2016

An Injectable Stem Cell Delivery System for Treatment of Musculoskeletal Defects

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Virginia Commonwealth University

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An Injectable Stem Cell Delivery System for Treatment of Musculoskeletal Defects

A Thesis Submitted
in the Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy at
Virginia Commonwealth University

By

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February, 2016
ACKNOWLEDGEMENTS

I would like to thank my parents Mr. and Mrs. D. Leslie whole heartedly for their continuous support throughout my graduate studies, without them by my side I would not have completed graduate school. My deepest gratitude goes toward my brother, one of my greatest supporters and cheerleaders, giving me numerous motivational speeches along my graduate school journey. To my remaining family members, I am very grateful for your various calls, and texts to offer encouragement and prayers for the protection of my sanity. Thanks to my friends across the globe with their never ending support, assuring me that the end is near. Chantel for her encouragement, chocolates to fuel the late nights. I am very grateful for Debbie providing encouragement and home cooked meals when I was not able to do so.

Dr. Boyan and Dr. Schwartz, thank you for your mentorship during graduate school and taking your time to coach me in developing my scientific skills. Thanks for providing funding for my graduate research as my thesis project developed and guiding me as I solved problems directly related to my thesis work by thinking outside of the box. Thanks to my committee members who have guided me in completing my thesis and played a major role in some experiments of the thesis. Dr. Sundaresan, thank you for aiding and conducting the analysis of the migration study. I am grateful for the support of the present and past members of the Boyan-Schwartz laboratory in conducting experiments and troubleshooting various experimental problems. Thanks to Sharon Hyzy for ensuring that all components of the experiments were ordered in a timely fashion. Thanks to Dr. Cohen for aiding in the in vivo experiments, MicroCT and processing histological samples. Thanks to my undergraduates Eric Pinski and Nathan Kirby for their work on projects that
contributed to the thesis. The remaining members of the laboratory thanks for helping with animal surgeries and editing my written work.
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<tr>
<td>ACAN</td>
<td>Aggrecan</td>
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<tr>
<td>AM</td>
<td>Adipogenic differentiation medium</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BMP2</td>
<td>Bone morphogenetic protein 2</td>
</tr>
<tr>
<td>CM</td>
<td>Chondrogenic differentiation medium</td>
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<tr>
<td>Col 1</td>
<td>Collagen Type I</td>
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<td>Col 2</td>
<td>Collagen Type II</td>
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<tr>
<td>Col 10</td>
<td>Collagen Type X</td>
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<tr>
<td>DiR</td>
<td>1,1’-dioctadecyltetramethyl indotricarbocyanine iodide</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor</td>
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<tr>
<td>LEPR</td>
<td>Leptin receptor</td>
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<tr>
<td>MicroCT</td>
<td>Micro computed tomography</td>
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<td>MG-63</td>
<td>Human osteosarcoma cell line</td>
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<tr>
<td>MSCGM</td>
<td>Mesenchymal Stem Cell Growth Medium</td>
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<td>OCN</td>
<td>Osteocalcin</td>
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<td>OM</td>
<td>Osteogenic Differentiation Medium</td>
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<td>OPG</td>
<td>Osteoprotegerin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Osx</td>
<td>Osterix</td>
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<tr>
<td>PBS</td>
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<tr>
<td>PCR</td>
<td>Polymer Chain Reaction</td>
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<td>PLGA</td>
<td>Poly-lactide co-glycolide</td>
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<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferation-activated receptor gamma</td>
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<tr>
<td>rASCs</td>
<td>Rat Adipose Derived Stem Cells</td>
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<td>RUNX2</td>
<td>Runt-related Transcription Factor 2</td>
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<tr>
<td>TGFβ-1</td>
<td>Transforming growth factor beta 1</td>
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<tr>
<td>TGFβ-2</td>
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<td>TGFβ-3</td>
<td>Transforming growth factor beta 3</td>
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<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase Nick-End Labeling</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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ABSTRACT

AN INJECTABLE STEM CELL DELIVERY SYSTEM FOR TREATMENT OF MUSCULOSKELETAL DEFECTS

By

Shirae K. Leslie, M.S.

A Thesis Submitted to in the Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy at Virginia Commonwealth University 2016

Director: Dr. Barbara D. Boyan, Dean, School of Engineering
Virginia Commonwealth University

The goal of this research was to develop a system of injectable hydrogels to deliver stem cells to musculoskeletal defects, thereby allowing cells to remain at the treatment site and secrete soluble factors that will facilitate tissue regeneration. First, production parameters for encapsulating cells in microbeads were determined. This involved investigating the effects of osmolytes on alginate microbead properties, and the effects of alginate microbead cell density, alginate microbead density, and effects of osteogenic media on microencapsulated cells. Although cells remained viable in the microbeads, alginate does not readily degrade in vivo for six months. Therefore, a method to incorporate alginate lyase in microbeads was developed and optimized to achieve controlled release of viable cells. Effectiveness of this strategy was determined through cell release studies and measuring proteins and expression of genes that are characteristic of the cell’s phenotype. Lastly, in vivo studies were done to assess the ability of alginate microbeads to localize microencapsulated cells and support chondrogenesis and osteogenesis. This project will provide insight to the tissue engineering field regarding cell-based therapies and healing musculoskeletal defects.
CHAPTER 1

Specific Aims

57 million musculoskeletal injuries occur each year, of which 26% are fractures [1]. Five to 20% of the total fractures result in nonunion [1]. A fracture results in non-union when the bone fails to heal completely with bone. Despite the prevalence of fractures resulting in non-unions, there are no methods that currently exist to prevent their occurrence.

Cartilage injuries can occur in ear, nose, and areas articulating joints. In addition, there is a high demand for cartilage replacement in musculoskeletal diseases and congenital birth defects. Unlike bone, cartilage is avascular and tissue regeneration is difficult to achieve.

For defects in both tissue types, current therapies include a surgical procedure involving the implanting of a cell-laden scaffold, an allograft, or a biomaterial. However, these therapies do not lead to total repair due to the inability for the delivered cells or biomaterial to integrate completely. In addition, donor site morbidity may limit the number of autologous cells available, necessitating the use of allogeneic cells, which may be rejected by the host. Injectable cell-based therapies would be especially advantageous for these patients since it’s less invasive and possess the potential to have greater success.

Cell-based therapies using adipose-derived stem cells (ASCs) provide one possible form of treatment for bone defects. ASCs are known to be multipotent [2], easily isolated, and readily available. One disadvantage of cell-based therapies is cells tend to disperse to other tissues [3]. It is critical that a method of delivery is developed to allow cells to remain at the injury site, maintain their ability to differentiate and release cells after a given period. One option is to use alginate, a
well-known biocompatible hydrogel. Alginate is known to maintain cell viability after injection and thus could be used as a delivery platform [4, 5]. In addition, alginate provides a system that allows for the exchange of nutrients, wastes and secreted factors. However, alginate does not readily degrade in vivo for six months. Therefore before it can be used to deliver cells, a method of controlled degradation must be established. Prior to this research, there were no methods to control the degradation of alginate hydrogels in vivo. Several attempts have been made by using gamma irradiation, oxidization and a bifunctional crosslinker, however they have not been successful [6, 7]. The objective of this project was to develop an injectable stem cell delivery technology for treatment of musculoskeletal defects.

**Specific Aim 1:** Determine production parameters for encapsulating stem cells in injectable alginate microbeads.

Task 1.1 Determine the effects of osmolytes on alginate microbead properties.

Task 1.2 Determine the effects of alginate microbead cell density and alginate microbead density on microencapsulated cells.

Task 1.3 Determine the effects of osteogenic media on microencapsulated cells.

**Specific Aim 2:** Develop a method of achieving a controlled release of rASCs from alginate microbeads.

Task 2.1 Develop and optimize a method of incorporating alginate lyase in alginate microbeads.

Task 2.2 Establish degradation parameters to achieve controlled release of cells.

Task 2.3 Determine the viability of rASCs released from alginate microbeads.

Task 2.4 Determine the effect of alginate degradation on the phenotype of released rASCs.

Task 2.5 Investigate the ability of alginate microbeads incorporating alginate lyase to degrade in vivo without an inflammatory response.
Specific Aim 3: To determine the ability of alginate microbeads to localize cells at the site of interest and support chondrogenesis and osteogenesis.

Task 3.1 Investigate the ability of alginate microbeads to localize cells.

Task 3.2 Determine the ability of microencapsulated rASCs to produce ectopic bone in vivo.

Task 3.3 Determine the ability of microencapsulated rASCs to regenerate bone in a unicortical diaphyseal defect.

Task 3.4 Illustrate the multipotency of rbASCs.

Task 3.5 Demonstrate the ability of rbASCs to initiate cartilage regeneration.

Locally treating defects with injections provides a less invasive repair method than surgical interventions for treating skeletal injuries. The use of these injectable hydrogels to deliver stem cells will improve the success of cell-based therapies.
CHAPTER 2

Background and Literature Review

2.1 Bone

Bone is a major organ in the body and a major part of the skeletal system. Over 200 bones are present in the human skeletal system. Bone is a tough supporting tissue that functions in both movement and structural stability in conjunction with muscles [8]. Bone is a dynamic tissue, which includes a mineralized matrix embedded with blood vessels, bone cells, and nerves. There are three types of bone specific cells – osteocytes, osteoblasts, and osteoclasts. Osteocytes sense mechanical stress and send signals to osteoblasts for bone remodeling. Osteoblasts are able to secrete collagen rich extracellular matrix that enables mineralization. Osteoclasts are responsible for bone remodeling by localized acidification and protease secretion. Bone is formed by endochondral ossification or intramembranous ossification. In endochondral ossification, cartilage is formed first by mesenchymal progenitor cells differentiating to chondrocytes. Chondrocytes go through hypertrophy and the extracellular matrix mineralizes. The invasion of blood vessels delivers cells that degrade the extracellular matrix and osteoprogenitor cells to form bone. Intramembranous ossification occurs directly where osteoprogenitors form bone directly. Long bones (femur, tibia) are formed by endochondral ossification while flat bones (bones of the skull) are formed by intramembranous ossification.

2.2 Stem Cells and Fracture Repair

Fracture repair is controlled by an appropriate cellular response and fracture mechanics, which include how much the ends of the bone can move relative to each other (stable or unstable).
There are three phases of fracture repair; inflammation, reparative and remodeling. During the acute period of fracture healing, inflammation leads to the release of bone-derived growth factors. These growth factors contribute to the chemotaxis and aggregation of mesenchymal stem cells (MSCs) to form a repair tissue known as a repair blastema. Acute inflammation results in vasodilation and exudation of plasma and leukocytes. Inflammatory cells begin debris removal. In the reparative phase, an increased cell division occurs first in the periosteum and tissues immediately surrounding it and later extends throughout the fractured bone [9]. A mechanically stable fracture has vasculature spanning the repair blastema and MSCs will differentiate into osteoblasts to form woven bone, which later becomes remodeled. In the case of mechanically unstable fractures, MSCs congregate in the repair blastema and form cartilage, which spans the gap thereby providing flexible stability. Simultaneously, MSCs from the periosteum will produce subperiosteal bone, which forms an outer bridge between the two ends of the fracture thus providing more stability. At this stage, the cartilage starts to undergo hypertrophy, veins begin to invade delivering resorptive elements to the hypertrophic cartilage and MSCs on the surface of the hypertrophied and resorbing cartilage. In the presence of the new vasculature, MSCs undergo osteogenic differentiation and new bone spicules are laid down to replace the cartilage. This cartilage is replaced by woven marrow filled bone, which undergoes remodeling to become cortical bone concluding the remodeling phase [10].

2.3 Non-unions

Long bone fractures are typically difficult and slow to heal (the initial bridging process) primarily due to the lack of establishing an intact bony bridge between the fragments and providing mechanical stability [9]. A rate of 5-10% of bone fractures result in the formation of a non-union.
A normal fracture heals within 6 to 8 weeks. Fractures in bones with thicker cortices may need a few additional weeks to heal. However, if no healing occurs after 6 months it can be considered a non-union. There are two types of non-unions: hypertrophic and atrophic non-unions. A hypertrophic non-union has a large broadened callus that is not capable of regenerating a bony union. The underlying reason for the hypertrophic non-union is the instability of the fracture, which allows multidirectional motion of the fracture fragments. An atrophic non-union is completely different. The underlying reasons for an atrophic non-union is a combination of biological impairment and mechanical factors. The impaired biological factors include damaged vascular supply and the destruction of the periosteum and endosteum. This impairment is also coupled with soft tissue damage and detraction of the surrounding tissue. The mechanical underlying reasons for atrophic non-unions include rigid fixation, insufficient compressive forces, and a fracture gap that is too wide for a bony bridge to be formed [10].

2.4 Stem Cells and Cartilage Repair

Tissues that are continuously being remodeled like bone and liver, easily heal unlike other tissues that do not constantly replace themselves. Complete repair of all types of cartilage is rarely seen. There are three types of cartilage - hyaline cartilage, elastic cartilage and fibrocartilage. Hyaline cartilage covers the bone in joints, elastic cartilage is present in the epiglottis and ear, while fibrocartilage is found between the discs in the spine and between the bones of the hips and pelvis. Once there is a lesion or break in the cartilage, there is no immediate presence of bioactive factors and stem cells. Instead, for cartilage damage that does not go all the way to the underlying bone, the cartilage rarely repairs. In contrast, in a full thickness cartilage injury, blood from the underlying bone marrow fills the injured site carrying osteochondrprogenitor cells, which forms fibrocartilage and restores the underlying bone [11].
2.5 Therapies for Fracture Repair

The gold standard for bone repair is using an autologous bone graft. The graft can take the form of cancellous or cortico-cancellous bone, and may include vasculature. In addition, osteoinductive materials like demineralized bone matrix (DBM) or osteoconductive synthetic materials can be used. Exogenous growth factors can supplement any deficiency of endogenous factors at the fracture site.

In cases where current bone graft substitutes fail, other interventions must take place. Primary intervention fails sometimes because of a lack of host stem cells or compromised health of the host. In some cases, implants can be used with modified surfaces to ensure that the bone integrates well. Alternatively, implanting scaffolds that allow cell invasion and proliferation as well as aiding in the regeneration process can be used [12]. These scaffolds may or may not be previously seeded with cells. In the cases where cell insufficiency is a problem, cell-based therapies would be an appropriate solution.

2.6 Cell-based Therapies for Fracture Repair

Cell-based therapies for bone repair are beneficial for patients where there is a diminished pool of progenitor cells or the host tissue is compromised [1]. There are many clinical trials underway to investigate the efficacy of cell-based therapies for bone regeneration and repair. These trials may take three forms – a cell suspension, a cell suspension coupled with a biomaterial or a cell suspension with a biomaterial previously conditioned in a non-bony site. For the clinical trials with cells only, stem cells may be derived from the bone marrow or adipose tissue. However very little success has been seen the injection of stem cells intravenously or directly to the defect site.
Most cells tend migrate to the lung [13]. Alternatively, some scientists have taken the approach of delivering a concentrated mass of cells solely or combined with platelet rich plasma [14].

To achieve optimum results using cell suspensions, the cell suspension must be coupled with a scaffold. Cell-based therapies have coupled cells with cancellous bone, platelet gel, β-tricalcium phosphate, calcium sulfate, DBM, collagen matrix or hydroxyapatite, as examples [14]. The results from these studies have shown greater bone formation with the presence of cells. In fact, tricalcium phosphate and hydroxyapatite loaded with rat MSCs placed within a segmental defect showed more radio opaque tissue within the defect compared to the scaffold alone [15]. For trials with animals, the scaffold is sometimes loaded with cells and cultured in a muscle or bioreactor for few weeks and later transplanted to an orthotopic site. This approach has led to good results.

A number of different types of cells have been delivered to bone sites including: unfractionated bone marrow stem cells, mesenchymal stem cells, differentiated osteoblasts or chondrocytes, or genetically modified cells [16]. Hydroxyapatite loaded with culture expanded progenitor cells was implanted in diaphyseal defects within humans. Most patients healed well with good integration of the implant into the bone and regained functionality [17]. Other human trials reporting the use of bone marrow-derived multipotent cells with macro-porous hydroxyapatite scaffolds were placed within defects. The results showed the progressive integration of implants in addition to more bone being present on the external surface. After a 7 year follow up a good integration of the implants were observed [18]. Most of the clinical trials investigating cell-based therapies for fractures have employed bone marrow derived mesenchymal stem cells. To date, results have shown the safety and effectiveness of the approach, however to
get the optimum results it is still unknown how many progenitors from the bone marrow are needed and raises the question if there is a large enough supply.

2.7 Cell-based Therapies for Cartilage Regeneration

There have been previous studies using cells for cartilage regeneration in animal models. However most studies have focused on hyaline cartilage where MSCs have been combined with biomaterials like polycaprolactone, polylactic acid, PLGA or fibrin gel. The results showed no adverse effects and some repair of the cartilage within osteochondral defects. Additional studies need to be carried out to determine if the new cartilage was indeed from the cells delivered. There is no evidence in literature that this approach has been used for elastic cartilage however studies have initiated that path for auricular cartilage by showing the presence of stem cells in the perichondrium [19].

2.8 Microencapsulation of Stem Cells for Therapy

The therapeutic nature of stem cells derives from their ability to self-renew as well as maintain the ability to differentiate along various lineages. In addition to differentiating into tissue specific cells and contributing directly to tissue repair, stem cells can create a microenvironment to support the regeneration of tissues or organs by secreting factors that elicit a regenerative response from surrounding cells. Both of these roles require stem cells to be delivered to the desired site and to remain at the site long enough to achieve the therapeutic goal.

The most common method of delivery is to infuse a stem cell suspension via the vasculature [20]. Even when cell suspensions are injected directly to the intended site, most of the cells are gone within 24 hours. There are a number of reasons for this, including cell migration and necrosis resulting from the harsh environment. Additionally, the host’s immune system may reject the
newly introduced stem cells. A delivery system that shields and creates a temporary home for the cells can improve their viability and increase their residence time at the delivery site.

Porous polymer hydrogels like alginate and gelatin have been used as cell-delivery vehicles for some time [21]. Cells can be incorporated into the gels by simple dripping, vibration assisted dripping, and emulsion based encapsulation [22]. These methods result in microbeads that are generally about 500 µm in diameter. Microbeads of this size can limit the exchange of nutrients and waste products and may not be injectable. To overcome these limitations, we developed an electrostatic encapsulation method that results in microbeads that are 200 µm in diameter, are easily injectable, and do not have reduced diffusivity of nutrients and waste products [23].

Alginate is an accessible, block co-polymer that consists of pure mannuronic acid (M) blocks or pure guluronic acid (G) blocks, or alternating MG blocks [24]. Due to its ability to instantaneously crosslink after exposure to divalent cations, alginate is used as a hydrogel. We have successfully produced microbeads using a variety of alginate formulations including: high mannuronate content alginate with low, medium and high viscosities [23] (Fig. 2.1).

![Figure 2.1: Microencapsulated ASCs derived from (A) rat and (B) human.](image)

Here we report the microencapsulation of cells using low viscosity, high mannuronate content alginate. The crosslinking solution contains calcium chloride and glucose and is buffered using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Calcium chloride provides
divalent cations to crosslink alginate between mannuronate residues. Glucose is added as nutrient osmolyte in order to maintain physiological osmolality of the crosslinking solution for the cells, thus enabling reduced Ca\(^{++}\) content and enhancing cell viability [23]. HEPES prevents \textit{in vivo} calcification by maintaining pH at or below pH 7.3 (Fig. 2.2) [5]. This method produces microbeads with a high surface area to volume ratio, which is beneficial for molecular transport. Using this method adipose stem cells (ASCs) retain high viability during microencapsulation and remain viable for up to 3 weeks in culture [5].

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{microencapsulation_diagram.png}
\caption{Schematic of electrostatic potential based microencapsulation.}
\end{figure}

Depending on the desired therapeutic use of the microencapsulated cells, the residence time for cells within the microbeads may vary. In order for this to be possible the material used to encapsulate the cells must be able to undergo controlled degradation \textit{in vivo}. For example, a non-degradable polymer can be used if the therapeutic use of microencapsulated stem cells is insulin production in the case of diabetic patients [25]. A degradable polymer can be used if it is required for the stem cells to be released to support tissue regeneration. To control the rate of cell release, we have developed a method by which the degradation of alginate can be modulated enzymatically (Fig. 2.3). Alginate lyase cleaves the glycosidic bonds in alginate specifically between the mannuronate and guluronate residues through a β-elimination reaction [26].
Figure 2.3: Release of ASCs from alginate (medical grade) microbeads with different formulations of alginate lyase (U): alginate (g) over a 12 day period.
CHAPTER 3

Development of a Cell Delivery System Using Alginate Microbeads for Tissue Regeneration

Summary

Alginate microbeads incorporating adipose-derived stem cells (ASCs) have potential for delivering viable cells capable of facilitating tissue regeneration. These microbeads are formed in calcium crosslinking solutions containing organic osmolytes to ensure physiological osmolality, but the comparative effects of these osmolytes on the microencapsulated cells are not known. In addition, delivery parameters needed to use microencapsulated cells for tissue regeneration remain unknown. We investigated the following parameters: 1) osmolyte effects on microbead diameter, cell viability and growth factor production; 2) the effect of the number of cells per microbead and the number of microbeads per unit volume on cell viability, growth factor production, and microbead degradation; 3) the ability of both degradable and non-degradable alginate microbeads to localize cells at the delivery site in vivo; and 4) whether alginate microbeads containing alginate-lyase elicit an inflammatory response after repeated exposure. Smallest microbead diameters were achieved using glucose as the osmolyte but cell viability and growth factor production did not depend on osmolyte type. As cell number per microbead or microbead number per well increased, growth factor production per cell decreased although percent cell viability was unchanged. The rate of cell release varied with the number of beads per well and with the number of cells per microbeads. At the highest microbead density and at the lowest density of cells per microbead, cell release was delayed. Therefore fewer microbeads may be sufficient for clinical applications. Both degradable (0.22 U/g) and non-degradable (0 U/g) alginate microbeads localized cells at the
delivery site. Degradable alginate microbeads delivered subcutaneously elicited a mild chronic inflammatory response on second exposure, but how this might impact repeated use of the technology remains to be determined.

3.1 Introduction

Optimized production and delivery parameters for cell-based therapies are critical to their regenerative capability. One goal for cell-based therapies is to use the minimal number of a patient’s cells while maintaining cell viability, phenotype and function. For some cell-based therapies it is important that the cells remain at the treatment site and that the material used as the delivery vehicle does not have an adverse effect on the surrounding tissue or elicit an inflammatory response.

We have shown that adipose-derived stem cells (ASCs) can be encapsulated in alginate microbeads and remain viable for at least three weeks in culture [4]. Alginate is a block co-polymer consisting of mannuronic acid, guluronic acid or alternating mannuronic and guluronic blocks [27]. A hydrogel is formed instantaneously once alginate is exposed to divalent cations by forming ionic bonds between the divalent cation and guluronic acid. Alginate hydrogels have been used in a variety medical applications to deliver therapeutic agents and to encapsulate cells to deliver endogenous proteins or aid in tissue regeneration and organ repair [28-30]. Typically, these microbeads have diameters of 500µm or more [31]. By generating microbeads using an electrostatic potential, 200µm diameters can be achieved, enabling the microbeads to be injected through 18 gauge needles and facilitating greater mass transport of nutrients and waste [32]. Subsequent studies showed that these smaller beads necessitated reduced Ca²⁺ content in the crosslinking solution than is commonly used to generate the larger diameter microbeads to preserve cell viability during the crosslinking process. To maintain osmolality of the cross-linking
solution at physiological range, the nutrient glucose was included as an osmolyte [23]. However whether other organic osmolytes could be used was not known, nor was it known what effect these osmolytes might have on the beads themselves or on ASCs within the microbeads. The presence of a nutrient osmolyte in the crosslinking solution also serves as an energy source, which can affect the production of growth factors by the encapsulated cells. Therefore, one goal of the present study was to compare the effects of glucose on microbead diameter as well as on cell viability and growth factor production with those of two non-nutrient sugar osmolytes, mannitol and trehalose, and an inorganic osmolyte - sodium chloride.

The number of cells required to achieve a therapeutic outcome is not known. Some studies have shown that using a high cell dose leads to favorable response while the opposite has also been observed [33]. The number of cells per microbead can be varied by altering the number of cells in the original suspension, but how this might impact the overall viability of the cells or the production of therapeutic factors per bead is not known. Moreover, it is not known whether the number of beads per unit volume can affect these parameters. Finally it isn’t known if the release of cells from the beads is affected by either the number of cells per bead or the number of beads per unit volume.

Alginate microbeads can be injected directly to the desired treatment site and retain cells at the site for at least 3 months [4]. Although alginate stability eventually decreases as Ca\(^{++}\) ions diffuse from the hydrogel, this can occur much slower than tissue regeneration requires. Recently we showed that alginate lyase can be used to provide controlled degradation of the hydrogel [34]. Here, we compared the ability of alginate microbeads that contain alginate lyase to localize rASCs within tissue with alginate microbeads that do not contain the enzyme. Finally, we examined whether the alginate lyase microbeads elicit an acute inflammatory response typical of many
biomaterials [35] or if they elicit a delayed hypersensitivity reaction. This is important because ultrapure alginate is not known to elicit an inflammatory response in vivo. However it is unknown if the by-products of alginate lyase mediated digestion elicit an inflammatory response.

This study explores production and delivery parameters for microencapsulated ASCs. Cell-based therapies should be efficient in using the minimum number of cells required to achieve regeneration, especially in cases where availability of autologous cells is low.

3.2. Methods

3.2.1 Adipose Derived Stem Cells

ASCs were isolated from six 100-125g male Sprague-Dawley rats under an Institutional Animal Care and Use Committee (IACUC) approved protocol at the Georgia Institute of Technology and Virginia Commonwealth University and cultured using a previously described method [2, 36]. Briefly, the inguinal fat pad was removed and transferred to a container with Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 3% sterile-filtered L-glutamine-penicillin-streptomycin (P/S). The tissue was washed three times with Hank’s balanced salt solution (HBSS) (Invitrogen) and then incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO) for 30 minutes on a rocker at 37°C. Following trypsinization, the supernatant was discarded. Adipose tissue was cut into small pieces and incubated in a digestive cocktail containing collagenase type I (365 units/mL) (Sigma Aldrich) and dispase (3 units/mL) (Gibco, Carlsbad, CA) for four hours. After incubation, the oily upper layer was removed and the digest was quenched with an equal amount of mesenchymal stem cell growth medium (MSCGM, Lonza Biosciences, Walkersville, MD). The cells were separated, plated in T-175 flasks at 5,000 cells/cm² and cultured in MSCGM at 37 °C and 5% CO₂. For the next two days the monolayers were washed with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) and fed with MSCGM every 24 hours. Thereafter media were
changed every 48 hours and cells were cultured to confluence. First passaged cells were used for studies.

Human ASCs (hASCs) were obtained from Lonza. The hASCs were cultured in MSCGM (Lonza) and passaged as needed until enough cells were obtained for microencapsulation.

For the localization study, ASCs were isolated from green fluorescent protein (GFP) positive rats (Rat Resource and Research Center, Columbia, MO) under IACUC approval at the Virginia Commonwealth University and cultured up to passage one.

3.2.2 Alginate Microbead Production

To investigate the effect of osmolyte on alginate microbead formation, sodium chloride (Sigma Aldrich), mannitol (Sigma Aldrich), glucose (Sigma Aldrich), and trehalose (Sigma Aldrich) were used in the calcium crosslinking solution. Medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginites, FMC BioPolymer, Sandvika, Norway) was dissolved in 0.9% (w/v) saline (Ricca Chemical, Arlington, TX at a concentration of 20 mg/ml, resulting in a 2% alginate solution. Microbeads were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 5 ml/hr flow rate, nozzle with an inner diameter of 0.12 mm, an electrostatic potential of 6 kV and a crosslinking solution containing 50 mM CaCl$_2$, 150 mM osmolyte (sodium chloride, mannitol, glucose, or trehalose) with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) at pH 7.3 [23, 32]. Alginate microbeads were allowed to crosslink for an additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca$^{++}$, and suspended in the culture media.

3.2.2.1 Degradable Alginate Microbead Production

Alginate lyase was incorporated into alginate microbeads by combining equal volumes of 4% medical grade LVM alginate and a 0.9% (w/v) saline containing 0.44 U alginate lyase/ml.
Prior to the combination, both components were kept at 4°C. The final 2% (w/v) alginate was mixed at 1000 rpm for one minute to produce 0.22 U alginate lyase/g alginate. The alginate/alginate lyase mixture was used to form microbeads using a crosslinking solution containing 75 mM CaCl₂, 90 mM glucose and 10 mM HEPES using the same flow rate, nozzle and electrostatic potential described above.

3.2.3 Effect of Osmolytes

ASCs were encapsulated in UV-sterilized medical grade alginate at a concentration of 10 x 10⁶ cells/ml and crosslinked in a calcium solution containing one of the four osmolytes. MG63 osteoblast-like cells (American Type Culture Collection, Rockville, MD) were used as a control population for comparison.

3.2.3.1 Alginate Microbead Morphometrics

Immediately after microbead production, images were made of microbeads suspended in crosslinking solution. A second set of images was made once the microbeads were transferred to the culture media (MSCGM for microencapsulated rASCs and DMEM supplemented with 10% fetal bovine serum for microencapsulated MG63s) for at least 24 hours. The diameter of the microbeads was analyzed with Image-Pro Plus software (version 4.5.1.22, Media Cybernetics). Three images per well were taken using a Leica DMIL microscope connected to a Leica DC 300 camera (Leica, Solms, Germany).

3.2.3.2 LIVE/DEAD Assay

Viability of microencapsulated rASCs was measured using a LIVE/DEAD Viability kit (Invitrogen). Samples (200 µl) were collected at weeks 1 and 2. Aliquots were transferred to a chamber slide and incubated for 30 minutes at 37°C in 0.9% (w/v) saline containing 4 µM ethidium homodimer-1 and 2 µM calcein-AM. Images were obtained using a Zeiss LSM 700-405 confocal
microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Three images were obtained for each well and then red and green cells were counted using Image-Pro Plus software.

3.2.3.3 Soluble Factor Quantification

To investigate the effects of osmolytes on factor production, microencapsulated hASCs (Lonza) were cultured for 3 days in MSCGM (GM) and then treated with chondrogenic differentiation media (CM) for 7 days. The CM consisted of high glucose DMEM (4.5 g/L glucose) with 110 mg/L sodium pyruvate (Sigma), 50 µg/mL ascorbic acid 2-phosphate (Sigma Aldrich), 10 nM dexamethasone (Sigma Aldrich), 1% ITS+ (Sigma Aldrich), 40 µg/mL proline (Sigma Aldrich), 100 ng/mL of the recombinant human bone morphogenetic protein 6 (BMP6, Rocky Hill, NJ), and 10 ng/mL recombinant human transforming growth factor beta-1 (TGFβ1, R&D Systems, Minneapolis, MN). Enzyme-linked immunosorbent assays (ELISA) were used to measure the levels of insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), TGFβ2, and TGFβ3 (R&D Systems) in the conditioned media and retained in the microbeads. To measure growth factor retention within microbeads, beads were collected, uncrosslinked in 82.5 mM sodium citrate, lyophilized, resuspended and digested in alginate lyase (1 U/ml) (Sigma Aldrich) for an hour and assayed using ELISAs [37]. All data were normalized by the DNA content of the cells.

3.2.3.4 DNA Quantification

Cells collected from the digested microbeads were used to determine DNA content of the microencapsulated cells (Quant-iTTM Picogreen® ds DNA Reagent, Invitrogen).

3.2.4 Cells/Microbead and Microbeads per Unit Volume

3.2.4.1 Microbeads per Unit Volume
To investigate the effect of the number of microbeads per unit volume, microencapsulated hASCs (10 x 10^6 cells/ml alginate) were suspended in CM resulting in densities of 60,000, 12,000, 6,000, and 1,200 microbeads per well. A control group of 12,000 microbeads per well was suspended in GM. CM or GM was added to each well, resulting in a total volume of 2 ml. On day 7 of culture, fresh media were added to each culture. Conditioned media were collected 24 hours later and growth factor content determined by ELISA. The microbeads were collected and any retained growth factor determined as well. Data were normalized to DNA content. Total growth factor levels were calculated by not normalizing by the DNA content. Cell viability was determined as described above.

3.2.4.2 Cells per Microbead

To investigate the effect of cell number per bead, hASC suspensions (20, 10, 5 and 2 x 10^6 cells/ml alginate) were microencapsulated to produce microbeads containing 71, 37, 21, and 7 cells respectively. Microbeads were cultured in CM. A control group (37 cells per microbead) was cultured in GM. Growth factor production was determined as described above. Cell viability was determined.

3.2.4.3 Cell Release

To determine if alginate lyase mediated degradation was altered by the number of cells per bead or the number of beads per well, hASCs were encapsulated in microbeads containing 0 or 0.06 units of enzyme per g of UV-sterilized medical grade alginate. The microbeads were suspended in 40 μm cell strainers (BD Falcon, Franklin Lakes, NJ) and cultured in CM in non-tissue culture treated 6-well plates to limit proliferation of released cells in the well. Media were changed every 48 hours and each cell strainer was washed twice with 0.9% (w/v) saline to ensure that all the cells released were collected. Any additional cells on the plate’s surface were collected.
by trypsinization. For the groups where alginate microbeads did not fully degrade by the end of the 12 day period, 82.5 mM sodium citrate was used to release the remaining cells. DNA was quantified as described previously. Alginate microbead degradation was monitored using light microscopy. At each time point one image per group was taken.

### 3.2.5 Retention of Cells In Vivo

#### 3.2.5.1 Cell Labeling

GFP-rASCs were labelled with DiR (1,1’-dioctadecyltetramethyl indotricarbocyanine iodide) fluorescent dye (PerkinElmer, Waltham, MA) to more effectively distinguish their fluorescence (Absorption\Emission: 748/780 nm) from the autofluorescence of the surrounding tissue which also fluoresces green [38]. The optimal dye concentration for labeling GFP-rASCs while maintaining viability was previously determined over a two week period with dye concentrations ranging from 4.2 µg/ml to 135 µg/ml. A million cells were incubated in the respective dye concentration for 30 minutes at 37°C. After labeling was complete, cells were washed with PBS twice. Immediately after staining with DiR, a portion of the cells were used for viability measurements. The viability was quantified with a cell counter (Countess, Invitrogen) and trypan blue (Lonza). The remaining cells were then plated at 5000 cells/cm² in 24-well plates and 96-well black with a clear bottom for viability and fluorescence measurements on days 7 and 14. At each time point, DiR fluorescence was quantified using Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) while the GFP fluorescence was quantified using a plate Synergy H1 reader (BioTek, Winooski, VT).

#### 3.2.5.2 Microbead Injection Protocol

Microencapsulated GFP-rASCs were delivered intramuscularly to athymic nude mice under a protocol approved by the IACUC at Virginia Commonwealth University. GFP-rASCs were labelled using 33.8 µg/ml DiR dye and then microencapsulated in microbeads containing 0
U, 0.06 U, 0.22 U and 1.75 U per g medical grade alginate microbeads. Microbeads were suspended in 1 ml saline and 100 µl were injected into the right gastrocnemius and left deltoid of each mouse using a 27 gauge needle. An equivalent number of cells in a suspension were injected as a control. (n=4, per variable)

3.2.5.3 Analysis of Results

Retention of microencapsulated cells at the injection site was followed with a Maestro 2 Imaging System (PerkinElmer, Waltham, MA) [38]. Images were taken on days 0 (immediately after the injection), 1, 2, 4, 6, 8, 10, 12 and 14. Animals were placed in a prone or either lateral recumbent positions on the imaging platform while under anesthesia (2% isoflurane in oxygen). On the final day, animals were euthanized and an ex vivo organ assessment conducted to identify the presence of labeled GFP-rASCs. Inguinal lymph nodes, sternum, heart, lung, liver, spleen, stomach, intestines, both kidneys, and testes were removed and imaged. Fluorescence was quantified and normalized to the fluorescence measured on day 0 for each animal’s arm or thigh. Images were converted to a scaled image by applying a threshold of 0.01 scaled counts/second. To quantify the fluorescence, an area of interest was selected and the threshold applied using Maestro 2.10 software (PerkinElmer, Waltham, MA). The viability of the same batch of cells delivered to the animals was measured in vitro at each imaging time point using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI).

3.2.6 Inflammation

In vivo studies were performed to determine if the degradation by-products generated by alginate lyase elicit a delayed hypersensitivity inflammatory response. Male C57 Black 6 mice were handled under a protocol approved by the IACUC at Virginia Commonwealth University. Prior to surgeries, mice were anesthetized using isoflurane gas. Non-degradable (0 U/g) and
degradable (0.22 U/g) alginate microbeads (0.1 ml) without cells were injected subcutaneously on the dorsum using a 27 gauge needle [4]. Each animal received one injection subcutaneously above the right gastrocnemius (n = 6 for each experimental condition). After 2 weeks each animal received another injection above the left gastrocnemius. Animals were euthanized by CO₂ inhalation after 6 weeks and a portion of the leg removed and processed for histology as described below.

After 48 hours of fixation in formalin, samples were dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin, and cut into 7-μm thick sections (Shandon Finesse 325, Thermo Scientific). The sections were stained with haematoxylin and eosin, and then imaged with a Leica DMLB microscope (Leica Microsystems, Bannockburn, IL). Images were analyzed for the presence of inflammatory cells.

3.2.7 Statistical Analysis

The results of the in vitro assays were calculated as the means ± SEM of six independent cultures per variable. The results from the in vivo study examining retention of cells were calculated as the means ± SEM of 4 animals per variable. Statistically significant differences between groups were determined by one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. P values ≤ 0.05 were considered significant. All experiments were repeated at least twice to ensure validity of results, with the exception of the in vivo studies. Data presented are from representative experiments.
3.3. Results

3.3.1 Effect of Osmolytes

3.3.1.1 Microbead Diameter

Microbeads had a spherical morphology that was unchanged by osmolyte type or presence of cells (Fig. 3.1A). Inclusion of cells increased microbead diameters by 30% regardless of osmolyte. All microbead formulations exhibited an increase in diameter when transferred from the crosslinking solution to culture medium, whether or not cells were present. Alginate microbeads without cells had the largest increase in diameter upon transfer to culture media (Fig. 3.1B). A smaller increase was observed in alginate microbeads incorporating rASCs or MG63 cells (Fig. 3.1C-D). Microbeads fabricated with glucose as the osmolyte had the smallest diameter. Furthermore, glucose maintained diameters below 200 µm in microbeads transferred to culture media.
Figure 3.1: The diameter of alginate microbeads crosslinked in calcium chloride solutions made with different osmolytes. (A) Alginate microbeads incorporating no cells suspended in the crosslinking solution and full media, and incorporating rASCs suspended in MSCGM under an inverted light microscope (mag. = 10x). (B) Microbeads incorporating no cells. (C) Microbeads incorporating rASCs. (D) Microbeads incorporating MG63s. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. calcium chloride, $ vs. sodium chloride, # vs. mannitol, + vs. glucose.

3.3.1.2 Effects of Osmolytes on ASC Viability

The type of osmolyte did not impact cell viability (Fig. 3.2A). No differences were noted in the ratio of live to dead cells amongst microbead types, nor was there a difference between 7 and 14 days in culture (Fig. 3.2B).
Figure 3.2: Viability of microencapsulated rASCs crosslinked in calcium chloride solutions made with different osmolytes (Scale bar = 50 µm). (A) LIVE/DEAD staining of released ASCs, where green represents live cells and red represents dead cells and (B) the percent viable cells. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. sodium chloride, # vs. mannitol, a vs. day 7.

3.3.1.3 Growth Factor Production

The osmolyte did not impact production of TGFβ2, TGFβ3, IGF-1 and VEGF, either secreted into the culture media or retained with the microbeads after treatment with CM for 7 days (Table 3.1).
Table 3.1. Growth factor secreted in media and retained in the microbeads by microencapsulated cells treated with chondrogenic media.

<table>
<thead>
<tr>
<th></th>
<th>Growth Factor in Media/DNA (ng/µg)</th>
<th>Growth Factor Retained in Microbeads/DNA (ng/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VEGF</td>
<td>IGF-1</td>
</tr>
<tr>
<td>NaCl</td>
<td>15 ± 1.6</td>
<td>8 ± 0.5</td>
</tr>
<tr>
<td>Mannitol</td>
<td>12 ± 0.7</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>Trehalose</td>
<td>14 ± 1.8</td>
<td>7 ± 0.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>12 ± 0.8</td>
<td>7 ± 0.4</td>
</tr>
</tbody>
</table>

3.3.2 Number of Microbeads per Volume

Growth factor production was sensitive to microbead number per unit volume. As the number of microbeads increased per well, IGF-1, VEGF, TGFβ2 and TGFβ3 production per cell decreased both inside the microbead and in the surrounding media (Fig. 3.3). Compared to beads cultured in MSCGM, microbeads cultured in CM produced and retained reduced levels of VEGF.

The total amount of growth factor produced per well was relatively insensitive to the number of microbeads per unit volume (Fig. 3.3). Compared to microbeads cultured in GM, microbeads cultured in CM produced slightly more IGF-1, but this was only statistically higher in wells with the lowest number of microbeads (Fig. 3.3E). Total VEGF levels in the microbeads and in the conditioned media was elevated when microbeads were cultured at the highest density (Fig. 3.3F). In contrast, the amounts of TGFβ2 and TGFβ3 were comparable at all microbead densities.
Figure 3.3: Effect of microbead density per unit volume on growth factor production by microencapsulated hASCs. Microbeads were cultured for 7 days in MSCGM or CM. Top row - protein levels normalized by DNA: (A) insulin growth factor 1 (IGF-1), (B) vascular endothelial growth factor (VEGF), (C) transforming growth factor beta 2 (TGFβ2), and (D) transforming growth factor beta 3 (TGFβ3) in conditioned media and retained in the microbead. Bottom row – total IGF-1 (E), VEGF (F), TGFβ2 (G), and TGFβ3 (H) in conditioned media and retained in the microbead. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. 12 GM, # vs. 60 CM, $ vs. 12 CM, and ^ vs. 6 CM.

3.3.3 Cells per Microbead

Alginate microbeads encapsulating the smallest number of cells produced the highest IGF-1, VEGF, TGFβ2 and TGFβ3 level per cell (Fig. 3.4). Control microbeads, which contained 37 cells per bead, produced similar levels of IGF-1, VEGF, TGFβ2 and TGFβ3 in MSCGM as microbeads cultured in CM. However, CM suppressed VEGF secreted into the medium although there were no differences in VEGF retention in the microbead.
The total amount of growth factor produced per well was relatively insensitive to the number of cells per microbead (Fig. 3.4). Compared to microbeads cultured in GM, cells cultured in CM retained more IGF-1 in the microbead, but the number of cells per microbead did not impact this result, nor was there an effect on the number of cells on the amount of IGF-1 in the media (Fig. 3.4E). Total VEGF levels in the microbeads and in the media were reduced in CM cultures (Fig. 3.4F). Only in microbeads containing 37 cells was there a statistically relevant increase in VEGF compared to other cell densities and this was only evident in the microbead. Cell number per bead had only minor effects on TGFβ2 and TGFβ3.

Figure 3.4: Effect of cell number per microbead on growth factor production by microencapsulated hASCs. Protein levels normalized by DNA: (A) insulin growth factor 1 (IGF-1), (B) vascular endothelial growth factor (VEGF), (C) transforming growth factor beta 2 (TGFβ2), and (D) transforming growth factor beta 3 (TGFβ3) in conditioned media and retained in the microbead, Total protein levels of IGF-1 (E), VEGF (F), TGFβ2 (G), and TGFβ3 (H) in conditioned media and retained in the microbead. Data were analyzed using one-way ANOVA followed by
Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. 37 GM, # vs. 71 CM, $ vs. 37 CM, and ^ vs. 21 CM.

3.3.4 Effect of Alginate lyase

Release of cells from the microbeads varied with the number of microbeads per well (Fig. 3.5A). At the highest number of microbeads cell release was delayed compared to the other groups. These microbeads released 59% of their cells on day 12. The number of cells per microbeads did not affect the rate of cell release (Fig. 3.5B).

Figure 3.5: (A) The effect of microbead density per well on cell release from microbeads containing 0.06 U/g alginate lyase/alginate over a 12 day period. (B) The effect of number of cells per microbead on cell release from microbeads containing 0.06 U/g alginate lyase/alginate.
Percent DNA of all cells released. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 2.

3.3.5 Cell Viability

Cell viability was greatest when microbeads were cultured in GM compared to CM (Table 3.2). However, there were no differences in viability as a function of the number of microbeads per unit volume amongst the cultures grown in CM. Similarly, cells grown in CM had reduced viability compared to GM, but no differences in cell viability as a function of cells per microbead were noted when microbeads were cultured in CM.

Table 3.2: Effects of microbead number per well and number of cells per microbead on cell viability. Microbead were cultures for 7 days in MSCGM (GM) or chondrogenic medium (CM), * p < 0.05 vs. GM.

<table>
<thead>
<tr>
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<th>CM</th>
<th>CM</th>
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<td>99 ± 0.2</td>
<td>89 ± 2.4</td>
<td>91 ± 1.4</td>
<td>87 ± 1.8*</td>
<td>82 ± 5.2*</td>
<td></td>
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<tr>
<td>Cells/Microbead</td>
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<td>71</td>
<td>37</td>
<td>21</td>
<td>7</td>
<td></td>
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<tr>
<td>% Viability</td>
<td>98 ± 0.4</td>
<td>91 ± 1.6*</td>
<td>90 ± 0.8*</td>
<td>93 ± 0.7</td>
<td>91 ± 2.9*</td>
<td></td>
</tr>
</tbody>
</table>

3.3.6 Degradable Microbeads and Cell Retention

Degradable microbeads were able to retain cells at the treatment site for the 14 day test period (Fig. 3.6). In contrast, sites treated with cell suspensions showed decreased fluorescence as early as day 1. Gastrocnemius muscle injected with microbeads containing 1.75 U enzyme/g alginate exhibited decreased fluorescence at day 8 whereas reduced fluorescence as observed in the deltoid by day 4.
Only the inguinal lymph node had a detectable fluorescence signal. The right inguinal lymph nodes for animals receiving the cell suspension had a significantly higher fluorescence compared to the left inguinal lymph node and right inguinal lymph nodes of animals in the other treatment groups (Fig. 3.7).
Figure 3.6: *In Vivo* localization of rASCs. (A) Fluorescent images indicating the location of delivered cells and (B) percent change in the fluorescence from day 0 in the right gastrocnemius, (C) fluorescent images indicating the location of delivered cells and (D) percent change in the fluorescence from day 0 in the left deltoid of animals receiving a cell suspension (CS), microencapsulated cells in 0 U/g, 0.06 U/g, 0.22 U/g, and 1.75 U/g microbeads on days 1, 2, 4, 6, 8, 10, 12 and 14. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 1 and # vs. day 2.

![Figure 3.6](image)

Figure 3.7: *Ex vivo* organ assessment (A) Fluorescent images of the inguinal lymph nodes, heart, sternum, and lung (B) quantification of the fluorescent signal from both right and left inguinal lymph nodes of animals receiving a cell suspension (CS), microencapsulated cells in 0 U/g, 0.06 U/g, 0.22 U/g, and 1.75 U/g microbeads in the right gastrocnemius and left deltoid on day 14. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test.
* p < 0.05 vs. right inguinal lymph node and # vs. the respective inguinal lymph node in the cell suspension group.

3.3.7 Inflammatory Response to Degradable Alginate Microbeads

Non-degradable microbeads were still present at 6 weeks and histological sections showed no infiltration of white blood cells (Fig. 8A). Degradable microbeads were not present in tissue sections at the end of the experiment and two of the 5 animals had an infiltration of foamy histiocytes. One animal from each group died during the length of the experiment. However, the cause of death was not linked to alginate microbeads or alginate lyase delivered.

Figure 3.8: H&E staining of subcutaneous site of alginate microbead implantation after 6 weeks: (A) alginate microbeads (0 U/g), scale bar represents 500 µm; (B) scale bar represents 100 µm, arrows indicate alginate microbeads; (C) alginate microbeads incorporating alginate-lyase (0.22
U/g), scale bar represents 200 µm, the arrow head indicates the implantation site; and (D) alginate microbeads incorporating alginate lyase (0.22 U/g), scale bar represents 20 µm.

3.4. Discussion

The results of this study confirm that injectable microbeads containing ASCs generated using an electrostatic potential will remain viable in vitro and in vivo. They also show that organic and inorganic osmolytes can be used as alternatives to glucose in the crosslinking solution without negatively impacting cell viability or production of key growth factors, although there are small changes in microbead diameter. The microbeads were transferred to culture medium 10 minutes after being exposed to the cross-linking solution so any positive effects of using a nutrient osmolyte like glucose versus non-nutrient osmolytes like trehalose, mannitol or NaCl was not addressed in this study.

The diameter of the alginate microbeads affects injectability and the mass transfer of nutrients to the encapsulated cells. The smallest diameters were observed when using glucose, but in all cases diameters were 200 µm or less prior to transfer into culture media. Whether cells were present or not, the microbeads increased in diameter once they were transferred from calcium crosslinking solution to the respective media. This may have resulted from a change in pH. It is known that once anionic polymers like alginate are placed in media with a pH higher than the pKa of the polymer’s ionic groups, the swelling ratio increases [39]. A change in the water gradient, produced by a low water concentration in the crosslinking solution (75 mM CaCl₂) and a higher water concentration in the media (1.8 mM Ca²⁺), may also play a role. In addition, alginate hydrogels have been known to swell once they are placed in tissue culture media due to the presence of chelators, monovalent ions, and non-crosslinking divalent cations like Mg²⁺, resulting in an increase in alginate porosity [40, 41]. Viability of cells in vivo is important to the regenerative
success of any cell-based therapy because it affects the ability of the cells to aid in tissue regeneration. The viability of rASCs microencapsulated in all four types of crosslinking solutions had a relatively high viability and this was at 80% across all groups even at two weeks. This confirms our previous observations and suggests that as long as the microencapsulated cells are in a nutrient rich environment, the nature of the osmolyte is not a critical factor. Further support for this is the fact that the osmolyte did not change the ability of the cells to produce growth factors. As a result using any of the osmolytes should be equally acceptable for hASCs. However this may change if other cell types are used.

Our findings indicate that growth factor production is more sensitive to the culture medium than to either the number of microbeads per unit volume or the number of cells per microbead. The results showed that where the smallest number of microbeads were present there was the largest amount of growth factor per cell being produced. This is not surprising as the supply of nutrients is greater when cells are competing for a finite resource. Similarly fewer cells per microbead correlated with greater production of growth factors. This suggests that a therapeutic result may be more effectively achieved using few beads containing fewer cells per bead. Assuming diffusivity to be constant, growth factors secretion into the medium would be sensitive to the medium, as was the case when microencapsulated rASCs were cultured in CM versus MSCGM.

Unlike growth factor production, alginate-lyase mediated cell release was affected by the number of microbeads. As the microbead density increased, the rate of degradation decreased. Microbead degradation involves two mechanisms. At early time points, the enzyme’s activity is more prominent, leading to cleavage of the glycosidic bonds. At later time points, however, the stability of the alginate decreases due to an influx of monovalent ions from the surrounding media.
and subsequent ion exchange with Ca++. Thus at lower microbead concentrations, there is a greater amount of monovalent ions present in each well to displace Ca++ and hence, an earlier release of cells [34]. The number of cells per microbead was only important in so far as the cells reduced the volume of alginate. Only at the lowest number of cells per microbead, was cell release negligible and this may reflect an inability to detect the released cells as much as it reflects the greater alginate content.

The microbeads successfully retain cells at the treatment sites in contrast to cells injected as a suspension. As we reported previously [34], microbeads containing 1.75 U alginate lyase per gram alginate degraded the most rapidly, and released cells from these microbeads migrated from the treatment sites by day 4 in the deltoid muscle and by day 8 from the gastrocnemius. Loss of cells from the muscles may have been affected by differences in vasculature or differences in loading [42]. In both cases however, even in the fast degrading microbeads, cells were effectively retained for up to a week. These results also show that cell retention can be achieved in a controlled manner by varying the enzyme content of the microbeads.

One concern is that the imaging method used in this study is detecting artifact remaining following cell death, as has been observed in studies using fluorescent nanoparticles to label cells [43]. It is unlikely that the GFP-labelled ASCs became distributed to other tissues, however. In the treatment groups where migration was observed, the lymph nodes were the only organs positive for the DiR dye used to stain the cells. A significantly higher fluorescence was present in the right inguinal lymph node, showing that cells may have migrated to that organ since the microbeads were delivered in close proximity to the right lymph node. The lymph nodes belonging to animals receiving the cell suspension had the highest fluorescence. Although the other groups had
detectable fluorescence additional studies must be done to confirm the presence of DiR labelled GFP cells at the treatment site.

Alginate is a biocompatible hydrogel; however, the biocompatibility of alginate lyase or the byproducts of alginate lyase mediated degradation is not known. Our previous results indicate that after a single exposure, no inflammation is present, even when alginate-lyase is included [34]. Here, we examined whether a delayed hypersensitivity reaction was possible. At 6 weeks after a second injection, alginate microbeads were still present whereas alginate microbeads incorporating alginate lyase were no longer detectable. Histological sections from animals receiving non-degradable alginate microbeads had no evidence of macrophages while 2 animals receiving degradable alginate microbeads had an infiltration of foamy histiocytes (macrophages with abundant cytoplasm), indicating the presence of inflammation. It is possible that the byproducts of alginate-lyase mediated degradation may have elicited an inflammatory response earlier in the time line of the study that was resolved. This illustrates that the byproducts of alginate lyase mediated degradation may elicit a mild chronic inflammatory response but comparatively less than the response elicited by sutures [44]. These results are inconclusive with respect to a delayed hypersensitivity reaction.

3.5. Conclusions

This study investigated a number of variables associated with the clinical use of alginate microbeads for tissue regeneration applications. Glucose was found to be the optimal osmolyte as it relates to the smallest alginate microbead diameter. Both microencapsulated cell viability and growth factor production were found to be osmolyte-independent. Only a small number of alginate microbeads and a low cell density may be required to achieve the same levels of growth factor
production as delivering a high number of alginate microbeads with a higher cell density. The rate of cell release can be modulated by the number of microbeads, the number of cells per microbead and the concentration of alginate lyase. Clinically, a low microbead density and a low microbead cell density can be used to achieve similar growth factor production as higher density groups; however the time of cell release may be delayed. Degradable alginate microbeads can be used to localize microencapsulated cells at the delivered site. However, alginate microbeads incorporating alginate lyase may elicit a mild chronic inflammatory response upon repeated exposures.
CHAPTER 4

The Production of Osteogenic and Angiogenic Factors from of Microencapsulated ASCs is Species Dependent and can be Modified by the Pretreatment of Media used.

Summary

Cell-based therapies can provide additional cells at an injury site to aid in regeneration especially when a large amount of tissue is removed or the host’s stem cells are compromised by disease or age. However, present modes of delivering stem cells lead to low viability and cell dispersal, limiting the contribution of the cells to the repair process. We previously showed that adipose stem cells (ASCs) can be localized in tissues by encapsulating them in injectable alginate microbeads generated using an electrostatic potential and Ca$^{++}$ crosslinking solution containing an organic osmolyte. The microencapsulated cells constitutively produce angiogenic factors but when treated with chondrogenic medium, support chondrogenesis in vivo. Here, we investigated the effects of osteogenic medium (OM) on encapsulated rat ASCs (rASCs) and determined the optimal combination of OM components that will lead to the production of both osteogenic and angiogenic factors. Our results showed that microencapsulated rASCs cultured in growth medium (GM) produced angiogenic (VEGF, FGF2) and osteogenic (BMP2) factors. OM, which contains dexamethasone (d) and ascorbic acid (aa), reduced VEGF in cultures of rASCs, mouse ASCs (mASCs) and human ASCs (hASCs), but not in cultures of rabbit ASCs (rbASCs). OM without dexamethasone (OM-d), but not OM without ascorbic acid (OM-aa), restored angiogenic factor production by rASCs to levels observed in GM. Unlike rASCs, BMP2 levels secreted by rbASCs,
mASCs, and hASCs were not affected by treatment media. In conclusion, microencapsulated ASCs can be treated to produce osteogenic and angiogenic factors to aid in the bone repair process, but the effects of the media are species specific.

4.1 Introduction

Paracrine signaling from multipotent mesenchymal stem cells (MSCs) has a therapeutic effect on wound repair. These cells are involved in the three phases of wound repair – inflammation, proliferation, and remodeling, by producing proteins that influence cell migration, proliferation, angiogenesis, and survival of surrounding cells [45]. In bone regeneration, MSCs contribute by producing osteogenic factors, like bone morphogenetic protein-2 (BMP2) and angiogenic factors like fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor (VEGF), in addition to modulating inflammation [46].

Given the important role MSCs play in the bone repair process, augmenting sites of wound repair and fractures at risk for forming non-unions with MSCs would be advantageous, especially in cases where the stem cell population is insufficient or otherwise compromised by disease, immunodeficiency, smoking and age. However, to harness the full potential of these therapies, the cells must be viable and maintain their phenotype after delivery. Unfortunately, delivery as an injectable suspension or on an implantable scaffold often leads to low cell viability, limiting the therapeutic effect of the cells.

We developed an injectable microbead technology using alginate hydrogels to encapsulate cells within 200 µm diameter spheres, thereby protecting the cells from shear during injection and providing a high mass to volume ratio, enhancing their mass transfer properties [23, 47]. Alginate is a co-block polymer that consists of pure mannuronic acid (M) blocks and pure guluronic acid (G) blocks, or alternating MG blocks [24]. The hydrogel is formed when M units form an ionic
bond with a divalent cation. In our system, an alginate-cell suspension is exposed to an electrostatic potential of 6kV to generate the microbeads, followed by cross-linking the alginate in a Ca\(^{++}\) solution containing glucose as an osmolyte. The number of cells per microbead is tunable and depends on the number of cells in the original suspension. Using this technology, we have shown that the encapsulated cells remain viable in cell culture for at least 3 weeks and \textit{in vivo} for at least 3 months [5]. Moreover, encapsulated adipose stem cells (ASCs) are able to produce factors that stimulate angiogenesis \textit{in vitro} and \textit{in vivo}, and when pretreated with chondrogenic medium, produce factors that support chondrogenesis [37, 48].

The goal of the present study was to determine if the microbead technology could be adapted for use in bone regeneration strategies. ASCs are multipotent and have the ability to differentiate into osteoblasts when treated with BMP2 or when cultured in osteogenic medium (OM) containing dexamethasone, ascorbic acid 2-phosphate, and beta-glycerol phosphate [2, 36]. It is not known if microencapsulated ASCs differentiate into osteoblasts when cultured in OM or if OM affects production of either osteogenic or angiogenic factors. We previously showed that growth factor production by monolayer cultures of ASCs was sensitive to treatment with dexamethasone and ascorbic acid 2-phosphate, two of the components of OM [37]. Dexamethasone decreased VEGF; ascorbic acid 2-phosphate increased FGF2. However, when the ASCs were cultured in chondrogenic medium, removal of dexamethasone resulted in an increase in VEGF and removal of ascorbic acid 2-phosphate resulted in a decrease in FGF2. These observations led us to hypothesize that treatment with OM would support osteogenic differentiation of microencapsulated ASCs and stimulate production of factors associated with osteogenesis, including BMP2 and VEGF. Moreover, production of these factors could be tuned by manipulating the dexamethasone and ascorbic acid 2-phosphate content of the OM.
4.2 Methods

4.2.1 Adipose Derived Stem Cells

ASCs were isolated from the inguinal fat pads of 100-125g male Sprague-Dawley rats, male New Zealand White rabbits, and C57 Black 6 (C57Bl/6) mice under Institutional Animal Care and Use Committee (IACUC) approved protocols at the Georgia Institute of Technology and Virginia Commonwealth University as described previously [2, 36].

The cells were separated, plated in T-175 flasks at 5,000 cells/cm² and cultured on tissue culture polystyrene (TCPS) in mesenchymal stem cell growth medium (GM, Lonza, Walkersville, MD) at 37°C and 5% CO2. For the next two days the monolayers were washed with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) and fed with GM every 24 hours. Thereafter media were changed every 48 hours and cells were cultured to confluence. First passage cells were used for all studies.

Human ASCs from three different donors were obtained from Lonza, and cultured to a passage where enough cells were present for each study. 4.2.2 Microencapsulated Cells

To form microbeads, ASCs were encapsulated in UV-sterilized medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginate, FMC BioPolymer) at a concentration of 10⁷ cells/ml. Medical grade alginate was dissolved in 0.9% (w/v) saline (Ricca Chemical, Arlington, TX) at a concentration of 40 mg/ml. Microbeads incorporating cells were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 5 ml/hr flow rate, nozzle with an inner diameter of 0.12 mm, and an electrostatic potential of 6 kV. The crosslinking solution contained 50 mM CaCl₂, 150 mM glucose with 15 mM HEPES [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid] (Sigma Aldrich) at pH 7.3 (Sigma Aldrich) [5, 23]. Alginate microbeads were allowed to crosslink for an
additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca++, and suspended in the respective culture medium.

4.2.3 Effect of Microencapsulation on Growth Factor Production

To investigate the effect of microencapsulation on the ability of ASCs to produce angiogenic and osteogenic factors, microencapsulated ASCs derived from Sprague Dawley rats (rASCs) were compared to monolayer cultures of rASCs grown on TCPS. Microbeads containing rASCs were cultured for five days in GM. At that time, one half of the cultures were maintained in GM and the other half switched to OM for an additional five days. rASCs were plated at 5000 cells/cm² and grown five days in GM, followed by five days in OM. In addition, rat osteoblasts (ROBs) were plated at 5000 cells/cm² and were grown for all 10 days in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) as a positive control.

4.2.4 Effects of Dexamethasone and Ascorbic acid 2-phosphate

To investigate the contributions of dexamethasone and ascorbic acid 2-phosphate to the response of microencapsulated ASCs to OM, microbeads incorporating rASCs (37 cells/microbead) were cultured in GM for 5 days. At that time, media were changed and microbeads were cultured for an additional 5 days in GM, OM, OM without ascorbic acid 2-phosphate (OM-aa), OM without dexamethasone (OM-d), or OM without ascorbic acid 2-phosphate and dexamethasone (OM-aa-d). In order to specifically address the role of dexamethasone, rASC microbeads were cultured in GM for 5 days and an additional 5 days in GM, GM with dexamethasone (GM+d), OM, or OM-d. To examine whether effects of dexamethasone on growth factor production by microencapsulated ASCs is species specific,
microbeads containing ASCs derived from mice, rats, rabbits and humans (37 cells/microbead) were cultured in GM for 5 days and an additional 5 days in GM, OM, and OM-d.

4.2.5 Soluble Factor Quantification

Enzyme-linked immunosorbent assays (ELISAs) were used to measure the levels of vascular endothelial growth factor A (VEGF), fibroblast growth factor 2 (FGF2), and bone morphogenetic protein 2 (BMP2) (R&D Systems, Minneapolis, MN) in the conditioned media of monolayer cultures and microbead cultures. We also assayed any growth factors retained within the microbeads. To do this, beads were collected, uncrosslinked in 82.5 mM sodium citrate, lyophilized, resuspended, digested in alginate lyase (1 U/ml) (Sigma Aldrich) for one hour and assayed.[37]

All ELISAs were normalized by the DNA content of the cells. Cells layers were lysed in 0.05% Triton-X100 (Sigma Aldrich). Cells were collected from the digested microbeads by centrifugation and lysed in Triton-X100. DNA content of the lysates was determined using the Quant-iTTM Picogreen® ds DNA Reagent (Invitrogen) according to the manufacturer’s directions.

4.2.6 RNA Extraction and Real Time PCR

For these analyses, cells were collected by centrifugation from the digested beads and RNA was extracted by adding TRizol [Invitrogen] to the resulting cell pellet, which was homogenized using a Qiashredder (QIAGEN, Valencia, CA). RNA was extracted using chloroform and isopropanol (Sigma Aldrich), and quantified with the Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were then converted into cDNA by the reverse transcription of 1 µg RNA with random primers (Applied Biosystems, Warrington, UK) and Multiscribe Reverse Transcriptase (Applied Biosystems). mRNA levels were measured by real-time PCR using the StepOne Plus PCR System (Applied Biosystems). mRNA levels for the following
proteins were measured; BMP2 (Bmp2), FGF2 (Fgf2), transforming growth factor beta-1 (Tgfβ1) and VEGF-A (Vegfa). In addition the mRNA levels for BMP antagonists, Noggin (Nog) and Gremlin 1 (Grem1) were measured. All primers (Table 4.1) were designed using the Beacon Designer 7.0 program and then synthesized by the Eurofins MWG Operon (Huntsville, AL). mRNA levels were quantified relative to a standard curve of known concentration, and results were normalized to the transcript levels of the housekeeping gene, 40S ribosomal protein S18 (Rps18).

**Table 4.1**: Primer sequences used for the analysis of mRNA levels.

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**4.3 Results**

rASCs cultured on TCPS and in alginate microbeads treated with OM had significantly lower DNA content compared to groups that received GM treatment (Fig. 4.1A). The DNA content of rat osteoblasts was significantly lower than rASCs in monolayer cultured in GM or OM. Microencapsulated rASCs receiving treatments with GM or various combinations of OM and its
components had similar DNA content. In some experiments, the OM-d group had lower DNA compared to OM-aa (Fig. 4.1B) or OM (Fig. 4.1C).

![Figure 4.1](image)

**Figure 4.1:** A) DNA content of confluent monolayer cultures of rASCs grown in GM and OM on tissue culture polystyrene (TCPs). DNA content of rat osteoblasts are shown for comparison. DNA content of microencapsulated rASCs (μB) cultured in GM and OM for 5 days. (B) Effect of removing ascorbic acid (aa) or dexamethasone (d) from OM on DNA content of microencapsulated rASCs. Microbeads were cultured in GM, OM, OM-aa, OM-d, OM-aa-d for 5 days. (C) Effect of dexamethasone on DNA content of microencapsulated rASCs. Microbeads were cultured in GM, GM+d, OM, or OM-d for 5 days. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. GM, $ vs. OM, # vs. TCPs.

Similar VEGF levels were produced by monolayer cultures and microbeads treated with GM. Rat osteoblasts produced 3 times less VEGF compared to rASCs cultured in GM. VEGF levels were reduced after OM treatment in both monolayer and microencapsulated cultures (Fig. 2A). A similar reduction in VEGF levels was seen after microencapsulated rASCs were treated with OM-aa and GM+d (Figs. 4.2B, 4.2C). However, similar VEGF levels were attained as in GM groups once microbeads were treated with either OM-d or OM-aa-d (Figs. 4.2B, 4.2C). OM treated microencapsulated rASCs produced significantly less VEGF compared to GM for many experiments (Fig. 4.2D). Little or no FGF2 was detected in the conditioned media from all cell types cultured on TCPS or microencapsulated rASCs independent of the treatment (Figs. 4.2E –
There was at least a 100% increase in BMP2 levels produced by microencapsulated rASCs cultured in GM or OM compared to their respective monolayer cultures (Fig. 4.2I). The BMP2 levels were equivalent for all cell types cultured on TCPS and regardless of the treatment (Fig. 4.2I). OM-aa, OM-d, and OM-aa-d treated groups secreted significantly less BMP2 compared to the GM treated group (Fig. 4.2J). However, BMP2 levels produced by microencapsulated rASCs treated with OM are similar compared to GM (Fig. 4.2L) despite the fluctuations observed (Figs. 4.2J, 4.2K). A treatment over control analysis for many experiments demonstrates that BMP2 levels between GM and OM are not different.

Figure 4.2: (A) VEGF in the conditioned media of monolayer cultures of rASCs and microencapsulated ASCs cultured for 5 days in GM and OM. Monolayer cultures of rat osteoblasts grown in their respective media for 5 days are shown for comparison. (B) Effect of removing ascorbic acid (aa) or dexamethasone (d) from OM on factor production by microencapsulated rASCs after 5 days in GM, OM, OM-aa, OM-d, OM-aa-d for 5 days. (C) Effect of dexamethasone
on factor production by microencapsulated rASCs cultured in GM, GM+d, OM, or OM-d for 5 days. (D) Effect of OM v. GM on VEGF, FGF2, and BMP2 in conditioned media of microencapsulated rASCs. GM values are indicated by the dashed line at 1.0. (E-H) FGF2 and (I-L) BMP2 were analyzed as described for VEGF. Data shown in A-C, E-G, and I-K are from a representative experiment. Each data point represents a single independent culture. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. GM, $ vs. OM, # vs. TCPS. Data in D, H and L are treatment/control ratios for N = 6 separate experiments and were analyzed using the Mann-Whitney test.

VEGF and FGF2 retained within the microbeads treated with OM were detectable but remained significantly lower than GM treated groups (Figs. 3A - 3H). A similar reduction was observed in the VEGF and FGF2 levels retained within microbeads treated with OM-aa, and GM+d compared to microbeads treated with GM. Removing dexamethasone from OM resulted in VEGF and FGF2 levels similar to those produced by GM treated groups (Figs. 3A – 3G). GM+d treatment led to a similar reduction in VEGF and FGF2 as observed with OM treatment (Figs. 3C, 3G). BMP2 levels retained by microbeads cultured in OM, OM-aa, and OM-aa-d were similar (Fig. 3J) however, a 3 fold increase was observed in OM-d treated groups compared to groups treated with OM (Figs. 3J, 3K). Although significantly higher levels of BMP2 were retained within microbeads treated with OM (Fig. 3I), a treatment over control analysis showed the BMP2 levels retained within microbeads are not significantly different between GM and OM.
Figure 4.3: (A) VEGF retained within the microbeads of microencapsulated rASCs after 5 days of culture in GM or OM. (B) Effect of removing ascorbic acid (aa) or dexamethasone (d) from OM on factor retention by microencapsulated rASCs after 5 days in GM, OM, OM-aa, OM-d, OM-aa-d for 5 days. (C) Effect of dexamethasone on factor retention by microencapsulated rASCs cultured in GM, GM+d, OM, or OM-d for 5 days. (D) Effect of OM v. GM on VEGF, FGF2, and BMP2 in retained in rASC microbeads. GM values are indicated by the dashed line at 1.0. (E-H) FGF2 and (I-L) BMP2 were analyzed as described for VEGF. Data shown in A-C, E-G, and I-K are from a representative experiment. Each data point represents a single independent culture. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. GM, $ vs. OM, # vs. TCPS. Data in D, H and L are treatment/control ratios for N = 6 separate experiments and were analyzed using the Mann-Whitney test.
OM treatment of cells grown on TCPS and in microbeads caused a decrease in Vegfa expression. Rat osteoblasts expressed similar levels of Vegfa as rASCs on TCPS cultured in GM (Fig. 4A). Vegfa mRNA levels (Figs. 4A - 4C) decreased after OM, OM-aa, and GM+d treatment and was restored when dexamethasone was removed from the media. While OM did not reduce Fgf2 expression in monolayer ASCs, it did reduce expression in the microencapsulated ASCs (Fig. 4D). Deletion of dexamethasone from OM or addition to GM increased expression of this factor (Figs. 4E, 4F). Tgfb1 expression was also decreased in microencapsulated ASCs but not affected in monolayer cultures (Fig. 4G). Deletion of dexamethasone from OM increased Tgfb1 expression but addition of dexamethasone to GM had no effect (Figs. 4H, 4I).
Figure 4.4: (A) Vegf expression in monolayer cultures of rASCs and microencapsulated ASCs cultured for 5 days in GM and OM. Monolayer cultures of rat osteoblasts grown in their respective media for 5 days are shown for comparison. (B) Effect of removing ascorbic acid (aa) or dexamethasone (d) from OM on Vegf expression by microencapsulated rASCs after 5 days in GM, OM, OM-aa, OM-d, OM-aa-d for 5 days. (C) Effect of dexamethasone on expression by microencapsulated rASCs cultured in GM, GM+d, OM, or OM-d for 5 days. (D-F) expression of Fgf2 and (G-I) Tgfb1 were analyzed as described for VEGF. Data shown are from a representative experiment. Each data point represents a single independent culture. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. GM, $ vs. OM, # vs. TCPS.

Bmp2 mRNA levels of rASCs grown on TCPS increased with OM treatment, however the opposite was observed in microencapsulated groups (Fig. 4.5A). Removal of dexamethasone from OM reversed the OM inhibition of Bmp2 expression in the microencapsulated cells (Fig. 4.5B) but addition of dexamethasone to GM inhibited Bmp2 expression (Fig. 4.5C). Expression of the BMP2 antagonist Grem1 was unaffected in monolayer cultures whereas it was blocked in microencapsulated ASCs (Fig. 4.5D). Removal of dexamethasone removed the OM inhibition of Grem1 expression but addition of dexamethasone to GM had no effect (Figs. 4.5E, 4.5F). Expression of Nog was increased more than 3-fold in monolayer cultures treated with OM but it was reduced by 50% in cultures of microencapsulated ASCs (Fig. 4.5G). Removal of dexamethasone from OM restored expression to GM levels (Fig. 4.5H) but addition of dexamethasone to GM had no effect.
Figure 4.5: (A) Bmp2 expression in monolayer cultures of rASCs and microencapsulated ASCs cultured for 5 days in GM and OM. Monolayer cultures of rat osteoblasts grown in their respective media for 5 days are shown for comparison. (B) Effect of removing ascorbic acid (aa) or dexamethasone (d) from OM on Bmp2 expression by microencapsulated rASCs after 5 days in GM, OM, OM-aa, OM-d, OM-aa-d for 5 days. (C) Effect of dexamethasone on expression by microencapsulated rASCs cultured in GM, GM+d, OM, or OM-d for 5 days. (D-F) expression of Grem1 and (G-I) Nog were analyzed as described for VEGF. Data shown are from a representative experiment. Each data point represents a single independent culture. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. GM, $ vs. OM, # vs. TCPS.
VEGF and BMP2 secreted into the conditioned media were differentially regulated by OM and by
dexamethasone. OM reduce secretion of VEGF and BMP2 by microencapsulated rat ASCs;
VEGF was restored to GM levels by dexamethasone removal but BMP2 was unaffected (Figs. 6A,
6B). Secretion of neither factor was affected by OM or by OM-d in cultures of microencapsulated
rabbit ASCs (Figs. 6C, 6D). VEGF secretion was blocked by OM in cultures of microencapsulated
mouse ASCs and partially restored by removal of dexamethasone (Fig. 6E), but OM completed
blocked secretion of BMP2 and dexamethasone removal had no effect on this inhibition (Fig. 6F).
For all three human donors, OM reduced secretion of VEGF and deletions of dexamethasone
restored secretion to GM levels but BMP2 in conditioned media was either not detected or was not
affected by OM or OM-d (Figs. 6G - 6I). Variability was also observed for factors retained within
the microbeads. VEGF retained in the rat microbeads was inhibited by OM and restored to GM
levels by removing dexamethasone from OM (Fig. 6M). BMP2 in the microbeads was low in
cultures grown in GM and OM had no effect, but removal of dexamethasone from OM caused a
marked increase in BMP2 in the microbeads (Fig. 6N). OM and OM-d had no effect on VEGF or
BMP2 in microbeads containing rabbit ASCs (Figs. 6O, 6P). VEGF was reduced by OM in
microbeads containing mouse ASCs and was increased by removing dexamethasone from the
medium (Fig. 6Q). No effects of media were observed in mouse microbead BMP2 (Fig. 6R).
Microbeads containing human ASCs all exhibited reduced VEGF when cultured in OM, which
was restored to GM levels by removal of dexamethasone, but no effect of media on BMP2 content
was observed (Figs. 6S - 6X).
Figure 4.6: Comparison of VEGF and BMP2 in the conditioned media microencapsulated ASCs and retained within the microbead after 5 days of culture in GM, OM or OM-d. Microbeads containing rASCs, New Zealand white rabbit ASCs, C57bl/6 mouse ASCs, or human ASCs from three donors were examined. (A–F) VEGF in conditioned media. (G-L) BMP2 in conditioned media. (M-R) VEGF in microbeads. (S-X) BMP2 retained within microbeads. Data shown are from a single representative experiment. Each data point represents 6 independent cultures. Data
were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test.
* \( p < 0.05 \) vs. GM, $ vs. OM.

4.4 Discussion

This study demonstrates that production of growth factors and other regulatory molecules by microencapsulated ASCs is sensitive to the culture medium used and is different from production by monolayer cultures over the same time period and in the same media. The effects of both culture media and microencapsulation vary with the factor being examined. In some cases, regulation occurs at the expression level. There is additional regulation due to the differential retention of proteins within the alginate bead or released into the conditioned media. Importantly, our results show that deletion or addition of individual media components can have a marked effect on expression, retention, and release of both VEGF and BMP2. Finally, our results suggest that species differences may exist. Our data indicate that microencapsulation modifies production of some, but not all factors. When rASCs were cultured in MSC growth medium, comparable levels of VEGF and FGF2 were present in the conditioned media of monolayer cultures and microencapsulated cells. In contrast, the conditioned media of microencapsulated rASCs had higher levels of BMP2 than monolayer cultures. Higher production of BMP2 by microencapsulated cells may be due to the microenvironment present in the 3D system of the microbeads, including cues related to cell shape [49]. MSCs in monolayer culture are flattened and spread in contrast to cells within the alginate hydrogel, which are spherical [50]. Other studies have shown that cytoskeletal arrangement can have profound effects on BMP2 gene expression and protein synthesis [51].

Oxygen tension within the microbead environment may also play a role. Others have shown a positive relation between hypoxia and BMP2 production [52, 53]. A study with
endothelial cells under hypoxia resulted in a 2-fold to 3-fold increase in Bmp2 expression [52]. We did not measure differences in oxygen tension within the bead in the present study. In previous work, we showed that microencapsulated ASCs generated angiogenic factors including VEGF and FGF2 both in vitro and in vivo [37, 48]. This would suggest that the cells within the beads are in a hypoxic environment. However, in the present study while we observed a marked increase in Vegf expression and noted Fgf2 expression, but we did not observe increased VEGF production by encapsulated ASCs compared to monolayer ASCs, nor did we detect FGF2 production. Moreover, expression and production of VEGF and FGF2 were decreased when the microencapsulated ASCs were incubated in OM. This was not due to a reduction in cell number as DNA content of the cultures was not affected by the difference in medium. Osteoblasts in a hypoxic microenvironment have been shown to produce VEGF, but osteoblast differentiation does not appear to be responsible for the results[53]. Although BMP2 was increased in cultures grown in OM, suggesting osteogenic differentiation of the cells, the decrease in VEGF and FGF2 argued against the hypothesis that significant differentiation had occurred under the conditions of the experiment.

This led us to consider if specific components of the OM might be responsible. We focused on two differentiation cues in OM: ascorbic acid 2-phosphate and dexamethasone [54, 55]. Our results showed that removing ascorbic acid 2-phosphate did not alter any of the parameters tested in the study. In contrast, removal of dexamethasone stimulated expression of Vegfa, Fgf2, Tgfβ1, and Bmp2 as well as its inhibitors Grem1 and Nog. There was a corresponding increase in VEGF and FGF2, but not of BMP2 in the conditioned media of the microbead cultures. When we added dexamethasone to GM, we observed comparable effects on expression and protein to OM-treated microbeads, validating the conclusion that dexamethasone was a key player in modulating
production of angiogenic factors, as has been shown by others [56]. Although dexamethasone inhibited expression and production of angiogenic factors in our study, Mostafa et al. demonstrated the importance of dexamethasone on the differentiation of stem cells along the osteogenic lineage and specifically, for the mineralization of cells [57]. It has also been shown that dexamethasone induces osteogenesis via Runx2 upregulation [58].

One explanation for the differences in our study with those reported in the literature is the experimental design. We examined effects on microencapsulated cells, not monolayer cultures as has been done previously. This may have altered the threshold concentration of dexamethasone needed to trigger differentiation. The reported dose of dexamethasone in the literature is 10 nM, [59] which is similar to the dexamethasone concentration in Lonza OM, suggesting that a different optimal dexamethasone concentration may exist for microencapsulated rASCs.

Time course of exposure is also a consideration. Whereas Bmp2 expression was reduced in microbeads cultured in OM compared to GM, it was increased by removal of dexamethasone from OM. Despite the increase in expression, BMP2 was not increased in the conditioned media of OM-d cultures. However, BMP2 was increased in the microbead itself. This suggests that protein production was stimulated but the rate of BMP2 diffusion through the microbead hydrogel was not as efficient as for VEGF or FGF2. Whether this was due to differential affinities of these factors for alginate or to differences in rate of synthesis was not examined. Our data support the conclusion that the overall effect of OM is due to interactions among its components. Removal of ascorbic acid 2-phosphate by itself did not modify the parameters we tested, but in cultures grown in media without ascorbic acid 2-phosphate and dexamethasone, the deletion of ascorbic acid 2-phosphate mitigated the effects of dexamethasone depletion. Expression of Fgf2 in OM-aa-d microbeads was double that seen in OM-d and was increased over levels in GM cultures. In
contrast, expression of Tgfb1 was 40% lower in OM-aa-d microbeads compared to OM-d microbeads. Bmp2 and Grem1 in OM-aa-d microbeads were more than 50% lower than OM-d microbeads but Nog was unaffected. Under the conditions of our study, we did not see a corresponding effect of ascorbic acid deletion on levels of VEGF, FGF2 or BMP2 in the conditioned media, but we did observe a 50% reduction in the amount of BMP2 retained in the microbead, supporting our hypothesis that the hydrogel properties are important considerations in determining the overall effect of medium composition on outcomes. Beta-glycerophosphate has been shown by others [60] to regulate osteoblastic differentiation of MSCs. It should be noted that we did not modify the beta-glycerophosphate content of the OM, so all of our observations are in the context of this important media component being present.

Our results also showcase potential differences in outcomes dependent on the source of ASCs. Microencapsulated ASCs from New Zealand white rabbits did not exhibit reduced VEGF in OM, nor did they show enhanced VEGF when cultured in OM-d. Microencapsulated C57Bl/6 mouse ASCs did not exhibit increased retention of BMP2 within the alginate hydrogel in OM-d media although they did respond similarly to Sprague Dawley rat microbeads to OM-d treatment with respect to BMP2 content of the conditioned media. Moreover, within species variation exists as well. Microbeads containing ASCs from three different human donors had similar responses to GM, OM, and OM-d, with one significant exception: production of BMP2. The findings support use of caution in making definite statements concerning complex cell populations typically present in ASC preparation.

4.5 Conclusions

Microencapsulated rASCs are able to produce osteogenic and angiogenic factors. The expression, production, secretion and retention of these factors are dependent on the culture media
used and on properties of the alginate hydrogel. Dexamethasone is a key factor in OM that regulates angiogenic factor production. In addition, factor production differs among species as well as among individual donors. Therefore, local factor production by microencapsulated ASCs can play an important role in bone regeneration. In the case of clinical applications, pre-screening of human donors would be recommended for their ASCs.
CHAPTER 5

The Development of a Degradable Alginate Microbead System for Bone Regeneration

Summary

Cell-based therapies have potential for tissue regeneration but poor delivery methods lead to low viability or dispersal of cells from target sites, limiting clinical utility. Here, we developed a degradable and injectable hydrogel to deliver stem cells for bone regeneration. Alginate microbeads <200µm are injectable, persist at implantation sites and contain viable cells, but do not readily degrade in-vivo. We hypothesized that controlled release of rat adipose-derived stem cells (ASCs) from alginate microbeads can be achieved by incorporating alginate-lyase in the hydrogel. Microbeads were formed using high electrostatic potential. Controlled degradation was achieved through direct combination of alginate lyase and alginate at 4°C. Results showed that microbead degradation and cell release depended on the alginate lyase to alginate ratio. Viability of released cells ranged from 87% on day 2 to 71% on day 12. Monolayer cultures of released ASCs grown in osteogenic medium produced higher levels of osteocalcin and similar levels of other soluble factors as ASCs that were neither previously encapsulated nor exposed to alginate lyase. Bmp2, Fgf2, and Vegfa mRNA in released cells were also increased. Thus, this delivery system allows for controlled release of viable cells and can modulate their downstream osteogenic factor production.

5.1. Introduction

Approximately 5 to 20% of the total bone fractures that occur yearly fail to heal completely resulting in a nonunion [1]. Treatment frequently involves resection of the nonunion tissue and use
of grafts augmented with autologous bone marrow. One of the factors contributing to non-union is the loss of multipotent stem cells capable of differentiating along the osteoblast lineage [61]. Injectable, cell-based therapies could be especially advantageous for these patients since they are less invasive than current surgical treatments and could aid in bone regeneration. Adipose-derived stem cells (ASCs), which are multipotent [2], are easily isolated, and can be induced to express an osteoblastic phenotype [36]. Recent reports indicate that ASCs are not as osteogenic as bone marrow stem cells, but their ready availability supports their use as a potential cell source [62].

One notable disadvantage of cell based therapies is that injected cells tend to disperse to tissues other than the intended site [3]. Moreover, those cells that do home to the intended site may not remain viable over a sufficient period of time or differentiate into the desired phenotype. This is particularly problematic in tissues like fracture non-unions, where vasculature is often not well developed. Therefore, the goal of this study was to develop a system of injectable hydrogels that would allow ASCs to remain viable at the injury site, maintain their ability to differentiate along the osteoblast lineage, and be released after a given time period.

Alginate hydrogels have gained special attention because of their ability to provide a temporary support for a variety of cell types [63-65] while facilitating the transport of nutrients, gases and metabolic wastes [66]. Alginate microbeads have been used to deliver insulin secreting cells for insulin dependent diabetes [28], parathyroid glands for hypoparathyroidism [29], and stem cells for organ repair [30]. Furthermore, alginate hydrogels can be modified to incorporate specific biochemical cues [24, 67]. Alginate is a block copolymer that consists of (1-4)-linked-β-D mannuronic acid (M units) and α-L-guluronic acid (G units) monomers, where each block may be repeating M units (poly (M)) or G units (poly(G)) or a random organization of M and G units (poly(MG)) [27]. It is present in the cell walls of brown algae and can also be synthesized by
bacteria. The advantage of alginate compared to synthetic polymers is its ability to form a hydrogel under relatively mild pH and temperature, and its ability to be sterilized [27]. Alginate forms a hydrogel once it is cross-linked between the G units of neighboring chains with divalent cations such as Ca\(^{++}\) or Ba\(^{++}\).

When ASCs are encapsulated using an electrostatic potential in a calcium crosslinking solution containing glucose as an osmolyte, the resulting microbeads contain a tunable number of cells in a bead diameter of 200\(\mu\)m. These microbeads were used successfully as a delivery vehicle for subcutaneous injection of ASCs in mice and the cells remained viable for two months [4, 5]. Mass transfer issues are obviated due their small size and high surface to volume ratio. These studies have also shown that encapsulated ASCs can be induced to express a chondrocytic phenotype and to produce chondrogenic factors, suggesting that similar approaches will be successful for bone regeneration [48, 68].

Despite the many advantages of alginate, a central challenge remains – alginate does not degrade enzymatically within the human body and non-enzymic breakdown occurs very slowly. In vitro, release of cells is achieved by chelating Ca\(^{++}\), thereby breaking the crosslinks between chains. There have been attempts to control the rate of degradation of alginate constructs in vivo by decreasing the molecular weight through oxidation and \(\gamma\)-irradiation, thus inducing hydrolytically labile groups [6]. After six months, these constructs maintained their structure but elastic modulus was decreased and cell migration was increased. Another approach involved the encapsulation of poly(lactide-coglycolide) (PLGA) particles loaded with alginate lyase [69], an enzyme that cleaves the \(\beta\)-1,4-glycosidic linkage between the monosaccharide units of the alginate through a \(\beta\)-elimination reaction producing uronate products [26]. Finally another strategy used covalent crosslinking with bifunctional groups to degrade alginate constructs [70]. These efforts
have been partially successful in larger alginate constructs (4mm), but there are no methods to control the degradation of smaller alginate microbeads (200µm – 500µm), which are important for injectable applications.

In the present study, we focused on developing a system of injectable hydrogels to allow the cells to remain at the area of injury, and secrete soluble factors that facilitate bone regeneration. We hypothesized that controlled degradation of alginate microbeads can be used to control the release kinetics of viable cells for regeneration. Our objectives were to (i) develop and optimize a method of incorporating alginate lyase in alginate microbeads; (ii) establish controlled degradation parameters using medical grade alginate; (iii) determine if alginate lyase-mediated alginate degradation has an effect on the viability and phenotype of the released cells; (iv) determine if alginate microbeads containing alginate lyase degrade \textit{in vivo}; and (v) identify if the degradation by-products are toxic and elicit an inflammatory response.

5.2. Methods

5.2.1 Alginate Degradation

To test our hypothesis that time controlled degradation can be achieved by the incorporation of alginate lyase into alginate microbeads, various ratios of alginate lyase (units [U] of enzyme activity) to alginate [g] were combined to form microbeads. Two types of alginates were used: “wound healing” grade alginate (55% to 60% mannuronate units) (Protonal® LF10/60LS, FMC BioPolymer, Sandvika, Norway) and medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginates, FMC BioPolymer). Low viscosity alginate was dissolved in 0.9% (w/v) saline (Ricca Chemical, Arlington, TX) at a concentration of 40 mg/ml. Microbeads were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 5 ml/hr flow rate, nozzle with an inner diameter of
0.175 mm (0.12 mm for microbeads used in the *in vivo* study), an electrostatic potential of 6 kV and a calcium crosslinking solution. The crosslinking solution contained 75 mM CaCl₂, 90 mM glucose with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.3 (Sigma Aldrich) (HEPES-buffered) [70]. Alginate microbeads were allowed to crosslink for an additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca⁺⁺, and suspended in saline supplemented with calcium chloride (1.8 mM Ca²⁺).

Two methods were used to incorporate alginate lyase (28000 U/g, Sigma Aldrich, St. Louis, MO) into the microbeads. Initially, the enzyme was first packaged in polylactide-co-glycolide (PLGA 50:50) (Sigma Aldrich) particles at a w/w ratio of alginate lyase to PLGA of 1:10. The particles were made using the solid in oil in water method as previously described [69]. The PLGA particles were then incorporated into alginate microbeads by encapsulating 1mg PLGA particles to 70 mg alginate. Once the beads were made they were suspended in saline with physiological levels of calcium (1.8 mM Ca²⁺) at 37°C. However this approach did not prove to be successful in degrading the alginate beads.

Alternatively, alginate lyase was incorporated into alginate microbeads by combining equal volumes of 4% low viscosity, high mannuronate (LVM) alginate and a known concentration of alginate lyase solution in 0.9% (w/v) saline. Prior to the combination, both components were kept at 4°C. The final 2% (w/v) alginate was mixed at 1000 rpm for one minute. A series of degradation experiments were done with various ratios of alginate lyase to alginate to achieve the optimal ratios that resulted in controlled degradation over 12 days. The final gradient was 14 U/g to 0.35 U/g for experiments assessing alginate degradation in the absence of cells and 1.4 U/g to 0.09 U/g for experiments assessing degradation in the presence of cells. Prior to assessing
degradation in the presence of cells, experiments assessing alginate degradation in mesenchymal stem cell growth medium (MSCGM, Lonza Biosciences, Walkersville, MD) were performed.

Microbead degradation was monitored by measuring the absorbance of the uronate products in optically clear 96-well plates (UV-Star® Microplate, Greiner Bio One, Frickenhausen, Germany) at 235 nm by using a Synergy H4 Hybrid Reader (BioTek, Winooski, VT) [71] every 48 hours. In addition, the diameter of the microbeads was measured every 48 hours over the 12 day period using images taken with a Leica DMIL microscope connected to a Leica DC 300 camera (Leica, Solms, Germany) and analyzed with Image-Pro Plus software (version 4.5.1.22, 2002, Media Cybernetics, Rockville, MD). The light microscope was used to follow the alginate microbead degradation and cell release from the alginate microbeads. At each time point one image per field was taken for each group.

5.2.2 Adipose Derived Stem Cells

ASCs were isolated from 100-125 g male Sprague-Dawley rats ($n = 6$) [2, 36], under an Institutional Animal Care and Use Committee (IACUC) approved protocol at the Georgia Institute of Technology. Briefly, the inguinal fat pad was removed and transferred to a container with Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 3% sterile-filtered L-glutamine-penicillin-streptomycin (P/S). The tissue was washed three times with Hank’s balanced salt solution (HBSS) (Invitrogen) and then incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) for 30 minutes on a rocker at 37°C. Following trypsinization, the supernatant was discarded. Adipose tissue was cut into small pieces and incubated in a digestive cocktail containing collagenase type I (365 units/mL) (Sigma Aldrich) and dispase (3 units/mL) (Gibco, Carlsbad, CA) for four hours. After incubation, the oily upper layer was removed and the digest was quenched with an equal amount of MSCGM. The cells were
separated, plated in T-175 flasks at 5,000 cells/cm² and cultured in MSCGM at 37 °C and 5% CO₂. For the next two days the monolayers were washed with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) and fed with MSCGM every 24 hours. Thereafter media were changed every 48 hours and cells were cultured to confluence. First passaged cells were used for studies.

ASCs were also isolated from green fluorescent protein (GFP) positive rats (Rat Resource and Research Center, Columbia, MO) under IACUC approval and cultured up to passage 6 for the cell release studies.

5.2.3 Cell Release Studies

To test our hypothesis that controlled release of cells can be achieved through the controlled degradation of alginate microbeads, ASCs were encapsulated in various ratios of alginate lyase to UV-sterilized medical grade alginate. ASCs were released from the culture plate by trypsinization, and collected by centrifugation after rinsing the cell pellet with MSCGM, the cells were suspended in the alginate lyase/alginate mixture at a concentration of 25 x 10⁶ cells/ml. Alginate microbeads were then formed as previously described. Microencapsulated ASCs and GFP-ASCs were suspended in 40µm cell strainers (BD Falcon, Franklin Lakes, NJ) and cultured in MSCGM in non-tissue culture treated 6-well plates to limit proliferation of released cells in the well. Media were changed every 48 hours and each cell strainer was washed twice with 0.9% (w/v) saline to ensure that all the cells released were collected. Additional cells on the plate’s surface were collected through trypsinization.

5.2.3.1 Cell Number

The number of cells released was counted using a Z1 particle counter (Beckman Coulter, Brea, CA). For the groups where the alginate microbeads did not fully degrade by the end of the 12 day period, 82.5 mM sodium citrate was used to release the remaining cells. All cells were
collected, suspended in 0.05% Triton-X100 (Sigma Aldrich) and used to determine the DNA content [Quant-iT™ Picogreen® ds DNA Reagent, Invitrogen, CA] and alkaline phosphatase specific activity. The percent cell release was calculated as a function of the total number cells in the 0 U/g group (control), which reflected any change due to proliferation or cell death over the incubation period. The total number of cells microencapsulated in the control group was determined by the number of cells released on the 12th day using 82.5 mM sodium citrate.

To determine if our observations were applicable to other cell types, microbeads were prepared containing human osteoblast-like MG63 cells (American Type Culture Collection, Rockville, MD). Microbeads were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 1% penicillin-streptomycin.

5.2.3.2 Alkaline Phosphatase Specific Activity

To measure alkaline phosphatase specific activity, released cells were suspended in 0.05% Triton-X100 and lysed by sonication. Enzyme activity was measured as the ability to convert p-nitrophenylphosphate to p-nitrophenol at pH 10.2. This was normalized by the total protein content (Pierce Macro BCA Protein Assay kit, Pierce Biotechnology, Rockford, IL) in the cell lysate.

5.2.3.3 Cell Viability

5.2.3.3.1 LIVE/DEAD Assay

Viability of cells within microbeads and of cells released from the microbeads (released cells) was measured using a LIVE/DEAD Viability kit (Invitrogen). Samples (200µl) were collected at each time point (days 0, 2, 4, 6, 8, 10, and 12) for microbeads without alginate-lyase and on the days where the greatest cell release occurred for the microbeads incorporating alginate lyase. The 200µl aliquots were transferred to a chamber slide and incubated for 30 minutes at 37°C.
in 0.9% saline containing 4μM ethidium homodimer-1 and 2μM calcein-AM. Images were obtained using a Zeiss LSM 700-405 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Three images were obtained for each well and then red and green cells were counted using Image-Pro Plus software (Media Cybernetics) to determine the percent viability.

5.2.3.3.2 TUNEL Assay

To determine the number of cells going through apoptosis, the integrity of the cell’s DNA was measured using the TiterTACS™ Apoptosis Detection Kit following the manufacturer’s protocol (Trevigen, Gaithersburg, MD). At each time point, 300,000 cells from each well were used for the assay.

5.2.4 Phenotype of Released ASCs

For these experiments ASCs that were never microencapsulated or exposed to alginate lyase were compared to ASCs released from microbeads containing either 0 U/g or 0.13 U/g alginate lyase. To investigate the osteogenic potential of the released cells, ASCs were collected at day 8 from the 0 U/g and 0.13 U/g groups, were plated at 5000 cells/cm² and cultured in MSCGM until confluent. ASCs that were never microencapsulated or exposed to alginate lyase were also cultured in MSCGM until confluent. Both cell types were treated with OM for 7 days followed by MSCGM for 24 hours.

5.2.4.1 Alkaline Phosphatase Specific Activity

Alkaline phosphatase specific activity was measured as described in 5.2.3.2.

5.2.4.2 Soluble Factor Quantification

Enzyme-linked immunosorbent assays (ELISA) were used to measure the levels of bone morphogenetic protein-2 (BMP2), fibroblast growth factor-2 (FGF2), osteoprotegerin (OPG), and vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN) in the conditioned
medium. The levels of osteocalcin were measured with a radioimmunoassay kit (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA). All ELISAs and the radioimmunoassay were normalized by the DNA content of the cells.

5.2.4.3 RNA Extraction and Real Time PCR

RNA was extracted 8 hours after the last medium change using TRIzol® [Invitrogen] and quantified with the Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were then converted into cDNA by the reverse transription of 1 µg RNA with random primers (Applied Biosystems, Warrington, UK) and Multiscribe Reverse Transcriptase (Applied Biosystems). The cDNA was analyzed for mRNA levels by real-time PCR using the StepOne Plus PCR System (Applied Biosystems). mRNA levels for the following proteins were measured; BMP2 (Bmp2), type I collagen (Col1), FGF2 (Fgf2), Osterix (Osx), Runx2 (Runx2), osteocalcin (Ocn), and VEGF-A (Vegfa). In addition the mRNA levels for BMP antagonists, Noggin (Nog) and Gremlin 1 (Grem1) were measured. All primers (Table 5.1) were designed using the Beacon Designer 7.0 program and then synthesized by the Eurofins MWG Operon (Huntsville, AL). The mRNA levels were quantified relative to a standard curve of known concentration, and results were normalized to the transcript levels of the housekeeping gene, 40S ribosomal protein S18 (Rps18).
Table 5.1. Primer sequences used for the analysis of mRNA levels.

<table>
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<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>CTT CCG CTG TTT GTG TTT GG</td>
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<tr>
<td>Col1</td>
<td>CGA GTA TGG AAG CGA AGG</td>
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<td>AGA GGC AGA AGT CAG AGG</td>
</tr>
<tr>
<td>Vegfa</td>
<td>GGA CAT CTT CCA GGA GTA CC</td>
<td>TCC AGG GCT TCA TCA TTA CG</td>
</tr>
</tbody>
</table>

5.2.6 Alginate Microbead Degradation *In Vivo*

*In vivo* studies were done to examine if alginate microbeads incorporating alginate lyase would degrade and determine if the degradation by-products results in an inflammatory response. Male C57 Black 6 mice were handled under a protocol approved by the IACUC at the Georgia Institute of Technology. Prior to surgeries, mice were anesthetized using isoflurane gas. Alginate microbeads (0.1 ml) with and without alginate lyase (1.75 U/ml and 0 U/ml) but without cells were injected subcutaneously in the dorsal side using a 16 gauge needle [4]. Each animal received 1 injection subcutaneously \((n = 6\) for each experimental condition). Animals were euthanized by CO\(_2\) inhalation after 2 weeks and a portion of the skin removed and processed for histology as described below.

5.2.6.1 Histology

After 48 h of fixation in formalin, samples were dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin, and cut into 8-μm thick sections.
(Shandon Finesse 325, Thermo Scientific). The sections were stained with haematoxylin and eosin, and then imaged with a Leica DMLB microscope (Leica Microsystems, Bannockburn, IL).

5.2.7 Statistical Analysis of Data

The results of the quantitative and morphometric analyses were calculated as the means ± SEM of six independent cultures per variable. Statistically significant differences between groups were determined by one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. P values ≤ 0.05 were considered significant. All experiments were repeated at least twice to ensure validity of results. Data presented are from representative experiments.

5.3 Results

5.1 Controlled Degradation of Alginate Microbeads

Controlled degradation of alginate microbeads made from both “wound healing” (Fig. 5.1) and medical grade (Fig. 5.2) alginate was a function of alginate lyase concentration. For both alginate types, as the ratio of alginate lyase to alginate increased, the time required for total degradation of the alginate microbeads decreased (Figs. 5.1A and 5.2A). Complete degradation was not observed in microbeads produced using 0.7 U/g in either alginate formulation over the 12 day time course. Degradation of the wound healing grade alginate (28 U/g) occurred within 4 days (Fig. 5.1B) whereas degradation of the medical grade alginate (14 U/g) required 6 days (Fig. 5.2B).
Figure 5.1: Degradation of alginate (wound healing grade) microbeads with different formulations of alginate lyase (U): alginate (g). (A) Visualization of alginate microbead degradation under an inverted light microscope (mag. = 10x), (B) absorbance of uronate products, and (C) diameter of
beads as they degrade over the 12 day period. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 0.

Microbead diameter changed overtime in an enzyme-dependent manner that was formulation specific. Newly generated microbeads had an average diameter of 400µm when placed in the degradation solution (saline with 1.8mM Ca$^{2+}$). Incorporation of 28 U/g alginate-lyase caused wound healing grade alginate bead diameters to decrease by >100µm within 2 days. In contrast microbeads containing 0.7 U/g did not decrease significantly until day 12. There were no changes in diameter in the absence of enzyme (Fig. 5.1C). Similarly, medical grade alginate beads did not change size in the absence of enzyme (Fig. 5.2C). However, diameters increased by 100µm in the presence of alginate lyase and this effect occurred more rapidly at higher enzyme concentrations.

Degradation of the medical grade alginate microbeads with no cells in MSCGM (data not shown) occurred faster compared to the same microbeads suspended in saline supplemented with physiological levels of calcium (1.8 mM Ca$^{2+}$) (Fig. 5.2D).
Figure 5.2: Degradation of alginate (medical grade) microbeads with different formulations of alginate lyase (U): alginate (g). (A) Visualization of alginate microbead degradation under an inverted light microscope (mag. = 10x), (B) absorbance of uronate products, (C) diameter of beads,
and (D) diameter of beads in MSCGM as they degrade over the 12 day period. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 v. day 0.

5.3.2 Release of Cells

Cell release kinetics varied as a function of alginate lyase content (Fig. 5.3A). Medical grade alginate microbeads with the highest concentration of alginate lyase (1.4 U/g) released the cells in the shortest period of time (2 days) (Fig. 5.3B). A similar result was seen in the percent release where the highest percentage of cells (63%) was released on day 2 from the 1.4 U/g microbeads (Fig. 5.3C). DNA content of the released cells confirmed these observations (Fig. 5.3D). Released cells had measureable alkaline phosphatase specific activity (Fig. 5.3F). The highest levels of enzyme activity were seen in cells that were encapsulated in beads with alginate lyase content varying from 0.09 to 0.24 U/g. Similar cell release kinetics were observed using GFP-ASCs (Figs. 5.4A and 5.4B), and confirmed by measuring fluorescence (Fig. 5.4C). Release of MG63 cells exhibited comparable kinetics (Figs. 5.4D and 5.4E). Unlike the ASCs, however, alkaline phosphatase specific activity of the released MG63 cells was low and did not vary with alginate lyase content of the microbeads (Fig. 5.4F).
Figure 5.3: Release of ASCs from alginate (medical grade) microbeads with different formulations of alginate lyase (U): alginate (g) over a 12 day period. (A) Visualization of the released ASCs from alginate microbeads under an inverted light microscope (mag. = 10x), (B) the number of
released ASCs, (C) percent cell release, (D) total DNA content of the released cells, and (E) total alkaline phosphatase in the cell lysate. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 2.

Figure 5.4: Release of GFP-conjugated ASCs and MG63 osteoblast-like cells from alginate (medical grade) microbeads with different formulations of alginate lyase (U): alginate (g) over 12 days. Top row – GFP-ASCs: (A) the number of released cells, (B) total DNA content, and (C) fluorescence. Bottom row – MG63 cells: (D) the number of released cells, (E) total DNA content, and (F) total alkaline phosphatase in the cell lysate. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 2.

5.3.3 Effect of Alginate lyase Mediated Degradation on Viability of Released Cells

Live/dead staining demonstrated that ASCs microencapsulated in alginate without alginate lyase remained viable throughout the culture period (Fig. 5.5A). On day 2, 64% of the cells were viable and on day 12, 79% were viable (Fig. 5.5B). The cells released from the microbeads
remained viable throughout the 12 days (Fig. 5.6A), ranging from 87% on day 2 to 71% on day 12 (Fig. 5.6B). As the cells were released they were not in the process of apoptosis (Fig. 5.6C).

**Figure 5.5**: Viability of ASCs in microbeads without alginate lyase. (A) Live/dead staining of ASCs within the 0 U/g alginate (medical grade) microbeads (Scale bar = 50 µm) on days 2, 4, 6, 8, 10, and 12, where green represents live cells and red represents dead cells, and (B) the percent viable cells. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 2.
Figure 5.6: Viability of ASCs released from alginate (medical grade) microbeads made with different formulations of alginate lyase (U): alginate (g) (Scale bar = 50 µm) (A) Live/dead staining of released ASCs, where green represents live cells and red represents dead cells, (B) the percent viable cells, and (C) TUNEL assay of released ASCs (the horizontal bar represents the positive control generated) at the respective times; 1.4 U/g on day 2, 0.35 U/g on day 4, 0.24 U/g on days 4 and 6, 0.18 U/g on day 8, 0.13 U/g on day 10, and 0.09 U/g on days 10 and day 12. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. day 2.

5.3.4 Effect of Alginate Lyase Mediated Degradation on Cell Phenotype

Microencapsulation in alginate microbeads containing alginate lyase did not alter the osteoblast differentiation potential of ASCs. When compared to ASCs that were never
encapsulated, the released cells exhibited lower DNA content following culture for 7 days in OM but there were no differences due to incorporation of alginate lyase in the microbeads (Fig. 5.7A). Alkaline phosphatase specific activity of the released cells was comparable to that of the never-encapsulated ASCs (Fig. 5.7B). In contrast, ASCs released from the microbeads produced higher levels of osteocalcin than the never-encapsulated cells, but again, no difference was observed as a function of alginate lyase content (Fig. 5.7C). Osteoprotegerin content of the conditioned media was comparable for never-encapsulated ASCs and ASCs released from microbeads containing 0.13 U/g enzyme, whereas cells released from the control microbeads exhibited lower production of this protein (Fig. 5.7D). None of the ASCs produced detectable VEGF following 7 days culture in OM, although all ASCs produced FGF2 (Figs. 5.7E, and 5.7F).
Figure 5.7: The osteogenic potential of ASCs released from alginate microbeads. ASCs that were not encapsulated (monolayer cells) (M), microencapsulated (µE) and released from 0 U/g and 0.13 U/g alginate (medical grade) microbeads were cultured in osteogenic medium for 7 days, followed by MSCGM for 24 hours. (A) DNA content, (B) alkaline phosphatase specific activity in the cell lysate, and levels of the following proteins in the conditioned media: (C) osteocalcin, (D) osteoprotegerin, (E) vascular endothelial growth factor (VEGF), and (F) fibroblast growth factor 2 (FGF2). Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. M.
mRNA levels were more sensitive to whether the ASCs had been microencapsulated and the type of microbead. Runx2 (Fig. 5.8A), Osx (Fig. 5.8B), and Col1 (Fig. 5.8C) mRNAs were comparable in all ASCs. Bmp2 levels were elevated in cells released from the microbeads and this effect was even greater in cells released from microbeads containing alginate lyase (Fig. 5.8D). mRNAs for the BMP inhibitors Gremlin-1 and Noggin were also increased in ASCs released from microencapsulated cells. Whereas Grem1 was highest in cells from microbeads containing enzyme (Fig. 5.8E), Nog was elevated to a comparable extent in cells that had been microencapsulated in both types of microbeads (Fig. 5.8F). Vegf was increased in a comparable manner to Nog (Fig. 5.8G) and Fgf2 was increased in a comparable manner to Grem1 (Fig. 5.8H). Osteocalcin mRNA was insensitive to the prior experience of the ASCs (Fig. 5.8I).
Figure 5.8: mRNA expression in ASCs released from alginate microbeads. ASCs that were not encapsulated (monolayer cells) (M), microencapsulated (µE) and released from 0 U/g and 0.13 U/g alginate (medical grade) microbeads were cultured for 7 days in osteogenic medium, followed by MSCGM for 8 hours. mRNA’s for (A) runt-related transcription factor 2 (Runx2), (B) osterix (Osx), (C) type 1 collagen (Col1), (D) bone morphogenetic protein 2 (Bmp2), (E) Gremlin 1 (Grem1), (F) noggin (Nog), (G) vascular endothelial growth factor (Vegfa), (H) fibroblast growth factor 2 (Fgf2), and osteocalcin (OCN) were measured. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p< 0.05 vs. M.

5.3.5 Alginate Microbeads without Cells Incorporating Alginate Lyase Degrade In Vivo

After two weeks of implantation, microbeads without alginate lyase were present in the tissue of all animals (Fig. 5.9A). There was no apparent immune response (Fig. 5.9B) and the size of the beads was not reduced. In contrast, beads that contained alginate lyase were not present (Figs. 5.9C and 5.9D). There was no immune cell infiltrate in response to the degradation products.
Figure 5.9: H&E staining of subcutaneous site of alginate microbead implantation after 2 weeks: (A) alginate microbeads (0 U/g), scale bar represents 500 µm; (B) scale bar represents 200 µm, arrows indicate alginate microbeads; (C) alginate microbeads incorporating alginate lyase (1.75 U/g), scale bar represents 500 µm, the arrow head indicates the implantation site; and (D) alginate microbeads incorporating alginate lyase (1.75 U/g), scale bar represents 200 µm. The outlined black box represents the area magnified 4 times on the right.

5.4. Discussion

Alginate hydrogels used to deliver stem cells must be degradable in vivo to allow for the release of cells, thus facilitating tissue regeneration. Moreover, the rate of degradation must be controllable in order to fit the clinical application. In order to achieve controlled degradation, our first approach incorporated PLGA particles loaded with alginate lyase in alginate microbeads, as
suggested by Ashton et al. [69]. However this approach did not result in degradation, for a number of reasons. The loading efficiency of the PLGA particles in the microbeads was low, and the amount of released enzyme was insufficient to degrade the alginate. The 4 mm diameter constructs described by Ashton et al. were not only significantly larger than the microbeads used in the present study, but they were fabricated with 50% less alginate, which leads to lower guluronate content resulting in a lower cross-linking density. Hydrogel structures with lower mechanical stability and glycosidc bonds may degrade easily with PLGA loaded with alginate lyase. Moreover, the high electrostatic potential used to generate the microbeads used in the present study causes formation of an alginate-rich shell, which may further limit degradation.

The second approach demonstrated that controlled degradation can be achieved by the incorporation of alginate lyase during microbead production using an electrostatic potential. The effects of the alginate lyase are concentration dependent. As the amount of incorporated enzyme is increased, the rate of microbead degradation is increased. This correlation between enzyme content and alginate degradation was found with two different alginate formulations used in medical applications but there were some differences. Wound healing grade alginate required greater units of alginate lyase to achieve degradation within the 12 day period. In contrast, medical grade alginate degraded over a longer period of time and the diameter of the microbeads increased. Despite both types of alginate having similar ratios of M units to G units, differences in purity as well as alginate chain length may have played a role in the degradation rate [70]. The presence of polyphenols in the wound healing alginate does not appear to be responsible, however, since polyphenols have been shown to reduce the activity of alginate lyase in aerobic degradation [72, 73].
Degradation of alginate microbeads via alginate lyase occurs by two mechanisms; enzymatic cleavage and ion exchange. In the first few days, degradation is primarily due to enzymic cleavage of the glycosidic bonds between the M units. In our system, this was evidenced by the production of uronate products in the first four days of incubation. Once the glycosidic bonds are broken, the Ca\(^{++}\) ion crosslinks become more susceptible to ion exchange. The alginate structure disintegrates as a result of the out flux of Ca\(^{++}\) ions, which are replaced by monovalent ions resulting in decreased cross-linking density and, thus, a weakened hydrogel structure associated with an increase in pore size [74]. This two part mechanism likely contributed to the changes observed in microbead diameter over the 12 day period. The reduced crosslinking density coupled with the inward diffusion of other molecules resulted in the swelling observed in medical grade alginate microbeads. It is possible that a similar process takes place with microbeads produced using wound healing grade alginate, but it is more likely that the rapid cleavage of glycosidic bonds caused erosion of the surface of the microbead resulting in a decreased diameter.

The controlled release of cells was illustrated with various cell types demonstrating the versatility of the delivery system. For all three cell types, 1.4 U/g alginate lyase released most of the cells on day 2, while 0.09 U/g caused a gradual release of cells up to day 12. Alginate degradation in the absence of cells occurred more slowly. The ratio of alginate lyase to alginate was adjusted to account for the difference in degradation rates in the presence of cells. This difference is due to the reduced alginate content in each microbead. Mammalian cells do not possess alginate lyase, ruling out the possibility that native cellular enzymes contributed to microbead degradation. Additionally, the cells begin to leave the microbeads before total degradation occurs as observed with 0.35 U/g group where the majority of the cells were released.
on day 4, but without cells the total degradation did not occur in 12 days. Also in the presence of the cells and as time increases, alginate has been shown to decrease in gel strength [74].

The viability of cells post-injection is critical to the success of injectable cell-based therapies. Injection of stem cells is known to result in low viability ranging from 1% to 32% because of the harsh environment at the injury site and the mechanical disruption of the cell membrane by extensional flow during ejection from the needle [47]. Delivering cells in crosslinked alginate by injection has been shown to improve cell viability because of its protective effects during ejection via “plug flow”. This plug flow occurs when the hydrogel near the wall acts as a lubricant, protecting the rest of the hydrogel by allowing it to stay intact and move through the needle [47].

Once the cells are injected, the alginate can provide further protection while retaining them at the injury site. Our results indicate that cells encapsulated in the microbead formulations tested in this study are viable in the microbead environment, indicating favorable mass transfer of nutrients. The small increase in the number of viable cells noted in microbeads produced without enzyme may have been due to proliferation or to retention of secreted factors within the microbead [48] or to limited apoptosis. Moreover, inclusion of alginate lyase in the alginate did not have a negative impact on viability of ASCs released from the microbeads. Indeed, viability of cells released from microbeads containing alginate lyase was greater than from microbeads without enzyme. This suggests that the weakened structure of the alginate microbead due to cleavage of glycosidic bonds may have improved nutrient transfer.

Importantly, ASCs released from the microbeads did not exhibit major differences from cells that had never been microencapsulated, at least as a function of growth in osteogenic medium. At the time of release, cellular alkaline phosphatase specific activity was comparable for all
formulations of microbead. Released cells cultured in monolayer to confluence and then for 7 days in osteogenic medium had comparable alkaline phosphatase activity to ASCs that had never been previously microencapsulated. Similarly, mRNA levels for the osteogenic transcription factors Runx2 and Osx were not different compared to the monolayer cells, nor were mRNA levels for Col1.

Our results also suggest that microencapsulation may enhance the osteogenic potential of the ASCs with respect to their ability to produce paracrine factors that stimulate bone formation. The released ASCs exhibited reduced DNA content and increased osteocalcin production compared to the never-encapsulated ASCs after 7 days in OM. This was correlated with increased levels of Bmp2 mRNA, suggesting that BMP2 might act as an autocrine/paracrine regulator of the microencapsulated cells. Interestingly, expression of Bmp2 was accompanied by expression of its inhibitors, Grem1 and Nog, but in a differential manner. The significance of this is not known at this time, but other studies have shown that these proteins are regulated independently [75].

There was not complete convergence between mRNA levels at 8 hours post-treatment with osteogenic medium and protein levels at 24 hours for a number of factors, including osteocalcin, VEGF and FGF2, which may reflect normal cellular transcription and translational controls. Interestingly, mRNA expression was more sensitive to differences in microbead formulation. Cells encapsulated in alginate containing alginate lyase had greater expression of Bmp2, Grem1 and Fgf2 than cells that had been encapsulated in alginate without enzyme. Similarly, for some mRNAs, having the experience of encapsulation caused greater expression than seen in ASCs that had not been encapsulated, even though the same starting population of ASCs was used for these experiments. Previous research has shown that glucocorticoids can decrease production of angiogenic factors [56], suggesting that the dexamethasone present in osteogenic medium may
have inhibited VEGF production by the cells. The fact that Vegf mRNA was not affected and that the ASCs produced both Fgf2 mRNA and FGF2 protein argues against this, however.

It is important that the results gathered in vitro can be obtained in vivo to ensure that the delivery system is capable of working. Therefore alginate microbeads without cells (0 U/g and 1.75 U/g) were placed subcutaneously to determine if degradation would occur and whether or not the by-products of alginate lyase mediated degradation would elicit an inflammatory response. At the end of two weeks the alginate microbeads incorporating alginate lyase were not present thus showing that degradation took place while in the control group alginate microbeads were present in all the samples. The histological analysis of the skin shows that there was no leukocyte infiltration thus indicating no inflammatory response.

5.5. Conclusions

In conclusion, we have developed a degradable and injectable delivery system for stem cells where the time of cell release can be modulated for specific applications. The controlled degradation was achieved through the incorporation of alginate lyase into alginate microbeads. This system has shown that it can be used with multiple cell types. The cells released from the microbeads remained viable and maintained their ASC phenotype with respect to growth in osteogenic medium. Importantly, the results also show that mRNA expression of released cells is more sensitive to microbead formulation than protein production at the time points assayed. The released cells express higher levels of mRNAs associated with paracrine regulation of osteogenesis, suggesting they may positively impact bone formation in vivo. These microencapsulated cells can be directly injected to the site of injury. Therefore, in cases where surgery is usually required for fractures or non-unions, this stem cell delivery method can be used,
allowing controlled release of cells and the production of factors thus enhancing tissue regeneration.
CHAPTER 6

Microencapsulated Rabbit ASCs supported Chondrogenesis in a Rabbit Auricular Defect Model

Summary

Cell-based tissue engineering can promote cartilage tissue regeneration, but cell retention in the implant site post-delivery is problematic. Alginate microbeads containing adipose stem cells (ASCs) pretreated with chondrogenic media (CM) have been used successfully to regenerate hyaline cartilage in critical size defects in rat xyphoid suggesting that they may be used to treat defects in elastic cartilages like the ear. To test this, we used microbeads containing rabbit (rb) ASCs in critical size defects in New Zealand White rabbit ear cartilage. Low viscosity, high mannuronate medical grade alginate microbeads were generated using a high electrostatic potential and a calcium crosslinking solution containing glucose. rbASCs were isolated from inguinal fat pads of male New Zealand White rabbits and their multipotency validated. Microencapsulated rbASCs cultured in chondrogenic media expressed mRNAs for aggrecan, type II collagen, and type X collagen. Microbeads were implanted in 3mm defects created using a trephine in the mid-cartilage located in the ear of each 6 skeletally mature male rabbits (empty defect; microbeads without cells; microbeads with cells; degradable microbeads with cells; and autograft). At 12 weeks post-implantation, cartilage regeneration was assessed by microCT and histology. Microencapsulated rbASCs induced cartilage regeneration as well as formation of bone-like tissue in the defects. Elastin, the hallmark of auricular cartilage, was not evident in the neocartilage. This
delivery system offers the potential for regeneration of auricular cartilage but factors that induce elastin and consideration of the vascularity of the treatment site must be considered.

6.1 Introduction

Cartilage replacement is one of the top five cosmetic surgical procedures performed each year involving the ear and nose, as well as congenital birth defects in the United States [1]. Cartilage is difficult to regenerate due to its avascular nature making any repair or replacement of this tissue slow or challenging to achieve. Therapies that repair elastic cartilage include implantation of cell-laden scaffolds [76] and reshaping cartilage harvested from another site within the body [1], but these approaches frequently do not lead to complete cartilage regeneration. Moreover, donor site morbidity and lack of available tissue in the case of grafting, and cell migration compounded with low cell viability in the case of tissue engineering techniques, limit success [77, 78].

A more focused approach using a cell-based therapy that would maintain the viability, retention, and phenotype of the cells once implanted should improve tissue regeneration. We previously showed that alginate microbeads containing adipose stem cells (ASCs) pretreated with chondrogenic media could be used successfully to regenerate hyaline cartilage in critical size defects in the rat xyphoid [48]. This suggested that a similar approach could be used to treat critical size defects in elastic cartilages like the ear.

Alginate is found in seaweed and is a block copolymer that contains blocks of guluronic acid, mannnuronic acid, or alternating guluronic-mannuronic acid residues [24]. This biopolymer instantaneously forms a hydrogel once it comes in contact with calcium forming an ionic bond between guluronic acid and Ca++. The material is currently used in biomedical engineering for drug delivery, regenerative medicine, and wound healing [79, 80]. Using a microencapsulator to
apply an electrostatic charge in combination with a calcium crosslinking solution containing glucose, cells can be incorporated into alginate microbeads that are ≤200 µm in diameter, with cell viability greater than 85% in vitro after 2 weeks [81]. Moreover, cells within the beads are viable after 3 months in vivo [4]. The hydrogel system provides small diffusion distances allowing the exchange of nutrients, wastes and secreted factors.

Despite the biocompatibility of alginate, the polymer does not degrade in vivo. Alginate may persist up to six months at the delivery site, thus preventing the release of cells and secreted factors [6]. Recently, we showed that controlled cell release from alginate microbeads can be achieved by incorporating alginate lyase [34]. This enzyme acts through a β-elimination mechanism to cleave the glycosidic bonds between mannuronate residues [26]. Controlling the rate of release by varying the rate of microbead degradation allows the encapsulated cells to aid tissue regeneration in addition to the secretion of factors, which affects cell signaling and induces endogenous progenitor recruitment to facilitate tissue repair [81].

In this study, we developed an auricular cartilage critical size defect model in adult male New Zealand White rabbits and used it to examine the utility of the microbead technology for regenerating elastic cartilage. We hypothesized that the controlled release of rbASCs and secreted factors will lead to induction of cartilage in an auricular critical size defect.

6.2. Methods

6.2.1 Adipose Derived Stem Cells

rbASCs were isolated from New Zealand White rabbits rats (n = 2) [2, 36], under an Institutional Animal Care and Use Committee (IACUC) approved protocol at the Georgia Institute of Technology. Briefly, the inguinal fat pad was removed and transferred to a container with
Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 3% sterile-filtered L-glutamine-penicillin-streptomycin (P/S). The tissue was washed three times with Hank’s balanced salt solution (HBSS) (Invitrogen) and then incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) for 30 minutes on a rocker at 37°C. Following trypsinization, the supernatant was discarded. Adipose tissue was cut into small pieces and incubated in a digestive cocktail containing collagenase type I (365 units/mL) (Sigma Aldrich) and dispase (3 units/mL) (Gibco) for four hours. After incubation, the oily upper layer was removed and the digest was quenched with an equal amount of MSCGM. The cells were separated, plated in T-175 flasks at 5,000 cells/cm² and cultured in MSCGM at 37 °C and 5% CO₂. For the next two days the monolayers were washed with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) and fed with MSCGM every 24 hours. Thereafter media were changed every 48 hours and cells were cultured to confluence. First passaged cells were used for the phenotype studies.

6.2.2 Phenotype of rbASCs

To investigate the multipotency of rbASCs, cells were plated at 5000 cells/cm² and cultured in MSCGM until confluent. rbASCs were treated with MSCGM, osteogenic differentiation medium (OM, Lonza), chondrogenic differentiation medium (CM) and adipogenic differentiation medium (AM, Lonza) for 18 days. The CM was made with high glucose DMEM (4.5 g/L glucose), 110 mg/L sodium pyruvate (Sigma Aldrich), 50 µg/mL ascorbic acid 2-phosphate (Sigma Aldrich), 10 nM dexamethasone (Sigma Aldrich), 1% ITS+ (Sigma Aldrich), 40 µg/mL proline (Sigma Aldrich), 100 ng/mL of the recombinant human bone morphogenetic protein 6 (PeproTech, Rocky Hill, NJ), and 10 ng/mL recombinant human transforming growth factor beta-1 (R&D Systems, Minneapolis, MN).
6.2.2.1 RNA Extraction and Real Time PCR

RNA was extracted 8 hours after the last medium change using TRIzol ® [Invitrogen] and quantified with the Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were then converted into cDNA by the reverse transcription of 1 µg RNA with random primers (Applied Biosystems, Warrington, UK) and Multiscribe Reverse Transcriptase (Applied Biosystems). The cDNA was analyzed for mRNA levels by real-time PCR using the StepOne Plus PCR System (Applied Biosystems). mRNA levels for the following proteins were measured; collagen type II (COL2), collagen type X (COL10), runt-related transcription factor 2 (RUNX2), peroxisome proliferator-activated receptor gamma (PPAR-γ), aggrecan (ACAN), leptin receptor (LEPR), and osteocalcin (bone gamma-carboxyglutamate protein, BGLAP). All primers (Table 6.1) were designed using the NCBI program and then synthesized by Integrated DNA Technologies (IDT). The mRNA levels were quantified relative to a standard curve of known concentration, and results were normalized to the transcript levels of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
Table 6.1. Primer sequences used for the analysis of mRNA levels

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</table>

6.2.2.2 Histological Stains

Multipotency of rbASCs was also demonstrated via the use of histological stains of cultured cells. P1 rbASCs were plated at 5000 cells/cm² in 6-well plates. At confluence, cells were treated with MSCGM, OM, and AM for 14 days. Cells differentiated along the osteogenic lineages were stained with alkaline phosphatase and Alizarin Red. Cells differentiated along the adipogenic lineage were stained with Oil Red-O. Cell differentiation along the chondrogenic lineage was performed using pellet culture. Briefly, 150,000 cells were spun at 5000 RPM for 10 minutes in a 15 mL conical tube. Pellets were cultured for 14 days in CM. The pellet was embedded in paraffin and sections were stained with Alcian Blue. All stains were compared to an equivalent sample cultured in MSCGM as the control. In addition, sections were examined for the presence of elastin as described below for histologic analysis of regenerated ear cartilage.
6.2.3 Microencapsulated Cells

Low viscosity medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginate, FMC BioPolymer) was dissolved in 0.9% (w/v) saline (Ricca Chemical) at a concentration of 40 mg/ml. Microbeads were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG) at a 5 ml/hr flow rate, nozzle with an inner diameter of 0.12 mm, an electrostatic potential of 6 kV and a calcium crosslinking solution. The crosslinking solution contained 75 mM CaCl$_2$, 90 mM glucose with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.3 (Sigma Aldrich) (HEPES-buffered) [70]. Alginate microbeads were allowed to crosslink for an additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca$^{++}$, and suspended in saline supplemented with calcium chloride (1.8 mM Ca$^{++}$) at 37°C.

Degradable alginate microbeads were made by incorporating alginate lyase in the microbeads following the methods described in Leslie et al. [34]. Briefly, equal volumes of 4% alginate and a known concentration of alginate lyase solution combined in 0.9% (w/v) saline. At confluence, rbASCs were treated with CM for 7 days. The cells were released from the culture plate by trypsinization and collected by centrifugation after rinsing the cell pellet with MSCGM. The cells were then suspended in the alginate lyase/alginate mixture at a concentration of 10 x 10$^6$ cells/ml. Non-degradable microbeads incorporating rbASCs were made similarly with the exception of adding alginate lyase. Alginate microbeads were then formed as previously described and suspended in MSCGM for 24 h.

6.2.3 Phenotype of Microencapsulated rbASCs

To investigate whether or not microencapsulation affects the chondrogenic effects of CM, microencapsulated rbASCs (10 x 10$^6$ cells/ml) were treated with MSCGM for 3 days then CM or MSCGM for 7 days.
6.2.3.1 Soluble Factor Quantification

Enzyme-linked immunosorbent assays (ELISA) were used to measure the levels of vascular endothelial growth factor A (VEGF), transforming growth factor beta 2 (TGFβ2), bone morphogenetic protein 2 (BMP2) and transforming growth factor beta 3 (TGFβ3) (R&D Systems, Minneapolis, MN) in the conditioned medium and retained in the microbeads. To measure growth factor retention within microbeads, beads were collected, uncrosslinked in 82.5mM sodium citrate, lyophilized and resuspended and digested in alginate lyase (1U/ml) (Sigma Aldrich) for one hour and measured with ELISAs. All ELISAs were normalized by the DNA content of the cells.

6.2.3.2 DNA Quantification

The digested microbeads were used to determine the DNA content (Quant-iT™ Picogreen® ds DNA Reagent, Invitrogen) of the microencapsulated cells.

6.2.3.2 mRNA Levels

ACAN, COL2, and COL10 mRNA levels were measured as previously described in section 6.2.2.1 and normalized by GAPDH.

6.2.4 Cartilage Ear Defect In Vivo

6.2.4.1 Rabbit Auricular Cartilage Defect Surgery

All surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology. Male New Zealand white rabbits (2 – 3 kg, n = 6) were induced with a ketamine and xylazine intramuscular injection; anesthesia was maintained with inhaled isofluorane gas. Each animal was placed prone on the operating table and a 6 cm incision was made extending from the tragus to the mid-helix. A flap was created both anteriorly and posteriorly by elevating the perichondrium from the auricular cartilage. To create a full thickness defect, a dermal biopsy punch (Miltex Inc., Plainsboro, New Jersey) was used. In each ear, defects were made by placing a surgical stainless steel ruler in the posterior flap for
support and a dermal biopsy punch was used to create a full thickness cartilage defect. The skin incision was closed using 5-0 PDS (polydiaxanone) suture (Ethicon, Somerville, NJ) using a simple running technique.

6.2.4.2 Determining Critical Cartilage defect size

Prior to assessing the ability of microencapsulated rbASCs (n = 3) to initiate cartilage regeneration, a rabbit auricular cartilage critical size defect model was developed. Cylindrical full thickness defects were created using 1, 3 and 4 mm dermal biopsy punches (Miltex Inc). The defects were separated by 1 mm. In one ear of each animal, the autograft for the respective defect size was used to fill the defect to compare healing. After six weeks, the defects were examined for the degree of healing using equilibrium partitioning of an ionic contrast agent microCT (EPIC-microCT) (Scanco Medical) [82, 83].

6.2.4.3 Implantation of Microencapsulated rbASCs

The protocol used to assess the ability of the microencapsulated rbASCs to regenerate auricular cartilage is shown in Figure 6.1. rbASCs were cultured in CM for 7 days and microencapsulated in low viscosity, high mannuronate alginate microbeads as described above. Five 3 mm defects were made in each ear. Four defects were separated by 1.5 cm and a fifth defect (autograft) was created medially 1.25 cm away from the most superior defect. A 4 mm x 4 mm piece of SepraFilm ® (Genzyme Corporation, MA) was placed posteriorly to each defect. Each defect was filled with the respective treatment as outlined in the experimental schematic (Fig. 6.1). The five treatments in each ear included empty defect, non-degradable alginate microbeads containing rbASCs, non-degradable alginate microbeads, degradable alginate microbeads containing rbASCs, and an autograft. Each defect with a microbead treatment received 100 µl of microbeads (approximately 2500 microbeads). Each defect was sealed with a 4 mm x 4 mm piece
of SepraFilm. After 12 weeks the posterior skin was removed and the defects were recovered. A total of 6 rabbits were used with each ear receiving the procedure, resulting in a total of 12 samples per group.

Figure 6.1. Schematic outlining the experimental method. rbASCs that were previously treated with chondrogenic media for 7 days were microencapsulated and delivered to the appropriated defect site: 1. empty, 2. non-degradable microbeads incorporating rbASCs, 3. non-degradable microbeads without rbASCs, 4. degradable microbeads incorporating rbASCs, and 5. autograft. After 12 weeks the defects were harvested and analyzed via microCT and histology.

6.2.4.4 Epic MicroCT to Assess Neocartilage Formation

To assess the degree of healing in each defect, contrast-enhanced microCT analysis technique based on equilibrium partitioning of an ionic contrast agent (EPIC-microCT) was used [82]. Using this method we quantified the tissue volume and density within each defect. Once the defects were harvested they were placed in 10% neutral buffered formalin (VWR) for 24 h and transferred to 40% Hexabrix and 60% phosphate buffered saline incubated for 12 h at 37°C. All
samples were scanned in air using a µCT 40 (Scanco Medical) at 45 kVp, 200 µA, 600 ms integration time, and a voxel size of 35 µm. Images were processed using the accompanying Scanco Medical software. A constant volume of interest representing the absolute volume centered at the defect cavity was used for the analysis of each sample. The diameter of the volume of interest was 3 mm by 3 mm. To observe three-dimensional distribution of sulfated glycosaminoglycan (GAG) in the tissue, color images were based on the X-ray attenuation. Low X-ray attenuation (green and blue) corresponded to regions of high proteoglycan concentration and high X-ray attenuation (red and yellow) indicated regions of low proteoglycan concentration.

6.2.4.5 Histology

After 48 h of fixation in neutral buffered formalin, samples were dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin, and cut into 8 µm thick sections (Shandon Finesse 325, Thermo Scientific). The sections were stained with haematoxylin and eosin, safranin O, or Masson’s trichrome and then imaged with a Zeiss Observer.Z1 axio microscope (Leica Microsystems, Bannockburn, IL).

In addition, we examined the tissues for the presence of elastin using ab150667 Modified Verhoeff’s stain kit (Abcam, Cambridge, UK). Deparaffinized, hydrated sections were incubated for 15 minutes in elastic stain containing haematoxylin solution, ferric chloride, and Lugol’s iodine solution. After rinsing in tap water, the sections were dipped 20 times in a differentiating solution, rinsed and incubated in sodium thiosulfate solution for 1 minute. After rinsing in tap water, they were stained using van Gieson’s solution for 2-5 minutes, rinsed twice in 95% alcohol, dehydrated in absolute alcohol, cleared and mounted. This method stains elastic fibers black to blue/black, nuclei are blue to black, collagen is red, and muscle and other tissues are yellow.
6.2.4.6 Histomorphometric Analysis

To analyze the ability of the microencapsulated rbASCs to induce cartilage regeneration, the area of the new cartilage and the defect width were measured using IMAGE J software (NIH, Bethesda, MD). The area of new cartilage was identified by a color change in the H&E staining - mature cartilage (dark purple) to newly formed cartilage (light purple). Percent healing was defined as the summation of all cartilage or bone-like tissue within the borders of the defect divided by the width of the original defect.

6.2.5 Statistical Analysis

Data are presented as the mean with standard error. Statistically significant differences between groups were determined by one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. P values ≤ 0.05 were considered significant.

6.3. Results

6.3.1 Multipotentiality of rbASCs

Gene expression was sensitive to the culture media in which the rbASCs were cultured. rbASCs treated with CM for 18 days had higher mRNAs for COL2, ACAN, and COL10 than cells cultured in GM (Figs. 6.2A,B,C), but did not produce elastin (data not shown). Similarly, rbASCs in OM had significantly higher mRNAs for RUNX2 and BGLAP compared to rbASCs treated with GM for the same period (Figs. 6.2D and 6.2E). Cells cultured in AM had significantly higher mRNAs for LEPR and similar levels of mRNA for PPAR-γ compared to those treated with GM (Figs. 6.2F and 6.2G).
Gene expression was confirmed by histologic staining of the cultures. rbASCs cultured as a pellet in CM for two weeks showed a higher presence of proteoglycans with Alcian Blue staining than cells in GM (Fig. 6.2H). rbASCs cultured in OM for two weeks had greater calcium deposition and alkaline phosphatase staining than cells in GM (Fig. 6.2I), while rbASCs treated with AM had a greater presence of oil droplets with Oil Red O staining (Fig. 6.2J).

Figure 6.2: Multipotency of rbASCs. mRNA levels for COL2, ACAN, COL10, RUNX2, BGLAP, PPAR-γ, and LEPR (A-G); Alcian Blue staining of a 3D culture of rbASCs cultured in MSCGM and CM (H); alkaline phosphatase (ALP) and Alizarin Red staining of rbASCs cultures in MSCGM or OM (I); and Oil Red O staining of monolayer cultures cultured in MSCGM or AM (J). Magnification 10x.
6.3.2 Chondrogenic Phenotype of Microencapsulated rbASCs

Microencapsulated rbASCs exhibited chondrogenic differentiation when cultured in CM. mRNAs for ACAN, COL2, and COL10 were increased compared to microencapsulated cells cultured in GM (Figs. 6.3A,B,C). Levels of TGFβ2 and TGFβ3 in the conditioned media and retained within alginate microbeads were significantly higher than in GM cultures (Figs. 6.3D,E,F,G). BMP2 levels were comparable when microbeads were cultured in CM or GM (Figs. 6.3H,I). VEGF levels in the conditioned media of microbeads cultured in CM were significantly lower compared to GM cultures, but there was no difference in the amount of VEGF retained within the microbeads (Figs. 6.3J, K).

![Phenotype After Microencapsulation](image_url)

Figure 6.3. Phenotype of rbASCs after microencapsulation. mRNA levels for ACAN, COL2, and COL10 (A-C); and production of TGFβ2, TGFβ3, BMP2, and VEGF by microencapsulated
rbASCs treated with MSCGM and CM for 7 days. Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. MSCGM.

6.3.3 Determination of Rabbit Auricular Cartilage Critical Size Defect

After six weeks, empty defects of 1 mm, 3 mm, and 4 mm in diameter did not regenerate, as shown in the histological sections (Figs. 6.4A,B,C). A similar result was observed in the microCT images of 1 mm and 3 mm diameter empty defects (Figs. 6.4A,B). Neocartilage was not observed at the margins of the autograft and defect suggesting the autograft did not integrate with the adjacent cartilage tissue (Figs. 6.4D,E,F). MicroCT confirmed the presence of cartilage in the autografted defect sites. Based on these results, all subsequent surgeries were performed using a 3 mm defect.

![Figure 6.4](image_url)

Figure 6.4. Critical size defect in auricular cartilage. H&E staining and microCT images of empty defects (A, B, C) and the corresponding autografts of 1, 3, and 4 mm in diameter (D,E,F).
6.3.4 EPIC-microCT Analysis

Analysis of the defect sites at 12 weeks post-surgery showed that sites receiving autograft had a significantly greater total volume of tissue compared to all other groups (Fig. 6.5A). The average attenuation of the defect site was significantly greater when defects were treated with degradable microbeads containing rbASCs compared to all other groups (Fig. 6.5B). Empty microbeads and autograft had similar levels of attenuation, and were significantly higher than empty defects. Representative images of the defect locations and attenuation maps are shown in Figures 6.5C and 6.5D respectively.

Figure 6.5. MicroCT analysis of tissue in defects at 12 weeks post-surgery: tissue volume within the defect site (A), average attenuation of the defect site (B), 3D images of ear with defect location (C); and attenuation map of defects (D). Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. empty defect, $ vs. µBs with rbASCs, ^ vs. degradable µBs with rbASCs.
6.3.5 Histomorphometric Analysis of Cartilage Ear Defects

Histological analysis showed the infiltration of fibrous tissue after 12 weeks in empty defects (Fig. 6.6A). In contrast, autografts had the appearance of normal auricular cartilage but as noted above, it was not integrated with the margins of the defect (Fig. 6.6B). Autografts did not lead to either partial or complete healing. While the autograft maintained the structural properties of cartilage, there was no reintegration of the autograft with the defect tissue. Empty microbeads were still observed in the defect site surrounded by fibrous connective tissue (Figs. 6.6C,D). Non-degradable alginate microbeads incorporating rbASCs initiated cartilage regeneration, and in one case resulted in complete regeneration of the cartilage within the defect (Fig. 6.6E). Large areas of newly formed cartilage were observed in these samples.

![Figure 6.6](image)

**Figure 6.6.** Defects after 12 weeks. H&E staining of: A – empty defect, B - autograft, C - non-degradable microbeads without cells, and D - magnified view of microbeads in C. E - H - Defects incorporating non-degradable microbeads containing rbASCs. Defects treated with non-degradable microbeads containing rbASCs exhibited: newly generated cartilage, cartilage interwoven with calcified tissue and immature bone-like tissue. Sections of one defect stained with H&E (F), Safranin O (G), and Masson’s trichrome (H). Black arrows point to the newly formed cartilage and yellow arrows point to the original cartilage.
Degradable alginate microbeads containing rbASCs produced a different tissue response. Bone-like tissue adjacent to the defect margins was evident in H&E stained sections of the defect sites (Fig. 6.7F). This region also positively stained with safranin O, indicating cartilage was present as well (Fig. 6.7G). Masson’s trichrome staining of these samples confirmed that immature bone-like tissue was present (Fig. 6.7H).

**Figure 6.7.** Defects filled with degradable microbeads incorporating rbASCs. Histological stains of different regions of the same defect: H&E (A), Safranin O (B), Masson’s trichrome (C). Inset on left shows a magnified version of newly formed bone (Magnification 40x). Green arrows point to newly formed bone/calcified tissues. Radiographic image of defect with calcified tissue and the corresponding H&E staining (D).

There was no evidence of elastin in the neocartilage formed within the defect sites (Fig. 6.8). Normal chondrocytes in the auricular cartilage adjacent to the defect exhibited the characteristic pericellular staining indicative of elastic fibers (Fig. 6.8A). However, chondrocytes
in the neocartilage in the defect sites showed mainly collagen in the extracellular matrix (Figs. 6.8B,C,D).

Figure 6.8. Normal chondrocytes have pericellular elastic fibers (A) while cells in the sites implanted with nondegradable microbeads containing rbASCs are surrounded mainly by collagen (B-D). Staining with Modified Verhoeff’s elastic stain; images taken at 40x magnification. Elastic fibers are stained black to blue/black, nuclei stained blue to black, collagen stained red, and any muscle and other tissue stained yellow.

Histomorphometry demonstrated that the defects that received non-degradable alginate microbeads with rbASCs had the greatest area of new cartilage (Fig. 6.9A, Fig. 6.6E). In contrast, the empty defects had significantly less cartilage than all other groups (Fig. 6.9A). New immature
bone-like tissue was also evident in all defects (Fig. 6.9B, Fig. 6.7C), but the area of new bone was small and to a comparable extent in all defect sites. This tissue was observed in microCTs of the tissue (Fig. 6.7D), indicating that it was mineralized.

![Histomorphometric Analysis](image)

**Figure 6.9.** Histomorphometric analysis of the area of new cartilage (A); area of immature bone-like tissue (B); percent healing with new cartilage (C); and percent healing with immature bone-like tissue (D). Data were analyzed using one-way ANOVA followed by Bonferroni’s modification of Student’s t-test. * p < 0.05 vs. empty defect, $ vs. μBs with rbASCs, ^ vs. degradable μBs with rbASCs.

Histology showed that neocartilage and bone like tissue were present both inside and outside the defect region. When examining only the cartilage and bone-like tissue within the defect, percent healing of new cartilage for both degradable and non-degradable microbeads with rbASCs were increased compared to other groups, but not significantly (Fig. 6.9C). The percent
healing of immature bone-like tissue found in all defects was comparable regardless of treatment (Fig. 6.9D).

There was considerable variation in tissue response among animals in the same treatment group (Table 6.2). Empty defects only exhibited cartilage in 1 of 6 rabbit ears but immature bone like tissue was present in 4 of the 6. One of these had both cartilage and bone in the defect tissue. When defects were treated with non-degradable microbeads containing rbASCs, 7 of 11 rabbit ears had cartilage and 7 of 11 had immature bone. All rabbit ears had either cartilage or bone, but only 3 had both cartilage and bone. Defects treated with microbeads that did not contain cells also had cartilage (4/10) and bone like tissue (6/10). Seven of the rabbit ears had either cartilage or bone but only 3 had both types of tissue within the defect site. In sites treated with degradable microbeads containing rbASCs, 4 of 8 rabbit ears had cartilage and 6 of 8 rabbit ears had bone. Seven of the 8 rabbit ears had either cartilage or bone but only 4 had both. In defects treated with autograft, 4 of 11 animals had neocartilage, 7 of 11 had bone-like tissue; nine of the rabbit ears had either neocartilage or bone but only 2 had both tissues in the defect site.

Table 6.2. Induction of cartilage and/or immature bone by microencapsulated rbASCs. Data are the number of samples with new tissue in the defect site v. the number of total sites in a given group.

<table>
<thead>
<tr>
<th>Defect</th>
<th>Cartilage</th>
<th>Bone-like Tissue</th>
<th>Cartilage or Bone-like Tissue</th>
<th>Cartilage and Bone-like Tissue</th>
</tr>
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<tbody>
<tr>
<td>Empty</td>
<td>1/6</td>
<td>4/6</td>
<td>4/6</td>
<td>1/6</td>
</tr>
<tr>
<td>uBs with ASCs</td>
<td>7/11</td>
<td>7/11</td>
<td>11/11</td>
<td>3/11</td>
</tr>
<tr>
<td>uBs without ASCs</td>
<td>4/10</td>
<td>6/10</td>
<td>7/10</td>
<td>3/10</td>
</tr>
<tr>
<td>Deg. uBs with ASCs</td>
<td>4/8</td>
<td>6/8</td>
<td>7/8</td>
<td>4/8</td>
</tr>
<tr>
<td>Autograft</td>
<td>4/11</td>
<td>7/11</td>
<td>9/11</td>
<td>2/11</td>
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There was no evidence of inflammation in any of the treatment sites. Alginate lyase enzyme and the byproducts of alginate lyase mediated degradation did not lead to the infiltration of leukocytes. Similarly, defects that received only alginate microbeads with and without rbASCs did not exhibit an inflammatory response after 12 weeks.

6.4. Discussion

The therapy presented in this work packaged ASCs into injectable alginate microbeads that are 200 µm in diameter and tested their ability to support cartilage regeneration in critical size auricular cartilage defects. The results indicate that neocartilage formation can be achieved but the chondrogenic regimen used to pretreat the ASCs led to a phenotype more typical of commitment to an osteochondral lineage than to an elastic cartilage lineage. Moreover, characteristics of the rabbit ear model, particularly the vasculature in the overlying skin, may have contributed to the formation of immature bone in the regenerated tissues.

rbASCs have been used in tissue regeneration models for bone, cartilage, and cornea, but it has not been explicitly demonstrated that these stem cells are multipotent [84-86]. Our results show that, like rat and human ASCs [2, 87], rbASCs are multipotent and are able to differentiate along osteogenic, adipogenic, and chondrogenic lineages. Expression of mRNAs for phenotypic marker proteins was significantly higher in the respective differentiation media compared to control cultures. Additionally, all histological stains confirmed that rbASCs are able to differentiate along all three lineages.

Microencapsulated rbASCs maintained their chondrocyte phenotype after encapsulation. They expressed mRNAs for aggregcan (ACAN) and type 2 collagen (COL2), both of which are extracellular matrix proteins found in hyaline cartilage. They also expressed mRNAs for type X
collagen, which is typically found in hypertrophic growth plate chondrocytes, suggesting that these cells primed the regenerating tissue to support endochondral bone formation. The microencapsulated rbASCs secreted TGFβ2 and TGFβ3, both of which are associated with chondrogenesis [88], indicating that their presence in the defect sites may have contributed to neocartilage formation. The CM-treated rbASCs also secreted BMP2, an osteogenic factor, but not to a greater extent than the GM treated cells. Moreover, treatment with CM lead to a marked reduction in VEGF secretion. Taken together, these data suggest a complex cocktail of factors was generated by CM treatment that caused an endochondral lineage commitment while at the same time, reducing the vasculogenesis in the repair tissue generally associated with ossification.

We selected the rabbit ear as a model to test the effectiveness of the microbeads in regenerating cartilage based on published reports using this method [89, 90]. We were able to define criteria for a critical size defect in the rabbit ear cartilage. Based on our findings, the 3 mm defect was chosen due to its size for microbead implantation and ease of manipulation during surgery.

However, our results using multiple end points indicate that there are limitations that must be taken into consideration in future study designs. It should be noted that although autografts maintained the structural properties of cartilage, there was no reintegration of the autograft with the cartilage bed. In addition immature bone like tissue was present in all of the treatment groups. This was unexpected, given that the rbASCs were pretreated with CM and produced factors associated with chondrogenesis. The CM-treated rbASCs did not produce elastin, the hallmark of elastic ear cartilage, suggesting that they had not fully differentiated along an auricular chondrocyte lineage prior to implantation. Our results indicate that factors present in the surrounding tissue were insufficient to support auricular chondrocyte differentiation of the
implanted microencapsulated cells, at least over the time frame of the study. Moreover, the tissue overlaying the auricular cartilage is highly vascularized and bleeding during microbead placement may negatively impact chondrogenesis. These results are consistent with other reports of bone formation in auricular cartilage defects from tissue engineered constructs [91].

Even with these limitations, our results show that the formatting of the rbASCs contributed to the degree of chondrogenesis that was observed in the defects. The greatest amount of neocartilage was generated in sites implanted with non-degradable microbeads containing CM-treated rbASCs. These cells not only secreted higher levels of TGFβ2 and TGFβ3 than GM-treated rbASCs, but they retained these factors within the microbead itself. Moreover, VEGF secretion was markedly reduced. This suggests that secreted factors remained in a concentrated area within the defect leading to the induction of new cartilage and suppression of vasculogenesis. The neocartilage was present in small islands of tissue within the defect, and in one case, nearly spanned the full width of the defect. Although we expected to see the microbeads remaining in the tissue after 12 weeks, they were no longer present in most samples, possibly due to enzyme activity from neighboring defects containing degradable microbeads.

Degradable microbeads containing rbASCs also supported neocartilage formation, but to a lesser extent than the non-degradable beads containing rbASCs. However, production of bone-like tissue was highest in this group. Highly attenuated tissue on microCT corresponded to immature bone-like tissue based on Masson’s trichrome stained sections [92]. One possibility is that the CM-treated rbASCs released from these microbeads had a more hypertrophic chondrocyte phenotype than those retained in a rounded hyaline cartilage cell shape within the non-degradable microbeads, and as such supported endochondral ossification within the defect. The degradable microbeads were designed to release cells approximately 4 to 6 days post-implantation [34]. In
contrast, the retention of cells within the non-degradable microbeads may have allowed the defect site to have more exposure to the chondrogenic factors, thereby affecting chondrogenic differentiation of surrounding cells.

6.5. Conclusions

Cell-based therapy will be beneficial to patients in need of cartilage regeneration for craniomaxillofacial problems. Multipotent rbASCs formatted in alginate microbeads 200 micrometers in diameter can be delivered to auricular cartilage defects to stimulate chondrogenesis. Our results using a rabbit auricular cartilage critical size defect model suggest that it may be necessary to specifically induce rbASCs along an auricular cartilage cell lineage to ensure elastic cartilage formation without new bone formation in a well vascularized site.
CHAPTER 7

7.1 Discussion

Approximately 5-20% of fractures end in non-union due to the lack of growth factors and stem cells to aid in regeneration and repair. In some cases, the defect gap is too large for the body to heal on its own with bone [1]. Bone regeneration requires three important factors – cellular components, growth and differentiating factors, and a scaffold matrix. Cellular components from either exogenous cell sources or the local environment can give rise to new structural tissue. Growth and differentiating factors can guide the development of cellular components. Both factors can be provided exogenously using purified proteins or implanted cells secreting proteins and endogenously from extracellular matrix deposits generated by resident tissue cells. Where there is insufficient endogenous matrix to provide a substrate for cellular attachment, proliferation and differentiation, it is also necessary to provide a scaffolding structure [16].

Three approaches have been applied to the tissue engineering of bone: (1) matrix-based therapies, (2) factor-based therapies, and (3) cell-based therapies [16]. Matrix-based therapies use an implant to provide structure and stability. This approach depends on the recruitment of endogenous osteoprogenitors to repair the fracture. The implant’s surface aids in the regeneration process by initiating MSC differentiation. Commonly used implants are constructed from titanium, ceramics or hydroxyapatite. These implants tend to be porous and facilitate ingrowth of bone into the pores; however, the lack of their ability to induce bone formation limits their use significantly. Although hydroxyapatite has been proven to be a good support for cell and matrix interactions in bone formation, it requires many years before complete resorption occurs. In contrast, factor-based therapies supply osteoinductive stimuli, which otherwise may not be present or insufficient. Delivering growth factors has been successful in forming bone but is limited by the difficulty in
translating results from rodent models to larger animals. In addition, the optimal method to deliver and present these factors remains a challenge.

Cell-based therapies use the approach of delivering cells directly to the defect site. It is challenging to determine which factors are needed in any injury site. The cell-based approach does not rely on endogenous stem cells to produce new bone or growth factors; instead these factors are produced by the cells that are delivered, thereby compensating for the growth factor deficiency [16]. Cell-based therapies are particularly advantageous where the host tissue beds are compromised and in cases where the patients suffer from severe trauma or diabetes or patients with a limited number of osteoprogenitor cells because of age or osteoporosis.

Cell-based therapies may employ various cell types, including unfractionated bone marrow, purified and cultured expanded MSCs, differentiated osteoblasts or chondrocytes, or cells modified genetically to produce factors like rhBMP. Fresh bone marrow aspirates contain osteogenic precursors, however a significant amount of bone marrow is required to acquire sufficient cells thus making it an impractical source for large defects. An alternative source is autologous MSCs that have been expanded ex vivo. MSCs that have been differentiated along the osteogenic or chondrogenic lineage may also be used. Studies with pre-differentiated osteoblasts combined with an implant have shown the production of significantly more bone. The last type of cells are used for the delivery vehicle of genetic material coding for BMP2 [16].

The cell-based therapy developed in this thesis is a combination of applying the factor-based and cell-based approach. The factor-based approach is demonstrated by the growth factors secreted by microencapsulated ASCs once they were cultured in MSCGM or differentiation media. In our application, the matrix, alginate, was primarily used for localizing the cells rather than to
provide an attachment substrate for the cells. However, others have modified the polymer to provide attachment sites as well as bioactive ligands [93, 94].

Alginate microbeads are used as a mode of cell delivery in a number of fields and has great potential for tissue regeneration. Our approach has differed from previous work by generating microbeads that are less than 200 μm in diameter, thereby ensuring adequate mass transport to retain cell viability. Using this technology as my base, I have shown that these microencapsulated cells are able to produce growth factors that will aid in tissue regeneration, thus displaying their therapeutic potential. I have developed a method to control the release of cells from alginate microbeads using alginate lyase. Furthermore when placed in vivo I have demonstrated that degradable and non-degradable microbeads localize cells at the delivered site.

To investigate the ability of the microbead cell delivery system to facilitate tissue regeneration, we used a rabbit ear cartilage critical size defect model using rbASCs. Delivery of CM-treated rbASCs in alginate microbeads supported chondrogenesis, particularly when nonresorbable microbeads were used. Immature bone-like tissue was present in most of the treatment sites, but this was especially the case in sites treated with degradable microbeads. This may have been due to higher VEGF levels secreted by rbASCs or the presence of many veins at the site. Therefore to fully achieve elastic cartilage regeneration rbASCs may require a specific culture regime that would prevent the cells from going along an endochondral ossification pathway. In addition, another animal model could be used to assess the ability of microencapsulated rbASCs to regenerated elastic cartilage.

Many human trials investigating the use of stem cells have not been completed or were unable to assess the full potential of the use of stem cells partly due to the loss of cells after delivery. Alginate microbeads are known to localize cells and therefore may enable the completion
of human clinical studies assessing the effect of cell-based therapies on musculoskeletal defects. The delivery system could be adapted for any other tissue once the appropriate cells are microencapsulated. Microencapsulated cells are great for the production angiogenic factors namely VEGF and FGF because of the minimally hypoxic microbead environment. Therefore, microencapsulated stem cells would be effective in delivering angiogenic factors where angiogenesis is needed. Angiogenesis is critical to wound healing, therefore microbeads could be used in wounds that are difficult to heal. Microencapsulated ASCs in the muscle resulted in the formation of adipose tissue and therefore could be useful in clinical settings where a void space is remaining after a surgical procedure. Additional applications of the alginate microbead system could be the delivery of any pharmaceutical products, even nanoparticles to aid in other treatments since the degradation kinetics are controllable.

This delivery system enables the use of cell-based personalized medicine by microencapsulating patient’s cells. This approach reduces the possibility for immune rejection. We have also shown that only a few number of microbeads would be needed containing a small number of cells which is particularly advantageous for patients with a small number of available stem cells.
References


APPENDIX A

Osteoinductive Nature of Microencapsulated ASCs

Summary

Bone regeneration can be aided by bone graft substitutes due to their osteoconductive properties. Some of the current bone graft substitutes are not osteoinductive. We have developed a system of degradable and injectable alginate microbeads to deliver stem cells. ASCs secrete osteogenic and angiogenic growth factors. ASCs treated with OM or OM-d have shown osteoblast like properties. Therefore, we investigated the ability of microencapsulated ASCs derived from different species to induce ectopic bone in different models. All ASCs were pretreated with MSCGM or OM or OM-d. Microencapsulated ASCs were not osteoinductive. However, we observed the formation of additional adipose tissue indicating that the ASCs delivered supported adipogenesis.

A.1 Introduction

The gold standard for bone repair is an autologous source of bone. Autografts have osteogenic properties and provide structural support for new bone growth, however it is not osteoinductive. Autografts can be used from the patient’s iliac crest but at least 25% of the patients still report the experience of pain two years after the procedure. In addition, autografts are associated with donor site morbidity. An alternative approach for bone formation is growth factors. BMP2 and BMP7 are Food and Drug Administration (FDA) approved growth factors for bone. These two members of the BMP2 family achieve bone formation by initiating a cascade of events resulting in the cells secreting extracellular matrix that is characteristic of bone. Osteopromotive growth factors such as VEGF and FGF aid in bone formation once the process begins and leads to
better healing, but by themselves are not osteoinductive. Growth factors can be derived from living tissue – human or bovine bone, recombinant proteins or gene therapy [95]. To harness the effectiveness of growth factors the dose, delivery method and release kinetics must be optimized.

Biomaterials induce bone in ectopic sites by initiating a cascade of events resulting in MSCs undergoing osteogenic differentiation [96]. The roughness, porosity or the chemical components of the biomaterial are responsible for initiating the cascade of events. The presence of calcium phosphate has been shown to play an important role in inducing bone [97, 98]. The bone may form by intramembranous ossification or endochondral ossification if BMP2 is present [97, 99, 100]. The cascade of events involve the chemotaxis of undifferentiated MSCs followed by cell proliferation. The next steps only occur in the presence of BMP2 - cells are differentiated into chondrocytes with cartilaginous extracellular matrix; chondrocytes go through maturation, hypertrophy and calcification; blood vessel and osteoprogenitor infiltration, cartilage removal, osteoid apposition and bone matrix production; bone marrow formation and bone remodeling [83].

ASCs are multipotent and upon induction with differentiation media, secrete osteogenic growth factors including BMP2. Therefore ASCs could be used a source of growth factors for orthopedic tissue engineering. However, ASCs delivered as a suspension will not remain in the site of delivery. Providing a reliable delivery system, ASCs could be delivered to the site of interest and aid in the regeneration process by secreting osteogenic factors.

ASCs can be microencapsulated into alginate microbeads as a mode of delivery. Microencapsulated ASCs remain viable for as long as three weeks. ASCs derived from mice, rats and rabbits have shown the production of VEGF and BMP2 with and without induction media. OM treatment with microencapsulated rASCs and mASCs display a reduction in VEGF, however VEGF production is regained once dexamethasone is removed from OM. rbASCs produced higher
amounts of VEGF than ASCs from mice or rats. Given the osteopromotive nature of VEGF we first investigated the ability of microencapsulated rbASCs to induce ectopic bone in male New Zealand white rabbits. We also investigated the ability of microencapsulated ASCs derived from mice and rats to induce ectopic bone in athymic nude mice (an American Society of the Interanational Association for Testing and Materials (ASTM) model) [101].

A.2. Methods

A.2.1 Adipose Derived Stem Cells

ASCs were isolated from 100-125g male Sprague-Dawley rats (n = 6) [2, 36], a male New Zealand rabbit, male C57 Black 6 mice (n=6) under an Institutional Animal Care and Use Committee (IACUC) approved protocol at the Virginia Commonwealth University. Briefly, the inguinal fat pad was removed and transferred to a container with Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 3% sterile-filtered L-glutamine-penicillin-streptomycin (P/S). The tissue was washed three times with Hank’s balanced salt solution (HBSS) (Invitrogen) and then incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) for 30 minutes on a rocker at 37°C. Following trypsinization, the supernatant was discarded. Adipose tissue was cut into small pieces and incubated in a digestive cocktail containing collagenase type I (365 units/mL) (Sigma Aldrich) and dispase (3 units/mL) (Gibco, Carlsbad, CA) for four hours. After incubation, the oily upper layer was removed and the digest was quenched with an equal amount of MSCGM. The cells were separated, plated in T-175 flasks at 5,000 cells/cm² and cultured in MSCGM at 37 °C and 5% CO₂. For the next two days the monolayers were washed with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) and fed with MSCGM every 24 hours. Thereafter media were changed every 48 hours and cells were
cultured to confluence. ASCs were then cultured in either MSCGM, OM or OM without dexamethasone for an additional seven days before microencapsulation.

A.2.2 Microencapsulated Cells

To form microencapsulated cells, rASCs were encapsulated in UV-sterilized medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginates, FMC BioPolymer) at a concentration of 10 x 10⁶ cells/ml and crosslinked in a calcium solution. Medical grade alginate was dissolved in 0.9% (w/v) saline (Ricca Chemical, Arlington, TX) at a concentration of 40 mg/ml. Microbeads incorporating cells were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 5 ml/hr flow rate, nozzle with an inner diameter of 0.12 mm, an electrostatic potential of 6 kV and a calcium crosslinking solution. The crosslinking solution contained 50 mM CaCl₂, 150 mM osmolyte with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.3 (Sigma Aldrich) (HEPES-buffered) [5, 23]. Alginate microbeads were allowed to crosslink for an additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca²⁺, and suspended in the respective media. Microencapsulated ASCs were cultures overnight in the media they were previously cultured.

A.2.3 Degradable Alginate Microbead Production

Alginate lyase was incorporated into alginate microbeads by combining equal volumes of 4% medical grade LVM alginate and a 0.9% (w/v) saline containing 0.44 U alginate lyase/ml. Prior to the combination, both components were kept at 4°C. The final 2% (w/v) alginate was mixed at 1000 rpm for one minute to produce 0.22 U alginate lyase/g. The alginate/alginate lyase mixture was used to form microbeads using a crosslinking solution containing 75 mM CaCl₂, 90 mM glucose and 10 mM HEPES using the same flow rate, nozzle and electrostatic potential.
described above. The 0.44 U/g alginate microbeads were made similarly by starting with 0.44 U alginate lyase/ml solution.

A.2.4 Osteoinductivity of ASCs

6.2.4.1 Rabbit Ectopic Study

rbASCs were cultured in MSCGM or OM for 7 days prior to microencapsulation. Non-degradable (0U/g) and degradable microbeads (0.22 U/g) incorporating these rbASCs were made on the day before implantation. Microencapsulated rbASCs were loaded into a gelatin capsule and implanted in the gastrocnemius and rectus femoris of both rabbit legs for 6 weeks.

A.2.4.1.1 Micro CT

All samples were scanned in air using a µCT 40 (Scanco Medical) at 45 kVp, 200 µA, 600 ms integration time, and a voxel size of 35 µm. Images were processed using the accompanying Scanco medical software. A constant volume of interest representing the absolute volume centered at the defect cavity was used for the analysis of each sample.

A.2.4.2 Athymic Nude Mice Ectopic Study (osteoinductive nature of microencapsulated rASCs)

rASCs were cultured in MSCGM, OM, or OM without dexamethasone for 7 days before microencapsulation. rASCs from each treatment group were microencapsulated into non-degradable (0 U/g) and degradable microbeads (0.22 U/g and 0.44 U/g) and stored overnight in the respective media that incorporated cells treatment. To investigate the osteoinductive potential of rASCs, microencapsulated rASCs (100 ul) were injected to the gastrocnemious of both legs for 8 weeks. In addition, to investigate the time of rASCs release on ectopic bone formation, microbeads of different release profiles were used.
A.2.4.3 Athymic Nude Mice Ectopic Study (osteoinductive nature of microencapsulated mASCs)

mASCs were cultured in MSCGM or OM for 7 days prior to microencapsulation. Cells were microencapsulated into degradable microbeads (0.22 U/g) and stored in MSCGM or OM overnight. The next day, alginate microbeads were loaded in gelatin capsules with very small amounts of demineralized bone matrix for 8 weeks.

A.2.5 Histology

After 48 h of fixation in formalin, samples were decalcified for 5 days using . Following that samples were dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin, and cut into 8-μm thick sections (Shandon Finesse 325, Thermo Scientific). The sections were stained with haematoxylin and eosin, and then imaged with a Leica DMLB microscope (Leica Microsystems, Bannockburn, IL).

A.2.6 Statistics

The results from the in vivo study were calculated as the means ± SEM of both legs of 4 animals per variable thus providing an N or 8 for all studies except the rabbit in vivo study where an N of was used. Statistically significant differences between groups were determined by Student’s t-test. P values ≤ 0.05 were considered significant. Data presented are from representative experiments.

A.3. Results

A.3.1 rbASCs are not osteoinductive

Demineralized bone matrix, a positive control did not induce ectopic bone (Fig. A.1). Both degradable and non-degradable microencapsulated rbASCs produced radio-opaque deposits after 6 weeks. Microbeads in the non-degradable groups were present after six weeks with no new bone tissue present in both GM and OM treated groups The OM pretreated groups produced significantly higher amounts of radio-opaque tissue. Degradable microbeads were not present after
6 weeks. Differently stained tissue with a non-muscle morphology was also observed in both GM and OM groups. None of the samples induced ectopic bone (Fig. A.2).

Figure A.1: H&E staining of DBM implanted in male White New Zealand rabbit after 6 weeks.
Figure A.2: H&E staining of microbeads in rabbits after 6 weeks, (A) GM treated rbASCs, (B) OM treated rbASCs microencapsulated in non-degradable alginate microbeads and (C) GM treated rbASCs, (D) OM treated rbASCs microencapsulated into degradable alginate microbeads.

Figure A.3: Micro CT analysis of osteoinductivity of rbASCs. (A) Volume of radio-opaque tissue.

A.3.2 rASCs are not osteoinductive

DBM induced ectopic bone (Fig. A.4). Non-degradable microencapsulated rASCs were still present after 8 weeks in all samples across all treatment groups. Although microbeads were injected in the gastrocnemius, the microbeads were present in other portions of the leg due to the volume injected compared to the size of the animal’s leg. Non-degradable microbeads did not induce the formation of ectopic bone.

Degradable microbeads (0.22 U/g) incorporating rASCs, were not present after 8 weeks. However remnants of the microbeads were present in very small amounts. In the immediate area of the degraded alginate microbead, tissue with different staining and morphology compared to muscle was observed, but samples did not induce the formation of ectopic bone (Figs. A.5D – A.5E).

Degradable microbeads with the fastest cell release (0.44 U/g) also did not lead to any ectopic bone. Similar new tissue was observed in the muscle with fewer remnants of degraded
alginate. Nonetheless these microencapsulated rASCs did not induce any ectopic bone. The different degradation time points did not have any correlation to the volume of new tissue formed.

Figure A.4: H&E staining of DBM implanted in male athymic nude mice after 8 weeks

![Image of H&E staining of DBM implanted in male athymic nude mice after 8 weeks]

Figure A.5: H&E staining of rASC osteoinductivity in athymic nude mice after 8 weeks, (A) GM treated rASCs, (B) OM treated rASCs, (C) OM-d treated rASCs microencapsulated in non-

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degradable alginate microbeads, (D) GM treated rASCs, (E) OM treated rASCs, (F) OM-d treated rASCs microencapsulated into degradable alginate microbeads (0.22 U/g), and (G) GM treated rASCs, (H) OM treated rASCs, (I) OM-d treated rASCs microencapsulated into degradable alginate microbeads (0.44 U/g).

A.3.3 mASCs are not osteoinductive

Regardless of the pre-treatment received, both microencapsulated groups resulted in the formation of new adipose tissue (Figs. A.6 and A.7). In addition another type of tissue with a non-muscle or bone-like morphology was also observed directly beside the inactive demineralized bone matrix. All the degradable microbeads were not present after 8 weeks.

Figure A.6: H&E of mASC osteoinductivity in the gastrocnemius of athymic nude mice after 8 weeks, (A) GM treated mASCs and (B) OM treated mASCs microencapsulated into degradable alginate microbeads (0.22 U/g).
Figure A.7: Area of adipose tissue after microencapsulated mASCs were implanted in the gastrocnemius for 8 weeks.

A.4. Discussion

The demand for osteoinductive materials is always present. Bone induction is achieved by queues that will lead to the recruitment of MSCs to the local area and subsequent osteogenic differentiation. The standard for testing the osteoinductive nature of biomaterials is implanting the biomaterial into the gastrocnemius (a non-bone site) of an athymic nude mice. Microencapsulated ASCs derived from rat, mouse, rabbit and human are able to secrete osteogenic growth factors including BMP2 once induced with OM. BMP2 is known to be a potent growth factor in initiating bone growth. However, other proteins are also important in the formation of bone. One of the advantages the cell-based approach to bone formation is cells are able to secrete a plethora of growth factors to aid in bone regeneration. We have quantified BMP2 and VEGF levels secreted by microencapsulated ASCs derived from four species. Results showed that rabbits were able to secrete higher total levels of VEGF compared to rASCs. Given the importance of angiogenic factors in bone formation, we first investigated the ability of microencapsulated rbASCs to induce ectopic bone. After 6 weeks our histological analysis did not confirm that the radio-opaque tissue was indeed bone. Although VEGF is important for bone formation it is not the only factor needed.
In addition, it is possible that a longer time for treatment could be required for rbASCs to induce bone \textit{in vivo}. However the formation of new tissue was observed in few samples. This new tissue was not similar to muscle or bone and would therefore requires additional analysis to determine the type of tissue formed. In the remaining samples, it is possible that the microbeads were displaced to other locations of the muscle since the inactivated demineralized bone matrix could not be located on histological sections.

In addition to VEGF and BMP2, microencapsulated rASCs are known to secrete osteocalcin, FGF and osteoprotegerin, all of which are important in bone formation. We have shown a reduction (not detectable in some cases) in angiogenic factor production once microencapsulated rASCs were treated with OM. OM-d treatment of microencapsulated rASCs showed the production of both angiogenic and osteogenic factors, which are both crucial for bone. Therefore, we investigated the osteoinductive nature of microencapsulated rASCs by using the ASTM method. After 8 weeks, microencapsulated rASCs previously treated with GM, OM, OM-d did not form ectopic bone. This could be due to the quantity of microbeads delivered. It is possible that a large number of microbeads incorporating cells need to be localized in the same area, however this was difficult to achieve because of the small muscle size and delivering the microbeads by an injection. In addition, a longer in vitro culture time may be necessary for the cells to secrete more growth factors to produce bone.

Having used the ASTM standard to determine the osteoinductive nature of microencapsulated rASCs which is a xenograft model, we investigated an allograft model using the ASTM standard by implanting microencapsulated mASCs. At the end of 8 weeks a large amount adipose tissue was formed. This is not surprising since ASCs were implanted. Even though mASCs were previously cultured in GM and OM. Similar amounts of adipose tissue was found in
both groups (Fig. A.7). This indicates that a longer treatment time with OM may be needed to achieve the desirable results. Studies have shown that mineralization occurs at later time points. Therefore it is possible that with a longer treatment time ectopic bone could be formed. There was newly formed tissue around the inactivated demineralized bone. Additional experiments must be done to investigate whether or not the newly formed tissue is bone and whether it was only due to the presence of the inactivated bone or delivered ASCs. Some studies have shown that with the presence of a rough surface alone can encourage new bone formation therefore it is possible that the inactivated DBM provides a rough surface for the ASCs. In that case the ASCs may need to be co-delivered with a material that has rough surface.

**A.5. Conclusions**

Microencapsulated ASCs isolated from mice, rats and rabbits did not form bone ectopically. Microencapsulated mASCs lead to the engraftment of adipose tissue.
APPENDIX B

Microencapsulated rASCs Support Osteogenesis in a Unicortical Diaphyseal Defect

Summary

Bone graft substitutes have been successful in aiding bone regeneration. Recently, it has been shown that based on the chemical composition, bone graft substitutes can alter the bone remodeling process and limit the bone marrow cavity regeneration. An alternative approach is to use cell-based therapies, however their effect on bone remodeling have not been investigated. One cell-based approach is to deliver microencapsulated ASCs using alginate microbeads. Microencapsulated ASCs have previously shown to remain viable up to 3 weeks in vitro. In this study we investigated the effect of microencapsulated rASCs that were previously pretreated with MSCGM or OM. Degradable and non-degradable microbeads incorporating rASCs were placed in a 3 mm unicortical diaphyseal defect. Some defects were left either empty or filled with fresh frozen allograft as a negative or positive control respectively. Our results showed that all defects healed, both cortical bone and bone marrow were recovered. Samples receiving degradable microbeads incorporating ASCs that were pretreated with OM produced more bone within the defect.

B.1. Introduction

Defects in the skeletal system due to injury or disease can be repaired with bone graft substitutes. Bone graft substitutes usually have one or more of the following components; osteoinductive proteins that are able to initiate bone formation among the surrounding cells,
osteocomductive matrix to allow the new bone formed to adhere, and osteogenic cells which have the ability to form bone [102]. The bone graft substitutes may be autologous, allografts or other materials comprised of hydroxyapatite, PLGA, tricalcium phosphate, and bioglass amongst others. An autologous bone graft is normally removed from the iliac crest. This is an excellent solution but can be difficult to harvest and may be coupled with complications, thus creating a need for alternative solutions. The bone graft substitutes are typically implanted into the defects which may be accompanied by external fixation depending on the size of the defect. Once the bone graft substitute is placed within the fracture a hematoma forms releasing cytokines and growth factors. An inflammatory reaction occurs to the trauma of the surgery and to the material of the substitute. This is very important as it leads to degranulation and the release of growth factors that will initiate migration and proliferation of MSCs and fibrovascular tissue in and around the graft. Blood vessels begin to invade through existing Haversian or Volkmans canals leading to osteoclastic resorption of graft surfaces. Lastly, bone formation occurs on the graft surfaces [12]. Depending on the type of material, it may contain inherent factors that will initiate bone growth. Success of bone graft substitutes have been achieved however previous studies have shown that the chemical composition is able to inhibit the remodeling of the bone marrow [103]. The bone marrow is normally remodeled by a series of capillary invasion in the area which carries the MSCs [104]. The main function of the bone marrow is to generate blood cells and store fat. In addition the bone marrow has many hemapoetic stem cells and mesenchymal stem cells. Therefore without the presence of the bone marrow the complete function of the bone is not possible.

Another approach to heal fractures is to use cell-based therapies which bring the advantage of stem cells secreting factors that may be absent at the fracture site or providing additional cells if the host’s stem cells are not enough. Previously, we have developed a system of
microencapsulating ASCs into alginate microbeads which allows the cells to remain viable for up to 3 weeks in vitro. The microencapsulated stem cells have also shown the ability to secrete osteogenic and angiogenic factors once they are treated with differentiation media. One of the advantages of this system is that the release of the stem cells can be controlled in a time dependent manner by using an enzyme known as alginate lyase. This enzyme works by cleaving the glycosidic bonds. As the cells are released from these microbeads they also maintain their viability and ability to secrete osteogenic and angiogenic growth factors after induction with differentiation media.

As a stem cell therapy it is important to determine whether the microencapsulated ASCs are able to aid in the bone regeneration process and whether or not it inhibits the remodeling of the bone marrow. Therefore in this study we created a unicortical diaphyseal defect in the femur and delivered microencapsulated rASCs pretreated with MSCGM, OM, or OM-d for 8 weeks to determine the effects of this cell-based therapy on osteogenesis and bone marrow remodeling.

**B.2. Methods**

**B.2.1 Adipose Derived Stem Cells**

ASCs were isolated from 100-125g male Sprague-Dawley rats \((n = 6)\) \([2, 36]\), under an Institutional Animal Care and Use Committee (IACUC) approved protocol at the Georgia Institute of Technology and the Virginia Commonwealth University. Briefly, the inguinal fat pad was removed and transferred to a container with Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 3% sterile-filtered L-glutamine-penicillin-streptomycin (P/S). The tissue was washed three times with Hank’s balanced salt solution (HBSS) (Invitrogen) and then incubated in 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) for 30 minutes on a rocker at 37°C. Following trypsinization, the supernatant was discarded. Adipose
tissue was cut into small pieces and incubated in a digestive cocktail containing collagenase type I (365 units/mL) (Sigma Aldrich) and dispase (3 units/mL) (Gibco, Carlsbad, CA) for four hours. After incubation, the oily upper layer was removed and the digest was quenched with an equal amount of MSCGM. The cells were separated, plated in T-175 flasks at 5,000 cells/cm² and cultured in MSCGM at 37 °C and 5% CO₂. For the next two days the monolayers were washed with Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen) and fed with MSCGM every 24 hours. Thereafter media were changed every 48 hours and cells were cultured to confluence. ASCs were then cultured in either MSCGM, OM or OM without dexametasone for an additional seven days before microencapsulation.

B.2.2 Microencapsulated Cells

To form microencapsulated cells, rASCs were encapsulated in UV-sterilized medical grade alginate (50% mannuronate units) (PRONOVA™ UP LVM, ultrapure sodium alginates, FMC BioPolymer) at a concentration of 10 x 10⁶ cells/ml and crosslinked in a calcium solution. Medical grade alginate was dissolved in 0.9% (w/v) saline (Ricca Chemical, Arlington, TX) at a concentration of 40 mg/ml. Microbeads incorporating cells were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 5 ml/hr flow rate, nozzle with an inner diameter of 0.12 mm, an electrostatic potential of 6 kV and a calcium crosslinking solution. The crosslinking solution contained 75 mM CaCl₂, 90 mM osmolyte with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.3 (Sigma Aldrich) (HEPES-buffered) [5]. Alginate microbeads were allowed to crosslink for an additional 10 minutes, washed with 0.9% (w/v) saline (Ricca Chemical) to remove the excess Ca⁺⁺, and suspended in the respective media. Microencapsulated ASCs were cultures overnight in the media they were previously cultured.
B.2.2.1 Degradable Alginate Microbead Production

Alginate lyase was incorporated into alginate microbeads by combining equal volumes of 4% medical grade LVM alginate and a 0.9% (w/v) saline containing 0.44 U alginate lyase/ml. Prior to the combination, both components were kept at 4°C. The final 2% (w/v) alginate was mixed at 1000 rpm for one minute to produce 0.22 U alginate-lyase/g alginate. The alginate/alginate lyase mixture was used to form microbeads using a crosslinking solution containing 75 mM CaCl$_2$, 90 mM glucose and 10 mM HEPES using the same flow rate, nozzle and electrostatic potential described above.

B.2.3 Unicortical Diaphyseal Defect

To determine if microencapsulated rASCs would prevent the remodeling process microbeads were placed in a unicortical defect for 8 weeks. A defect was created on the proximal portion of the femur with a trephine (3 mm inner diameter). To reduce the blood flow from the marrow cavity, pressure was applied using gauze to the newly created defect before implanting microbeads in the defect.

B.2.4 Histology

After 48 h of fixation in formalin, samples were decalcified for 5 days using . Following that samples were dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin, and cut into 8-μm thick sections (Shandon Finesse 325, Thermo Scientific). The sections were stained with haematoxylin and eosin, and then imaged with a Leica DMLB microscope (Leica Microsystems, Bannockburn, IL).

B.2.5 Histomorphometrics

A region of interest around the defect was chosen for all samples. The area of connected cortical bone was measured and unconnected bone in the marrow cavity was also quantified as
trabecular bone. The remaining area within the bone marrow was quantified as bone marrow cavity.

B.2.6 Statistics

The results from the in vivo study were calculated as the means ± SEM of both legs of 4 animals per variable thus providing an N or 8. Statistically significant differences between groups were determined by Student’s t-test. P values ≤ 0.05 were considered significant. Data presented are from representative experiments.

B.3. Results

B.3.1 Both bone marrow and cortical bone were recovered

Defects that were not filled with microbeads (empty) during surgery healed within 8 weeks with both cortical and bone marrow recovering. The application of fresh frozen allografts to defects resulted in cortical bone recovery in 7 of 8 samples, however the bone marrow was not fully recovered in most samples where pieces of allograft were still remaining after 8 weeks.

Figure B.1: H&E staining of the defect site in the femur after 8 weeks.

Non-degradable microbeads as expected were present after 8 weeks in the cortical bone, bone marrow cavity, and area directly outside of the defect (opposite to the marrow cavity). In non-degradable groups, all samples healed with both cortical bone and bone marrow recovering
independent of rASC pretreatment. In the cortical and trabecular bone, bone formed around the microbeads making complete contact with the microbead’s surface.

Degradable microbeads were not present within the defects after 8 weeks. All the defects healed completely with one sample either in the GM and OM group generating additional bone attached to the femur. Microbeads were not present after 8 weeks and no indications of an inflammatory response. The area of new bone in the defect site was significantly higher in the OM group compared to the empty.

Figure B.2: H&E staining of unicortical defects receiving non-degradable microencapsulated rASCs pretreated with A) GM, B) OM, C) OM-d or degradable microencapsulated rASCs pretreated with D) GM, E) OM, F) OM-d after 8 weeks.
B.4. Discussion

Therapies for fractures or non-unions have the primary goal of facilitating bone regeneration without inhibiting the bone remodeling process. Previously, it has been shown that the chemical composition of bone graft substitutes can inhibit the bone remodeling process by delaying the marrow recovery [103]. Stem cells are multipotent and can be delivered in vivo by using alginate microbeads. The paracrine effect of growth factors produced by stem cells can be used to facilitate bone regeneration. This cell-based therapy involves alginate microbeads, ASCs and alginate lyase for degradation. However the effects of alginate lyase and the by-products of alginate lyase mediated degradation on bone remodeling are known. As this cell-based therapy is developed it is important that it does not inhibit the bone remodeling process. Therefore we investigated the effect of microencapsulated rASCs on bone remodeling.

The results showed that microencapsulated rASCs did not inhibit the bone remodeling process. All non-degradable sample as healed well more importantly newly formed bone was able to form around the alginate microbeads indicating that the surface of alginate beads are osteoconductive. There was also a trend of more bone being present in the samples compared to empty defect which may be due to the growth factors secreted by encapsulated stem cells.
Naturally derived hydrogels have been used for bone repair due to their biocompatibility and tunability. The mechanical properties of these hydrogels are not well suited for bone.

All samples receiving degradable alginate microbeads healed well and reflected a similar trend of more bone being present within the region of interest. The osteogenic media pretreated groups produced more bone which could be due to the cells differentiating along the osteogenic path and enhancing the bone healing. In one sample extra bone was present in the GM and OM group. This may have resulted from the factors secreted from the encapsulated cells localizing in the area just outside the defect and initiating bone formation.

**B.5. Conclusions**

Microencapsulated ASCs supported osteogenesis and did not alter the bone remodeling process. The alginate microbeads were able to deliver the cells and their secreted factor to the area however they did not alter the bone remodeling process.