INVESTIGATING TREATMENTS OF ADIPOSE STEM CELLS TO MAXIMIZE TROPHIC FACTOR PRODUCTION FOR CARTILAGE REGENERATION

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INVESTIGATING TREATMENTS OF ADIPOSE STEM CELLS TO MAXIMIZE TROPHIC FACTOR PRODUCTION FOR CARTILAGE REGENERATION

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TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF ABBREVIATIONS

acan	Aggrecan
comp	Cartilage oligometric matrix protein
col2	Collagen II
colX	Collagen X
fgf2	Fibroblast growth factor 2
igf1	Insulin growth factor 1
nog	Noggin
pthlh	Parathyroid like hormone
tgfb1	Transforming growth factor beta 1
tgfb2	Transforming growth factor beta 2
tgfb3	Transforming growth factor beta 3
vegfa	Vascular endothelial growth factor a
CM	Chondrogenic media
MSCGM	Mesenchymal stem cell growth media
uB	Microbead

SUMMARY

Osteoarthritis is a leading cause of disability in the United States. Current methods of treatment aim to replace cartilage as opposed to regenerating tissue. There is interest in using stem cells as a source of growth factors to stimulate cartilage cells to produce extracellular matrix and an environment conducive to cartilage regeneration. The purpose of this study is to assess the effect of chemical and biological stimulation and a 3 dimensional structural environment on mRNA levels of adipose-derived stem cells *in vitro* and on cartilage regeneration *in vivo*. Adispose-derived stem cells (ASCs) were isolated from rats, microencapsulated, and treated with chondrogenic media. Chondrogenic media (CM) increased mRNA levels of many genes involved in chondrogenesis, including *comp*, *acan*, *colX*, and *tgfb3*, and decreased levels of the angiogenic factors *fgf2* and *vegfa*. Microencapsulation increased mRNA levels of *pthlh* and *tgfb3*, and there was little difference on mRNA levels between calcium and barium crosslinks. The chemical components, ascorbic acid 2-phosphate (AA2P) and dexamethasone (Dex), and biological components, growth factors TGF-B1 and BMP-6, or chondrogenic media were assessed for their effect on ASC gene profile. Removing growth factors reduced mRNA levels of all genes assessed, whereas removing Dex and AA2P had variable effects on mRNA levels. ASCs were microencapsulated and preconditioned with CM prior to transplantation in a 2 mm cartilage defect in rats. The *in vivo* results displayed moderate cartilage infiltration into the defect. These data suggest that microencapsulation and treatment with CM can alter the gene profile to be conducive to cartilage regeneration and may regenerate cartilage *in vivo*.

CHAPTER 1

INTRODUCTION

Osteoarthritis, a joint disorder characterized by the wearing down of cartilage and underlying bone, is the leading cause of chronic disability in the United States [1]. There is no cure for osteoarthritis, and current treatments aim solely to alleviate pain or surgically replace the cartilage [2]. However, treatments using direct implantation of cartilage cells have shown minimal success. Over time cartilage loses the ability to regenerate itself. Therefore, there is interest in stimulating the existing chondrocytes to secrete growth factors and produce extracellular matrix proteins that could assist in regeneration.

Stem cells have shown to be a promising source of cartilage regeneration. Adipose-derived stem cells (ASCs) are useful for cartilage regeneration due to their chondrogenic potential, production of chondrogenic trophic factors, and abundance in the body [3]. ASCs, however, have the potential to produce factors involved in angiogenesis, which is detrimental to cartilage growth [4]. Current studies in gene therapy have been able to increase production of certain growth factors that stimulate chondrogenesis one at a time [5]. Cartilage regeneration, however, is a multifactorial process in which many growth factors contribute to condrogenesis, and gene therapy does not alter the entire secretory profile [6]. Thus, chondrogenic media, including ascorbic acid-2-phosphate, dexamethasone, and the growth factors BMP-6, and Tgfb-1, may be used to differentiate ASCs towards a chondrogenic phenotype and stimulate production of many chondrogenic growth factors, while inhibiting production of angiogenic factors [7].

It is known that chemical and biological factors, such as chondrogenic media, and the

cell's environment, such as a 3D scaffold, may promote chondrogenesis. It is unknown, however, what the effect of different components of chondrogenic media and differing structural environments are on the secretory profile of ASCs. Therefore, the purpose of this study was to determine the effect that chondrogenic media has on ASC growth factor production in a 3D environment, the effect of the different components of chondrogenic media on growth factor production, and if ASCs preconditioned in chondrogenic media can regenerate cartilage *in vivo*. It was hypothesized that treatment of ASCs with chondrogenic media in a 3D structural environment would promote expression and secretion of chondrogenic growth factors *in vitro*, and cartilage regeneration *in vivo*.

CHAPTER 2

METHODS AND MATERIALS

Cell Isolation

ASCs were isolated from male Sprague-Dawley rats and cultured in Lonza Mesenchymal Stem Cell Growth Medium (MSCGM). ASCs were isolated using fluorescence activated cell sorting to be negative for CD45 and positive for CD73 and CD271. Resting chondrocytes were isolated from the ribs of male Sprague Dawley rats and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/ml ascorbic acid until fourth passage prior to experimental analysis.

Microencapsulation

When primary ASCs reached 90% confluence, cells were trypsinized and microencapsulated in low molecular weight (~150 kDa) alginate with a high mannuronate to guluronate ratio. The alginate (20 mg/mL) was dissolved in sterile-filtered saline. Cells were suspended in the alginate solution at a concentration of 25×10^6 cells/mL. Microbeads were formed using a Nisco Encapsulator VAR V1 LIN-0043 and a cross-linking solution of 50 mM $CaCl_2$, 150 mM glucose, and 15 mM HEPES buffer. The microbeads were washed three times in MSCGM prior to cell culture studies.

Cell Culture

Once first passage ASCs reached 90% confluence, ASC monolayers and microbeads were then treated for 5 days with either MSCGM or chondrogenic medium (CM) consisting of DMEM containing 4.5g/L glucose with 1 mM sodium pyruvate, 40 μg/ml proline, 50 μg/ml ascorbic acid 2-phosphate (AA2P), 1% ITS+, 100 nM dexamethasone (Dex), 10 ng/ml

recombinant human transforming growth factor beta-1 (TGF-β1) and 100 ng/ml recombinant human bone morphogenic protein 6 (BMP-6). In subsequent studies, ASC monolayers were treated for five days with either MSCGM supplemented with different combinations of 50μg/mL AA2P, 100nM Dex, 10ng/mL TGF-β1, and 100ng/mL BMP-6 or with CM that lacked different combinations of 50μg/mL AA2P, 100nM Dex, 10ng/mL TGF- β1, and 100ng/mL BMP-6. Once media were changed on the fifth day, RNA was collected after 8 hours as described below. Conditioned media and ASCs were collected after 24 hours, and ASCs were lysed in 0.05% Triton X-100.

RNA Isolation and Reverse Transcription

Alginate microbeads were uncross-linked in 82.5 mM sodium citrate (Sigma), pelleted at 500×g for 10 minutes and washed two more times in sodium citrate to remove any residual alginate. TRIzol reagent (Invitrogen) was added to the resulting cell pellet, homogenized using a QIAshredder (QIAGEN, Valencia, CA, USA), and RNA was isolated using chloroform and an RNeasy Kit (QIAGEN) as previously described [28]. 1 μg RNA was then reverse transcribed to cDNA using a High Capacity Reverse Transcription cDNA kit (Applied Biosystems, Carlsbad, CA, USA).

Microarray Analysis

cDNA was converted into cRNA using a RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY, USA). Biotin labeled cRNA was cleaned up using a GeneChip Sample Cleanup Module (Affymetrix Inc., Santa Clara, CA, USA) and fragmented at 94°C in Fragmentation Buffer for 35 minutes. Following fragmentation, 15 μg biotinylated cRNA was hybridized to an Affymetrix Rat Genome GeneChip (Rat 230_2.0) at 45°C for 16 hours, washed, stained with streptavidin phycoerythrin (Fluidics Station 400, Affymetrix), and scanned

according to manufacturer's guidelines. The GeneChips were then assessed for data quality using Affymetrix-developed quality controls. Data analysis was performed using GeneSifter (Geospiza, Seattle, WA) with significant differences in expression of a single gene being defined as a 3-fold change in mRNA levels. Significant differences in mRNAs of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were defined as z-scores greater than 2.0. RNA isolated from rat liver tissue served as a reference control.

Growth Factor mRNA Levels and Production

Chondrogenesis is a complex process orchestrated by a wide array of growth factors. Therefore, mRNA levels and production of paracrine factors involved chondrocyte proliferation, proteoglycan synthesis, hypertrophic differentiation, and vascular invasion were quantified. Along with these paracrine factors, mRNAs for chondrocytic markers were also quantified as previously described using real-time PCR with gene-specific primers using the Step One Plus Real-time PCR System and Power Sybr® Green Master Mix (Applied Biosystems) [29]. All primers were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Eurofins MWG Operon (Huntsville, AL, USA), unless otherwise noted (Table 1). Growth factor production over the last 24 hours of culture was quantified using ELISA (R&D Systems) and normalized to DNA content measured with a Quant-iT PicoGreen kit (Invitrogen). To measure growth factor retention within the microbead, cultures were uncrosslinked in 82.5mM sodium citrate and the supernatants and cells were frozen at -80^{o}C. Samples were then lyophilized for 24 hours and the resulting dried constructs were digested in 1 unit/mL alginate lyase (Sigma) for 1 hour and measured with ELISA.

Xiphoid Defect

To assess the effects of ASCs on cartilage regeneration, 2 mm cylindrical defects were made in the xiphoids of 125g male Sprague-Dawley rats as previously described [30] under a protocol approved by the Institutional Animal Care and Use Committee of the Georgia Institute of Technology. ASC microbeads preconditioned with MSCGM or CM were implanted into the defect and immobilized with a hydrogel mixture consisting of 25 mg/mL RGD-conjugated alginate (NOVATACH M RGD, FMC BioPolymer) in DMEM to promote cell infiltration and 20 mg/mL $CaSO_4$ (Sigma) mixed at a 4:1 volume ratio. Empty defects with the hydrogel mixture and re-implanted excised cartilage (autografts) served as controls. All groups were tested in 7 rats. 35 days post operation, the xiphoids were excised and fixed in 10% phosphate-buffered formalin (Sigma) for 48 hours and embedded in paraffin. Sagittal sections of the defect were stained with safranin-O using a fast green counter stain to evaluate proteoglycan present.

Statistical Analysis

Statistical differences among all experimental groups were determined via ANOVA with a post hoc Tukey test (GraphPad Prism, La Jolla, CA, USA). Statistical differences between control and experimental groups were determined via Student's t-test. Differences in means were considered to be statistically significant if the p-value was less than 0.05. All *in vitro* experiments had six independent cultures per treatment group to ensure sufficient power to detect statistically significant differences and were conducted multiple times to validate the observations. However, only data from a single representative experiment are shown and are expressed as means ± standard errors.

CHAPTER 3

RESULTS

Effect of chondrogenic media and structural environment on chondrogenic phenotype

To assess effects of chondrogenic media and different structural environments on mRNA levels of genes involved in chondrogenesis, ASCs were microencapsulated in alginate and treated with chondrogenic media (CM). CM increased mRNA levels of various genes involved in chondrogenesis, including *comp*, *acan*, *colX*, and *tgfb3*, compared to monolayer cells treated with MSCGM alone (Figure 1). *Nog*, a bone morphogenic protein (BMP) inhibitor, showed highly increased expression in the monolayer ASCs treated with CM, but this effect was diminished when the cells were microencapsulated. CM also decreased mRNA levels of the angiogenic factors f*gf2* and *vegfa*. Microencapsulation alone increased mRNA levels of *tgfb3*. *Col2*, an important component of the extracellular matrix of chondrocytes, showed highest expression when ASCs in microencapsulated cells were treated with CM.

To determine if the interaction between CM and microencapsulation was significant, a 3-Way ANOVA was conducted on data of mRNA levels of 13 genes quantified for CM, microencapsulation, and time. The interaction between chondrogenic media (CM) and microencapsulation (uE) was for mRNA levels of *nog*, *pthlh*, and *tgfb3* (Figure 2). Over a two week span, time had no significant effect on any mRNA levels assessed.

Different crosslinkers, calcium and barium, were used to determine if altered chemical composition of the microbeads would have an effect on mRNA levels of ASCs. Microencapsulation in both barium and calcium crosslinks increased mRNA levels of *tgfb3* and

pthlh, and decreased levels of *fgf2*, compared to monolayer ASCs (Figure 3). Barium crosslinks decreased expression of *tgfb3* compared to calcium crosslinker.

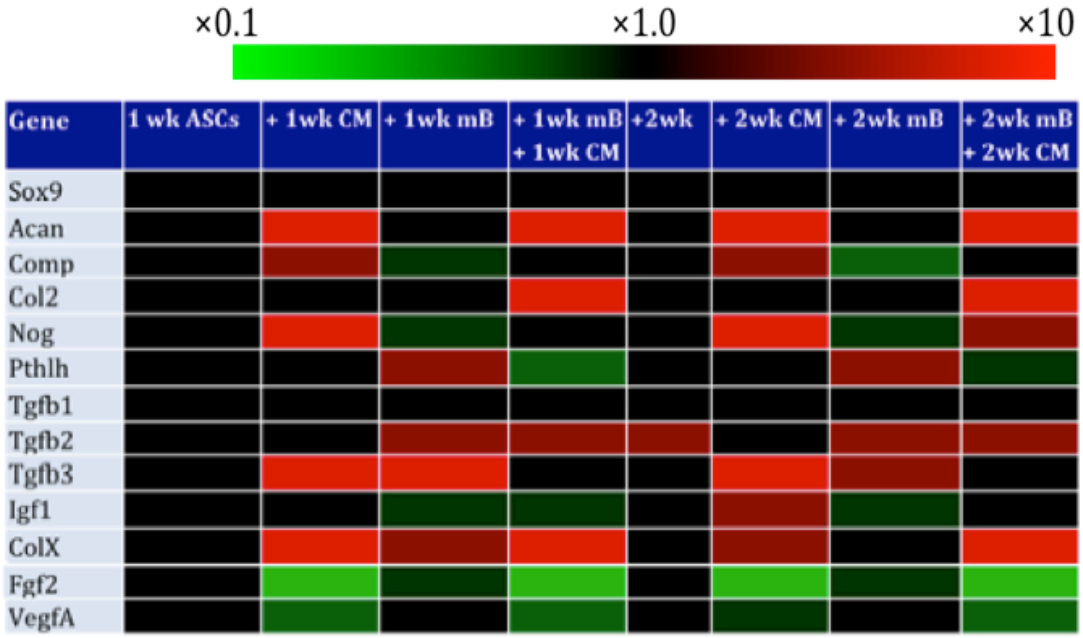

Figure 1. Heat map displaying relative quantities of each of the 13 genes quantified for treatment over control (monolayer cells with MSCGM).

Gene	Media	uE	Time	Media + uE
Sox9	**			
Acan	***			
Comp	***	***		
Col2		**		
Nog	***	***		*
Pthlh	***			**
Tgfb 1				
Tgfb 2		**		
Tgfb 3				**
Igf1	*	**		
ColX	***	***		
Fgf2	***	***		
VegfA	**			

Figure 2. 3-Way ANOVA of to assess effect of chondrogenic media, microencapsulation (uE), time and interactions on mRNA levels (*p<0.05, **p<0.001, ***p<0.0001).

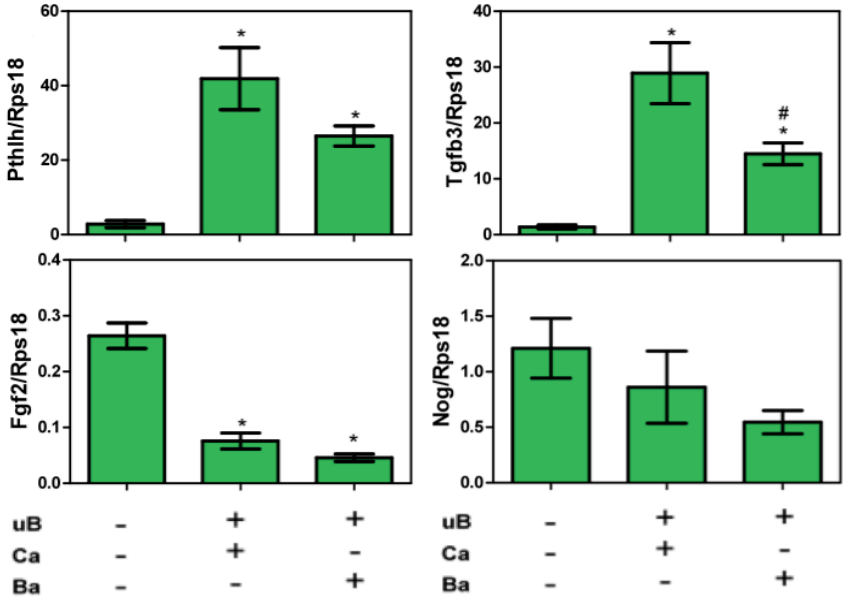

Figure 3. Gene expression of hASCs microencapsulated (uB) using a barium (Ba) or calcium (Ca) crosslinker (*p<0.05 vs. plated ASCs).

Effect of chondrogenic media components on chondrogenic phenotype

To determine the effect of individual components of chondrogenic media, monolayer ASCs were treated with all combinations of the components of chondrogenic media: 50μg/mL ascorbic acid 2-phosphate (AA2P), 100nM dexamethasone (Dex), 10ng/mL TGF-β1, and 100ng/mL BMP-6. Removing Dex from CM significantly increased mRNA for *comp* and decreased mRNA levels for *bmp2* and *nog* (Figure 4). Eliminating both Dex and AA2P increased mRNA levels of *pthlh*, *tgfb3*, and *vegfa*. Removing ascorbic acid 2-phosphate decreased mRNA levels of *acan*, *igf1*, and *bmp2*. Eliminating both growth factors decreased expression of most genes investigated.

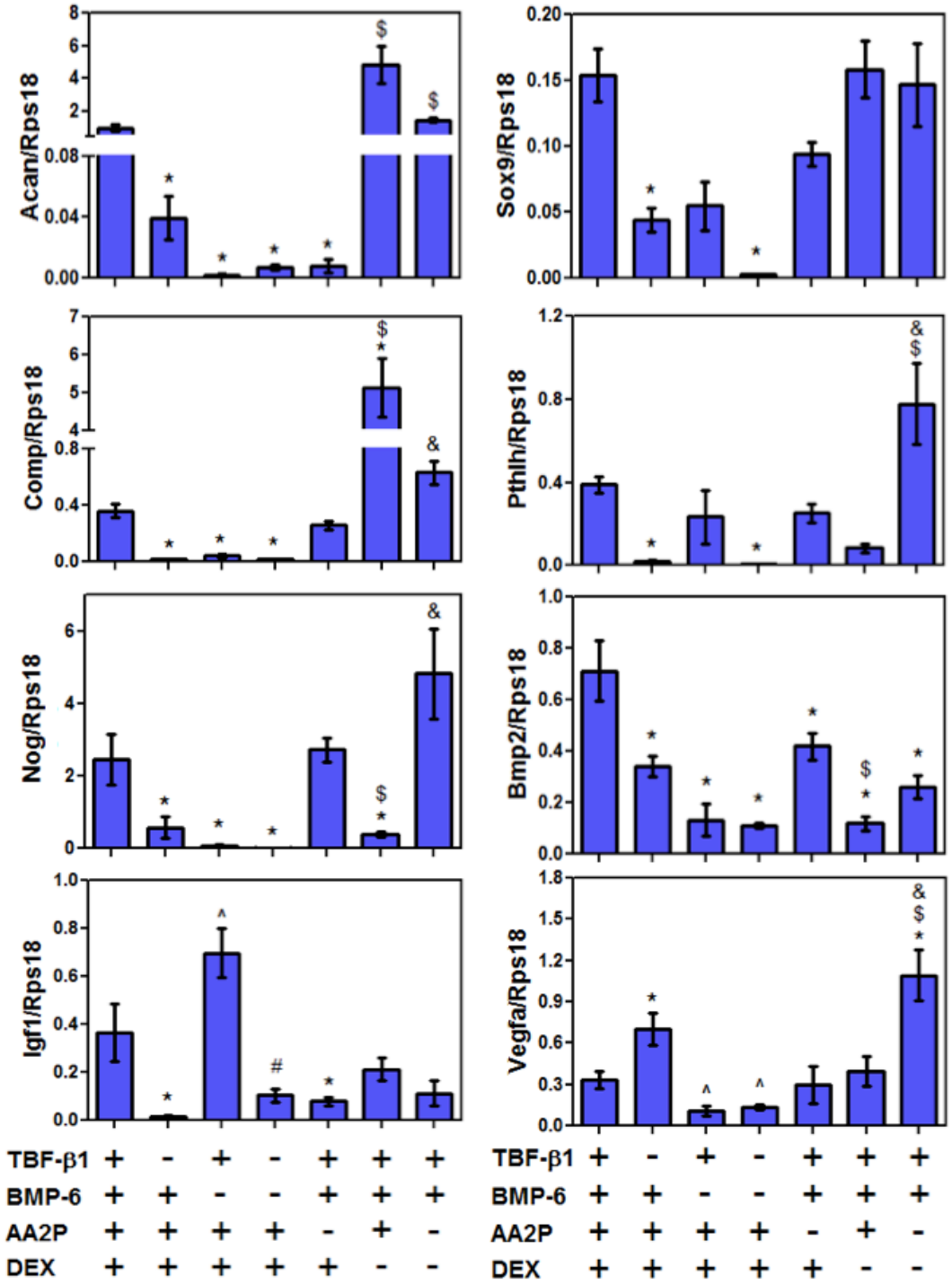

Figure 4. mRNA levels of monolayer ASCs treated with combination of TGF-B1, BMP-6, dexamethasone (Dex), and ascorbic acis 2-phosphate (AA2P). *p<0.05 vs. complete chondrogenic media (CCM), ^p<0.05 vs. -TGF-B1, #p<0.05 vs. -BMP-6, $p<0.05 vs. –AA2P, &p<0.05 vs. -Dex.

Effect of microencapsulation and CM on cartilage regeneration *in vivo*

To confirm the results shown *in vitro*, that microencapsulated ASCs treated with chondrogenic media demonstrate a chondrogenic phenotype, a 2 mm cartilage defect was created in rats. Microencapsulated ASCs preconditioned with chondrogenic media were injected into the defect, with a cartilage autograft, microencapsulated ASCs treated with standard growth media, and an empty defect serving as controls. Histological samples of the defects were prepared and stained for chondrocytes. Defects treated with hydrogel alone showed no cell infiltration into the defect and a lack of fast green staining (Figure 5A). Microencapsulated ASCs preconditioned in MSCGM (Figure 5B) and CM (Figure 5C) showed little cell infiltration into the defect. However, fast green staining revealed protein deposition throughout the extent of the defect.

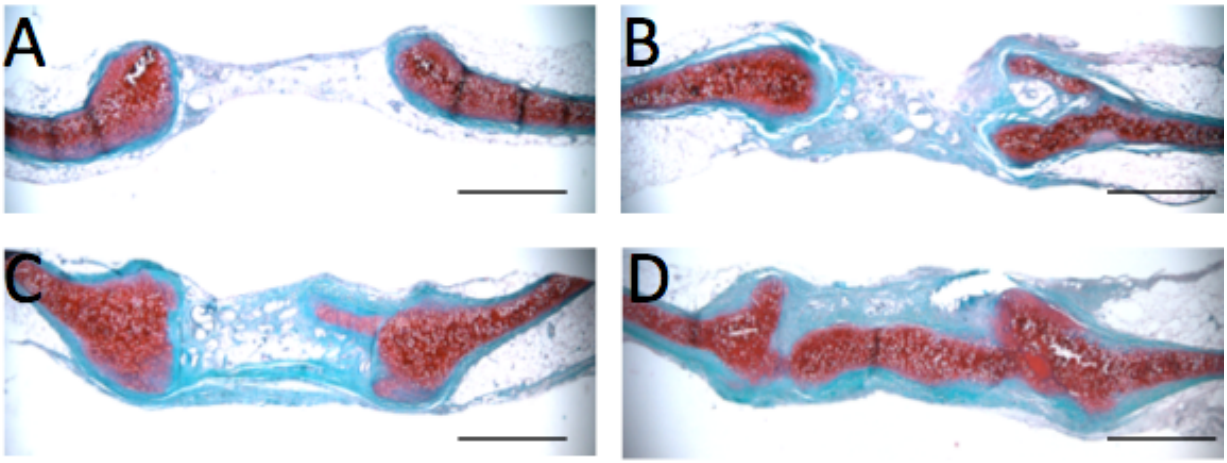

Figure 5. (A) RGD-conjugated hydrogel, (B) ASC microbeads preconditioned with the GM and RGD-conjugated hydrogel, (C) ASC microbeads preconditioned with the CM and RGD-conjugated hydrogel, and (D) autograft. Sections were stained with safranin-O and counter stained with fast green. Scale bar represents 1mm.

CHAPTER 4

DISCUSSION

Stem cell therapies have been investigated as a method of tissue regeneration. Current therapies to differentiate stem cells into the desired tissue have shown promising results, but have been yet to approved by the FDA. A potential method of regeneration is stimulating stem cells to release trophic factors to promote an environment of tissue regeneration. This is the first extensive study to demonstrate that microencapsulation and chondrogenic media components have different effects on the gene profile of ASCs and that microencapsulated ASCs preconditioned in CM can promote cartilage regeneration *in vivo*.

Chondrogenic media was shown to increase mRNA levels of a variety of genes involved in chondrogenesis and maintaining the cartilage phenotype. *Acan*, which is responsible for giving cartilage its compressive properties, and *comp*, which is a significant component in the extracellular matrix of cartilage, were both upregulated when treated with CM. *Nog*, a BMP inhibitor, was shown to be upregulated with CM treatment, which may assist in chondrogenesis by inhibiting cells from undergoing osteogenesis after chondrogenesis. The growth factor *tgfb3*, which stimulates cartilage proliferation and differentiation, was upregulated after CM treatment. CM treatment and microencapsulation decreased angiogenic factors, which is also important in stimulating ASCs to differentiate into avascular chondrocytes instead of vascular osteoblasts. The interaction between microencapsulation and CM was significant in three of thirteen genes quantified, suggesting that there could be an additional benefit in using both treatments as opposed to microencapsulation or CM alone. It should be noted, however, that microencapsulation alone increased expression of some angiogenic factors, suggesting that the

structural environment alone may promote ASCs to secrete angiogenic factors and deter the process of chondrogenesis.

Different microbead crosslinks did not have an effect on mRNA levels, suggesting that it is the 3D environment alone, as opposed to the barium or calcium, responsible for altering gene expression. The time points investigated did not have an effect on mRNA levels. Thus, there could be sustained expression of these growth factors over a two-week period, which would be translatable to therapeutic treatment.

In regards to individual components of CM, growth factors were shown to have a large impact on mRNA levels of chondrogenic genes. Dexamethasone, a potent steroid, was important in reducing levels of *vegfa*, a growth factor involved in angiogenesis. Inhibition of this gene would promote chondrogenesis by reducing the likelihood of osteogenesis occurring. The effect of AA2P was variable amongst different genes. This could be explained by the fact that AA2P, a derivative of Vitamin C, is involved in a variety of pathways, which could have variable downstream signaling effects on mRNA levels of different genes.

Microencapsulation alone of cells treated with MSCGM showed some extracellular matrix deposition throughout the defect, evidenced by fast green staining. This could be explained by the fact that the 3D structural environment alone may induce growth factor production. Preconditioning with CM appears to have a similar effect, increasing protein deposition throughout the defect. Complete cartilage infiltration and regeneration, however, was not seen in the defect at 35 days. This suggests that it may have been necessary to leave the microbeads for longer time points to assess the extent of regeneration. Furthermore, growth factors may have been secreted early in the regeneration process, and therefore cease to have an effect over the extent of the 35 day period. A method of controlled release of the factors from the

microbeads may better promote chondrogenesis by providing a constant stream of growth factors.

Adipose derived stem cells have shown their capability to produce a variety of chondrogenic factors, particularly when preconditioned with chondrogenic medium. Alginate microbeads, which are non-immunogenic, enable localized transport of these ASCs to the site of regeneration. These ASCs may not only differentiate into chondrocytes, but also secrete therapeutic factors that promote extracellular matrix growth. Together these results suggest a promising method of cartilage regeneration for diseases such as osteoarthritis.

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