mesenchymal stem cells (MSCs) and delivering bioactive molecules. Challenges associated with inefficient use of stem cells, scaffolding techniques and poor understanding of the role of growth factors hinders the success of tissue engineering strategies.

A major challenge with the use of autologous stem cells is its low proliferation and migratory capacity. Our lab previously established that using a biomaterial implant, autologous MSCs can be directed and recruited in large numbers at the implantation site using signaling molecules. Preserving bioactivity of these molecules was one major concern. To eliminate this problem, our lab fabricated albumin MB based scaffolds that were able to preserve the bioactivity and deliver growth factors.

As albumin was associated with poor cell attachment and infiltration, we made use of gelatin MB in this study as it is known for its cell adhesion properties. Gelatin MB concentration

in polymer solutions was optimized based on numerous studies that assessed the physical, mechanical, and cell interaction properties. Out of 5% w/v gelatin, 10% w/v gelatin, and 20% w/v gelatin used, 10% w/v gelatin scaffold fared better in terms of load bearing capacity, dispersion of gelatin in scaffold matrix, and its pore size. Various growth factors like SDF1-α, BMP-2, and Epo were tested for potentiating MSC migration and differentiation *in vitro. Among them,* Epo came out to be a highly potent recruiter of MSCs and an osteoinductive agent *in vitro.* They were then loaded into gelatin MB PLGA scaffolds and the ability of such scaffolds to deliver growth factors over time was evaluated. Scaffold with these growth factors alone or in combination was also evaluate *in vivo* for bone regenerative applications *in vivo* in an animal model for bone regeneration. Our results show that Epo was a highly potent recruiter of MSCs and an osteoinductive factor. In fact the effect of Epo alone was more pronounced than BMP-2 and surprisingly Epo+BMP-2.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

DEVELOPMENT OF GELATIN BASED SCAFFOLD FOR RECRUITMENT OF MESENCHYMAL STEM CELLS (MSCS)

1.1 Introduction

The drawbacks of the existing treatment modalities has led to the development of tissue engineering approaches that have the potential for serving as a suitable alternative. It has already made tremendous strides in the past couple of decades in dealing with tissue and organ loss. The idea of developing a new tissue comes from mimicking the replication of the natural tissue present in the living system. Engineered tissues offer a number of advantages over the transplantation of autografts, eliminating donor site morbidity, and tissue compatibility. In recent years, several tissue engineering strategies have been approved by US Food and Drug Administration (FDA) for clinical application that includes various cells, biomaterial scaffolds, and growth factors.[1, 2] As shown in Figure 1.1, tissue engineering involves the combination of scaffolds to provide an adequate micro-environment for cells which are able to differentiate and maintain the specific phenotype. The addition of bioactive factors like growth factors, cytokines or hormones provide suitable signals for cell differentiation into specific lineages and proper biomechanical environment.[3]

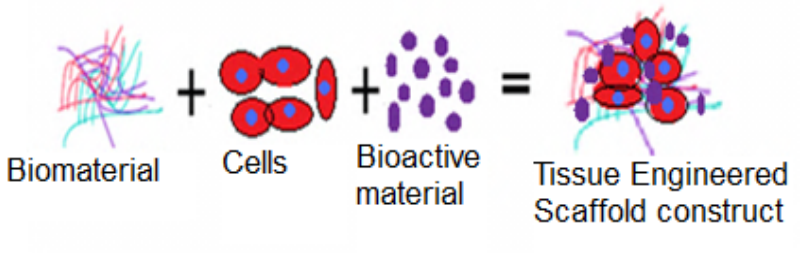

Fig.1.1 Illustration depicting the major components used in tissue engineering technology. Tissue engineering comprises of 3 major materials: cells, biomaterial for cell substrate, and bioactive molecules which help cells to proliferate and differentiate.

The scaffold matrix that provides initial structural support and retains cells in the defective area is subsequently degraded as the cells secrete their own matrix. Scaffolds also act as delivery systems for bioactive agents, such as cytokines and chemokines. As mentioned above, cell based tissue engineering has opened new prospects for regeneration of damaged tissue. Cell source play a major role in deciding the type of tissue to be regenerated. Primarily, cell source can be classified in three types:

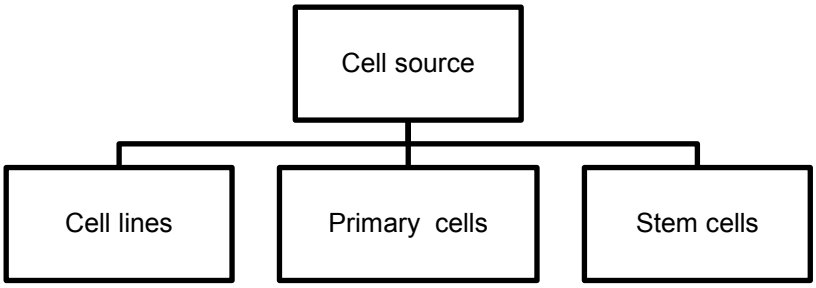

Figure 1.2 Hierarchy of Tissue Engineering cell source type. Classification depends on method of cell extraction and can be divided into cells lines, primary cells, and stem cells.

Figure 1.2 depicts major cells source used in tissue engineering applications. Cells lines also known as allogenic cells are cells derived from autologous cells altered in terms of uniformity, standardization, quality control, and cost effectiveness. They are easily available but have problems associated with immune rejections. Primary cells on the other hand are extracted directly from the tissue and hence eliminate problems with immunogenicity but are difficult to obtain. They involve in long extraction process and are painful for the patients. Stem cells eliminate these challenges and their uses have approached as a potential alternative in cell based therapies.

This can be, in many ways; attributed to the progress in stem cell biology.[4] Stem cells have gained in importance because of their unique biological properties and ability to self-replicate. Also, stem cells can differentiate into multiple lineages upon receiving proper signals.[5] Recently, stem cells have been seen as a potential therapeutic alternative for repair of damaged

adult organs.[6] Stem cells types currently available are embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs).[6]

Embryonic stem cells, derived from the inner cell mass of the blastocyst are totipotent and capable of forming almost any tissue. However, their use is limited by ethical concerns, immunological incompatibility, heterogeneous differentiation, and potential teratoma formation.[7] iPSCs derived from non-pluripotent cells by forced genetic expression are capable of differentiating into multiple lineages. However, use of iPSCs for tissue engineering is still in the early stages and concerns of safety still remain.[8] Taking this into account, adult stem cells [9-12] are extremely significant for tissue engineering applications. Adult stem cells have various sources like bone marrow, adipose, umbilical cord, placenta, peritoneum, and even tissue stem cells. Adult stem cells can be classified as either mesenchymal stem cells (MSCs) or hematopoietic stem cells (HSCs). MSCs arise from the supporting structures in bone marrow and can act as feeder layer for the growth of hematopoietic stem cells in culture.[13] These cells are highly proliferative forming fibroblasts like colonies and were initially called colony-forming units-fibroblasts (CFU-F).[1] They are characterized by their capacity to adhere to plastic, their phenotype (CD73+, CD90+ , CD105+, CD14– or CD11b–, CD19– or CD79a–, and CD45–).[1] MSCs possess the capacity to differentiate into various tissues of the mesenchyme like bone, muscle tendon, ligaments, and cartilage as shown in Figure 1.3.

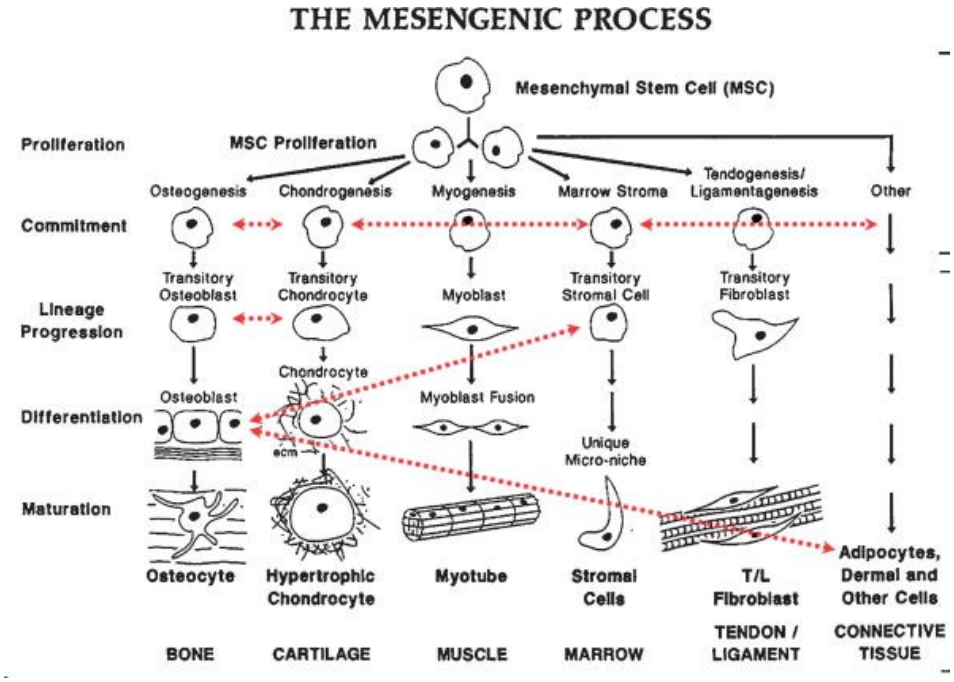

Figure 1.3 Mesenchymal stem cell differentiation hierarchy. [14] MSCs possess multidifferentiation capacity and can be differentiated into osteogenic, chondrogenesis, myogenesis, and other lineages capable of regenerating various tissues.

Stem cells are commonly cultured *in vitro* prior to therapeutic application. A major problem associated with adult stem cells is the painful isolation procedure and laborious and time consuming *in vitro* expansion procedures. Another common problem with use of stem cells cultured *in vitro* is that they might lose their phenotype and might not differentiate exactly to desired cell type. There is also a possibility of rejection of allogenic stem cells by host immune cells upon transplantation. Hence to circumvent this problem; use of autologous stem cells would be a potential viable approach. Autologous stem cells would eliminate painful extraction and expansion conditions. However, obtaining a large number of cells for transplantation is a limitation of autologous bone marrow stem cells. To eliminate this problem, our laboratory has recently established a method for the recruitment of autologous stem cells using biomaterial implant.[15] We showed that biomaterial implantation leads to recruitment of inflammatory cells and a substantial number of MSCs and HSCs to the implantation site.[15]

Although, we can direct autologous stem cells to a specific site in the body, without proper signals, these cells can differentiate into myofibroblasts. Hence, it is possible that delivery of suitable signaling cytokines could enhance the recruitment and differentiation of autologous stem cells into a specific lineage. A tissue engineering scaffold has the capability to serve as a signaling molecule releasing device that allows cell infiltration, attachment, and differentiation. In fact, a recent study by our group has shown that delivering a stem cell homing cytokine from a commonly used salt leached scaffold leads to enhanced stem cell engraftment.[16] However, salt leached scaffolds and many of the other commonly used scaffolds are incapable of releasing growth factors in as sustained manner. More importantly, they seldom are able to preserve the bioactivity of the loaded growth factor. To overcome these limitations, our group had previously developed albumin microbubble porogen based scaffolds, in which protein microbubbles act as a porogen that can also protect the growth factor from damage. Although, albumin was used as a carrier protein, a major challenge associated with these scaffolds was the poor cell adhesion and cell infiltration.[17]

In this regard, a protein that is biocompatible, biodegradable, capable of better cell attachment and that helps in cell proliferation is desired. Collagen is one such natural protein. collagen is one of the major natural protein found in the extracellular matrix of the body, but due to its high cost, its denatured form gelatin; was used in this study . Gelatin is known to biodegradable, biocompatible, non-immunogenic, better cell attachment.[20] Previously, gelatin has been used as a composite material in scaffolds that are blended with polymer solution or made as hydrogels for soft tissue engineering applications.[19] One problem associated with use of gelatin for in vivo is its rapid breakdown.[21] This can be minimized by using crosslinking agents, but this has a significant effect on its biocompatibility.[21]

In this study, to test gelatin for use in tissue engineering applications, PLGA scaffolds were fabricated using gelatin microbubbles as a protein carrier. PLGA being hydrophobic; have poor cell adherence property. Denatured from of collagen, gelatin; a significant component in the

extracellular matrix of human body was used as a carrier protein because it is shown to have high cell adhesion property, have similar biological properties to collagen and blends well with PLGA.[22]

1.2 Hypothesis

Gelatin based scaffold can be fabricated with improved cell affinity and proliferative property.

1.3 Materials and methods

1.3.1 Fabrication of gelatin microbubble scaffolds

Based on our previous experience, Poly (DL-lactic acid-co-glycolic acid) (PLGA) (75:25) was used in this study.[17] 75:25 PLGA with a molecular weight of 113 kDa was purchased from Medisorb (Lakeshore Biomaterials, Birmingham, AL). 1,4-dioxane solvent to dissolve PLGA, was purchased from Sigma Aldrich (Milwaukee, WI) and Gelatin from Sigma (St Louis, MO) As shown in Figure 1.4; 5% w/v (A), 10% w/v (B), and 20% w/v(C) gelatin was used for microbubble (MB) fabrication to test for optimal gelatin concentration needed for MB that blends well with PLGA solution. This was added to two different amount of PLGA. In one group, PLGA and gelatin MB solution content was kept equal (1:1), whereas in other group, PLGA content was kept double the amount of gelatin MB solution (2:1).

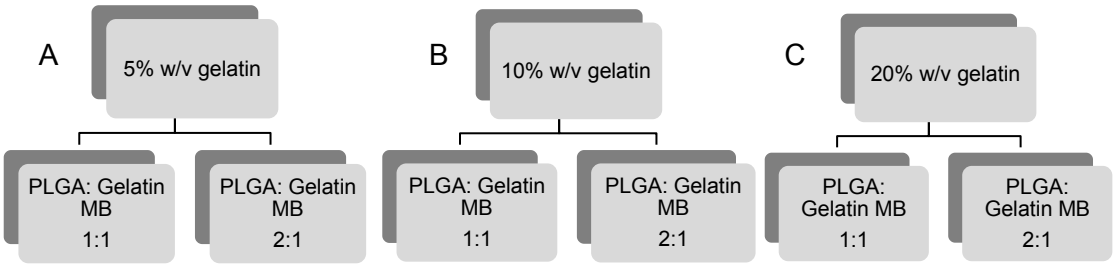

Figure 1.4 Schematic illustration showing gelatin concentration and PLGA: Gelatin MB ratio used. 5% w/v (A), 10% w/v (B), and 20% w/v (C) gelatin concentration MB were added to two different PLGA amount.

In this study, gelatin microbubbles were used based on previous studies done in our lab.[23] Here, PLGA scaffolds were fabricated using a modification of a procedure previously established in our lab.[17] Gelatin with different concentrations 5% w/v, 10% w/v, and 20% w/v were mixed in DI water and allowed to dissolve at 40^{o}C. Once gelatin was completely dissolved, the solutions were overlaid with nitrogen gas and sonicated using a probe sonicator (Ultrasonix, Bothell, WA) at 20 kHz for 10 seconds. They were quenched in liquid nitrogen before lyophilizing for 72 hours at 0.03 mBar vacuum in a Freezone 12 lyophilizer (Labconco, Kansas City, MO, and USA).

1.3.2 Surface morphology and characterization of scaffolds

Scanning electron microscopy (SEM) analysis was done to characterize the surface of the PLGA: Gelatin scaffolds prepared by the above mentioned procedure. Small square – pieces of scaffold were bonded to a steel stub using a colloidal silver adhesive tape. Scaffold pieces were coated with silver using a sputter coater (CRC – 100 Sputtering Systems, Plasma Sciences Inc.) for 15 minutes at an Argon (Ar) gas pressure of 8 mTorr and 50 mA, in order to minimize overcharging. They were subsequently observed under a HITACHI300 SEM which was operated under an accelerating voltage of 12 kV.

Scaffolds were also embedded in OCT for analysis of protein and pore size comparison. For coomassie blue assay, the scaffolds were immersed in paraformaldehyde for 15 minutes and then dipped in coomassie blue staining solution for 10 minutes. The scaffolds were then placed in OCT compound and put in vacuum chamber for 1 hr. for proper infiltration of dye within the scaffold pores.

For measuring the pore size, scaffold embedded in optimal cutting temperature compound (OCT) were placed under vacuum for 1 hr. to ensure proper infiltration of OCT compound inside scaffold pores. Both these scaffolds were then allowed to freeze in -80^{0}C for 24 hr. before sectioning. 8 um thin sections were cut for each group using Leica cryostat and placed on poly-l-lysine coated slides for further analysis.

Also, essence of gelatin in PLGA scaffold was qualitatively measured by staining scaffold sections by coomassie blue assay. Coomassie blue dye was purchased from EMD Biosciences (Darmstadt, Germany). Coomassie blue dye binds to protein indicating presence of protein in the scaffold matrix. Scaffolds were immersed in coomassie blue dye solution before embedding them in OCT to ensure proper dye uptake and infiltration inside scaffolds. Scaffolds placed in OCT were first put in vacuum for dye to penetrate in the scaffolds and then were frozen at -80^0C overnight before sectioning using Leica cryostat.

1.3.3 Scaffold porosity measurement

Scaffold porosity was calculated based on ethanol displacement method that works on basis of Archimedes Principle.[24] Scaffolds of 3mm X 3mm X 4mm (L X B X H) were used for porosity calculation. The scaffolds were weighed as $W_{S1,}$ W_{S2}, and W_{S3} respectively. A density bottle with ethanol (density: 0789 g/cm^3) to a known volume was measured as $W_{1\text{-}1,}$ $W_{1\text{-}2,}$ and $W_{1\text{-}3}$. Nearly half of the ethanol was removed from density bottle and was kept inside vacuum chamber to evacuate air from the bottle. Density bottle was refilled with the original volume and weighed as $W_{2\text{-}1,}$ $W_{2\text{-}2,}$ and $W_{2\text{-}3}$ respectively. The saturated scaffold was removed from the ethanol bottle and contents of remaining bottle were measured as $W_{3\text{-}1,}$ $W_{3\text{-}2,}$ and $W_{3\text{-}3}$. Calculation was carried out using the following formulas:

$$\text{Volume of scaffold pores:}(V_p)=(W_2\text{-}W_3\text{-}W_s)/\rho_e$$

$$\text{Volume of scaffold skeleton:}(V_s)=(W_1\text{-}W_2+W_s)/\rho_e$$

$$\text{Scaffold porosity:}(\varepsilon)=V_p/(V_p+V_s)$$

Average of three replicates was taken for final calculation and standard deviation was calculated for plotting of error bars.

1.3.4 Scaffold mechanical strength

Samples were cut into approximately (6.3 mm width X 6.5 mm thickness) using a sharp razor blade for analysis mechanical stability. MTS Insight 2 machine fitted with 500N load cell was used for compression testing of fabricated scaffolds with a deflection rate of 2 mm/ min and

at 10 % compressive strain. Compressive strength (MPa) of the scaffolds was evaluated for analyzing mechanical strength based on our earlier publication.[17]

1.3.5 Scaffold degradation rate in vitro

Scaffold degradation study was carried out based on a previous study.[25] Briefly, weight loss and water uptake of scaffolds was measured for evaluating its degradation rate. Dry samples were weighed and noted as W_i. Samples were immersed in phosphate buffer saline (PBS) for measuring its degradation. PBS was changed every day and at the end of third day samples were blotted for excess removal of PBS and further air dried and then vacuum dried for one day for measuring its weight loss was noted as W_f. % weight loss was calculated using the following formula:

$$\% \text{ Weight Loss} = ((Wi - Wf)/Wi) \times 100$$

W_i = weight of scaffold before immersing in PBS

W_f = weight of scaffold removed from PBS, dried for 1 day and then weighed.

This procedure was carried out for 3 weeks and samples were taken in three replicates for having standard deviation.

1.4 Results

1.4.1 Characterization of Gelatin Microbubble

Gelatin microbubbles were synthesized from 5%, 10%, and 20% w/v gelatin solutions and their MB size was evaluated. We found that the average microbubble size for 5%, 10% and 20% gelatin was around 50 µm, 76 µm, and 120µm respectively. Increase in gelatin concentration showed increase in average MB size as shown in Figure 1.5 and this was consistent with our earlier publication with albumin MB. For 5% gelatin, very small MB were observed overall and while for 10% and 20% gelatin, average MB size was observed high. The microbubble reveals a core- shell structure wherein, protein forms a coating around nitrogen gas bubble. Table 1.1 summarizes gelatin MB size range for all groups.

Table 1.1 Microbubble size range for gelatin groups.

Gelatin concentration	5% w/v gelatin	10% w/v gelatin	20% w/v gelatin
Microbubble size range	40-69 µm	57-89 µm	55-175 µm

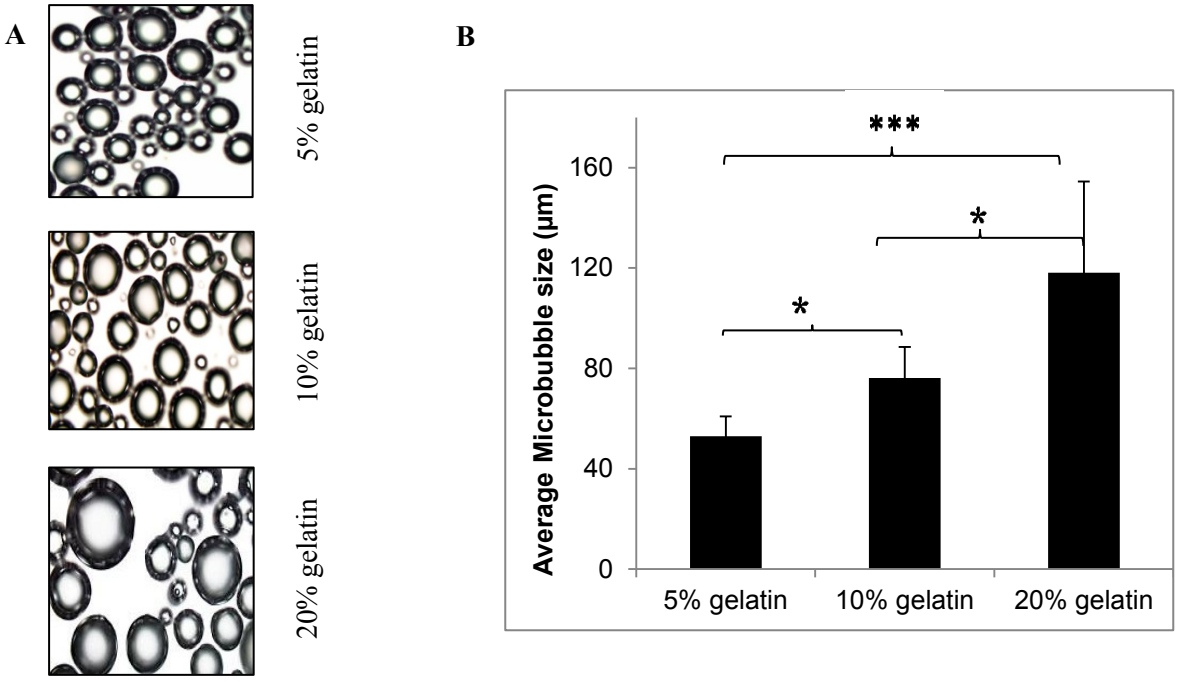

Figure 1.5: Gelatin microbubble average size for different gelatin concentrations. (A) Optical images of gelatin microbubbles for analyzing microbubble average size (Mag 200X). (B) Average size of gelatin microbubbles (*: $p<0.05$, ***: $p< 0.001$).

1.4.2 Characterization of PLGA: Gelatin scaffolds using SEM

Scanning electron microscope was used to analyze pore structure of scaffolds fabricated with varied PLGA: Gelatin ratio consisting of different gelatin concentration. For control scaffolds, as anticipated, very small pores were present due to phase separation and with introduction of gelatin; large pores were formed on the scaffold as seen in Figure 1.6. For scaffolds having PLGA: Gelatin MB (1:1), with increase in gelatin concentration (Figure 1.6 A), the pores formed were higher. Similar trend was observed (Figure 1.6 B) in scaffolds with higher PLGA content,

PLGA: Gelatin MB (2:1). Large pores found on the scaffolds might be because of gelatin MB and micropores due to solvent crystals.

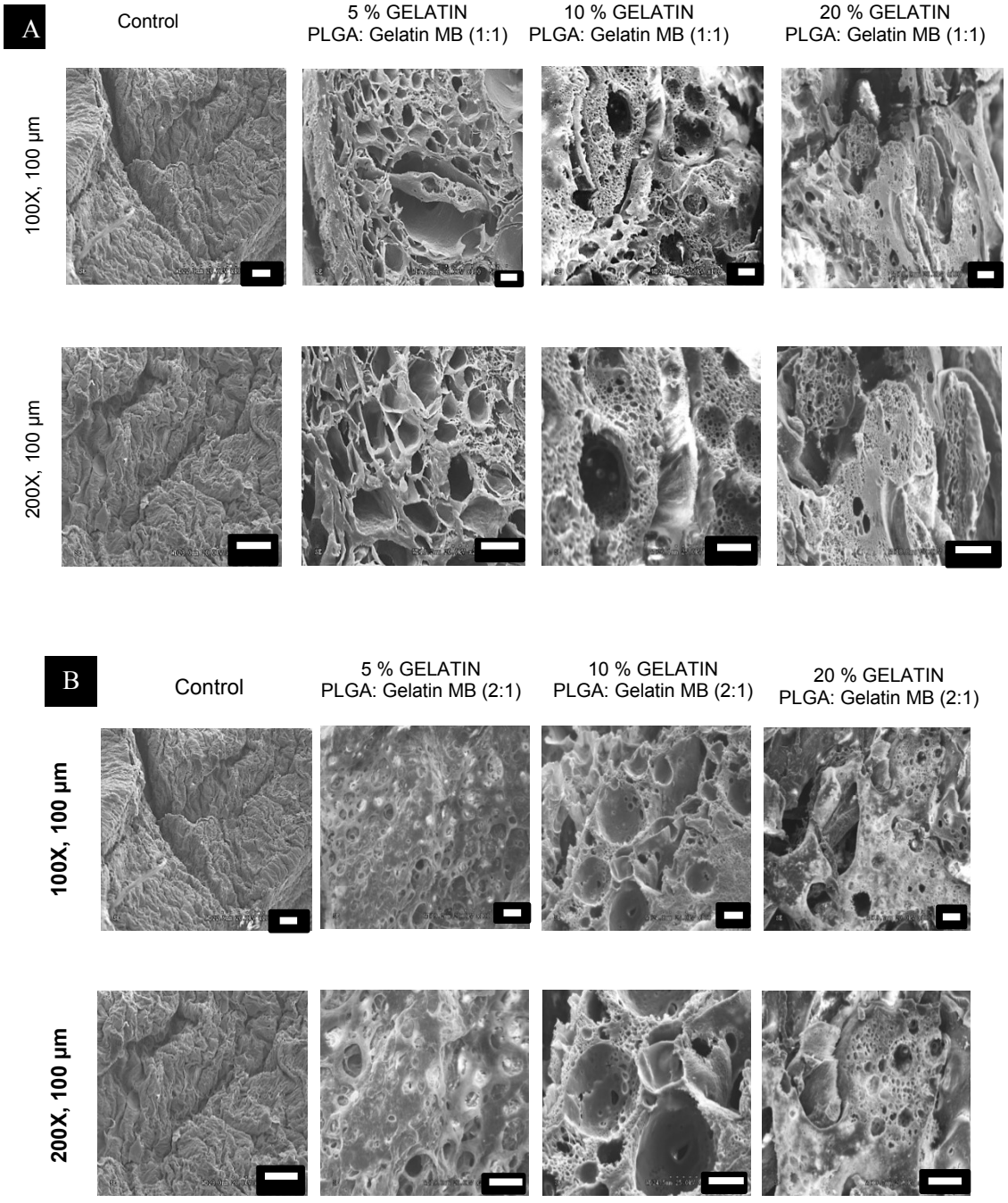

Figure 1.6 SEM analysis of PLGA: Gelatin scffolds. Panel (A) shows SEM images of scaffolds of PLGA: Gelatin MB (1:1) group. Panel (B) shows SEM images of scaffolds of PLGA: Gelatin MB (2:1) group.

Average pore size of the PLGA phase seperated scaffolds as expected was very low and with introduction of gelatin MB, overall pore size increased. For scaffolds with PLGA: Gelatin MB (1:1) group (Figure 1.7 A) , 20% gelatin group showed lower average pore size compared to 10% group, but the difference was not significant. Similar trend was observed in scaffodls with PLGA: Gelatin MB (2:1) group (Figure 1.7 B) wherein, increasing gelatin concentration increased the average pore size.

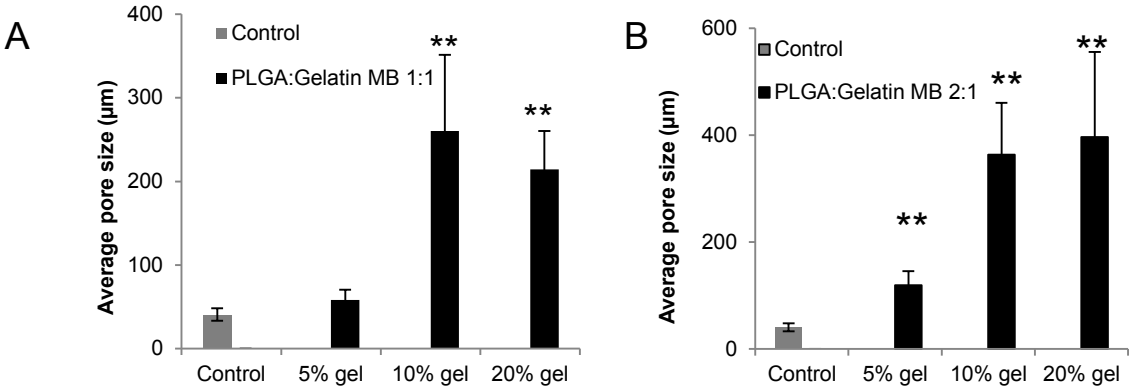

Figure 1.7 Average pore size of the scaffolds. Figure A shows average pore size for the group PLGA: Gelatin MB 1:1. Figure B shows avergae pore size for group PLGA: Gelatin MB 2:1. (***: p< 0.001). All groups are compared with respect to control.

1.4.3 Protein localization in the scaffold using coomassie blue stain

In order to determine the location of gelatin in the scaffold, coomassie blue staining technique was used. With the exception of control and 5% gelatin groups, all other groups had a dense distribution of the dye indicating the presence of gelatin both along the pores and throughout the matrix. It also points towards the role played by gelatin microbubbles in creating pores in the scaffold (Figure 1.8).

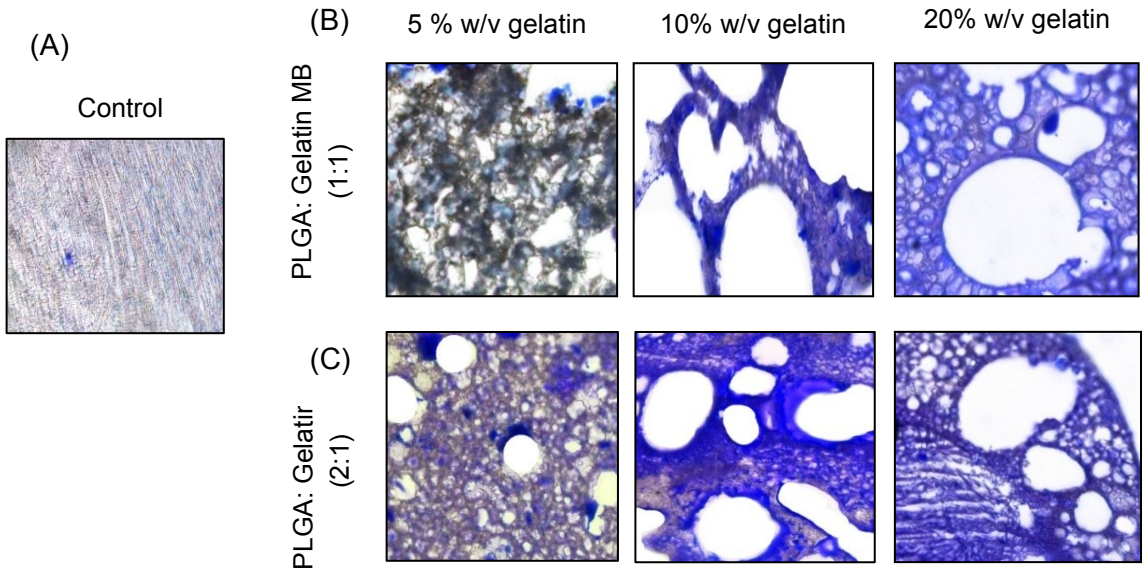

Figure 1.8 PLGA: Gelatin scaffold sections stained with coomassie blue dye. Blue color indicates the dye uptake by gelatin protein present in the scaffold. All groups (A,B, & C) show uniform distribution of gelatin inside scaffold matrix except in 5% gelatin with PLGA: Gelatin 1:1 ratio.

The internal structure of the scaffolds show open porous structure with pores in the range as reported below (Table 1.2).

Table 1.2 Pore size range of PLGA: Gelatin scaffolds

Scaffold Group	**Pore size range (µm)**	
Gelatin conc. (w/v)	PLGA: Gelatin MB 1:1	PLGA: Gelatin MB 2:1
Control	34-53	
5%	40-69	98-163
10%	146-293	156-437
20%	115-583	144-471

Scaffold sections also show internal pores of scaffolds. Pore size range (Table 1.2) for control was found to be consistently low between 34-53 µm. For 5% gelatin having equal PLGA and gelatin content was found between 40-69 µm which was relatively low compared with a uniform pore distribution in 10% gelatin group with a range of 146-293 µm. Pore size distribution for 20% gelatin group was found to be high between 115-583 µm. With increase in gelatin

concentration, scaffold pore size was found to increase and this reflects the MB size increase with gelatin concentration. Similar trend was observed for PLGA: Gelatin MB (2:1) group.

1.4.4 Porosity of PLGA: Gelatin scaffolds

Scaffold porosity was calculated using ethanol displacement method and is shown in Table 1.3. As anticipated, control showed consistently lower porosity of 69 %, which is very low and due to the phase separation. With introduction of gelatin in PLGA, overall porosity was found to be increased but interestingly despite of varied pore size. Porosity for all scaffold groups was found nearly the same. Though, an increase in porosity was observed among the groups with increase in gelatin concentration with very slight increase. The results indicate that porosity of scaffold is not affected by gelatin concentration or polymer to protein ratio.

Table 1.3 Porosity of PLGA: Gelatin scaffolds calculated using ethanol displacement method

Scaffold Group	Average Porosity (%)	
Gelatin conc. (w/v)	PLGA: Gelatin MB (1:1)	PLGA: Gelatin MB (2:1)
Control	69.06±2.15	
5%	76.06±3.65	74.06±1.84
10%	76.81±3.47	76.93±2.91
20%	80.43±1.16	80.33±1.90

1.4.5 Mechanical strength of PLGA: Gelatin MB scaffolds

Mechanical strength of the control scaffolds as expected was high around 0.4 MPa as it did not contain any gelatin in it. As the gelatin MB was added to the scaffolds (Table 1.5), the compressive strength of the scaffolds decreased. Interestingly for 5% gelatin, compressive strength was found to be very low (Table 1.4) and this can be attributed to the aggregation of gelatin MB on PLGA solution. Comparing 10% and 20% groups, 10% groups showed relatively higher compressive strength and indicate that with increasing gelatin concentration, the overall compressive strength of the scaffold reduces.

Table 1.4 Scaffold mechanical strength expressed in terms of compressive strength (MPa).

Scaffold Group	Compressive strength (MPa)	
Gelatin conc. (w/v)	PLGA: Gelatin MB1:1	PLGA: Gelatin MB2:1
Control	0.42±0.16	
5%	0.12±0.05(* p<0.05)	0.07±0.01(** p< 0.01)
10%	0.30±0.10	0.14±0.06(* p<0.05)
20%	0.17±0.07(* p<0.05)	0.06±0.03(* p<0.05)

1.4.6 Comparison of degradation of PLGA: Gelatin scaffolds

Figure 1.9 shows % weight loss of scaffolds after immersing in PBS. As shown in figure; control scaffolds showed low degradation rate. While scaffolds having gelatin MB in them degraded faster. Scaffolds having equal PLGA: Gelatin MB (1:1), 10% gelatin MB scaffolds showed lower degradation rate compared to 20% gelatin MB scaffolds. Similar trend was observed in scaffolds with higher PLGA amount.

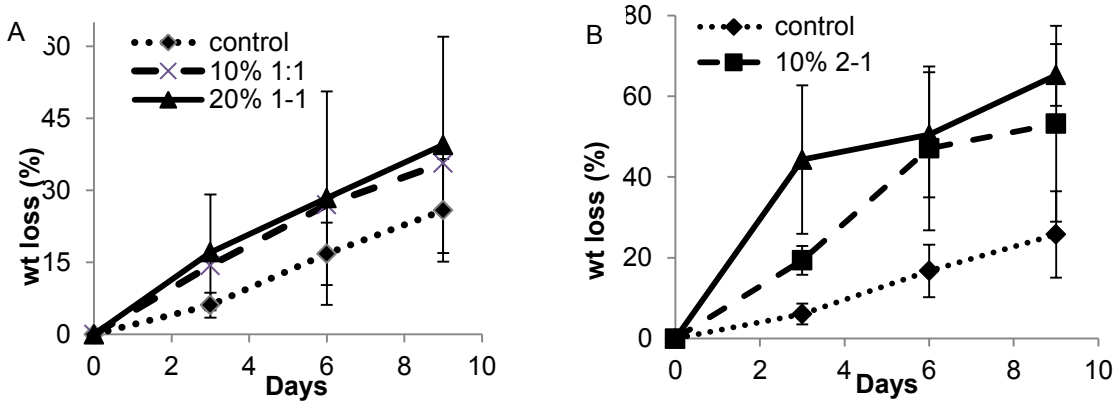

Figure 1.9 Percent Weight Loss of scaffolds over a period of 2 weeks. Samples (n=3) were tested every 3 days.

1.4.7 Comparison with Albumin MB scaffolds

Out of all the scaffold groups 10% gelatin scaffolds with PLGA: gelatin MB 1:1 showed similar characteristics to our previously fabricated albumin scaffold. Table shows a brief comparison of both scaffold properties.

Table 1.5 Comparison for 10% gelatin MB scaffolds v/s albumin MB scaffolds

Properties	Gelatin scaffold (10% w/v)	Albumin scaffold
Average MB size (μm)	76 μm	76 μm
Average pore size (μm)	146-293 μm	100- 150 μm
Porosity (%)	75.8%	92%
Compressive modulus (MPa)	2.5 MPa (control: 2.9 MPa)	1.5 MPa (control: 2.7 MPa)

Table 1.5 shows that though average MB size for both scaffolds was found to be 76 μm, average pore size was found to be different. For albumin MB scaffolds, a homogenous pore size of 100- 150 μm was obtained while gelatin MB showed very varied pore size of 146-293 μm. Interestingly, porosity of gelatin MB scaffolds (75.8%) was found to be low compared to albumin MB scaffolds (92%). But compressive modulus of gelatin MB scaffolds was found to be much higher compared to albumin MB scaffolds.

Now that we have gelatin MB scaffolds having properties comparable to albumin MB scaffolds with higher mechanical strength and better cell attachment properties, we wanted to test whether these scaffolds are capable for releasing chemokines in a sustained manner or not. For that we first test chemokines capable of recruiting MSCs, help proliferating them, and also help differentiating them to osteogenic lineage.

1.5 Discussion

PLGA is a widely used polymer for fabrication of porous scaffolds for cell infiltration, proliferation and differentiation. Over the years, various fabrication processes like porogen leaching, freeze drying, gas foaming, and thermally induced phase separation have been developed for preparing porous scaffolds.[26] These techniques have produced scaffolds with different extents of porosity and physical properties but have been lacking in the areas of cell infiltration, proliferation, and differentiation. This problem was eliminated to some extent with cell friendly proteins like fibronectin, collagen, and glycosaminoglycan (GAG) by either blending them in polymer solution or coating the surface of scaffold.[17, 27] Addition of protein showed

increased cell attachment, but for a shorter duration.[17] A further consideration is the incorporation of bioactive molecules in the scaffold for enhancement of cell proliferative and differentiation properties. Scaffold fabrication processes involving high temperature and harsh organic solvents can denature the proteins and thus have limited the use of proteins in scaffolds.[28]

Keeping these criteria in observance, our recently published study has revealed that Albumin MB are good carrier protein and preserves bioactivity of loaded growth factors. Such growth factor loaded microbubbles could be incorporated into polymer solutions to create a novel set of scaffolds that could protect and release bioactive growth factors.[17] However, one problem with use of Albumin was that such microbubble scaffolds exhibited poor cell attachment and infiltration capacity. We believe that to overcome this, cell friendly proteins like collagen and gelatin can be used. Based on published studies that have shown gelatin to be more conducive for scaffold-related applications and our own pilot studies, we used gelatin to synthesize microbubbles.

It was seen that microbubble size increased with increase in gelatin concentration. Interestingly our observation of an average size of 76 μm for 10% w/v gelatin agrees well with the average BSA microbubble size that was found adequate in our earlier study. These MBs were further used to fabricate PLGA based scaffolds wherein gelatin MBs was used as porogen. Further we analyzed role of MBs in porosity and pore formation in scaffolds when introduced in fabrication process. Interestingly, all scaffolds exhibited high porosity (almost 70%-80%) comparable to control phase separated scaffolds which are beneficial for cell in growth and higher metabolic activity within scaffolds.[29] However, despite similar porosity, surface analysis by SEM revealed that addition of gelatin microbubbles in the scaffold fabrication process had pores formed on the surface with varied pore size that might be advantageous for cell infiltration depth and proliferation.[30] It must also be noted that the inherent microstructure provided by conventional phase separation technique is also preserved as we

observed micropores as well. These pores are created by the solvent 1-4 Dioxane crystals. After sublimation of solvent crystals, microporous scaffolds similar to geometry of solvent crystals are obtained. The pore size on the surface does indicate that the microbubbles could have a role to play in creating porosity. This has in fact been tested in the past and most recently in our publication. Indeed, as earlier, scaffold surface morphology coincided with internal structure of scaffolds. Scaffolds sections stained with coomassie blue dye showed similar pore structure as seen in SEM. A significant difference ($p<0.01$) in average pore size between 20% w/v gelatin and control was found. 10% w/v gelatin also showed similar results. Gelatin distribution as seen from coomassie blue sections was not uniform in 5% w/v gelatin groups and was similar to control scaffolds.

10% w/v and 20% w/v gelatin groups showed highly distributed gelatin MB in scaffold matrix. Coomassie blue dye was seen distributed throughout the lines of pores in entire scaffold for 10% w/v and 20% w/v gelatin. This indicates that pores are formed by gelatin MB. So if growth factors are incorporated in the MB, they would be uniformly distributed in the scaffold and would be able to release in a controlled fashion as shown in next section.

Scaffolds internal pore size was assessed for all gelatin groups and for 10% gelatin PLGA: Gelatin MB (1:1) group average pore size range of 146-293 μm was obtained which is lies well in the range of 100 μm-300 μm found optimum for most tissue engineering applications.[31] Optimum pore size depends on cell type to be used and for the application it is to be used. 5% w/v gelatin scaffolds had low pore size which are not beneficial for cell migration, that might result in formation of a cellular capsule around the edges of scaffolds limiting diffusion of nutrient and waste removal, whereas; 20% gelatin groups had very large pores resulting in a small surface area preventing cell adhesion.[32, 33] This led us to narrow our further experiments to 10% w/v and 20% w/v gelatin groups as 5% w/v gelatin scaffolds showed similar properties as control scaffolds having low pore size and very low distribution of gelatin in the scaffold matrix.

Earlier we saw that how porosity and pore size possibly can affect cell infiltration and proliferation. Along with it, a certain amount of mechanical strength for scaffold is also necessary when implanted in vivo to ensure that scaffolds do not get crushed and can be used for load bearing applications like bone regeneration and also for maintaining a proper tissue layer for vascularization.[34, 35] Compressive strength for 10% w/v and 20% w/v gelatin scaffolds was evaluated to measure effect of pore size and gelatin MB concentration on overall mechanical strength of scaffolds. Overall scaffold synthesized using gelatin MB showed lower mechanical strength when compared to PLGA phase separated scaffolds as was found in BSA MB scaffolds.[17] Scaffolds with higher gelatin concentration and higher internal pore size showed lower compressive strength compared to low gelatin concentration and low average pore size. It was seen that mechanical strength of PLGA: Gelatin MB (1:1) scaffolds were high compared to PLGA: Gelatin MB scaffolds with ratio 2:1 for its respective gelatin concentration groups. Another possible reason for decrease in mechanical strength of scaffolds with higher polymer content can be its viscosity. Highly viscous polymer might not allow proper settlement of gelatin microbubble solution deep inside the polymer rendering proper blending of gelatin MB inside PLGA solution. For all the tests conducted, scaffold with 10% gelatin having equal contents of PLGA and gelatin was found to have higher mechanical strength; one with intermediate porosity.

Further we tested degradation rate of 10% w/v and 20% w/v gelatin scaffolds over a period of 3 weeks. For groups PLGA: gelatin (1:1) content, degradation was found much lower compared to PLGA: gelatin MB (2:1) indicating higher PLGA content is not favorable for long term applications. Rapid morphological changes of scaffolds are not advantageous when implanted in vivo with cells seeded on them as they would not get proper substrate to attach. This can also be attributed with high pore size. Highly porous scaffolds might facilitate exchange of aqueous fluid between inside and outside of scaffold. One key point necessary for the scaffold to support cell proliferation and differentiation in vivo is its degradation rate. Cell

proliferation is highly dependent on the scaffold composite material and its degradation behavior that includes the pH change, change in molecular weight, its water absorption capacity and its weight loss. The tissue engineering scaffolds should have proper degradation time and rate as they are particularly essential at the later stage of implantation when the cells start to migrate deep into the scaffold.[25] Appropriately, degradation of 10% gelatin with PLGA: gelatin (1:1) was found similar to control indicating that 10% gelatin with PLGA: gelatin (1:1) has much lower degradation rate sustaining longer release of growth factors with proper cell proliferation.

Thus, gelatin microbubble seems to be an excellent carrier for release of growth factors as it circumvents all possible defects mentioned earlier. Also, 10% w/v gelatin with PLGA: gelatin MB (1:1) scaffold proves to have better mechanical properties, highly interconnected pores and its degradation rate is also appropriate.

CHAPTER 2

STRATEGIES FOR MSCs RECRUITMENT AND DIFFERENTIATION USING VARIOUS CYTOKINES IN VITRO AND IN VIVO

2.1 Introduction

The use of stem cells has led to a paradigm shift in tissue engineering strategies. Stem cells are cultured in vitro, expanded and seeded on scaffolds to allow proliferation and then implanted in vivo. One problem with such technique is that cells tend to come out of the scaffolds easily and the actual seeding density is not obtained. Consequently, the number of cells needed is obtained less. Instead, use of autologous stem cells would help eliminate this problem as it negates uses of seeding scaffolds prior implantation.

During a routine evaluation of the inflammatory response to various biomaterial implants, our group stumbled upon cells which expressed cell surface markers found on stem and progenitor cells.[15] To determine whether these cells were indeed stem cells, a subcutaneously implanted wound chamber model was employed using polymeric tubes. While a large number of cells expressed CD45+ and CD11b+ markers indicative of hematopoietic and inflammatory cells, a significant number of cells collected from the wound chamber were established as stem cells based on the following three conditions: (1) they expressed various stem cell markers (MSCs (CD73+/CD105+/CD90+/CD45-+) and HSCs (Lin-/ Sca-1+/c-kit+)) that were confirmed using flow cytometry analysis; (2) they adhered to cell culture dish and (3) they differentiated into various lineages like, bone, fat and nerve cells when given specific signals. Various biomaterial implants with different physical and chemical properties were tested subcutaneously for their influence on autologous stem cell recruitment. This led to the first ever documentation of autologous stem cells around implants in the tissue space. Interestingly, it

was found that the stem cell numbers increased over time. The study brought forth an important link between the inflammatory response evoked by a material and the subsequent stem cell response. These findings are important as recent studies have shown new bone formation with use of Adipose derived adult stem cells wherein a scaffold system was used to heal the bone defect.[9]

A wide range of proteins are available that play a key role in cell proliferation and differentiation. These proteins are endogenously secreted in the body by cells themselves (autocrine) or as a result of communication with surrounding cells (paracrine).[36] These proteins (or cytokines) and growth factors (sub-class of cytokines) are hormone like group of regulatory proteins classified on basis of their receptor structure. Cytokines, also known as interleukins, interferons, monokines, and lymphokines are secreted by a wide variety of cells upon activation or stimulation. Cytokines help in the cross talk between the cells. The activation of cytokines depends on the stimulus signals from immunogenic response.[37] Cytokines bind to the target specific receptors, activating various signal transduction pathways in turn leading to the activation of other cytokines.[37-39] Cytokines are associated with various cellular activities like cell survival, proliferation, differentiation, apoptosis, and adhesion.[40] The type of cytokine secreted depends on the genetic level and cellular response.[40]

The cytokines that have frequently been applied for bone tissue engineering include bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF or FGF-2), vascular epithelial growth factor (VEGF), insulin like growth factors (IGF-β), and transforming growth factor-β (TGF-β). Bone contains numerous growth factors, IGFs being most abundantly found are associated binding proteins responsible for bone remodeling. TGF-β and BMPs are known to be potent chemotactic for bone cells.[41] The significant capability of cytokines may be imagined from the fact that BMP and FGF alone can induce bone and vascular tissue regeneration, respectively, without the assistance of scaffold or seeded cells. Apparently, an addition of proper cytokines to a cell–scaffold construct must further promote the tissue

regeneration compared with no use of cytokines.[36] IGFβ1, FGF, BMPs, TGFβ have been known to stimulate osteoblast proliferation and promote bone formation. Recently, it has been known that growth factors play an important role during early bone formation process.[42]

MSCs are found at various sites in the body like bone marrow, spleen, and adipose tissues.[43] Out of these, bone marrow derives stem cells have proved as a potential source of circulating stem cells that gets recruited from the blood to the peripheral solid organs at the time of tissue injury and with the help of chemokines, the ability of the MSCs to migrate and differentiate into desired phenotype can be potentially increased.[44] The MSC migration efficiency may depend on factors such as: (1) Specific receptors or ligands upregulated by injury tissues that facilitate trafficking, adhesion, and infiltration of MSCs, and also provide MSCs with a specialized microenvironment or niche to support their self-renewal and maintain their multi-potentiality. (2) Integrins, selectins, and chemokine receptors expressed on MSCs are involved in migration of MSCs across the endothelium. (3) MSCs are passively arrested in capillaries or microvessels including arterioles and post-capillary venules, and then directly interact with accessory cells and the release a wide array of soluble growth factors and trophic cytokines.[45]

Our group then tested the role of chemokines and growth factors in enhancing the phenomenon of recruitment of autologous stem cells around biomaterial implants. A study involving subcutaneous implantation of PLGA scaffolds soaked with stromal derived factor1-α (SDF-1α) showed that we could enhance the recruitment of autologous stem cells at the implantation site.[16] SDF-1α was incorporated into PLGA salt leached scaffold, and it was found that a higher number of stem cells got recruited at the implantation site and improved wound healing. Furthermore, the numbers of recruited stem cells increased with sustained delivery of stem cell chemokines. We also found that Epo was associated with the recruitment of various progenitor cells like endothelial progenitor cells and MSCs.[46, 47] It is known that the MSCs get attracted near resorption pits and proliferate before differentiating into osteoblasts during bone

formation process.[48] These series of steps are known to be controlled by various growth factors such as TGF-B, BMPs, and IGFs.[48] VEGF is a known growth factor for its angiogenic property. VEGF helps recruiting endothelial cells and is known to induce proliferation and differentiation of osteoblasts by stimulating endothelial cells.[49] TGF-B are known to be a coupler between bone formation and bone resorption, and its receptors are found on osteoblast cells [50]. Epo shares similarity with VEGF in terms of its analogical pathway. They both share similar mechanism when stimulated by hypoxia. Also, like VEGF; Epo is known to promote angiogenesis upon binding with MSCs through Epo receptors (Epo-R).[51]

So far, most studies have been carried out using MSCs differentiated into osteoblasts in vitro and then transplanting them in vivo. And these derived osteoblast cells have shown similar characteristics to osteoblasts and hence have been considered a standard method for use of MSCs for bone formation.

Besides the point discussed above for considering autologous MSCs for this study, we wanted to check whether autologous MSCs possess similar potential to differentiate themselves in osteoblasts and play an active role in bone formation similarly to that found in in vitro differentiated MSCs.[52]

In this study, we hypothesized that MSC has a potential to migrate to the host tissue and differentiate upon systemic delivery. We conducted *in vitro* test for studying MSCs migration, proliferation, and differentiation in response to various growth factors as described below.

2.2 Hypothesis

Cytokine-releasing scaffolds can be fabricated to recruit and then to differentiate MSCs for repairing bone defect.

2.3 Materials and Methods

2.3.1 Isolation of MSCs and in vitro culture

MSCs were cultured from 3 week old Balb/C mice. The use of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Arlington. MSC isolation was carried out similar to our earlier publications.[53] Briefly, bone marrow was collected from femur and tibia by flushing the bones using Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were then transferred to 25 mm^2 culture flasks. Next day, non-adherent cells were washed thoroughly with PBS and replaced with fresh media in order to get pure MSCs in culture (Figure 2.1). Media was supplemented with 0.1 % penicillin and streptomycin for avoiding any bacterial ingrowth.

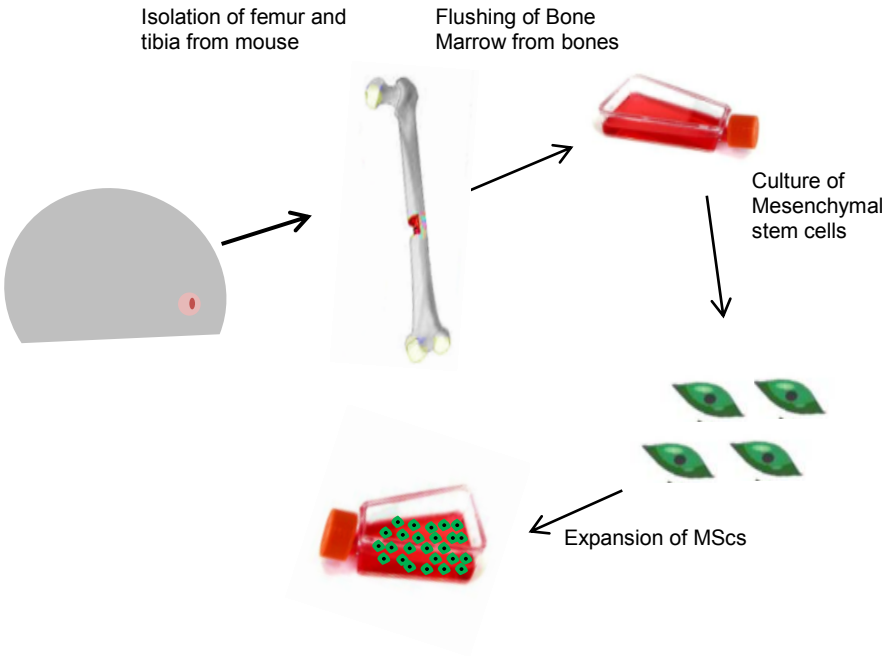

Figure 2.1 Schematic of stem cell isolation and culture *in vitro*. Under aseptic condition, bone marrow is flushed out from femur and tibia of mouse and the cells obtained are kept in culture. After day 1, suspended cells are removed from the flask in order to obtain pure MSCs.

2.3.2 Chemotaxis assay

The migration assay was carried out as discussed in earlier publication[54]. The assay was performed in the transwell plates having 6.5 mm diameter with 8um pore filter as shown in Figure 2.2. The transwell were fitted onto 24 well plates. Unstimulated P2 BMSCs (5 X 10^5) cells were added to the upper chamber of the transwell and in the lower chamber; 600uL of migration media (DMEM) with the chemotactic factors was added. After overnight incubation of the transwell at 37 ^oC, 5% CO_2, the upper side of the well was carefully washed with PBS. Then the apparatus was disassembled, the cells on the upper side of the membrane were wiped off with cotton swab, and the migrated cells were visualized using DAPI staining. Migration was quantified by counting the nuclei that passed through the filter. Stained nuclei from a minimum of 6 fields of view (200X) for 3 replicates were counted and the data was expressed as the average number of migrated cells. The chemotactic activity of cytokines BMP-2 (200ng/mL), EPO (200IU/mL), and SDF-1 (10ng/mL) was evaluated.

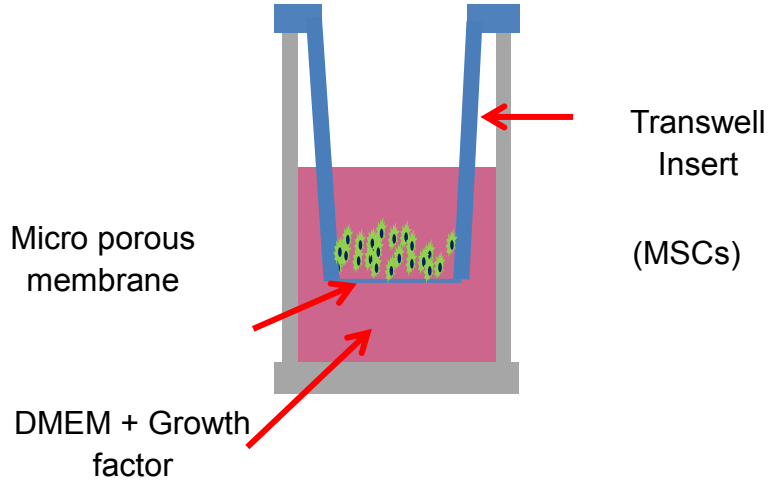

Figure 2.2 Transwell migration mechanism. The cells are seeded onto upper chamber of transwell insert and media containing growth factors are added below the transwell in well plate. Number of cells migrated across the membrane were counted.

2.3.3 *Cell proliferation rate of MSCs in effect of growth factors.*

MSCs were isolate from mouse femur and tibia as described earlier. Passage two cells were used for entire study. Briefly, cells were allowed to proliferate at 90% confluency and then were trypsinized, counted using hemocytometer to obtain a cell density of100, 000 cells/ml. A 12 tissue culture well plate was used for seeding 4000 cells/ well. Cell were incubated in DMEM media supplemented with 10% FBS and 1% penicillin and streptomycin. For cell proliferation, SDF-1α, BMP-2, Epo, and combination of Epo + BMP-2 growth factors were studied. Growth factors were added to the cells 24 h after seeding to ensure proper cell attachment. The dose used for study was as mentioned earlier. Media was changed every two days and cells were allowed to be in culture for 1 week. Cells were incubated with DMEM media and 10% Alamar blue dye for 24 h in 37 ºC before reading the absorbance values using plate reader and cell number was calculated based on standard curve. Alamar blue is a cell proliferation assay based on redox reaction. When dye interacts with cells, it becomes reduced and turns red to pink depending on cell density.[55] Absorbance were measured at 570 nm and 600 nm based on published protocols. Media color changes from blue to pink associated with AB reduction.

2.3.4 Osteogenic differentiation of MSCs in presence of growth factors

Cell culture was carried out as described earlier. After trypisinization, cells were allowed to attach to tissue culture plate for 24 h in DMEM media supplemented with 10% FBS and 1% penicillin and streptomycin before culturing cells in osteogenic media. Osteogenic media was made using DMEM, 10% FBS, 1% antibiotics, 10nM dexamethasone, 10mM B-Glycerophosphate, and 50 µg/ml 2-phosphate-ascorbic acid. Growth factors SDF1-α, BMP-2, Epo, and combination of Epo + BMP-2 were studied for differentiation. Growth factors were added along with osteogenic media 24 hr. post cell seeding. Cells were kept in culture changing media and growth factors every three days and then stained with Alizarin Red S dye for evaluating calcium deposits on MSCs at the end of 3 weeks.

2.3.5 Fabrication of growth factor loaded scaffolds

Epo (500 IU), DSF1-α (10 µg/ml), and BMP-2 (0.04 µg/mm^3) was found to be effective based on pilot studies done in our lab. Growth factors were mixed with 10% w/v gelatin solution to form chemokine loaded gelatin MB and scaffolds were fabricated as described earlier.

2.3.6 In vitro growth factor release from scaffold

The scaffolds were tested for release of bioactive molecules. Erythropoietin (Epo) and stromal derived factor-1 alpha (SDF-1α) were used as model growth factors. Growth factors were labeled with Oyster 800 fluorescence dye in order to measure fluorescence intensity. Scaffolds were immersed in Hank's buffer solution with 5% FBS incubating at 37'C with partial agitation throughout the study. The buffer solution was collected and replaced with fresh solution every day. Fluorescence intensity was measured using UV-Vis photo spectrometer with 760 mm excitation wavelength and 794 mm emission wavelength. Fluorescence intensity was then converted to concentration (µg/mL) using a standard curve and plotted in terms of % cumulative release.

2.3.7 In vivo animal model for bridging mouse calvarial critical size defect

The idea of choosing mouse calvarial model came from the fact that ratio of defect size to total cranial volume in CSD of mouse when projected onto a human skull gives predictable human capacity to regenerate a 2.3 cm diameter defect.[1] Also, the mouse calvarial resembles the cranial of infants. This would help establishing a well-defined therapeutic means for healing CSD. Figure 2.3 depicts an overview of entire procedure

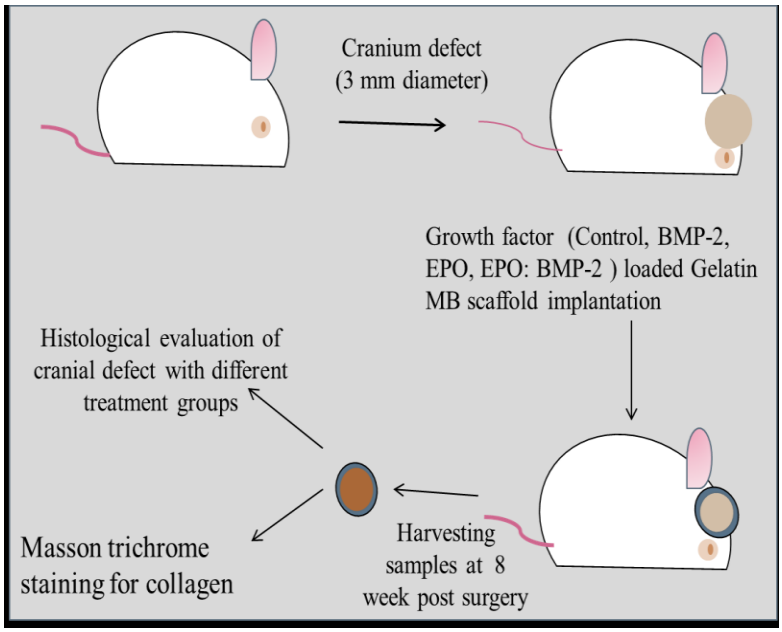

Figure 2.3 In vivo animal model for bone regeneration in mouse calvarial critical size defect

A total of 16 female, 4 weeks old mice (Balb-c) were used for this experiment. The use of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Arlington. The animals were placed individually in plastic cages (22 ºC RT). They were fed with standard laboratory mouse food pallet and had *ad libidum* supply to drinking water. Mouse calvarial defect was created as per earlier published data.[57]

2.3.8 In vivo implantation of growth factor loaded gelatin scaffolds

Circular calvarial defect (3mm, full thickness) was created in 10 week old Balb-C mice. The surgery was performed under general anesthesia using isoflurane inhalation. Following anesthesia, a skin flap was raised to expose the underlying bone and a trephine was used to create the defect. The wound was irrigated with phosphate buffered solution (PBS) continuously while drilling. The calvarial disk was removed carefully so as to avoid injury to the underlying dura or brain. The implants were cut to the defect size (3mm diameter) using a dermal punch and were carefully placed in the defect area using forceps. The animals were randomly divided into 4 experimental groups that received the following manner: (1) No scaffold implantation, (2)

MB: BMP-2 scaffold, (3) MB: Epo scaffold and (4) MB: Epo+BMP-2 scaffold. The untreated cranium with just the defect served as a control. The skin was sutured over the implant using 5-0 Vicryl suture. The mice were placed individually in cages under aseptic conditions. Animals were sacrificed 8 weeks post-surgery for assessment of defect.

2.4 Results

2.4.1 Analyzing MSCs migration in effect of growth factors in vitro

Chemotactic agents SDF-1, BMP-2, and Epo were utilized to examine response of primary BM-MSCs isolated from murine bone marrow femur and tibia in a transwell system. SDF1-α, BMP-2, and Epo were loaded in the bottom chamber of the transwell system with DMEM media containing 10% Fetal bovine serum (FBS). Evaluation of MSCs migration showed that Epo evoked the highest and most significant chemotactic activity (Figure 2.4), followed by SDF1-α. BMP-2; although a known osteogenic differentiation agent; was not a potent MSC chemotactic agent.

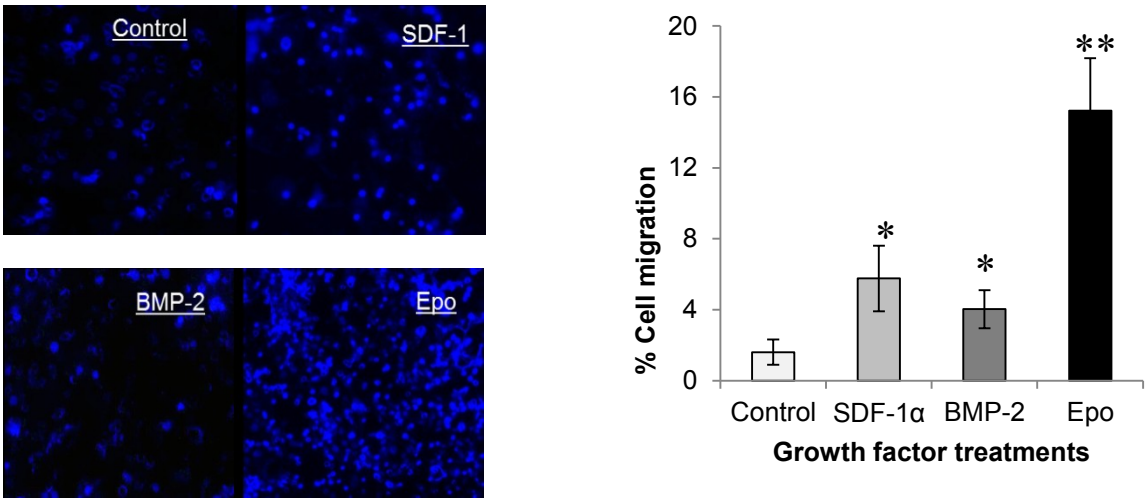

Figure 2.4 Images depicting migration capacity of bone marrow (BM) MSCs towards cytokines DAPI stained transwell membrane. Representative stained filters of MSCs toward medium containing 1) no growth factors, 2) SDF-1(* $p < 0.05$), 3) BMP-2, and 4) Epo (** $p < 0.01$). (B) Represents % of cell migration towards specified growth factors.

2.4.2 Effect of growth factor on MSC proliferation at 1 week

Cells were allowed to proliferate in presence of growth factors and its proliferation rate was measured using Alamar Blue assay. It was found that cell proliferation rate in response to various growth factors was almost similar (Figure 2.5) and no difference was found among the groups except Epo being slight higher.

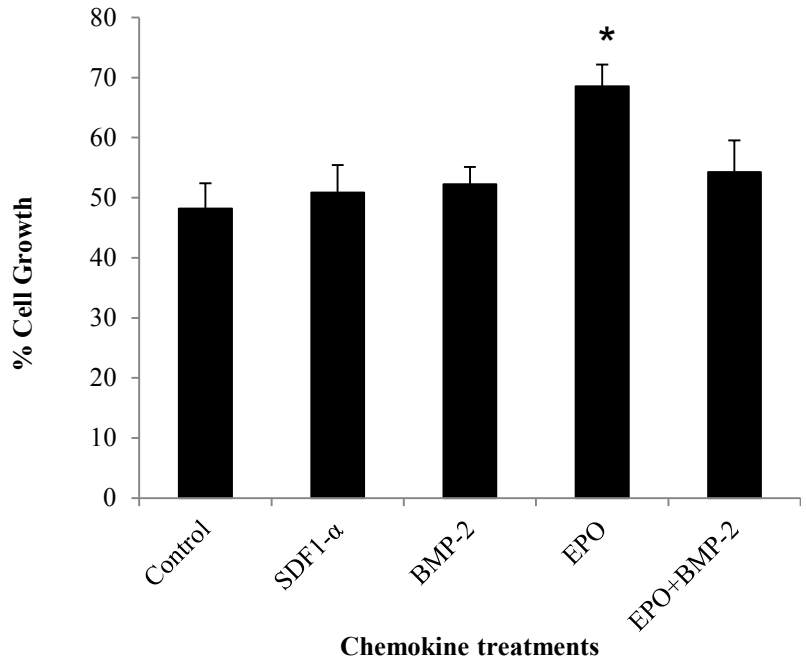

Figure 2.5 Analysis of BM-MSC proliferation rate in response to growth factors. SDF-1, BMP-2, Epo and Epo+BMp-2 were chosen for cell proliferation. Cells were kept in culture for 1 week and then the absorbance was measured using Alamar Blue assay. (*: $p<0.05$).

2.4.3 In vitro MSC differentiation to osteogenic lineage in effect of various growth factors

Isolated BM MSCs showed spindle like and globular morphology in various groups after keeping them in culture added with differentiation media and growth factors for 3 weeks. Alizarin Red S stain was used for confirming mineralization of apparently differentiated cells. Figure 2.6 (A and B) shows difference in morphology and cell number within groups.

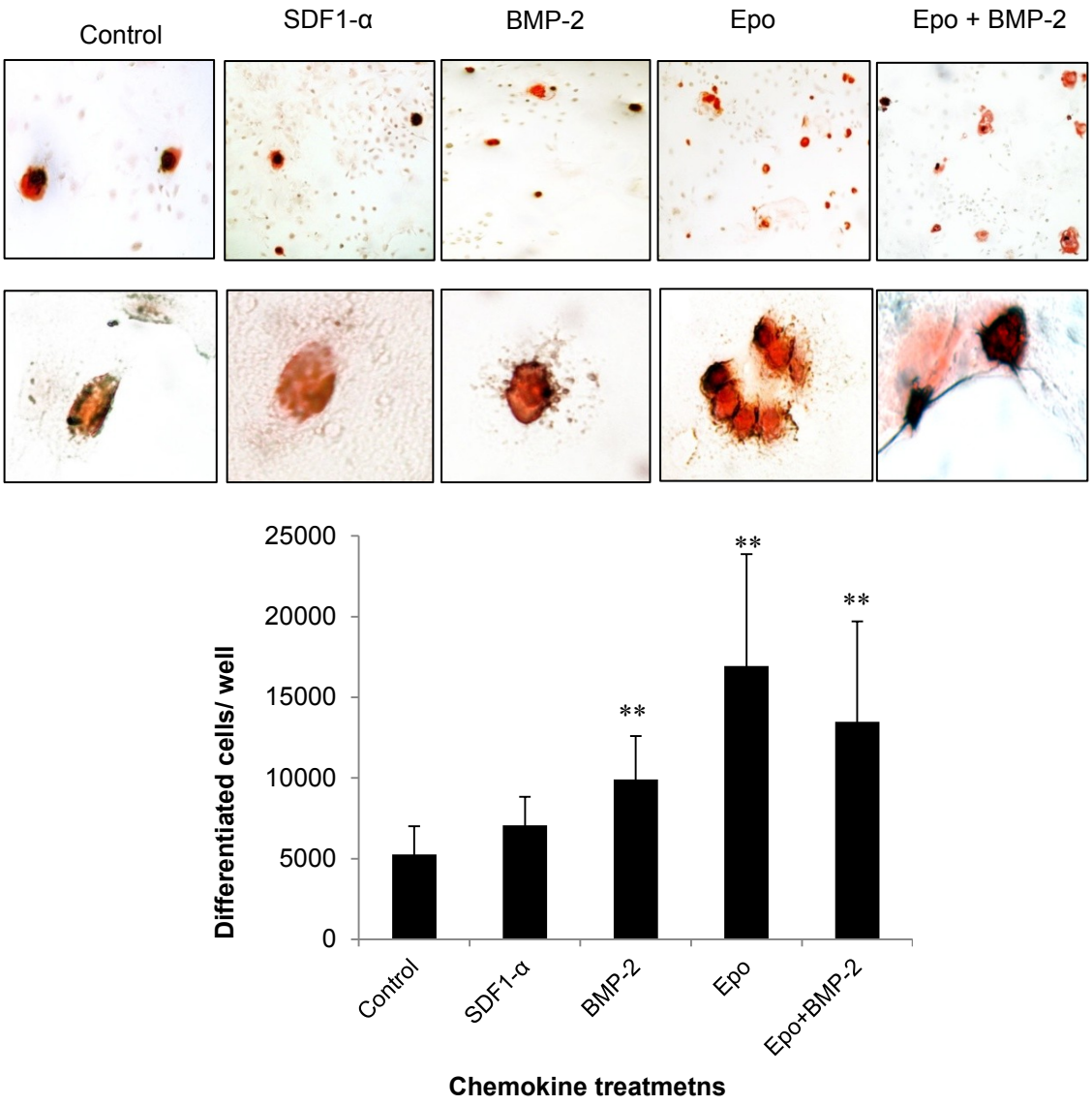

Figure 2.6 Alizarin Red S staining for mineralization and representation of differentiated cell number. Images shows differentiated MSCs when stained with Alizarin Red S dye 3 weeks after kept in culture. (**: $p<0.01$)

Control cells (with no growth factors added) and SDF1-α showed similar cell morphologies and number of cells differentiated. While for BMP-2, Epo, and Epo+ BMP-2; cells were globular is shape and showed presence of mineralized nodules. To our surprise, BMP-2 showed significantly less number of differentiated cells compared to Epo. Also, Epo showed formation of differentiated cell clusters which was not found in any other group. Epo + BMP-2

showed low number of differentiated cells compared to Epo. Surprisingly Epo fared better indicating its possible role in osteoinductivity

2.4.4 Studying growth factor release from scaffold

Epo was used as model drugs to evaluate release of growth factors from the scaffold constructs. Based on previous results and earlier done studies in our lab, scaffold with 10% gelatin having 1:1 PLGA: gelatin ratio was considered optimum for further studies. As seen in figure 2.8, a cumulative release for 20 days was calculated. Epo was found to be released from the scaffold at a much higher rate compared to control. As compared with control scaffolds Epo loaded MB scaffolds showed an almost 2X increase in drug release that was sustained over at least 3 weeks.

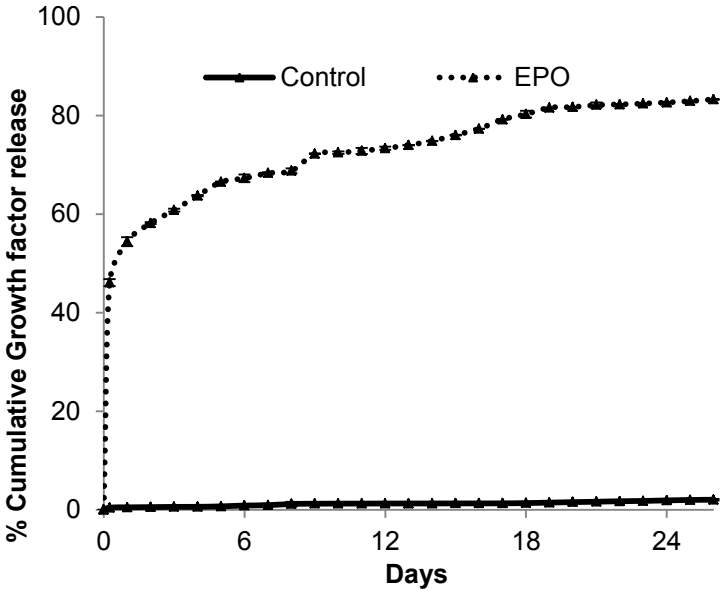

Figure 2.7 Growth factor release profile from 10% gelatin scaffold having equal polymer to protein ratio. Epo and SDF-1α were used as model growth factors to study the release rate. Epo showed an initial burst release and then a gradual release over period of 3 weeks.

Control on the other hand, showed consistently low release throughout the study. This indicates that gelatin MB scaffolds are capable of releasing growth factors in a sustained manner.

2.4.5 Histological evaluation of the defect 8 week post implantation

Craniums were carefully removed and the area with implant was cut with precision for histological assessment. Cranium were embedded in OCT and freeze in -80'C overnight before sectioning using a cryostat. 8 µm thin sections were collected on poly-l-lysine coated slides and subject to Masson trichrome staining. Figure 2.8 represents healing of defect 8 weeks after implantation showing actual defect area and new bone growth in presence of BMP-2, Epo, and Epo+ BMP-2 growth factors respectively. Bone healing, as seen by collagenous tissue ingrowth into the scaffold implant was most prominent in Epo group followed by Epo+BMP-2 group and BMP-2. As expected, untreated controls showed the least defect coverage with only formation of fibrous tissue. Interestingly, combinational growth factor use (Epo + BMP-2) showed less bone formation compared to Epo alone. Epo group showed collagen deposits well within the scaffold area while in other groups scaffold probably washed away.

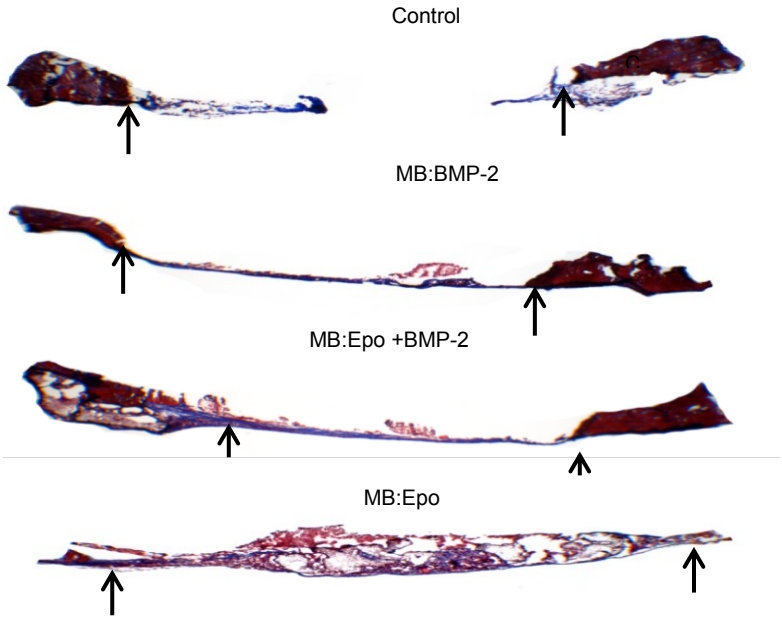

Figure 2.8 Histological evaluation of bone defect healing in vivo post 8 week implantation. Staining was done using Masson Trichrome. Blue color indicated formation of collagen tissue in newly grown bone. Images taken at 40 X (8µm section thickness). Arrows indicate the defect area margins. Defects were treated with Tissue Engineered MB Scaffold with different treatment groups. Defect area with no scaffold, MB scaffold loaded with BMP-2 growth factor, MB scaffold loaded with Epo growth factor and MB Scaffold loaded with Epo+Bmp-2 growth factors.

Deposition of collagen on newly formed bone was analyzed using ImageJ software. In agreement with the histological images, quantitative assessment of collagen formation per entire tissue area was found to be highest in Epo and Epo+BMP-2 followed by BMP-2 (Figure 2.9). Bone ingrowth was found maximum in Epo and collagen staining.

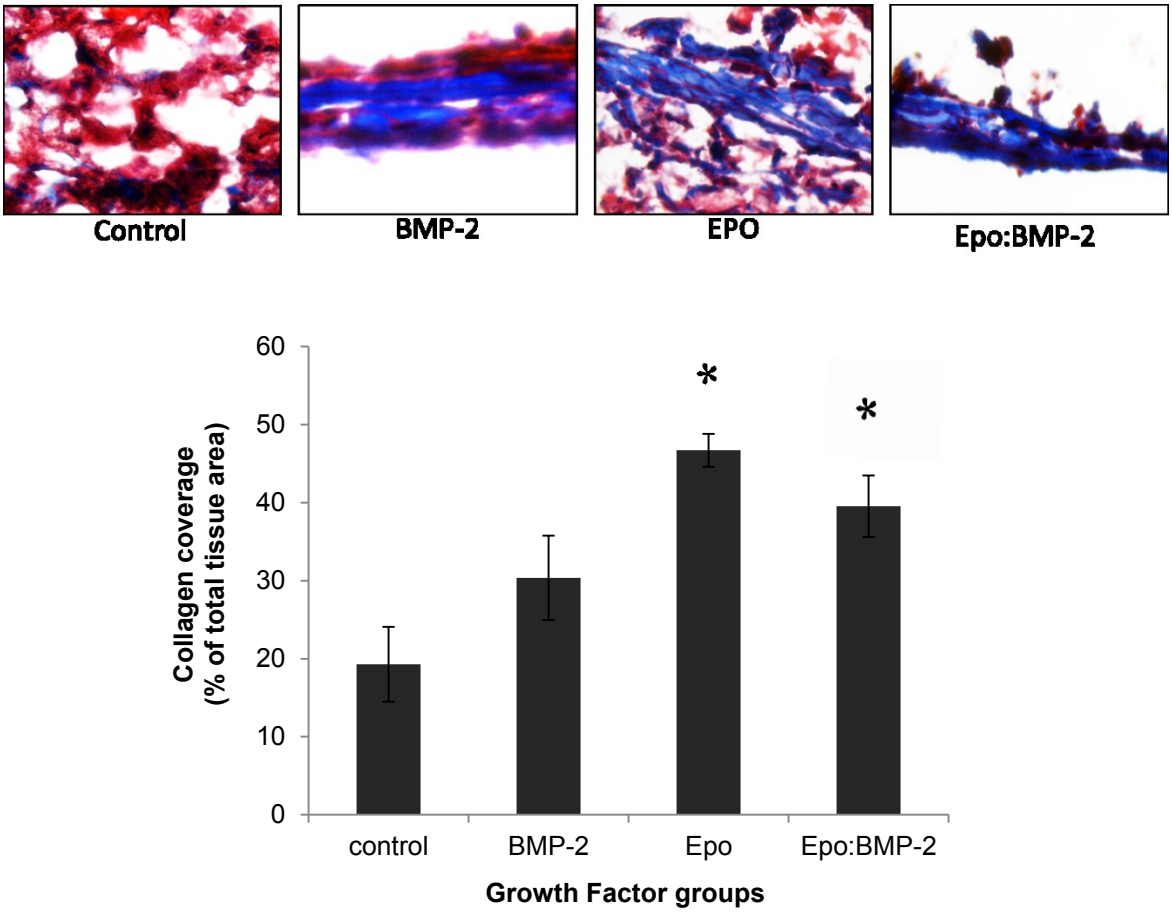

Figure 2.9 Analysis of collagen depositions on newly formed bone using Masson Trichrome staining. Collagen area over entire bone growth was evaluated and it was found maximum in Epo (*: $p<0.05$), followed by Epo + BMP-2 (*: $p<0.05$).

2.5 Discussion

Recent studies have shown that biomaterial implant trigger recruitment of MSCs and help in healing process although requiring mass recruitment of MSCs. Recruiting MSCs in large number has been a challenge and it highly depends on (1) specific receptors or ligands

upregulated by injury tissues that facilitate trafficking, adhesion, and infiltration of MSCs, (2) Integrins, selectins, and chemokine receptors expressed on MSCs involved in migration of MSCs.[45, 58] One way of increasing MSCs at the implantation site would be with help of locally present signaling molecules that can attract MSCs upon interaction with their respective receptors present on MSC surface. A major *in vivo* stem cell homing agent, SDF-1α; also expressed during the inflammatory process, was first tested along with other cytokines like BMP-2 and Epo for its ability to recruit MSCs.[16] These signaling molecules are known for their migratory, proliferative, or differentiation activity on MSCs.[54]

Chemotactic activity of SDF1-α was found to be more compared to BMP-2 and goes with earlier studies. SDF1-α is known homing agent for MSCs *in vivo* and also has been proved in our earlier publications.[59, 60] But to our surprise, Epo fared better compared to SDF1-α. A possible reason for such observation could be linked with *in vitro* expression of CXCR4; SDF1-α receptors present on MSCs surface. Studies have shown that MSCs expanded *in vitro* lack CXCR4 receptors and thus a decreased MSCs homing in stimulation of SDF1-α has been observed.[61, 62] Earlier publications have shown that MSCs migrate in presence of SDF1-α *in vitro* only if they are pre stimulated in TNFα.[54] Our observations also agree with earlier results where SDF-1 was not able to stimulate MSC migration to a great extent.[61, 63] The same study also found that effect of SDF-1 highly depends on its dose and its effect increases under hypoxia condition.[61]

At the same time, Epo emerged as a potent MSC chemotactic and this is in fact in concurrence with numerous other studies including our own earlier observations. We found Epo to be associated with progenitor and MSC recruitment in a peritoneal setting.[23, 46] In fact studies have shown Epo to be a potent stem cell chemotactic that is significantly more potent than VEGF as well.[64] Epo is known to increase chemotaxis, migration of MSCs, but also activation of Metalloproteinase - 9 and production of pro-angiogenic factors which are necessary for ECM remodeling and for formation of new blood vessels respectively.[65] It has been found that Epo

could express receptors at the chondrocytes, but also induce better bio-mechanical strength, and callus formation.[66] BMP-2 although not known as a MSC recruiter, is well established as a potent differentiation agent. Earlier studies have shown that BMP-2 is capable of differentiating MSCs into osteoblast progenitor cells[48], and is most commonly used osteogenic growth factor for healing ectopic and orthotropic bones.[67] BMP-2 is known to bind with its serine/threonine kinase receptors and activates intracellular receptor-regulated Smad proteins (R-Smads, Smad1/5/8) and the mitogen-activated protein kinase (MAPK) components Erk1/2, which subsequently transmit the BMP-2 signal to the nucleus where the transcription of osteoblast genes are regulated.[68] BMP-2 expression has been seen in early stages of MSCs recruitment and is known to persist till their differentiation to osteogenic lineage and its expression decreases during later stages of bone formation in more differentiated cells.[7]

Further, effect of these growth factors was tested on MScs proliferation rate. Surprisingly, all growth factors showed almost similar effect of cell proliferation with Epo showing a slight increase. The results suggest that these growth factors have a significant effect on MSC migration but possess similar ability for cell proliferation. After evaluating these growth factors for MSC recruitment, it was necessary to test their differentiation potential of growth factors on MScs in vitro. For this, we allowed the cells to differentiate in culture for 3 weeks. It was seen that cells cultured in presence of Epo showed highest mineralized nodules and possible mechanism of Epo effects on BM MSC differentiation are discussed later.

Localized growth factor delivery using scaffolds in general is carried out in 2 ways: (1) locally injecting growth factors at the scaffold site, (2) directly incorporating it into the scaffold.[69] When injected locally, growth factor does not have a tendency to stay there for a prolonged period rendering its effect in long term applications. Incorporation can either be done using hydrogels, blending it with polymer solution, or incorporating in microparticles and nanoparticles.[70, 71] Use of microparticles does not provide greater loading efficiency which reduces overall growth factor release. Problem associated with blending growth factor inside

polymer scaffold is that it often denatures the growth factor upon exposure to harsh solvents used in scaffold fabrication process.[17] Also, it is known that a high burst release rate is observed in first two days when scaffold are soaked in growth factors as it is bound only on the surface.

Gelatin scaffold fabricated in this study alleviated the problem faced by BSA scaffolds in terms of cell adhesion and proliferation. Gelatin has been used as a carrier for drug delivery in form of microparticles and hydrogels.[20] One problem associated with it is that it has low mechanical strength and high initial burst release. Instead, using gelatin as a carrier protein in form of a porogen would eliminate the problem of release rate. Gelatin was used as a carrier for delivery of model for growth factors like Epo. Epo was found to be released at a much higher rate compared control scaffolds. Studies have shown that growth factor release depends on the electric charge of growth factor as well as it carrier protein.[70] Electrostatic interaction play role in release of growth factors.[72] If the growth factor to be released is positive in charge, then carrier protein should be chosen which has positive charge to maintain interaction and release of growth factor.[70] Gelatin has an isoelectric point of 5.0 which is acidic in nature.[73] Epo has acidic isoelectric point of 3.7.[74, 75] Growth factor dose also might have an overall effect on their release.[76]

So far it was observed that growth factors used in this study had a potential for MSC recruitment and differentiation *in vitro* where Epo emerged as a more MSC migratory and also a better osteoinductive agent along with development of gelatin MB based scaffolds capable of MSC proliferation and releasing growth factors sustainably. A noteworthy point here is that, though these growth factors showed excellent signs of osteoinduction and MSC migration *in vitro*, they do not necessarily have similar enhanced properties when used *in vivo* due to different *in vitro and in vivo* biochemical environmental conditions. Henceforth to test their potency on MSCs *in vivo,* we used mouse calvarial model and tested for growth factor loaded gelatin MB scaffolds for recruitment of autologous MSCs and its osteoinductive properties.

The idea of choosing BMP-2 and Epo as growth factors for this model is that both cytokines apparently showed good signs of differentiation into osteogenic lineage and they appear in the early stages of bone formation we wanted to compare and analyze effect of both these growth factors in vivo and their possible involvement in bone formation. It has been known that BMP-2 is involved in differentiation of MSCs to osteogenic lineage and promotes cell proliferation *in vivo*. This has resulted in MSCs migrating from bone marrow to the defect site and new bone formation in vivo at the defect site.[77]

We loaded our gelatin MB scaffolds with various cytokines like Epo, BMP-2 and Epo+BMP-2 and applied it in a calvarial defect model in mice. Critical size defect has been defined as the smallest in situ bone defect that cannot heal in a shorter duration of time span. Schmitz and Hollinger (1986) first defined a CSD as the "smallest size intraosseous defect in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal". For assessing osteogenic activity for a bone defect model, we chose to use Critical size defect (CSD); defect that cannot be healed by itself and that heals with fibrous tissue rather than bone tissue.[78] Various tissue engineering approaches have been established for healing CSD in different animal models with use of transplanted cells but this has a risk of maintaining cell phenotype inside the body, along with that, growth factors and platelet rich plasma have been used for treatment of CSD.[79] Selection of animal models highly depends on type of application being taken into consideration. Choice of animal model includes (1) method by which CSD is created, (2) skeletal maturation state and size, (3) physiological state of the model, and (4) capacity of bone wound repair.[1] Mouse calvarial model was chosen because ratio of defect size to total cranial volume in CSD of mouse when projected onto a human skull gives predictable human capacity to regenerate a 2.3 cm diameter defect.[1]

Also, based on our in vitro findings and those reported by a number of studies published earlier by us and others, we assumed that delivery of both Epo and BMP-2 could enhance the effect of recruiting and differentiating MSCs. Surprisingly, we found that to the

contrary, that although potent, the effect of dual delivery was not cumulative . In light of this finding, although not clear, we believe that the signaling pathways involved in potentiating cellular changes could play a major role. It is known that BMP-2 acts *via* the Smad pathway (References) and recent studies have shown that Epo is a known suppressor of the pathway (References). Although this result was contrary to what we expected, it was interesting to observe that Epo alone was able to both recruit and differentiate stem cells.

We believe Epo is highly potent for the following reasons. A recent study has shown that addition of Epo to medium with MSCs triggers their differentiation to osteoblasts. It has also been shown that Epo acts on HSCs to trigger production of BMP-2.[80] Epo is known to activate JAK/STAT signaling in HSCs triggering secretion of BMP-2 for bone formation.[81] Also, since our earlier study showing autologous recruitment of MSCs and HSCs around implants, we believe that Epo could possibly have acted on MSCs and triggered BMP-2 production from the HSCs to stimulate osteogenesis. This might also explain why we don't see any greater signs of osteogenesis in Epo+BMP-2 group.

Our results indicate the potential of using Epo in bone regenerative processes for which we developed a scaffold that could serve as a good substrate. Our results augur well for the future of bone tissue engineering and spine fusion therapies and do have the potential to make tremendous impact in clinically relevant conditions.

CHAPTER 3

PROSPECTIVE WORK AND LIMITATIONS

In this study we showed how gelatin microbubble scaffolds can be modified for better cell attachment, cell proliferation, and how release rate of different cytokines can be controlled using gelatin microbubble scaffolds in vitro and how these cytokines potentially can signal autologous MSCs to get attracted, proliferate, and differentiate into osteogenic lineage.

Though, we tested for few potential chemokines for osteogenic differentiation, other chemokines which are already known to have a better effect on stimulating stem cells into osteogenesis needs to be done. For example, vascular endothelial growth factor (VEGF) is a known angiogenic agent. In fact, Epo is known to have similar effects as VEGF for angiogenesis with a difference that Epo receptors are expressed at an early stage on MSC differentiation which is not the case with VEGF. Comparison of Epo and VEGF have been done in terms of cell migration in vitro under hypoxic conditions but studies need to be carried out for comparing direct effect of Epo and VEGF on autologous MSC migration and differentiation *in vivo.* [64] Also, BMP-7 is known for bone formation but little is known about its effect on MSC migration. Along with it, comparison of BMP-2, BMP-4, and BMP-7 in bone formation is not studied much. Parathyroid hormone (PTH), interleukins, tumor necrosis factor (TNF-α) are some possible differentiating agents that can be studied.

In our study, combined effect of Epo and BMP-2 was low compared to Epo alone at 8 week. It is necessary to test whether similar results are obtained at an earlier stage and later than 8 week of data that we had. In one study, wherein they used BMP-2 and VEGF for bone regeneration in vivo, they found that at 4 week, combination of BMP-2 and VEGF showed higher bone formation compared to VEGF, but for 12 week, they had results showing BMP-2 and VEGF effect lower than VEGF which coincides with our study [42]. Hence further investigation

needs to be done for analyzing effect of combined growth factors having similar or different mechanism for stimulating stem cells.

It is also important for these cytokines to have same similar effect on cells like progenitor cells, osteoblast cells, osteoclasts, HSCs, and adipose derived adult stromal cells (ADAS). The mechanism by which the cytokines behave in accordance to cell type can be investigated. The healing period with each of the cell type may vary according to the environment and cytokines behavior. For example, to differentiate embryonic stem (ES) cells cardiomyocytes, ES cells needs differentiating agent like Interleukin-6 and BMPs to differentiate into cardiomyocytes.

The ECM which provides cells the required substrate for proliferation and should mimic the natural tissue. In this study we used gelatin as it is one major component found in natural ECM of the body. Instead fibronectin can be used as it also has better cell attachment properties. Depending on the type of tissue to engineer ECM can be chosen close to that found in natural tissue. For example, for cartilage tissue engineering, Glycosaminoglycan and hyaluronic acid can be used as ECM

This study was carried out to show long term application of autologous stem cells in tissue regeneration applications and how use of MSCs can circumvent conventional problems associated with stem cell extraction and expansion. This study was carried out to set a platform for future work for engineering tissues of different kinds with use of autologous stem cells.

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BIOGRAPHICAL INFORMATION

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