Alginate Hydrogel as a Three-dimensional Extracellular Matrix for In Vitro Models of Development

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ALGINATE HYDROGEL AS A THREE-DIMENSIONAL EXTRACELLULAR MATRIX FOR IN VITRO MODELS OF DEVELOPMENT

By

Catherine N. Sargus-Patino

A THESIS

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Under the Supervision of Professor Angela K. Pannier

Lincoln, Nebraska

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Tissue engineering, involving the use of three-dimensional (3-D) scaffolds for cell and tissue culture, is a promising technique for establishing in vitro culture models that mimic in vivo environments. In vitro models present ethical and cost advantages over in vivo models, and allow for controlled, mechanistic studies on factors that regulate normal and abnormal tissue development. Alginate, a linear polysaccharide derived from brown algae, has properties that make it a favorable material as a 3-D extracellular matrix for in vitro cell and tissue models. After demonstrating alginate’s tunable physical and chemical properties with respect to gel formation, stiffness, and cellular interactions, alginate hydrogel was employed in two separate culture systems that share in common the ultimate goal of serving as in vitro models of tissue development and function: 1) an in vitro model of pig embryo elongation to develop strategies for improving pregnancy outcomes, and 2) an in vitro model of growth plate cartilage to discover factors necessary for inducing native cartilage architecture for tissue engineering applications. In regards to pig embryo culture, embryos encapsulated within alginate hydrogels exhibited greater survival and morphological changes than non-encapsulated control embryos, along with increased expression of steroidogenic transcripts and estrogen production, consistent with
in vivo elongation. For the growth plate model, alginate hydrogels were used for the encapsulation of mouse growth plate chondrocytes in vitro to study the effects of soluble parathyroid hormone (PTH), a signaling factor that regulates growth plate structure and function in vivo, along with the effects of an arginine-glycine-aspartic acid (RGD) peptide conjugated to the alginate. PTH and RGD peptide treatment resulted in decreased collagen X and Indian hedgehog transcript expression in encapsulated chondrocytes, demonstrating the role of these factors on the regulation of chondrocyte hypertrophy in vitro. Overall, information gained from utilizing these in vitro models as research tools can be used to advance the field of developmental biology and enhance tissue engineering therapies for the treatment of degenerative diseases.
ACKNOWLEDGEMENTS

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Lastly, to my mom and dad, thank you both for your immense love and support, for encouraging my curiosity as a child, and for teaching me that a positive attitude can go a long way.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2-D</td>
<td>Two-dimensional</td>
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<tr>
<td>3-D</td>
<td>Three-dimensional</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BMPs</td>
<td>Bone morphogenic proteins</td>
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<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>Calcium sulfate</td>
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<tr>
<td>ColX</td>
<td>Type X collagen</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>Aromatase</td>
</tr>
<tr>
<td>CT</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>Cytochrome P450 side chain cleavage</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol 17-β</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-(dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF7</td>
<td>Fibroblast growth factor 7</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>G</td>
<td>α-L-guluronic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GRGDSP</td>
<td>Glycine-arginine-glycine-aspartic acid-serine-proline</td>
</tr>
<tr>
<td>hBMSCs</td>
<td>Human bone marrow-derived mesenchymal stem cells</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Heparin sulfate proteoglycans</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>IL1B</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>KRT18</td>
<td>Cytokeratin-18</td>
</tr>
<tr>
<td>LN</td>
<td>Laminin</td>
</tr>
<tr>
<td>LSM</td>
<td>Least-squares mean</td>
</tr>
<tr>
<td>M</td>
<td>β-D-mannuronic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone-related protein</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RCS</td>
<td>Rat chondrosarcoma</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantity</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>STAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>N-hydroxy-sulfo succinimide</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TGFBs</td>
<td>Transforming growth factor-betas</td>
</tr>
<tr>
<td>USDA</td>
<td>U.S. Department of Agriculture</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIM</td>
<td>Vimentin</td>
</tr>
<tr>
<td>wcRNA</td>
<td>Whole-cell RNA</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

CHAPTER 1: Thesis Introduction and Background ........................................... 1

1.1. Introduction .............................................................................................. 1

1.2. Tissue Engineering .................................................................................. 2

1.3. Alginate .................................................................................................... 3

1.3.1. Structure and properties ...................................................................... 3

1.3.2 Applications ............................................................................................ 7

1.3.3. Chemical modifications ......................................................................... 8

1.4. In vitro models of development ............................................................... 10

1.5. Pre-implantation pig embryo development ............................................. 12

1.5.1. Developmental hallmarks .................................................................... 12

1.5.2. Embryonic loss in the pig ..................................................................... 14

1.5.3. Previous studies on in vitro elongation of embryos ............................ 15

1.6. Architecture and development of growth plate cartilage ...................... 16

1.6.1. Zonal arrangement of chondrocytes ..................................................... 16

1.6.2. Signaling pathways and transcription factors that regulate growth plate

function ........................................................................................................... 18

1.6.3. Cartilage injury and disease ................................................................. 21

1.6.4. Previous studies on engineering growth plate cartilage .................... 22

1.7. Objectives of thesis .................................................................................. 23

CHAPTER 2: Alginate gel design and characterization .................................. 24

2.1. Introduction .............................................................................................. 24

2.2. Methods .................................................................................................... 26
2.2.1. Gel formation ........................................................................................................... 26
2.2.2. Chemical modification of alginate ................................................................. 31
2.2.3. Confirmation of peptide conjugation ............................................................ 32
2.2.4. Determination of Young’s modulus .............................................................. 33

2.3. Results ...................................................................................................................... 35
2.3.1. Gel morphologies ............................................................................................ 35
2.3.2. Confirmation of peptide conjugation ............................................................ 37
2.3.3. Young’s modulus values .................................................................................. 39

2.4. Discussion ................................................................................................................ 41

CHAPTER 3: In vitro development of pre-implantation porcine embryos using alginate hydrogels as a three-dimensional extracellular matrix .......... 47

3.1. Introduction ............................................................................................................. 47

3.2. Materials and methods ........................................................................................ 49
3.2.1. Production and collection of embryos ............................................................ 49
3.2.2. Embryo encapsulation and culture ................................................................. 50
3.2.3. Characterization of embryo viability and morphology .................................. 52
3.2.4. Transcript expression analysis ....................................................................... 53
3.2.5. Estradiol-17β and protein assays ................................................................. 56
3.2.6. Statistical Analysis ......................................................................................... 56

3.3. Results ...................................................................................................................... 57
3.3.1. Evaluation of embryo viability and morphology ............................................. 57
3.3.2. Transcript expression levels .......................................................................... 59
3.3.3. Estradiol-17β and protein analysis ................................................................. 61
CHAPTER 4: The effect of parathyroid hormone and RGD peptide ligands on the 
in vitro behavior of growth plate chondrocytes within 3-D alginate

matrices

4.1. Introduction

4.2. Methods

4.2.1. Preparation of alginate solutions

4.2.2. Isolation of growth plate chondrocytes

4.2.3. Chondrocyte encapsulation and culture

4.2.4. Characterization of cell viability and proliferation

4.2.5. Transcript expression analysis

4.2.6. Statistical analysis

4.3. Results

4.3.1. Evaluation of chondrocyte viability and proliferation

4.3.2. Transcript expression levels

4.4. Discussion

CHAPTER 5: Future studies and conclusions

5.1. Introduction

5.2. Future directions for the in vitro pig embryo model

5.3. Future directions for the in vitro growth plate model

5.4. Conclusions

REFERENCES
LIST OF FIGURES

Fig. 1-1. Chemical structures of G-block, M-block, and alternating block in alginate (Reprinted from Lee and Mooney, *Progress in Polymer Science*, 2012). .......................................................... 4

Fig. 1-2. Alginate hydrogels prepared by ionic cross-linking (egg-box model). (Reprinted from Lee and Mooney, *Progress in Polymer Science*, 2012). .......................................................... 5

Fig. 1-3. Functional zonal arrangement of the growth plate within long bones. ............. 18

Fig. 1-4. Schematic illustration of the Ihh/PTHrP feedback loop........................................... 19

Fig. 2-1. Schematic illustration of the formation of alginate hydrogel beads, in conjunction with the encapsulation of embryos. .................................................................................. 28

Fig. 2-2. A) Mesh mold used for creating cylindrical gels. B) Example of a gradient gel pre-crosslinking. ............................................................................................................. 29

Fig. 2-3. Mold for creating alginate gel tubes, consisting of a cylindrical mesh frame with glass tube through the center.................................................................................................. 30

Fig. 2-4. The setup for the formation of alginate wells. ......................................................... 31

Fig. 2-5. Representative images of alginate beads................................................................. 35

Fig. 2-6. A) Cylindrical alginate hydrogel, B) Gradient gel consisting of different concentrations of alginate, dyed with different colors, C) Alginate gel tube prior to removing glass tube, D) Resulting alginate gel tube. ............................................................................................................. 36

Fig. 2-7. Representative image of alginate wells, with dimensions of 1 mm x 5 mm........... 37
Fig. 2-8. Representative bright field images of D1 ORL UVA cells adhered to unmodified alginate hydrogel gels (A and C), and RGD-alginate gels (B and D), 24 hr and 72 hr post-seeding. .......................................................... 38

Fig. 2-9. $^1$H-NMR spectra of unmodified alginate (red) and GRGDSP-alginate (blue) in D$_2$O.......................................................... 39

Fig. 2-10. Representative stress-strain curve of a 1.5% RGD-alginate gel. ....................... 40

Fig. 2-11. Young’s modulus as a function of alginate concentration for unmodified (black circle) and RGD-modified (red triangle) alginate gels.......................................................... 41

Fig. 3-1. Schematic illustration of the double encapsulation method.......................... 51

Fig. 3-2. Representative illustration of morphological changes observed over 96 h of culture in embryos double encapsulated in 0.7% alginate hydrogels or remaining as non-encapsulated control embryos, with corresponding cellular survival and death after 96 h of culture..... 53

Fig. 3-3. Expression levels of A. STAR, B. CYP11A1, C. CYP19A1 and D. IL1B mRNA measured by qPCR from in vivo-produced control embryos and in vitro-cultured CONT (non-encapsulated control), ENC- (encapsulated with no observable morphological changes) and ENC+ (encapsulated with observable morphological changes) embryos.......................... 60

Fig. 3-4. A. Estradiol-17β (E2) concentrations (pg/mL) in culture media collected at 24-, 48-, 72- and 96-h of culture containing only media (MEDIA), non-encapsulated control embryos (CONT), encapsulated embryos with no observable morphological change (ENC-) and encapsulated embryos with observable morphological changes (ENC+). B. E2 concentrations (pg/mL) in uterine flushings from pregnant gilts at d 9, 10 and 11 of gestation.......................... 62
Fig. 3-5. A. Total protein (mg) in culture media collected at 24-, 48-, 72- and 96-h of culture containing only media (MEDIA), non-encapsulated control embryos (CONT), encapsulated embryos with no observable morphological change (ENC-) and encapsulated embryos with observable morphological changes (ENC+). B. Total protein (mg) in uterine flushings from pregnant gilts at d 9, 10 and 11 of gestation. ................................................................. 63

Fig. 4-1. Schematic illustration of zonal arrangement of chondrocytes and Ihh/PTHrP feedback loop within the growth plate. ........................................................................... 72

Fig. 4-2. Live/dead staining at Day 1 of culture of chondrocytes encapsulated in the first (A.) and last (B.) beads that were prepared from the RGD/no PTH condition, and representative beads from this batch on Day 2 (C.) and Day 3 (D.) of culture. .................................................. 81

Fig. 4-3. Representative images of nuclear (blue) and EdU (green) staining of chondrocytes cultured in unmodified alginate gels without PTH (Unmodified/no PTH), unmodified gels with 1 μM PTH (Unmodified + PTH), RGD-alginate gels without PTH (RGD/no PTH), and RGD-alginate gels with 1 μM PTH (RGD + PTH). ................................................................................. 82

Fig. 4-4. Expression levels of A. collagen X (ColX), B. Indian hedgehog (Ihh), and C. Sox9 (Sox9) mRNA from chondrocytes cultured in unmodified alginate gels (Unmodified), unmodified gels with 1 μM PTH (Unmodified + PTH), RGD-alginate gels (RGD), and RGD-alginate gels with 1 μM PTH (RGD + PTH). ................................................................................. 85

Fig. 5-1. Expression levels of CYP11A1 (blue), CYP19A1 (red), and STAR (green) mRNA measured by qPCR from Enc d 9 (encapsulated embryos cultured in d9 flushes), Enc d 10 (encapsulated embryos cultured in d10 flushes) and Enc serum (encapsulated embryos cultured in serum media). ......................................................................................... 94
Fig. 5-2. Representative images of Alg, RGD-Alg, and Alg + OPN embryos that changed morphologically by 96 hours of culture, along with a control embryo that did not change morphologically, other than swelling. .................................................................................................................. 98

Fig. 5-3. Schematic illustration of the approaches for co-culturing uterine cells with encapsulated pig embryos. ........................................................................................................................................... 100

Fig. 5-4. Confocal microscopy images of in vivo-derived Day 9 embryo with actin and nuclei stained with phalloidin and DAPI, respectively. ........................................................................................................ 103

Fig. 5-5. Expression levels of A. Collagen X and B. PTHrP measured by RT-PCR from chondrocytes encapsulated in RGD-alginate and cultured with, 0 ng/mL, 0.2 ng/mL, or 0.5 ng/mL sonic hedgehog (Shh). ........................................................................................................................................ 105

Fig. 5-6. Schematic illustration of the encapsulation of chondrocytes and formation of mechanical/chemical gradients within alginate hydrogels via co-culture with factor-expressing cells (A,C) or incorporated signaling factors (B,D). ........................................................................................................ 108

Fig. 5-7. 1.5% RGD-alginate bead with two gel layers, consisting of Ihh-expressing HEK293T cells (HEK) encapsulated within the inner bead and rat chondrosarcoma cells (RCS) encapsulated within the outer layer. ........................................................................................................ 109
LIST OF TABLES

Table 2-1. Young’s modulus values for unmodified alginate and RGD-alginate hydrogels of increasing concentrations. ................................................................. 41

Table 3-1. Summary of embryo survival and observed morphological changes following 96 h of culture of either non-encapsulated control porcine embryos (CONT) or porcine embryos double encapsulated (ENC) in 0.7% alginate hydrogels................................................................. 59

Table 4-1. Overview of number of beads per culture condition and per endpoint analysis ........................................................................................................ 77

Table 4-2. Description of the designed primers. ................................................................. 79

Table 4-3. Proliferation results of chondrocytes cultured in unmodified or RGD-alginate gels, with or without PTH added to the culture media. ................................................................. 83

Table 5-1. Summary of embryo survival and morphological changes observed following 96 h culture of day 9 porcine embryos encapsulated in 0.7% alginate gels. Encapsulated embryos were assigned to be cultured in day 9 uterine flushes (D 9), day 10 uterine flushes (D 10), or in base serum media (Serum). ......................................................................................................................... 94

Table 5-2. Summary of embryo survival and morphological changes observed following 96 h culture of day 9 porcine embryos encapsulated in unmodified alginate (Alg), alginate blended with 1 µg/mL osteopontin (OPN), alginate conjugated with GRGDSP, and non-encapsulated control embryos (Control) ........................................................................................................ 98
CHAPTER 1

Thesis Introduction and Background

1.1. Introduction

The tissue engineering approach to cell and tissue culture, involving the use of three-dimensional (3-D) scaffolds, is a promising technique for establishing in vitro culture models that mimic in vivo environments. Alginate, a linear polysaccharide derived from brown algae, has properties that make it a favorable material as a 3-D extracellular matrix (ECM) for in vitro cell or tissue models. Alginate forms a hydrogel by ionic-crosslinking in the presence of a divalent cation, allowing for gentle encapsulation of cells or tissue. In addition, alginate hydrogel can be chemically modified to promote specific interactions between cells or tissue and the surrounding hydrogel, or can be left unmodified to solely provide mechanical support for tissue morphogenesis and/or cellular responses. For this thesis, alginate hydrogel was used to develop two separate culture systems that share in common the ultimate goal of serving as in vitro models for tissue development and function. For the first project, alginate hydrogel was investigated as a 3-D matrix for the in vitro development of pre-implantation pig embryos. Between day 10 and 12 of gestation in the pig, embryos undergo a dramatic morphological change, known as elongation. Deficiencies in elongation contribute to approximately 20% of embryonic loss, but exact mechanisms of elongation are poorly understood. In order to provide an in vitro tool for evaluating mechanisms of elongation, the first objective of this thesis was to utilize alginate hydrogels as 3-D scaffolds for the encapsulation and support of porcine embryo elongation in vitro. Secondly, alginate hydrogel was investigated as a 3-D matrix for the establishment of an in vitro growth...
plate model. Physical damage or congenital disorders that disrupt growth plate cartilage result in skeletal abnormalities that are often associated with long-term intensive healthcare needs due to impact on the joints, central nervous system, and ocular system. Studying the generation of growth plate architecture in vitro is critical for understanding mechanisms that regulate normal development of growth plate cartilage and how these mechanisms are altered in abnormal growth plates, in addition to enhancing tissue engineering strategies. Therefore, the second objective of this thesis was to determine the extracellular factors required to induce native growth plate structure and function within 3-D alginate hydrogels. Overall, in addition to advancing the field of developmental biology, the physiological information gained from using these in vitro models as research tools can be used to enhance fertility and pregnancy outcomes as well as tissue engineering and cell-based therapies for the treatment of degenerative diseases.

1.2. Tissue Engineering

The use of autologous or allogeneic grafts in traditional transplantation surgeries has serious limitations, including a lack of available donor tissues and severe immune responses, respectively [1]. Tissue engineering is a relatively new field that combines engineering with the life sciences in order to develop functional tissue substitutes, and has the potential to revolutionize medicine by providing optimal replacement tissues for those damaged due to injury or disease [2]. Advantages of tissue engineering include the use of autologous or allogeneic cells to overcome the limitation of donor tissue availability, as well as the ability to directly control in vitro culture conditions to model those that occur in vivo [3]. The basic principle of tissue engineering involves three main
components [4]. First, living cells are needed in order to produce the desired tissue. The cells are often derived from donor tissue or from stem or progenitor cells [2, 4]. Secondly, a 3-D scaffold is needed to provide structural support for the growing tissue and maintenance of cell-to-cell communication. These scaffolds often mimic the in vivo environment of the tissue by setting appropriate mechanical properties and chemical signals. Tissue engineering scaffolds can be made of synthetic materials, such as the polyglycolic or polylactic family of polymers, or they can be made of natural materials, such as collagen, alginate, or chitosan [2-5]. The final essential component of tissue engineering is the inclusion of bioactive signals, as they are used to establish cell-scaffold interactions and provide the necessary chemical stimuli to promote cell growth and differentiation [2-4]. Taken together, these three components of tissue engineering can act synergistically to develop functional tissue substitutes for the replacement of diseased or damaged tissue.

1.3. Alginate
1.3.1. Structure and properties

Hydrogels, i.e. networks of hydrophilic polymer chains with high water content, are highly appealing as 3-D scaffolds for in vitro cell and tissue culture due to their ability to mimic the physical properties of native soft tissue [6], as well as their high porosity for efficient diffusion of proteins and nutrients [7]. Alginate hydrogel, in particular, is widely used in tissue engineering applications due to its desirable properties as a biomaterial [8]. Alginate is a linear polysaccharide produced as an ECM component in brown algae and some bacteria. The polysaccharide is composed of repeating units of
β-D-mannuronic acid (M) and α-L-guluronic acid (G) [9] (Fig. 1-1) and forms a hydrogel by ionic cross-linking of the G residues in the presence of a divalent cation (Fig. 1-2) [10].

**Fig. 1-1.** Chemical structures of G-block, M-block, and alternating block in alginate (Reprinted from Lee and Mooney, *Progress in Polymer Science*, 2012).

Calcium chloride (CaCl₂) solution is commonly used as the crosslinking reagent for alginate and provides an external gelation method for creating alginate beads. Alginate solution can be dropped into CaCl₂, immediately forming a 3-D network of highly hydrated gel [11]. Sodium chloride (NaCl) is often added to the CaCl₂ solution for greater uniformity and higher porosity of the resulting alginate gel by providing anti-gelling cations; if NaCl is not added, the alginate at the surface of the bead becomes tightly packed, limiting further diffusion of the calcium ions into the center of the gel [7]. Hindered diffusion leads to an alginate bead with higher mechanical stiffness at the
surface and lower stiffness in the center. In addition, radial channels develop in the gel when the alginate is crosslinked in CaCl₂ solution without the addition of NaCl [12]. Alginate hydrogels can also be formed using internal gelation methods, in which a calcium slurry, usually calcium carbonate (CaCO₃) or calcium sulfate (CaSO₄), is mixed into the alginate solution and gelation occurs as the calcium slowly leaks from within the alginate [11]. In this case, release of the calcium is caused by a reduction in pH that may be harmful to cells that are within the alginate solution during gelation. However, the internal gelation method may be useful for seeding cells on pre-formed alginate scaffolds [11].

![Fig. 1-2. Alginate hydrogels prepared by ionic cross-linking (egg-box model). Only guluronate blocks participate in the formation of a corrugated egg-box-like structure with interstices in which calcium ions are placed (Reprinted from Lee and Mooney, Progress in Polymer Science, 2012).](image)

The external gelation process, which commonly involves CaCl₂, allows for gentle encapsulation of cells and tissue without negatively affecting cell viability. The alginate gel forms a mesh-like structure, with pore sizes ranging from 5 to 200 nm, that permits diffusion of proteins and hormones essential for cell and tissue growth and development [7, 13]. Increasing the concentration of alginate in the gels decreases the rate of diffusion of proteins through the pores [13]. Gels made of alginate with a high G content have the most open pore structure and therefore allow for the greatest rate of protein diffusion [7].
In addition to its gentle encapsulation process and diffusion capabilities, alginate promotes negligible non-specific protein adsorption and cell adhesion, leading to minimal interaction between encapsulated cells/tissue and the surrounding alginate hydrogel [14]. This feature allows alginate to act as a “blank slate”. If left unmodified, alginate gels can be used to solely provide mechanical support to cells or tissues; varying the concentration of alginate alters the mechanical properties of the resulting matrix and can be used to evaluate the effect of matrix mechanics on cell or tissue growth [15]. Otherwise, specific extracellular factors can be added to the alginate to control the interaction between encapsulated cells/tissue and the surrounding alginate hydrogel.

Degradation of alginate hydrogels does not readily occur in physiological conditions [16, 17], although some de-crosslinking can occur due to the gradual diffusion of calcium ions out of the matrix [18]. Increased degradation occurs in the presence of a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), lactate, citrate, or phosphate, which increases the removal of calcium ions from the matrix [13]. No known alginate-degrading enzymes exist in humans, but alginate lyases have been isolated from marine algae and a wide range of microorganisms [11]. Alginate lyases cause degradation of alginate via a β-elimination mechanism, in which the glycosidic $\text{1} \rightarrow \text{4}$ O-linkage between monomers is cleaved [19]. The lack of degradation in physiological conditions by alginate hydrogels makes alginate an appropriate material for maintaining structural integrity during relatively long culture times when used for in vitro cell or tissue culture. Alginate lyase or a chelating agent such as EDTA can then be used to easily retrieve the cells or tissue from the alginate bead if necessary for further assays. Due to these unique physical and chemical properties of alginate, along with its innate biocompatibility,
alginate has been employed in biomedical and food industries, as described below, including its recent popularity as a matrix for tissue engineering applications.

1.3.2 Applications

Alginate is commonly used in the food industry as a thickening agent to increase the quality of foods, such as ice cream and dressings, demonstrating its biocompatibility and low toxicity [11]. It has also been widely used in the pharmaceutical industry as a drug or protein delivery agent; alginate gels can release macromolecules in a controlled manner and can be orally administered or injected in a non-invasive approach [20].

Examples of alginate research for pharmaceutical applications include studies on composite alginate/poly(lactic-co-glycolic) microparticles for insulin delivery [21], coated chitosan-alginate beads for oral delivery of the antibiotic drug cefaclor [22], and tamoxifen-loaded nanoparticles for the treatment of breast cancer [23]. Recently, alginate research has shifted to tissue engineering applications due to its biocompatibility, relatively low cost, and gentle gelation process [11, 20]. Specifically, alginate hydrogels are favorable for cell transplantation or immobilization and can be used to deliver cells to a desired site in vivo or provide a controlled 3-D environment for new tissue formation in vitro [20]. Alginate gels have previously been used for the encapsulation and culture of a variety of cell types, including articular chondrocytes [24, 25], skeletal myoblasts [26, 27], neural stem cells [28], and mouse embryonic stem cells [29].

In addition to cell encapsulation, alginate hydrogels have demonstrated success as a 3-D extracellular matrix for the in vitro culture of organs and embryos, for the purpose of developmental studies [30], disease simulation [31], and advancing in vitro
fertilization techniques [32]. In regards to organ culture, alginate hydrogels have previously been used to support *in vitro* development of ovarian follicles in mice [15, 32, 33] and nonhuman primates [34], cryopreserved-thawed human cortical follicles [35], and sections of ovaries and oviducts to model specific changes that might lead to ovarian cancer [36]. The studies involving mouse ovarian follicle culture have demonstrated that encapsulation of follicles within alginate hydrogels enhances follicle maturation *in vitro* and can lead to subsequent fertilization *in vitro* [37]. For embryo culture, alginate hydrogels have previously been successful for the *in vitro* development of mouse pronuclear stage embryos [30] and bovine embryos up to the blastocyst stage [38]. The results of these previous studies provide evidence that alginate has great potential as an artificial 3-D extracellular matrix that can support *in vitro* culture of cells, tissues, and organs, making it the scaffold of interest for the *in vitro* models established in this thesis. However, despite the intrinsic properties of alginate that make it a favorable material for tissue engineering applications, chemical modifications are often required to promote desirable cellular functions, provide a greater range of mechanical properties, and/or provide controlled release of encapsulated factors.

1.3.3. *Chemical modifications*

Alginate’s free hydroxyl and carboxyl groups along its molecule chain provide numerous sites that are ideal for chemical functionalization. Properties such as degradability, hydrophobicity, and biological characteristics can be altered by functionalizing available hydroxyl and/or carboxyl groups, or interfering with carbon-carbon bonds [39]. For example, to decrease alginate gel stiffness and increase
degradability in physiological conditions, oxidation of alginate is often employed, which lowers the molecular weight (MW) of the polysaccharide [15, 39]. Sodium periodate, which is used for the oxidation reactions, breaks carbon-carbon bonds of the guluronate group yielding an adduct that does not form ionic bridges with divalent cations [40]; therefore, less crosslinking occurs, leading to lower mechanical stiffness of the resulting gel. Furthermore, partial oxidation of alginate creates acetal groups that are susceptible to hydrolysis. Consequently, increasing the degree of oxidation increases degradability of the resulting alginate gel in physiological conditions [41].

Another useful chemical modification of alginate is sulfation, prepared through the reaction of alginate with chlorosulfuric acid in formamide [39]. This process gives alginate a structural similarity to heparin and therefore promotes high blood compatibility. Sulfation of alginate also mimics heparin’s high affinity for certain growth factors; therefore, alginate-sulfate hydrogels have been used to provide protection and sustained release of heparin-binding growth factors, such as basic fibroblast growth factor, which is valuable for in vivo delivery systems and tissue engineering applications [42].

A common modification of alginate, especially for cell encapsulation purposes, is amidation, in which carbodiimide chemistry is used to form amide linkages between amine-containing molecules and the carboxylic acid functional groups of the alginate polymer backbone [39]. This type of chemistry is useful for covalently linking specific peptides to the alginate backbone to promote cell-matrix interactions, as cell adhesion is often a strict requirement for cell viability and proliferation [14]. The arginine-glycine-aspartic acid (RGD) peptide sequence, which is found in extracellular matrix proteins
such as fibronectin and laminin, is the most effective and commonly used peptide sequence to promote cell adhesion [43]. When covalently linked to alginate, RGD peptide sites serve as ligands to cell integrins to mediate cell migration and adhesion, and the ligand density can be varied to produce a desired degree of adhesion [14]. Overall, the tunable physical and chemical characteristics of alginate make it an ideal candidate as a 3-D matrix for tissue engineering strategies, including those used for the establishment of \textit{in vitro} models of tissue development and function, as described below.

1.4. \textit{In vitro} models of development

While traditional tissue engineering studies have focused on using scaffolds for tissue regeneration for transplantation therapies, as described earlier, the field of tissue engineering, in particular the use of scaffolds, is now being explored for other applications, such as studies on drug efficacy and toxicology as well as investigations on tissue development and morphogenesis. For these applications, an \textit{in vitro} tissue model presents ethical and cost advantages over \textit{in vivo} models, and allows for mechanistic studies on simple, controlled systems [44]. Traditional two-dimensional (2-D) culture systems result in a disruption of cell-to-cell interactions as cells attach to the substrate and migrate away from the tissue [33, 45]. Consequently, cells and tissue often behave very differently when cultured in two dimensions and experience major differences in gene expression due to the lack of a natural 3-D environment [46]. Three-dimensional culture systems can maintain embryo or tissue architecture and allow for direct physical interaction with the surrounding environment, which better mimics \textit{in vivo} development.
Therefore, the tissue engineering approach provides an exciting, more physiologically natural alternative to traditional 2-D systems as \textit{in vitro} models of development.

The use of tissue engineering principles to establish \textit{in vitro} models that closely resemble \textit{in vivo} conditions provides a means for researchers to evaluate normal and abnormal embryo or tissue development, as well as factors that affect development. Understanding native embryo and tissue development is important for advancing the field of developmental biology, which focuses on uncovering mechanisms involved in cell growth, differentiation, and morphogenesis. Knowledge of developmental biology mechanisms, specifically those involving gene regulation of cell differentiation and tissue specification, can be integrated with other biological fields to help solve major questions that still exist in those areas \cite{47}.

Examples of \textit{in vitro} culture systems that have employed the tissue engineering approach include models for ovarian follicle \cite{32-34} and embryo development \cite{48-51}, as well as \textit{in vitro} organ models such as kidney \cite{31}, cardiac \cite{52}, skin \cite{53}, and oviduct models \cite{36}. Human skin models, in particular, have served as \textit{in vitro} test systems for pharmaceutical research \cite{53}, and cardiac tissue models have provided a means for electrophysiological studies of the heart \cite{52}, a complicated task to evaluate by \textit{in vivo} measures. In addition, \textit{in vitro} tumor models have recently become highly appealing for studying the efficiency of chemotherapy drugs \cite{54}, as well as factors involved in tumor initiation and progression \cite{55}. The physiological and pathophysiological information gained from using these \textit{in vitro} models as research tools can be used for enhancing fertility and pregnancy outcomes as well as tissue engineering and cell-based therapies for the treatment of degenerative diseases. The research presented in this thesis
specifically focuses on the establishment of two separate *in vitro* models of development using alginate hydrogel as the 3-D matrix: a model for the development of pre-implantation pig embryos and an *in vitro* growth plate model. The context and impact of these two culture models are presented in the following sections.

### 1.5. Pre-implantation pig embryo development

#### 1.5.1. Developmental hallmarks

In the pig, the pre-implantation period of pregnancy is characterized by several developmental hallmarks that have significant impacts on embryo survival, uterine capacity, and subsequent pre-weaning survivability. Between day 10 and 12 of gestation, the embryo is transformed from a spherical structure (~1-2 mm) to a long thin, filament (>100 mm) in a process referred to as elongation [56]. During elongation, the embryo produces and secretes estrogen, which serves as the key molecule for maternal recognition of pregnancy and also modulates the production and secretion of proteins and growth factors within the uterus [56]. Elongation of the pig embryo is also characterized by significant changes in gene regulation as the embryo undergoes this transition [57, 58]. In conjunction with the increase of estrogen production and secretion by the embryo during elongation, several transcripts involved in steroidogenesis, such as steroidogenic acute regulatory protein [59], cytochrome P450 side chain cleavage (*CYP11A1*), and aromatase (*CYP19A1*), are increased in a similar pattern as estrogen production during embryo elongation [58, 60]. Unlike elongation in other domestic animals, rapid elongation of the pig embryo has been primarily associated with cellular remodeling and
differentiation rather than cellular hyperplasia [61]. As a result, potential markers for differentiation of the trophectoderm (i.e., cytokeratin-18 [KRT18]) and mesoderm (i.e., vimentin [VIM]) have been identified in the elongating pig blastocyst [62-64].

Proper interactions between the embryo and receptive uterine endometrium are essential for supporting embryonic development and subsequent implantation [65]. These interactions are initiated by the immune responsive cytokine, interleukin-1β (IL1B), which is produced by the embryo and uterine endometrium during elongation and may be responsible for suppressing the maternal immune response to prevent rejection of the embryo [66]. The increase in IL1B expression during trophoblastic elongation, and its association with the initiation of estrogen production by the embryo, suggests that IL1B may have a critical role in pre-implantation embryo development [67].

Many uterine specific factors have been shown to be up-regulated within the uterine milieu as the embryo initiates elongation and subsequently transitions to its superficial implantation on the uterine endometrium [68]. For instance, fibroblast growth factor 7 (FGF7), transforming growth factor-betas (TGFβs), osteopontin (OPN), fibronectin (FN), and laminin (LN) have been suggested to play a role in embryo-maternal interactions during elongation and implantation [69, 70]. OPN, in particular, is an ECM glycoprotein secreted by uterine epithelial cells that plays a role in the porcine implantation cascade during pregnancy [71]. Previous evidence has suggested that the RGD peptide of OPN binds to integrins alpha 5 and beta 3 on the embryonic trophectoderm, inducing cytoplasmic reorganization during embryo elongation by stimulating cell-cell adhesion and promoting cell migration [71]. OPN has been shown to enhance development of early porcine embryos to the blastocyst stage in vitro when
added exogenously to culture media [72]. The surface of the oviduct and uterus contains several other ECM components that have been found to influence embryonic development, morphogenesis, and function by stimulating cell-cell interactions [73, 74]. Two ECM glycoproteins involved in cellular adhesion, FN and LN, have previously been shown to increase the hatching rates of cultured human embryos when added to the culture media [75]. A study involving the culture of porcine blastomeres on FN-coated culture dishes demonstrated that FN enhanced development to the blastocyst stage compared to control treatment groups [76], suggesting its role in early embryonic development. Given the potential involvement of these growth factors and ECM components during early embryonic development in the pig, further investigations are warranted to elicit the exact mechanisms of pig embryo elongation. The alginate culture model established in this thesis for pre-implantation pig embryo development can serve as the necessary tool for studying the affects of these factors on embryo development and elongation in a controlled, *in vitro* environment. In addition to advancing knowledge in reproductive biology, the mechanisms uncovered with our *in vitro* culture model can be used to address critical issues with embryonic loss, as described below.

1.5.2. *Embryonic loss in the pig*

In the pig, 30-50% of ova released from the ovary do not result in a piglet, despite a typically high fertilization rate (>95%) [77, 78]. Therefore, low pregnancy rates are primarily the result of early embryonic or fetal loss. Approximately 20 percent of embryonic loss occurs during the pre-implantation period of pregnancy in the pig, with a significant proportion of these losses occurring due to deficiencies in embryo elongation.
Furthermore, adequate embryo elongation is necessary for successful implantation and ultimately affects embryo spacing, placental development, and fetal growth, which have implications on uterine capacity, litter size, and even postnatal piglet health [79]. Better understanding of factors that regulate elongation is needed in order to assess mechanisms contributing to embryonic loss. Knowledge of these mechanisms can then be used to develop strategies (e.g. marker-assisted selection, nutrient or hormone supplements that may improve reproductive efficiencies by decreasing embryo losses in early gestation) to improve pregnancy outcomes in the pig and potentially other domestic agricultural species. Therefore, an in vitro model that can facilitate embryo elongation is essential for evaluating factors that promote or impede elongation. However, past efforts to establish such models have not been met with success.

1.5.3. Previous studies on in vitro elongation of embryos

Previous attempts to culture embryos at later stages, incorporating a type of supporting structure, have been largely unsuccessful. In cattle, limited elongation of embryos in vitro was demonstrated using an agar gel tube system [48, 49, 80]; however, embryos cultured in this system had deficiencies in the development of the embryonic disk [49, 81]. This agar system did not provide for complete encapsulation and support of the embryos. Instead, the tubes merely served as reservoirs for growth, and the embryos grew to fill these tubes via physical induction. Thus, the shape of the tube may have forced growth that appeared as elongation, and the high percentages of agar used may have restricted optimal embryonic development. Furthermore, high levels of media supplements may have resulted in rapid cell growth, further contributing to the
appearance of embryonic elongation. Nevertheless, attempts to replicate these results using pig embryos have been unsuccessful [81].

Moreover, a previous study that investigated morphological changes of in vivo-produced bovine embryos within the agar gel tube system reported that only 3% of the in vivo-produced embryos initiated morphological changes following in vitro culture [82]. These findings highlight the dramatic environmental change that in vivo-produced embryos experience when transitioning from the uterine environment to an in vitro environment. Therefore, more efficient in vitro embryo culture systems are needed that can ease the embryo’s transition from the uterine environment and facilitate morphological changes that are consistent with in vivo elongation. The in vitro alginate culture system developed for this thesis (described in Chapter 3) addresses this critical need by serving as a model that can facilitate embryo morphological changes and other corresponding hallmarks of elongation. This alginate culture system was then translated to serve as an in vitro matrix for the establishment of a growth plate model in the second part of this thesis, the context of which is described in the following sections.

1.6. Architecture and development of growth plate cartilage

1.6.1. Zonal arrangement of chondrocytes

Longitudinal growth of long bones occurs at the ends of the epiphyseal plate, also known as the growth plate, which is a distinct cartilage region located at each end of a long bone [83]. The complex development of the growth plate, involving an array of signaling pathways for controlled patterning and growth of long bones, can serve as a
template for natural tissue regeneration, which has become a major focus of tissue engineering studies [84]. Within growth plate cartilage, chondrocytes differentiate in a spatially regulated manner that results in morphologically and functionally distinct zones of cells, forming a continuum from the long bone’s epiphysis towards the diaphysis (Fig. 1-3) [83, 85]. At the epiphyseal end, small, round progenitor cells make up the resting zone. These relatively quiescent cells enter into the cell cycle and begin to differentiate into rapidly proliferating, flattened chondrocytes that organize into columns as they divide, making up the proliferative zone of about 10-20 cells per column [83, 84]. The chondrocytes of the resting and proliferating zones secrete structural proteins, predominately type II collagen, forming a hyaline cartilage matrix [83, 85]. Continuing towards the diaphysis, the chondrocytes stop dividing and begin to differentiate further into prehypertrophic cells, adopting a more spherical morphology [84]. The prehypertrophic chondrocytes terminally differentiate into hypertrophic chondrocytes that swell, i.e. hypertrophy, increasing their volume about 5-10 fold during the process [84]. This increase in size is a principal contributor of longitudinal bone growth and therefore bone lengthening is primarily driven by the production of hypertrophic chondrocytes from the proliferating columns [83, 84]. Chondrocytes in the hypertrophic zone primarily synthesize type X collagen and remodel the cartilage matrix into a calcifying matrix [84, 85]. At terminal differentiation, chondrocytes induce blood vessel invasion through the secretion of specific proteins such as vascular endothelial growth factor (VEGF) and attract bone-forming osteoblasts [83, 85]. Finally, hypertrophic chondrocytes undergo apoptotic cell death and osteoblasts invade the mineralized cartilage matrix, leading to the initiation of bone formation [83, 85]. One distinction between engineered and native
cartilage is that engineered cartilage lacks the proper zonal arrangement of cells and extracellular matrix despite having normal gene expression profiles, suggesting that certain factors for inducing normal tissue architecture are yet to be determined. Several signaling pathways, which are described below, have been discovered to play a role in regulating growth plate architecture and function; however an in vitro growth plate model is warranted in order to determine additional factors that are required to induce normal growth plate structure and function.

![Fig. 1-3. Functional zonal arrangement of the growth plate within long bones.](image)

1.6.2. *Signaling pathways and transcription factors that regulate growth plate function*

The orderly progression of cell proliferation and differentiation in growth plate cartilage is regulated by a complex network of opposing signaling pathways. Chondrocyte proliferation in the growth plate is partly regulated by a feedback loop involving the interactions of Indian hedgehog (Ihh), a member of the hedgehog family of
secreted ligands, and parathyroid hormone-related protein (PTHrP) [83-85] (Fig. 1-4). Prehypertrophic chondrocytes exiting the proliferative columns express Ihh, which stimulates the production of PTHrP by resting cells and perichondral cells, i.e. chondrocytes located at the bone ends near the articular surface [83, 85]. Ihh diffusion to the perichondral cells is regulated by heparin sulfate proteoglycans (HSPGs) in the extracellular matrix [86]. The PTHrP binds to its parathyroid hormone/PTHrP receptor expressed by proliferating chondrocytes and suppresses chondrocyte hypertrophy, retaining the chondrocytes in the proliferative zone [83, 85]. As chondrocytes in the proliferative columns become too distant from the PTHrP-producing cells located at the bone ends, PTHrP concentration becomes too low to stimulate them; this lack of stimulation causes the chondrocytes to stop proliferating, enter the pre-hypertrophic phase, and begin to synthesize Ihh [84]. This feedback loop therefore regulates the length of the proliferative columns in growth plate cartilage, defining individual zones of chondrocyte maturation.

Fig. 1-4. Schematic illustration of the Ihh/PTHrP feedback loop.
Other signaling pathways that regulate endochondral development of bones include bone morphogenic proteins (BMPs), transforming growth factors, Wnts, and Fibroblast growth factors. These pathways, in addition to the Ihh-PTHrP signal pathway, influence growth plate function partially via the regulation of key transcription factors, most notably Sox9 and Runx2 [83, 85]. Sox9 acts at every stage of chondrocyte differentiation in the growth plate. It is expressed in mesenchymal cell condensations and is essential for converting them into chondrocytes [83, 84]. Sox9 is also expressed in proliferating chondrocytes but not in hypertrophic chondrocytes, and is necessary for restricting chondrocyte differentiation, preventing cells from converting to hypertrophic chondrocytes prematurely [83]. Furthermore, Sox9 is critical for stimulating the expression of many ECM components, including Collagens type II, IX, XI and aggregan [84, 87]. Runx2, on the other hand, is necessary for driving proliferative chondrocytes to differentiate further into hypertrophic chondrocytes [83]. In addition, Runx2 promotes the differentiation of mesenchymal progenitor cells into osteoblasts [84]. It is expressed in all osteoblasts as well as in hypertrophic chondrocytes [84].

Although significant advances have been made in discovering signal pathways and signaling gradients that regulate growth plate function, it is unclear whether these factors alone are sufficient to induce normal growth plate structure and function because current methods for in vitro culture of naïve chondrocytes do not generate native cartilage architecture. An in vitro model of the growth plate can therefore serve as the necessary tool for evaluating these factors. Defining the microenvironmental parameters that generate cartilage architecture is a crucial step toward the development of systems to
regenerate native cartilage, with the ultimate goal of addressing critical health issues that are described in the following section.

1.6.3. Cartilage injury and disease

Physical damage or genetic disorders that disrupt normal growth plate function can result in skeletal abnormalities. Osteochondrodysplasias, a group of disorders characterized by abnormal growth and remodeling of bone and cartilage, affect between 1/3000 to 1/5000 individuals and occur with over 200 different phenotypes [88]. Mutations responsible for these dysplasias may cause defects in the synthesis of extracellular matrix proteins, specific growth factors, receptors, and transcription factors [89]. These dysplasias often result in disorders of decreased or increased bone density leading to proportionate short stature in the case of osteodysplasias, or disproportionate short stature with short trunk or short limbs in the case of chondrodysplasias [88]. In addition to affecting quality of life, short stature and craniofacial defects are often associated with long-term intensive health care needs as a result of impact on the joints, the central nervous system, and the ocular system [90, 91]. Therefore, along with advancing the field of developmental biology, studying the generation of growth plate architecture in vitro is critical for enhancing tissue engineering strategies to provide optimal replacement tissue for abnormal or damaged growth plates. While an in vitro growth plate model has the potential to serve as a tool for these studies, proper zonal arrangement of chondrocytes, in particular the proliferating columnar zone, has not yet been established by previous efforts, which are described in detail below.
1.6.4. *Previous studies on engineering growth plate cartilage*

Previous attempts to establish an *in vitro* growth plate model have failed to induce native growth plate architecture, in particular a distinct zone of proliferating columnar chondrocytes. A study involving the encapsulation of porcine growth plate chondrocytes in 1.25% alginate beads demonstrated that expression patterns of hormone receptors and matrix proteins in chondrocytes cultured in alginate beads were more similar to that of native growth plates than chondrocytes grown in 2-D monolayers [92]; however, there was no report of proliferating chondrocytes forming columns within the alginate gels. Another study involving the encapsulation of proliferating chondrocytes from fetal bovine chondrocytes demonstrated that a 102-mM calcium chloride (CaCl$_2$) cross-linking solution, without the addition of sodium chloride, caused radial channels to form within the alginate beads, and that proliferating chondrocytes became aligned within these channels [12]. Although this alignment appeared to mimic the columnar arrangement of proliferating chondrocytes *in vivo*, no further characterization of the chondrocytes other than electron microscopy was performed. The chondrocytes may have simply been filling these channels due to physical restriction; no evidence was reported of the encapsulated chondrocytes maintaining the appropriate genotype of native proliferating chondrocytes of the growth plate. More recently, a study was conducted to differentiate human bone marrow-derived mesenchymal stem cells (hBMSCs) within layered agarose constructs in order to promote zonal arrangement of chondrocytes [93]. Zonal cartilage did develop to an extent in this study due to the mechanical gradient of the scaffold, with small collagen type II negative proliferative cells at the base of the constructs and hypertrophic chondrocytes towards the surface. However, although proliferation was detected, a
distinct zone of proliferating columnar chondrocytes was not observed [93]. Therefore, a need still exists to establish a 3-D in vitro growth plate model that exhibits normal growth plate structure and function in order to elucidate mechanisms that regulate formation of proliferative chondrocyte columns, which in turn can be used to enhance tissue engineering strategies. The alginate hydrogel system established for this thesis involving the 3-D encapsulation of growth plate chondrocytes has the potential to address this need.

1.7. Objectives of thesis

The overall objectives of this thesis are to employ alginate hydrogels as a 3-D matrix to establish in vitro models of development and then evaluate factors that regulate growth and development in these systems. Specifically, this thesis focuses on two developmental models: 1) an in vitro culture system that can support porcine embryo elongation, and 2) an in vitro model of growth plate cartilage. These in vitro models can be used as tools for studying mechanisms of development and identifying factors that regulate or enhance tissue growth and function. Chapter 2 will detail hydrogel design and characterization that was explored for these projects. Chapter 3 will describe studies focused on porcine embryo development within alginate gels and Chapter 4 will focus on the preliminary studies involving the growth plate model. Finally, Chapter 5 will conclude the findings of this thesis and describe plans for future investigations.
CHAPTER 2

Alginate gel design and characterization

2.1. Introduction

As described in Chapter 1, the fundamental elements of tissue engineering include cells, bioactive signals, and a 3-D scaffold to simulate the natural cellular environment by maintaining cell/tissue architecture and providing stimuli for growth and differentiation [4, 5]. The in vitro culture models established in this research project predominantly capitalize on the scaffold element of the tissue engineering approach to cell and tissue culture. Scaffold design is dependent on several factors, including physical properties (e.g. shape and mechanical integrity), mass transport properties (e.g. protein diffusion rate), and biological properties (e.g. cell adhesiveness), which greatly affect the success of tissue organization and development [1, 5]. Biomaterials that can be easily tuned to address these properties are ideal for tissue engineering applications so that optimal properties can be obtained for the desired outcomes. Hydrogels, i.e. networks of hydrophilic polymer chains with high water content, are a class of biomaterials that are widely used as tissue engineering scaffolds due to their ability to mimic the properties of native soft tissue [6], as well as their high porosity for efficient diffusion of proteins and nutrients [7]. Synthetic hydrogels such as polyethylene glycol [94] and natural hydrogels such as chitosan [95] and alginate [14] can be modified both physically and/or chemically to promote mechanical and biological cues, making them favorable as scaffolds for in vitro cell and tissue growth. This chapter will specifically focus on the design and characterization of alginate hydrogels as scaffolds for tissue engineering applications, in particular for use as in vitro culture models.
The principal purpose of a tissue engineering scaffold is to provide a framework in which tissue growth can occur by filling a space otherwise occupied by a native tissue or environment [5]. Physical properties that are essential to the success of the scaffold include gel formation characteristics and mechanical properties. As gel formation can dictate the direction in which tissue growth proceeds and how cell/tissue architecture is maintained, the first objective of this study was to investigate several methods for creating alginate gels of different forms, specifically focusing on designs that involve the external method of gelation (i.e. the use of calcium chloride [CaCl\textsubscript{2}] as the crosslinking agent). Mechanical properties of the gel should also be considered when exploring the optimal scaffold design for a specific application. The alginate gel must have enough structural integrity to withstand manipulation and maintain 3-D architecture of the encapsulated tissue for adequate cell-to-cell communication [5]; however, the gel must also be lenient enough to guide, not inhibit, cell and tissue growth. In addition, when cell integrins are coupled to the ECM, mechanical forces can be transduced into biochemical signals that influence cell behavior and function within the scaffold, a phenomenon known as mechanotransduction [96, 97]. Therefore, the stiffness of the artificial in vitro ECM (i.e., the scaffold) with which the cells are in contact can be an important regulator of basic cellular processes of proliferation, differentiation, and remodeling [96]. Stiffness was evaluated in the present study by determining Young’s modulus as a function of alginate concentration.

In addition to physical properties, bioactivity is another design variable to consider when establishing a tissue engineering scaffold, as the scaffold must promote desired cellular functions and therefore proper tissue development [1, 5]. For example,
biomaterials can be modified to present specific ligands for cell adhesion, which can influence cell-matrix interactions and can lead to altered gene expression and cellular behavior [1]. Due to their very hydrophilic nature, many hydrogels, including alginate, do not readily promote protein adsorption or cell adhesion unless chemically modified [5, 14]. The amino acid sequence arginine-glycine-aspartic acid (RGD), derived from numerous ECM proteins such as fibronectin, laminin, and collagen, is the most effective and commonly used peptide sequence for hydrogel modification to promote cell adhesion [5, 43]. Therefore, covalent attachment of an RGD peptide to the alginate polymer backbone and subsequent characterization of the conjugation were investigated in this study.

Overall, the objective of these studies was to demonstrate alginate’s ability to be modified with respect to physical properties (i.e., gel formation and stiffness) as well as bioactivity (i.e. cell adhesiveness). Each of these gel design variables can then be used to investigate its influence on porcine embryo development and growth plate development with the in vitro culture models established and described later in this thesis.

2.2. Methods

2.2.1. Gel formation

This section details several methods that were developed for creating alginate gels of different morphologies. The crosslinking solution used for each of these methods was prepared by dissolving calcium chloride (CaCl₂; Sigma Aldrich, St. Louis, MO) and sodium chloride (NaCl; Sigma Aldrich) in sterile MilliQ water to obtain a 50 mM
CaCl$_2$/140 mM NaCl solution. Alginate solutions were prepared by dissolving lyophilized alginate powder (Pronova UP MVG, >60% guluronic acid, 200,000 – 300,000 g/mol; NovaMatrix, Sandvika, Norway) in sterile MilliQ water. The gel formation methods described below were tested with alginate concentrations ranging from 0.375% to 1.5% alginate (w/v).

**Alginate beads:**

A simple “drop” method, based on methods described by other investigators [33, 98], was developed through our studies for the encapsulation of cells and tissues within alginate hydrogel beads (Fig. 2-1A) [50]. To create alginate beads on a scale large enough for the *in vitro* pig embryo model described in Chapter 3, 25-µL drops of alginate solution were slowly released from the edge of a wide-orifice pipette tip and gently touched to the surface of crosslinking solution (50 mM CaCl$_2$/140 mM NaCl) to pull the droplet off the tip and into the solution. After 3 minutes of crosslinking time to ensure complete gelation, the beads were removed from the crosslinking solution and diameters were measured using a caliper. For cell encapsulation, this method was modified to produce smaller beads using 10-µL drops of alginate for the *in vitro* growth plate model investigated in this project. The “drop” method was then extended to allow for the addition of multiple layers of alginate gel around the first bead to ensure complete encapsulation of pig embryos, which was utilized in the studies described in Chapter 3 (Fig. 2-1B). This double encapsulation method was also developed for future mechanical/chemical gradient or co-culture studies. To demonstrate the addition of a second layer, the 25-µL beads prepared during the first encapsulation were placed
individually into 45-µL drops of alginate. These drops containing the beads were then pipetted into crosslinking solution (50 mM CaCl$_2$/140 mM NaCl) using a wide-orifice pipet tip, and were allowed to gel for an additional 3 minutes, resulting in double-layered alginate beads. The double-layered bead was observed with a Leica DMI 3000B inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 5x magnification.

![Fig. 2-1. Schematic illustration of the formation of alginate hydrogel beads, in conjunction with the encapsulation of embryos. A) Single encapsulation, resulting in an embryo encapsulated within one layer of alginate hydrogel. B) Double encapsulation, resulting in an embryo encapsulated within two layers of alginate hydrogel.](image)

**Cylindrical alginate gels:**

An alginate hydrogel with a cylindrical morphology could provide extended space for tissue growth, while still maintaining a small enough radius for adequate diffusion of nutrients to the cells or tissue located within the gel. To create cylindrical alginate
hydrogels, a custom mesh mold was developed, consisting of a polypropylene mesh piece (0.021” x 0.027” opening size; McMaster Carr, Elmhurst, IL) held together with stainless steel syringe connectors (McMaster Carr) on each end (Fig. 2-2A). Approximately 500 µL of alginate solution was pipetted directly into the cylindrical mold via the opened slit at the top of the mesh. The mold was then lowered into crosslinking solution (50 mM CaCl₂/140mM NaCl) for 10 minutes to allow for complete gelation. After 10 minutes, the mold was removed from the crosslinking solution, the syringe connectors were taken off the ends of the mesh, and the resulting gel was gently peeled from the mesh using forceps. The diameter of the resulting gel was measured with a caliper. To create a cylindrical hydrogel for gradient and/or co-culture studies, alginate solutions of various concentrations (0.375% to 1.5%; dyed with food coloring) were consecutively added to the mesh mold (Fig. 2-2B) to demonstrate the formation of a mechanical gradient gel.

![Fig. 2-2. A) Mesh mold used for creating cylindrical gels. B) Example of a gradient gel pre-crosslinking; each dye color represents a different concentration of alginate solution.](image)

**Alginate gel tubes:**

A different approach to maintaining tissue architecture is to provide a reservoir for growth, in contrast to directly encapsulating the tissue within the hydrogel. Therefore, a method for creating a tubular alginate hydrogel was investigated and developed for this approach. Briefly, a glass microdispenser tube (Drummond, Broomall, PA) with a 1.5-
mm outer diameter was added through the center of the mesh mold that was described above for creating cylindrical gels (Fig. 2-3). Alginate solution was then pipetted into the mesh mold, filling the space around the glass tube. After placing this mesh system into crosslinking solution for 10 minutes, the metal syringe connectors were removed from the ends of the mesh and the glass tube was slowly drawn out from the center of the gel. Finally, the resulting gel was carefully peeled from the mesh using forceps.

![Fig. 2-3. Mold for creating alginate gel tubes, consisting of a cylindrical mesh frame with glass tube through the center.](image)

**Alginate wells:**

In addition to the hollow gel tube method described above, another method of creating a reservoir for tissue growth was developed, involving the creation of wells within alginate hydrogels. To create the wells, a long, narrow mesh mold was constructed, with an opened top in which to pipette the alginate solution. Alginate solution was then pipetted into the mesh mold until full, followed by the insertion of a gel electrophoresis comb into the alginate solution. This system was then placed into a basin of crosslinking solution (50 mM CaCl$_2$/140 mM NaCl) (Fig. 2-4) for 1 hour to ensure stability of the resulting gel. After being removed from the crosslinking solution, the
comb was carefully removed from the alginate, and the resulting gel was peeled from the mesh mold.

![Image](image1)

**Fig. 2-4.** The setup for the formation of alginate wells. A) Mesh system containing alginate, with gel electrophoresis comb inserted. B) Close-up of comb inserted into mesh mold.

2.2.2. Chemical modification of alginate

The RGD amino acid sequence is the most effective and commonly used peptide sequence for hydrogel modification to promote cell adhesion [5, 43]. To create bioactive alginate hydrogels, alginate was covalently modified with a glycine-arginine-glycine-aspartic acid-serine-proline (GRGDSP) peptide (Anaspec, Fremont, CA) to a concentration of 4 µmol/g alginate using carbodiimide chemistry, as previously described [14]. Briefly, two grams of sodium alginate (NovaMatrix) were added to 200 mL of MES buffer (0.1M MES, 0.3M NaCl, pH 6.5) and the alginate was allowed to dissolve for 7-8 hours on a stir plate. 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC; Sigma Aldrich) was added to activate 5% of the uronic acids of the alginate polymer backbone in order to form amide linkages with the peptide. To stabilize the reactive EDC intermediate against a competing hydrolysis reaction, N-hydroxy-sulfosuccinimide (sulfo-NHS; Pierce,
Rockford, IL) was also added to the solution at a 2:1 EDC to NHS ratio. Lastly, the GRGDSP peptide was incorporated into the solution, conjugating to the alginate backbone via the terminal amine of the peptide. After 20 hours of reacting, the conjugation reaction was quenched through the addition of hydroxylamine hydrochloride (Sigma Aldrich). The alginate solution was then dialyzed against decreasing salt solutions and finally ddH$_2$O over the course of 3 days. The final dialyzed solution was lyophilized to dryness and stored at -80°C until use.

2.2.3. **Confirmation of peptide conjugation**

Because unmodified alginate does not readily promote cell adhesion, a cell adhesion experiment was performed with unmodified alginate gels compared to GRGDSP-conjugated alginate (RGD-alginate) gels as an indirect method of confirming successful conjugation of the peptide. Briefly, 200 µL of 1% alginate or RGD-alginate solution was pipetted into wells of a 48-well plate. Next, 20 µL of 100 mM calcium chloride (CaCl$_2$) solution was pipetted into the center of each well of alginate or RGD-alginate solution. After 20 minutes of crosslinking, the gels were removed from each well and placed into a corresponding well of rinse media (Dulbecco’s Modified Eagle’s Medium [DMEM]; Gibco/Invitrogen, Carlsbad, CA) to remove excess calcium. After the rinse step, each gel was transferred to the bottom of a well of a 96-well plate immediately prior to cell seeding.

Mouse bone marrow mesenchymal stem cells (D1 ORL UVA; ATCC, Manassas, VA) were cultured in T-75 flasks in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). For seeding, cells were counted
using a hemocytometer and trypan blue staining for viable cells after being dissociated with 0.05% trypsin-EDTA. Cells were then seeded onto each gel (n=3 gels per alginate condition) at a density of 20,000 cells/cm² in 150 uL of cell culture media and cultured for 3 days at 37 °C and 5% CO₂. Gels were imaged 24 and 72 hours post-seeding to observe cellular adhesion using a Leica DMI 3000B inverted microscope (Leica Microsystems). Three images were taken per gel at 10x magnification and representative images are reported. Cells were then stained at 72 hours with 2 µM calcein AM/4 µM ethidium homodimer-1 and imaged with fluorescence microscopy to evaluate live and dead cells, respectively.

To further verify successful conjugation of the GRGDSP peptide to the alginate polymer backbone, NMR spectroscopy was used to characterize the samples. Unmodified alginate and GRGDSP-alginate were dissolved in deuterium oxide (D₂O; Sigma Aldrich) and placed in separate NMR tubes. The ¹H-NMR spectra of the samples were recorded on a Bruker DRX-500 500 MHz NMR spectrometer at an operating frequency of 500.13 MHz.

2.2.4. Determination of Young’s modulus

Sodium alginate (NovaMatrix) was dissolved in sterile MilliQ water to final concentrations of 0.375%, 0.5%, 0.7%, 1.0%, and 1.5% (w/v). For each concentration, alginate solution was pipetted into cylindrical mesh molds (3.5 mm diameter), as described above in section 2.2.1 for cylindrical alginate gels, and the molds were placed into crosslinking solution (50 mM calcium chloride/140 mM NaCl) for 10 minutes to allow for complete gelation of the alginate. Following removal from the mesh molds, the
gels were cut into 5-mm pieces for mechanical testing. Exact height and diameter of the cylindrical gels were measured with a caliper and recorded for use in analysis. Young’s modulus values of the alginate gels were determined using a TMS-Pro texture analyzer (Food Technology Corporation, Sterling, VA). Samples were compressed 1 mm at a rate of 1 mm/min while force and displacement were recorded over time. Average Young’s modulus values were determined from the linear regions of the stress-strain curves, at strain values ≥ 0.1. These methods were then repeated with 0.5%, 1.0%, and 1.5% RGD-alginate gels to any determine any effects of peptide conjugation on Young’s modulus of the resulting gels.

2.2.5. Statistical Analysis

All values are reported as mean ± standard error of the mean (SEM). For Young’s modulus studies, one-way analysis of variance (ANOVA) was used to determine if the mean Young’s modulus values between the different alginate concentrations were statistically different. Tukey’s multiple comparison test was then used to perform pairwise comparisons. Student’s t-test was used to compare the Young’s moduli of the alginate gels with those of the RGD-alginate gels to determine any significant differences between the two groups. Linear regression analyses were used to describe the relationship between alginate concentration and Young’s modulus. For these analyses, the $r^2$ value (coefficient of determination) is reported, giving a measure of the reliability of the linear relationship.
2.3. Results

2.3.1. Gel morphologies

A method involving 25-µL alginate drops was developed to produce gels of an appropriate size for the in vitro pig embryo model. The 25-µL drops resulted in beads of approximately 3 mm in diameter, which is suitable for 0.5-1.0 mm embryos or tissue samples (Fig. 2-5A). For cell encapsulation for the growth plate model, the method was adjusted to make smaller beads, using 10-µL alginate drops. The 10-µL drops resulted in beads with a diameter of approximately 2.5 mm (Fig. 2-5A). A method for adding multiple gel layers was then investigated for pig embryo studies, as well as future studies involving gradients and co-cultures. Encapsulating the first bead within a second drop of alginate resulted in a double-layered alginate gel, with a second layer gel thickness ranging from ~200-400-µm around the first bead (Fig. 2-5B).

![Fig. 2-5. Representative images of alginate beads. A) Alginate beads made from a 25-µL alginate drop (left) and 10-µL alginate drop (right), B) Representative 5x bright field image of a double-layered alginate bead: ‘1’ denotes the inner bead and ‘2’ denotes the second alginate layer (scale bar = 200 µm).](image)

The method developed for creating cylindrical alginate hydrogels, employing a custom mesh mold, resulted in 3-cm long gel with a diameter of approximately 3.5 mm (Fig. 2-6A). However, the length of the gel can easily be varied with this mold by
utilizing mesh pieces of a greater length. This method was then employed to produce a cylindrical gel with a mechanical gradient. The consecutive addition of alginate solutions of varying concentrations into the mesh mold resulted in a unified gel post-crosslinking (Fig. 2-6B). The mesh mold was then modified to produce a hollow tubular gel by adding a glass capillary tube through the center of the mold, prior to the addition of alginate. The result was an alginate gel tube with an inner and outer diameter of approximately 1.5 mm and 5 mm, respectively (Fig. 2-6C and D).

![Fig. 2-6. A) Cylindrical alginate hydrogel, B) Gradient gel consisting of different concentrations of alginate, dyed with different colors, C) Alginate gel tube prior to removing glass tube, D) Resulting alginate gel tube.](image)

The last gel method that was developed and described in the present study was for the creation of wells within alginate. Using a thin mesh mold and a gel electrophoresis comb, the result was a long, thin alginate gel consisting of a row of hollow wells (Fig. 2-
7). These gels in particular consisted of wells with dimensions of 1 mm x 5 mm, with a gel thickness of approximately 2 mm in between each well.

2.3.2. Confirmation of peptide conjugation

Cell adhesion studies were used as an indirect method of confirming successful conjugation of the RGD peptide to the alginate. D1 ORL UVA cells seeded onto RGD-alginate hydrogels demonstrated cell adhesion at the 24-hr time point, while little to no cell adhesion was observed on the unmodified alginate gels (Fig. 2-8A and B). Cell number on the RGD-alginate gels appeared to be greater at 72 hours compared to 24 hours, as cells began to form aggregated clusters (Fig. 2-8D). Live/dead staining confirmed viability of these cell clusters (Fig. 2-8F). In contrast, adhered cells remained nearly undetected on the unmodified alginate gels at 72 hours post-seeding (Fig. 2-8C and E). These results therefore confirmed successful peptide conjugation to the alginate polymer backbone.
$^1$H NMR results further confirmed conjugation of the peptide to the alginate polymer backbone. Figure 2-5 presents the $^1$H NMR spectra of unmodified alginate (red spectrum) and GRGDSP-conjugated alginate (blue spectrum). New peaks between δ4 and δ5 were distinctly detected in the spectrum of the RGD-alginate compared to that of the unmodified alginate, which can be attributed to the α-hydrogens of the amino acids of the peptide (Fig. 2-9). Based on previous $^1$H NMR studies involving this peptide, these peaks
have been labeled according to the amino acid to which they are most likely attributed (Fig. 2-9) [99, 100]. Additional peaks that were specifically detected in the GRDSP-alginate spectrum compared to the unmodified alginate spectrum between δ1.8 and δ2.8 may be derived from β-hydrogens of the peptide (Fig. 2-9).

2.3.3. Young’s modulus values

Young’s moduli of alginate and RGD-alginate gels were determined from the slopes of the linear regions of the stress-strain curves that were plotted after compression testing. A representative stress-strain curve is depicted in Fig. 2-10. The results of the compression testing indicated average Young’s modulus values of 2.59 ± 0.20, 3.00 ± 0.15, 7.64 ± 0.51, 7.75 ± 0.46, and 17.3 ± 0.85 KPa for 0.375%, 0.5%, 0.7%, 1.0%, and 1.5% unmodified alginate, respectively (n=20 per concentration; Table 2-1). No
significant difference was observed between the Young’s modulus values of the 0.375% and 0.5% alginate gels, as well as between the 0.7% and 1.0% gels. All other pairs had significantly different Young’s moduli.

The Young’s modulus values calculated for the 0.5%, 1.0%, and 1.5% RGD-alginate gels were 5.10 ± 0.33, 12.78 ± 0.62, and 21.72 ± 2.00, respectively (n=20 per concentration; Table 2-1). All three concentrations of RGD-alginate gels had significantly greater Young’s modulus values than the corresponding concentrations of unmodified alginate (p<0.05). The Young’s moduli for the unmodified alginate gels and RGD-alginate gels are plotted together in Fig. 2.11, along with their corresponding linear equations of best fit. A strong linear relationship was observed between Young’s modulus and alginate concentration for both the unmodified and RGD-conjugated alginate gels, with r² values of 0.9375 and 0.9981, respectively (Fig. 2-11).

Fig. 2-10. Representative stress-strain curve of a 1.5% RGD-alginate gel. The linear region is denoted at strain values ≥ 0.1.
Table 2-1. Young’s modulus values for unmodified alginate and RGD-alginate hydrogels of increasing concentrations. Values with different letters as superscripts differ significantly \((p<0.05)\).

<table>
<thead>
<tr>
<th>Alginate concentration (% w/v)</th>
<th>Young’s Modulus (KPa)</th>
<th>RGD-Alginate Concentration (% w/v)</th>
<th>Young’s Modulus (KPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375%</td>
<td>2.59 (± 0.20)(^a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5%</td>
<td>3.00 (± 0.15)(^a)</td>
<td>0.5%</td>
<td>5.10 (± 0.33)(^d)</td>
</tr>
<tr>
<td>0.7%</td>
<td>7.64 (± 0.51)(^b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0%</td>
<td>7.75 (± 0.46)(^b)</td>
<td>1.0%</td>
<td>12.78 (± 0.62)(^e)</td>
</tr>
<tr>
<td>1.5%</td>
<td>17.3 (± 0.85)(^c)</td>
<td>1.5%</td>
<td>21.72 (± 2.00)(^f)</td>
</tr>
</tbody>
</table>

Fig. 2-11. Young’s modulus as a function of alginate concentration for unmodified (black circle) and RGD-modified (red triangle) alginate gels. The plotted lines display the linear equations of best fit for both alginate conditions (Unmodified alginate: \(r^2 = 0.9375\); RGD-alginate: \(r^2 = 0.9981\)).

2.4. Discussion

In the present study, several aspects of alginate gel design were investigated and characterized, demonstrating alginate’s potential as a 3-D matrix for \textit{in vitro} cell and
tissue models. First, various methods of gel formation were developed to produce alginate gels of distinctive forms. Alginate has been widely used in cell/tissue encapsulation studies, but previous studies have mainly involved microencapsulation processes, producing alginate beads on the micro scale for cell [101-103] and small tissue [33, 36, 38] encapsulation. In contrast, the methods described here produced gels on a millimeter scale, which can be used for large tissue models such as the pig embryo model [50] and growth plate model described later in this thesis. Previous studies investigating the uniformity of 500-µm alginate beads created by the external gelation method used fluorescently labeled alginate to demonstrate that bead formation results in a radially inhomogeneous distribution of alginate, with the least amount of crosslinking located in the gel core [104, 105]. Adding a non-gelling salt, such as sodium chloride, to the CaCl$_2$ crosslinking solution results in greater uniformity, yet an inhomogeneous distribution of alginate still occurs [104]. This inhomogeneous distribution is most likely increased in the beads made using the methods of this study, due to the large size of the beads (2-3 mm). However, for the pig embryo project, which requires a large gel for encapsulation, this result is preferred due to a more permissive core for growth and a stiffer outer region for stability.

A method for creating cylindrical alginate gels was also established in this study. Previous studies involving cylindrical alginate gels have employed the internal gelation method to create the gels [15, 106, 107], in which a calcium slurry, usually calcium carbonate or calcium sulfate (CaSO$_4$), is mixed into the alginate solution and gelation occurs as the calcium slowly leaks from within the alginate [11]. In this case, release of the calcium is caused by a reduction in pH that may be harmful to cells that are within the
alginate solution during gelation [11]. The method described in this report for creating cylindrical alginate gels instead uses the external gelation method, involving CaCl₂, which would not have the harmful effect on cell viability. Furthermore, like the bead method, the cylindrical gel method in this study involves radial diffusion of calcium ions; however, the radial diffusion only occurs transversely to the center of the gel. Therefore, the cylindrical gels created in this study most likely contain an inhomogeneous distribution of crosslinking in the transverse direction, with a core axis throughout the length of the gel that has the lowest degree of crosslinking due to hindered diffusion. This permissive central area may serve to guide embryo/tissue growth in the longitudinal direction when cells or embryos are encapsulated within the gel.

Another approach to maintaining tissue architecture is to provide a physical reservoir to direct tissue or embryo growth by creating an environment within the gel in which the tissue or embryo can be inserted post-crosslinking. With the both alginate tube and well methods, the surrounding hydrogel tube or well can serve as a physical structure to potentially direct tissue growth within the hollow cavity. The tubular gels, in particular, could serve in multiple applications, as cells can be seeded inside the lumen and/or encapsulated within the walls of the tube. Previously, studies establishing methods for the creation of tubular alginate gels on a similar scale as the ones presented in this study have involved complex mechanisms, such as laser-assisted printing [108] and electrodeposition [109]. In contrast, the method described in this study offers a simple, gentle process for the creation of alginate tubes. Furthermore, this report is the first to demonstrate the formation of wells within alginate. Using the row of wells, embryos could be cultured in individual alginate wells yet within the same culture dish to observe
any embryo-embryo interactions in vitro without direct contact. Additionally, the row of wells can be easily cut to produce separated wells for individual embryo culture.

In addition to gel formation, chemical modification of alginate was investigated in the present study, specifically the covalent attachment of an RGD peptide to the alginate polymer backbone. The RGD peptides serve as ligands to cell integrins, which are cell-surface receptors that integrate with the ECM to mediate cell migration and adhesion [1]. As alginate is naturally an inert polymer that is not conducive to cell adhesion [14], a cell adhesion study can serve as an indirect method of confirming successful conjugation of the peptide. The GRGDSP-modified alginate gels promoted cell adhesion and slow proliferation of D1 ORL UVA cells, forming aggregated cell clusters over time. In contrast, the unmodified alginate gels promoted little to no cell adhesion. These results suggest that the peptide was successfully conjugated to the alginate backbone. The results of the GRGDSP-alginate gels support previous studies that used similar methods of RGD peptide conjugation to promote cell adhesion [14, 27]. These studies found that low RGD peptide densities promoted cell adhesion, slow proliferation, and aggregation into clusters, as seen with the gels of the present study; however, spreading and proliferation increased with increasing RGD peptide densities [27]. Therefore, future studies could investigate higher RGD peptide densities to increase proliferation effects and overcome clustering. Successful peptide conjugation was further confirmed by ^1^H-NMR spectroscopy, which has previously been used to confirm RGD peptide conjugation to polymers [100]. For tissue engineering applications, the unmodified alginate gels can serve to solely provide 3-D support for cell/tissue growth, while the RGD-alginate gels
can function to promote cell-matrix interactions via cellular adhesion and mechanotransduction [110].

Stiffness, in particular Young’s modulus, of alginate gels was another property that was investigated in this study. Stiffness is the most common mechanical cue experienced by cells, with a range of 0.1 kPa to 40 kPa for native soft tissues [111]. The results of the present study demonstrate that alginate gels made with our methods are within this stiffness range, validating the use of alginate as a 3-D matrix to mimic native soft tissues [6]. Furthermore, the results of this study demonstrate that Young’s modulus increases with increasing alginate concentration following a linear trend, which supports results found in previous studies [15]. Surprisingly, we found that GRGDSP conjugation to alginate resulted in Young’s moduli that were significantly greater than unmodified gels of the same concentrations, despite the fact that RGD peptide conjugation lowers the amount of carboxyl groups available for ionic crosslinking. These results were unexpected, as a previous study demonstrated that low RGD peptide concentrations did not have a significant effect on compression modulus and that higher amounts of peptide conjugation resulted in a lower modulus [112]. The Young’s moduli for both alginate conditions, however, are all within the stiffness range of native soft tissue and the largest difference detected between the unmodified and modified alginate gels was only ~5 KPa. It is also important to note that the unmodified alginate used in these studies was not subjected to the same buffer dissolution and subsequent lyophilization process as the peptide-conjugated alginate, which could serve as a reason for the difference in mechanical properties between the resulting gels.
In conclusion, this study demonstrates that alginate hydrogels can be easily manipulated in regards to gel formation, bioactive properties, and mechanical properties, making it a suitable material for use as a 3-D matrix in in vitro culture models. Each of the characteristics explored in this study can be tested for its effect on embryo development for the in vitro pig embryo model described in Chapter 3, as well as for its effect on chondrocyte function and arrangement for the in vitro growth plate model described in Chapter 4. Optimal gel conditions can then be determined in order to establish in vitro models that best mimic in vivo conditions, in order to evaluate factors and mechanisms involved in normal and abnormal development; knowledge of these mechanisms could be used to enhance strategies for increasing pregnancy outcomes (pig embryo project) and for engineering growth plate cartilage with proper architecture and function (growth plate project).
CHAPTER 3

*In vitro* development of pre-implantation porcine embryos using alginate hydrogels as a three-dimensional extracellular matrix

3.1. Introduction

In the pig, the pre-implantation period of pregnancy is characterized by several developmental hallmarks. Between day 10 and 12 of gestation, the porcine embryo undergoes a process known as elongation, transforming from a ~1-mm spherical structure to a long, thin filament >100 mm in length [113, 114]. Unlike elongation in other domestic species, rapid elongation of the pig embryo occurs via cellular remodeling and differentiation of the trophectoderm rather than cellular hyperplasia [113, 114]. During elongation, the embryo produces and secretes estrogen, which serves as a key molecular signal for maternal recognition of pregnancy and modulates the production of proteins and growth factors within the uterus [115]. Adequate elongation and successful implantation of the porcine embryo subsequently affect embryo spacing in the uterus, placental development, and fetal growth, which have implications on uterine capacity, litter size and postnatal piglet health [78, 116].

Approximately 20 percent of embryonic loss occurs during the pre-implantation period of pregnancy in the pig, with a significant portion of these losses occurring due to deficiencies in embryo elongation [77]. Early embryonic loss is poorly understood because exact physiological mechanisms by which the embryo elongates, and how these mechanisms are altered during embryonic loss, are not clear. Developing details of the mechanisms that regulate embryo elongation can allow for identification of specific biophysical, biomechanical, and molecular factors that could serve as targets for
improving pregnancy outcomes and neonatal piglet survival. An effective *in vitro* culture system that supports pig embryo elongation could be used to evaluate specific mechanisms of elongation. To date, attempts to initiate elongation in porcine embryos *in vitro* have been unsuccessful compared to other livestock species [81]. In cattle, limited elongation of embryos *in vitro* via physical induction was demonstrated using an agarose gel tube system [48, 49, 80]. However, embryos cultured in this system had deficiencies in the development of the embryonic disk [49, 81], which may have been the result of forced growth of the embryos to fill the tubes. Nevertheless, attempts to repeat these results using porcine embryos have been unsuccessful [81].

Alginate is a commonly used biomaterial, often employed as an artificial extracellular matrix in tissue engineering applications [8]. Alginate is a linear polysaccharide derived from brown algae composed of repeating units of β-D-mannuronic acid and α-L-guluronic acid [117]. One of the favorable properties of alginate as a biomaterial is its ability to form a hydrogel by ionic cross-linking of the guluronic residues in the presence of a divalent cation [118]. The alginate gel forms a mesh-like structure that permits diffusion of nutrients and hormones essential for cell and tissue growth and development [117]. Furthermore, alginate promotes negligible non-specific protein adsorption and cell adhesion, making it a suitable material for solely examining mechanical influence on tissue morphogenesis [14, 119]. We hypothesize that previous failure of porcine embryos to elongate *in vitro* is, at least to an extent, due to inadequate culture systems lacking three-dimensional (3-D) structure for proper biomechanical support of embryo elongation. Traditional two-dimensional (2-D) cultures result in a disruption of cell-to-cell interactions as cells attach to the substrate and migrate away
from the tissue [33, 45]. Three-dimensional culture systems, alternatively, can maintain embryo architecture and allow for direct physical interaction with the surrounding environment, which better mimics in vivo development. Therefore, our objective was to use alginate hydrogels to establish an in vitro culture system based on tissue engineering principles (i.e. 3-D scaffolds) that can mechanically support the culture of pre-implantation porcine embryos, ensure proper internal biomechanics, and initiate morphological changes. In the present study, in vitro development was evaluated by characterizing cellular survival, morphological changes, gene expression of steroidogenic and immunological transcripts, and estradiol 17-β (E2) production from porcine embryos encapsulated (ENC) in alginate hydrogels or remaining as non-encapsulated controls (CONT).

3.2. Materials and methods

3.2.1. Production and collection of embryos

All animal protocols were approved by the USMARC Animal Care and Use committee and met the USDA guidelines (1995) for the care and use of animals. Fifteen, normally cycling White crossbred gilts consisting of Landrace, Yorkshire and Duroc genetics were checked daily for estrus. Following first detection of estrus (designated as d 0), gilts were artificially inseminated with semen from a single Duroc sire with three sires represented across the replicate collections (International Boar Semen, Inc., Eldora, IA) and again 24 h later with the same sire. In three independent replicate collections, five gilts were slaughtered at the USMARC abattoir at d 9, 10, or 11 of gestation.
Following slaughter, reproductive tracts were removed immediately and each uterine horn was flushed with 20 mL (~37°C) of Hepes-buffered RPMI-1640 media (Invitrogen, Carlsbad, CA) containing 1x antibiotics/antimycotics (Sigma-Aldrich, St. Louis, MO). Embryos were recovered and classified according to size and morphology using a standard stereomicroscope. Embryos were then washed with Hepes-buffered RPMI-1640 media containing 1x antibiotics/antimycotics and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). From homogenous litters, spherical embryos (~1 mm in diameter with a range of 0.5 to 1.5 mm) recovered from gilts at d 9 of gestation (n = 3 gilts per replicate) were randomly assigned to the *in vitro* culture treatments (ENC or CONT); a subset of these embryos were collected and snap frozen individually in liquid nitrogen to serve as initial *in vivo*-produced spherical control embryos for transcript expression. From homogenous litters, ovoid (5-10 mm in diameter) or tubular (>10 mm in diameter with a range of 11 to 20 mm) embryos from the remaining gilts were recovered at d 10 or 11 of gestation, respectively (n = 1 gilt per day per replicate) and snap frozen individually in liquid nitrogen to serve as later-stage *in vivo*-developed control embryos for transcript expression. Uterine flushings from all pregnant gilts were collected, centrifuged to remove cellular debris and stored at -20°C for later E2 and protein analysis.

3.2.2. **Embryo encapsulation and culture**

Embryos assigned to the ENC group were encapsulated within alginate hydrogels, using a double encapsulation method (Fig. 3-1). Briefly, sodium alginate (Pronova UP MVG, >60% guluronic acid, 200,000 – 300,000 g/mol, NovaMatrix, Norway) was
dissolved in sterile MilliQ water to a final concentration of 0.7% (w/v). Droplets of warmed, sterile alginate solution were placed onto polystyrene culture dishes. A single embryo was pipetted into each droplet of alginate in a minimal amount of media as not to dilute the alginate solution. Each droplet was then pipetted into a warmed, sterile cross-linking solution (50 mM CaCl₂, 140 mM NaCl) and allowed to gel for 3 minutes.

![Fig. 3-1. Schematic illustration of the double encapsulation method. 1) Embryo is transferred into alginate droplet. 2) Droplet is pipetted into calcium solution. 3) Alginate bead forms around embryo. 4) Alginate bead is transferred to larger droplet of alginate. 5) Droplet with bead is pipetted into calcium solution. 6) Second layer of alginate forms around embryo. Following encapsulation, all embryos were washed and cultured individually in a single well within 4-well NUNC plates.](image)

The resulting alginate beads with encapsulated embryos were then rinsed twice in Hepes-buffered RPMI-1640 media containing 1x antibiotics/antimycotics and 10% FBS. Following the rinse, each bead was encapsulated in a second layer of alginate, using identical methods as described above for the initial encapsulation, to ensure complete
encapsulation of each embryo. The final resulting beads were then washed once with pre-gassed culture media containing RPMI-1640, 1x antibiotics/antimycotics and 10% FBS. ENC and CONT embryos were cultured in individual wells in 4-well NUNC plates (Thermo-Scientific, Rochester, NY) with 1 mL of culture media for 96 h at 38.5°C and 5% CO₂ in air with 100% humidity.

3.2.3. Characterization of embryo viability and morphology

Every 24 h, an image was recorded of each embryo by photographing with a SMZ1500 stereomicroscope (Nikon Instruments, Inc., Melville, NY). Based on visual appraisal of these images, embryos were classified into one of three in vitro treatment groups: CONT (non-encapsulated control embryos), ENC- (encapsulated embryos with no observable morphological change) or ENC+ (encapsulated embryos with observable morphological changes). Embryos undergoing morphological changes were characterized by a tubular formation of the embryo within the gel and subsequent flattening of this tube with many of these embryos migrating out of the gel forming a secondary spherical structure once freely in the culture media (Fig. 3-2C). At the termination of culture, embryo survival was determined by assessment of blastocyst fragmentation. Surviving embryos were characterized with no apparent sign of cellular degeneration, whereas dying embryos had significant cellular degeneration resulting in blastocyst compaction and darkening of the embryo. Embryo survival and death were confirmed in a subset (n = 10) of embryos using a fluorescein/ethidium bromide live/dead staining protocol previously reported [120]. Live/dead staining (green or red, respectively) was examined and imaged using an Axioplan2 fluorescence microscope.
3.2.4. Transcript expression analysis

At the termination of the 96-h culture period, all embryos were imaged prior to...
snap freezing in liquid nitrogen. CONT embryos were frozen in a small volume of culture media (~5 µL), whereas ENC embryos were frozen within the alginate hydrogels. Preliminary analysis of RNA quality following extraction demonstrated no difference when embryos remained encapsulated in the hydrogel vs. de-encapsulating the embryos prior to collection and extraction (data not shown). Whole-cell RNA (wcRNA) was extracted from individual embryos that survived (i.e., remaining intact with no cellular degeneration observed) throughout the duration of culture using the RNeasy Microkit (Qiagen, Valencia, CA) with on-column DNase-I treatment as described by the manufacturer. At the same time, wcRNA was extracted from in vivo-developed control embryos (i.e., spherical, ovoid and tubular) that had previously been collected. Quantification of the wcRNA was performed with RiboGreen as indicated by the manufacturer (Molecular Probes, Inc.; Eugene, OR).

Using porcine-specific primers previously validated to amplify mRNA specific for the long form variant of steroidogenic acute regulatory protein (*STAR*), cytochrome P450 side chain cleavage (*CYP11A1*), aromatase (*CYP19A1*), and interleukin-1β (*IL1B*) [121, 122], transcript expression levels were assessed using a two-step, real-time quantitative PCR (qPCR) method with a Chromo4 real-time PCR detection system (Bio-Rad, Hercules, CA). Briefly, reverse transcription was performed with 1 ng of wcRNA from the in vivo-developed controls (i.e., spherical, n = 7; ovoid, n = 11; tubular, n = 6), CONT (n = 7), ENC- (n = 13), and ENC+ (n = 9) using the iScript cDNA Synthesis kit (Bio-Rad) according to the protocol of the manufacturer. In addition, a pooled sample of wcRNA consisting of 1 ng of wcRNA from all embryo treatment groups represented was reversed transcribed to generate a pooled cDNA sample that was used to generate a
relative standard curve of cDNA for determining interassay CVs and relative quantity (RQ) of transcript expression. Each qPCR was assayed in duplicate and consisted of 50-pg equivalents of cDNA, 0.25 µM of the appropriate forward and reverse primers, and 12.5 µL of 1x iTaq SYBR Green Supermix with ROX (Bio-Rad) in a 25-µL reaction. All PCR conditions included denaturation (95°C for 2 min) followed by amplification (95°C for 15 sec, 60°C for 15 sec and 70°C for 45 sec) for 40 cycles. Melt-curve analysis and gel electrophoresis were used to confirm amplification of a single product of the predicted size.

Expression levels for each transcript were based on the threshold cycle (C_T) values determined using the Opticon Monitor 3 software (Bio-Rad). For each transcript, two assays were performed containing equal representation of all embryo treatment groups and a standard serial dilution of pooled cDNA consisting of 200-pg, 50-pg, 12.5-pg, 3.12-pg and 0.78-pg equivalents of cDNA. After converting the exponential C_T to the linear C_T using the formula \(2^{-C_T}\) [123], the interassay CV for each transcript was determined using the pooled cDNA samples from each assay and the intraassay CV for each transcript was an average of all samples from both assays. The inter- and intra-assay CVs for STAR were 19.3% and 18.8%, respectively; CYP11A1 were 16.1% and 18.0%, respectively; CYP19A1 were 14.9% and 20.0%, respectively; and IL1B were 7.3% and 20.4%, respectively. Calculation of the RQ value was determined using a relative standard curve method by plotting treatment C_Ts against the logarithmic values of standard amounts of pooled cDNA [124]. RQ values are expressed as arbitrary units.
3.2.5. *Estradiol-17β and protein assays*

Every 24 h, half the culture media (500 µL) in each well was collected, stored at -20°C for hormone and protein analysis, and exchanged with fresh pre-gassed culture media (500 µL). In addition, wells of culture media alone (MEDIA) were included in each replicate and processed similarly to wells containing embryos. Estradiol-17β and protein analyses were performed on culture media at 24, 48, 72 and 96 h from MEDIA (n = 8) and cultures containing embryos represented in the qPCR analysis (i.e., CONT, n = 7; ENC-, n = 13; ENC+, n = 9). In addition, independent analyses for E2 and protein were performed on uterine flushings collected from pregnant gilts at d 9 (n = 9), 10 (n = 3) and 11 (n = 3). Culture media and uterine flushings were measured for E2 using a radioimmunoassay procedure previously described and validated in swine [121, 125]. Culture media were ether extracted and measured for E2 in duplicate in one assay with an intraassay CV of 12.1%. Uterine flushings were ether extracted and measured for E2 in duplicate in one assay with an intraassay CV of 13.0%. In addition, culture media and uterine flushings were measured for total protein content using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein content in culture media was measured in duplicate in six assays with inter- and intra-assay CVs of 9.3% and 7.7%, respectively. Protein content from uterine flushings was measured in duplicate in one assay with an intraassay CV of 2.4%.

3.2.6. *Statistical Analysis*

Proportional data for embryo survival and morphological changes following culture were analyzed using the Chi-square test and were reported as least-squares means
± standard error of the mean (LSM ± SEM) after calculating with GLIMMIX model procedures [126, 127]. Transcript expression data, culture media E2 and protein levels, and uterine flushing E2 and protein levels were analyzed using MIXED model procedures and results are reported as LSM ± SEM [126, 127]. When a significant F-statistic was generated, means were separated using Dunnett’s multiple comparison test [126, 127]. Means were considered statistically different at \( P \leq 0.05 \) and tendencies between \( P = 0.06 \) and \( P = 0.10 \). Transcript expression data were categorized into 6 treatment groups corresponding to \textit{in vivo}-developed spherical, ovoid and tubular embryos and \textit{in vitro}-cultured CONT, ENC- and ENC+ embryos and the model included the fixed effects of treatment, replicate and the random effect of gilt within treatment. Culture E2 and protein data were categorized into 4 treatment groups (i.e., MEDIA, CONT, ENC- and ENC+) and the model included the fixed effects of treatment, culture time, replicate, treatment by culture time interaction, and the random effect of gilt within treatment by culture time interaction. Uterine flushing E2 and protein data were analyzed with the model including day of gestation, replicate and the random effect of sire within day of gestation. To remove skewness and normally distribute the data, all transcript expression and E2 data were log-transformed prior to statistical analysis and back-transformed to report observed means.

3.3. Results

3.3.1. Evaluation of embryo viability and morphology

Figure 3-2 displays representative morphological changes observed over 96 h of
culture of CONT and ENC embryos and their corresponding cellular survival and death observed by live/dead staining after 96 h of culture. The assessment of blastocyst degeneration at the termination of culture indicated that there was no significant difference ($P = 0.22$) in survival between the CONT and ENC embryos (Table 3-1). Embryo survival and death were further confirmed by live/dead staining a subset of embryos, which demonstrated no observed differences in cellular survival or death between treatment groups. Embryos classified as undergoing cellular degeneration had a greater proportion of dead cells (red) while surviving embryos had a greater proportion of live cells (green) (Fig. 3-2). Morphological changes were only observed in the ENC embryos (Fig. 3-2C), while all CONT embryos remained spherical throughout the culture period (Fig. 3-2A; Table 3-1). The percentage of embryos that underwent morphological changes was significantly different between CONT and ENC groups when analyzing all cultured embryos ($P < 0.05$) and only those embryos surviving ($P < 0.001$; Table 3-1). The morphological changes observed in the ENC embryos were characterized by a tubular formation of the embryo within the gel and subsequent flattening of this tube with many of these embryos migrating out of the gel forming a secondary spherical structure once freely in the culture media (Fig. 3-2C). Although 32% of the surviving ENC embryos underwent morphological changes, a significant proportion of ENC embryos remained spherical throughout culture (Fig. 3-2B). As a result, encapsulated embryos were further classified as encapsulated embryos with no morphological changes (ENC-) or encapsulated embryos with morphological changes (ENC+).
Table 3-1. Summary of embryo survival and observed morphological changes following 96 h of culture of either non-encapsulated control porcine embryos (CONT) or porcine embryos double encapsulated (ENC) in 0.7% alginate hydrogels.

<table>
<thead>
<tr>
<th></th>
<th>CONT</th>
<th>ENC</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (embryos)</td>
<td>24</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Embryo survival (%)²</td>
<td>33.3 ± 9.6 (8)</td>
<td>47.8 ± 5.9 (34)</td>
<td>0.22</td>
</tr>
<tr>
<td>No morphological change from all embryos (%)</td>
<td>100</td>
<td>83.1 ± 4.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Morphological change from all embryos (%)</td>
<td>0</td>
<td>16.9 ± 4.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Morphological change from surviving embryos (%)</td>
<td>0</td>
<td>32.3 ± 8.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹Values are reported as least-squares means ± SEM as determined using GLIMMIX analysis for the main effect of treatment (i.e., control vs. encapsulated embryos). Significance for the effect of treatment was determined using Chi-square analysis.

²Assessed by lack of blastocyst degeneration. Numbers in parentheses indicate the number of surviving embryos for each treatment group.

3.3.2. Transcript expression levels

The expression of STAR mRNA was increased (P < 0.05) in all in vitro treatment groups (CONT, ENC- and ENC+) compared to the initial in vivo spherical control (Fig. 3-3A). However, STAR expression was greater (P < 0.05) in ENC+ embryos compared to ENC- embryos and tended to be greater (P = 0.07) in ENC+ embryos compared to CONT embryos (Fig. 3-3A). As a result, the expression pattern of STAR in CONT and ENC- embryos was more similar to in vivo ovoid embryos, whereas ENC+ embryos displayed an expression pattern of STAR more similar to that of in vivo tubular embryos (Fig. 3-3A). Expression levels of CYP11A1 mRNA were increased (P < 0.05) in all in vitro treatment groups (CONT, ENC- and ENC+) compared to their initial in vivo spherical controls (Fig. 3-3B). Furthermore, CYP11A1 expression was greater (P < 0.05) in ENC+ embryos compared to CONT and ENC- embryos. Again as observed with
STAR, the expression pattern of CYP11A1 in CONT and ENC- embryos was more similar to in vivo ovoid embryos, whereas ENC+ embryos displayed an expression pattern of CYP11A1 more similar to that of in vivo tubular embryos (Fig. 3-3A).

![Graphs showing expression levels of STAR, CYP11A1, CYP19A1, and IL1B mRNA](image)

**Fig. 3-3.** Expression levels of A. STAR, B. CYP11A1, C. CYP19A1 and D. IL1B mRNA measured by qPCR from in vivo-produced control embryos and in vitro-cultured CONT (non-encapsulated control), ENC- (encapsulated with no observable morphological changes) and ENC+ (encapsulated with observable morphological changes) embryos. Data were log-transformed before analysis and back-transformed to observable values. Least-squares means ± SEM values are expressed as a relative quality (RQ). Statistical analysis demonstrated that embryo treatment had significant effects on the expression level of A. STAR (P < 0.01), B. CYP11A1 (P < 0.001), C. CYP19A1 (P < 0.01) and D. IL1B (P < 0.01). RQs with different superscripts for each transcript are significantly different (P < 0.05).

Expression levels of CYP19A1 mRNA were greater (P < 0.05) in ENC+ embryos compared to CONT and ENC- embryos (Fig. 3-3C). In addition, ENC+ embryos had an expression level of CYP19A1 significantly greater (P < 0.05) than in vivo spherical controls, whereas CYP19A1 expression levels were not different between CONT, ENC-
and in vivo spherical controls (Fig. 3-3C). Furthermore, expression of CYP19A1 was decreased ($P < 0.05$) in CONT and ENC- embryos compared to in vivo ovoid and tubular embryos (Fig. 3-3C). In contrast, CYP19A1 expression did not differ between ENC+ and the later-stage in vivo ovoid and tubular embryos, although the expression pattern was numerically more similar in ENC+ and in vivo ovoid embryos (Fig. 3-3C). No significant differences were detected in expression levels of IL1B mRNA between CONT, ENC- and in vivo spherical controls (Fig. 3-3D). However, IL1B expression was increased in the ENC+ embryos compared to CONT and in vivo spherical controls (Fig. 3-3D). Furthermore, expression of IL1B did not differ between ENC+ and in vivo ovoid embryos, but was significantly ($P < 0.05$) decreased in ENC+ compared to in vivo tubular embryos.

### 3.3.3. Estradiol-17β and protein analysis

A significant time-dependent increase ($P < 0.001$) in E2 levels in the culture media of encapsulated embryos (both ENC+ and ENC-) was identified compared to culture media from CONT embryos and culture media alone (Fig. 3-4A). Culture media from all embryo groups (i.e., CONT, ENC- and ENC+) had greater ($P < 0.05$) E2 levels than media alone after 96 h of culture (Fig. 3-4). Culture media containing ENC+ embryos had significantly greater levels ($P = 0.05$) of E2 compared to CONT and ENC-embryos at 72- and 96-h time points (Fig. 3-4A). In contrast, culture media containing ENC- embryos displayed an intermediate but significantly different ($P < 0.05$) level of E2 between ENC+ and CONT embryos at 96 h (Fig. 3-4A). A similar pattern of increased ($P < 0.05$) E2 production was observed in uterine flushings as pregnancy progressed from
9, 10 and 11 of gestation with the greatest levels of E2 in uterine flushings at d 11 of gestation (Fig. 3-4B).

Fig. 3-4. **A.** Estradiol-17β (E2) concentrations (pg/mL) in culture media collected at 24-, 48-, 72- and 96-h of culture containing only media (MEDIA), non-encapsulated control embryos (CONT), encapsulated embryos with no observable morphological change (ENC-) and encapsulated embryos with observable morphological changes (ENC+). **B.** E2 concentrations (pg/mL) in uterine flushings from pregnant gilts at d 9, 10 and 11 of gestation. Data were log-transformed before analysis and back-transformed to observed values and presented as least-squares means ± SEM. Statistical analysis demonstrated a significant \( P < 0.001 \) culture media treatment by culture time interaction for E2 concentration in which culture media containing ENC+ embryos had greater E2 concentration compared to MEDIA, CONT and ENC- groups at 72- and 96-h. Statistical analysis demonstrated a significant \( P < 0.05 \) gestational day effect for E2 concentration in uterine flushings.

Results from the protein assay indicate that no significant differences were detected for total protein levels in culture media containing MEDIA alone, CONT, ENC-, or ENC+ embryos (Fig. 3-5A). In contrast, there tended \( P = 0.09 \) to be a time-dependent increase in total protein levels from uterine flushings as pregnancy progressed from d 9, 10 and 11 of gestation (Fig. 3-5B).
In the present study, alginate hydrogels were employed as 3-D matrices to support in vitro culture of porcine embryos during the pre-implantation period. Previously, alginate hydrogels have been used to support in vitro development of early blastocysts in cattle [38], as an artificial zona pellucida for mouse morulae [128] and for the culture of ovarian follicles in mice [15, 33, 37, 129] and nonhuman primates [34]. However, this report is the first to demonstrate the use of alginate hydrogels for porcine embryo culture. For those studies involving the encapsulation of early bovine embryos, 1.5% alginate hydrogels supported similar blastocyst rates as 2-D controls; however, the alginate system resulted in decreased blastocyst hatching rates, suggesting that the percentage of alginate may have formed a matrix that was too rigid to allow for hatching [38].

3.4. Discussion

In the present study, alginate hydrogels were employed as 3-D matrices to support in vitro culture of porcine embryos during the pre-implantation period. Previously, alginate hydrogels have been used to support in vitro development of early blastocysts in cattle [38], as an artificial zona pellucida for mouse morulae [128] and for the culture of ovarian follicles in mice [15, 33, 37, 129] and nonhuman primates [34]. However, this report is the first to demonstrate the use of alginate hydrogels for porcine embryo culture.
gels of varying concentrations and demonstrated that decreasing alginate matrix stiffness enhanced follicle growth [15], linking biomechanical environmental factors with cell/tissue development and function. Specifically, these studies demonstrated that 0.7% alginate, the lowest concentration of alginate that was tested, formed a low-stiffness hydrogel that was the most permissive to mouse follicle growth, cellular differentiation and corresponding steroid production [15]. Based on these findings, 0.7% alginate was used in the present study in order to provide sufficient mechanical support to properly maintain porcine embryo development, without inhibiting growth or survival.

Our in vitro culture system utilizing alginate hydrogels was able to facilitate morphological changes of pre-implantation porcine embryos by applying a tissue engineering approach to embryo culture. Although survival rates did not differ between the encapsulated and non-encapsulated embryos, morphological changes were only observed with embryos encapsulated in alginate hydrogels. The inability of the non-encapsulated control embryos to undergo morphological changes in vitro suggests that a 3-D matrix is required to maintain appropriate embryo architecture for proper development during the elongation stage of gestation. These results support the findings of previous studies that demonstrated that both mechanical forces, generated from the stiffness of surrounding tissues or substrates, and cell-generated contractility regulate differentiation during embryonic development [96]. Our in vitro culture system employing alginate hydrogel as a 3-D matrix may be initiating embryo morphological change by stimulating biochemical signals within the embryo via mechanotransduction [96, 97], or by simply providing support for the 3-D structure of the embryo to maintain its cell-to-cell communication, a feature that traditional culture systems have been
lacking. The requirement of the 3-D structure as a necessity to support morphological changes can further be supported by the observation that when ENC+ embryos within our culture system exited the alginate hydrogel, they formed a secondary spherical structure likely due to fluid transport into the trophectoderm, which was observed in the non-encapsulated CONT embryos that did not have 3-D support.

Though the percentage of total encapsulated embryos that exhibited morphological changes (17%) may seem lower than ultimately desired, it is important to emphasize that if the encapsulated embryo survives within our newly developed in vitro system, it has a relatively high chance of initiating morphological changes (32%) in simple basal media conditions. In a previous study involving in vitro culture of bovine embryos in agarose gel tunnels, high levels of glucose in the culture medium may have resulted in rapid cell growth, contributing to the appearance of embryonic elongation [49]. Conversely, in the present study, no particular growth factors or nutrients were added to the culture medium, indicating that limited embryonic morphological changes can occur in vitro within alginate hydrogels without the addition of special media supplements. While the percentage of porcine embryos initiating morphological changes in the current study is significantly less than reported in studies culturing bovine embryos in the agarose gel tunnel system, ranging from 54% to 88% [48, 49, 80], it is important to highlight that these bovine studies with a high percentage of morphological changes were performed using embryos that were strictly produced in vitro and these embryos were selectively chosen as high quality in vitro-produced embryos prior to being placed into the agarose gel tunnel system [48, 49, 80]. In contrast, the current study only used in vivo-produced embryos collected at d 9 of gestation in the pig. A previous study that
investigated morphological changes using *in vivo*-produced bovine embryos within the agarose gel system reported a very low percentage (3%) of *in vivo*-produced bovine embryos that initiated morphological changes following *in vitro* culture in the agarose gel system [82]. This finding, in conjunction with the findings of the current study, illustrates that *in vivo*-produced embryos are not as efficient as *in vitro*-produced embryos at initiating morphological changes within *in vitro* culture systems, which is likely the result of the dramatic environmental change that *in vivo*-produced embryos experience going from the uterine environment to an *in vitro* environment. Therefore, the percentage of embryos that underwent morphological changes in the current study (i.e., 17%) is relatively high for a study involving *in vivo*-produced embryos within an *in vitro* culture system, with only basal culture media.

Several transcripts, such as steroidogenic transcripts (i.e., *STAR*, *CYP11A1* and *CYP19A1*) and immune responsive transcripts (i.e., *IL1B*) are increased in a similar pattern as estrogen production during embryo elongation [130, 131]. According to the results of the present study, *STAR* expression levels for ENC+ embryos were similar to tubular *in vivo* embryos, but greater than ovoid *in vivo* embryos. In contrast *STAR* expression in CONT and ENC- embryos remained similar to ovoid *in vivo* embryos. *STAR*, which is a major initiator of steroidogenesis [132], has previously been found to be more abundantly expressed in elongated filamentous embryos than in ovoid embryos [131]. Therefore, our *STAR* expression results suggest that embryos undergoing morphological changes within alginate hydrogels exhibit similar *STAR* expression trends as those that occur during initiation of elongation *in vivo*. Similar gene expression patterns were found with *CYP11A1* and *CYP19A1* within our *in vitro* culture system,
which are the two additional transcripts encoding for rate-limiting proteins required for estrogen synthesis [131]. *CYP11A1* and *CYP19A1* expression levels were increased in ENC+ embryos compared to CONT and ENC- embryos. These findings further suggest that encapsulated embryos that transformed morphologically during culture also changed at the level of gene expression, corresponding to the morphological transition and differential steroidogenic gene expression that occurs during the onset of elongation *in vivo*.

Proper interactions between the embryo and receptive uterine endometrium are also essential for supporting embryonic development and subsequent implantation [67]. These interactions are initiated by the immune responsive cytokine *IL1B*, which is increased by the embryo during elongation and may be responsible for preventing conceptus rejection via suppressing the maternal immune response [67]. Although our results demonstrate that the expression of *IL1B* was greater in ENC+ embryos compared to CONT embryos, the expression pattern was decreased in the ENC+ compared to tubular *in vivo* embryos. This outcome may suggest that direct interaction with the maternal endometrium is likely required for up-regulation of *IL1B* in the embryo, while up-regulation of steroidogenic transcripts may be independent of maternal-embryonic cross-talk.

*In vivo* estrogen production by the porcine embryo increases during the elongation stage, stimulating the synthesis and release of numerous endometrial secretory proteins, which is essential for establishing maternal recognition of pregnancy [115, 130]. Our results indicated that the ENC+ embryos produced and secreted a greater amount of E2 by 72 and 96 h of culture compared to the CONT and ENC- embryos. In addition, this
up-regulation of E2 in the culture media of ENC+ embryos was independent of increased production of total protein within the culture media. These results correspond with the increased expression of steroidogenic transcripts STAR, CYP11A1, and CYP19A1 in the ENC+ embryos, which supports previous findings of the direct correlation between these transcripts and steroid synthesis [131, 132]. Therefore, the E2 results further suggest that embryonic morphological changes induced by the alginate hydrogel culture system are indications of initiation of elongation due to the corresponding increase in estrogen production. The time-dependent increase in E2 observed in the culture media of ENC+ embryos followed a similar trend as observed for E2 levels in the uterine flushings as pregnancy advanced through this time period, with the greatest production of estrogen observed at d 11 of gestation when tubular embryos were present. Furthermore, protein increased in the uterine flushings as pregnancy advanced. Given that protein increases within the uterine milieu regardless of pregnancy status [133], increased expression of steroidogenic transcripts and E2 production by ENC+ embryos without a difference in protein levels within the culture media further suggests that up-regulation of steroidogenesis and subsequent estrogen production may be independent of maternal-embryonic cross-talk. It is important to highlight that there was a 48-h delay for embryos cultured within alginate hydrogels to initiate production and secretion of E2 into the culture media, as well as for significant morphological changes to occur, which indicate a delay in development for in vitro-cultured embryos compared to in vivo-developed embryos. A similar delay in morphological changes was observed in bovine embryos cultured in the agarose gel system compared to in vivo-developed embryos [82].
This delay is likely due to the embryos recovering from the encapsulation technique and adjusting to the *in vitro* microenvironment.

Unlike elongation in other domestic animals, rapid elongation of the pig embryo has been primarily associated with cellular remodeling and differentiation rather than hyperplasia [61]. As a result, follow up studies investigating potential markers for differentiation of the trophectoderm and mesoderm would be useful for measuring the level of differentiation from embryos undergoing morphological changes within our *in vitro* culture system. Furthermore, embryonic disc establishment, which was not monitored in the current study, will be included in follow-up studies to further evaluate the efficiency of our culture system. Monitoring the formation of the embryonic disc will be a useful technique for measuring the synchrony between trophoblast and embryonic disc development as we move forward to improve our *in vitro* culture system using alginate hydrogels.

In conclusion, the present study demonstrates an *in vitro* culture system that can support porcine embryo development during initiation of elongation. Our culture system, which employs alginate hydrogel as a 3-D extracellular matrix for porcine embryo culture, has proven to facilitate embryonic morphological changes with corresponding increases in steroidogenic transcript expression and estrogen production, in a similar pattern as observed in *in vivo*-developed embryos. Our findings advocate the potential of our alginate culture system as an *in vitro* tool for evaluating and understanding specific mechanisms of embryo elongation. Further investigations with our 3-D *in vitro* culture system can focus on the effects of certain growth factors, hormones, ligands, and uterine epithelial cell co-culture on development of pre-implantation porcine embryos. Specific
factors regulating pig embryo elongation can be identified to develop strategies to improve pregnancy outcomes in the pig. Identified factors could then be manipulated through either direct intervention or marker-assisted selection of genomic markers to improve early embryonic survival, uterine capacity, and pre-weaning piglet survival, which could have a significant impact on the profitability of swine production. Furthermore, our alginate culture system could be applied to embryos of other ungulate species, such as cattle and sheep that also undergo embryo elongation during the pre-implantation period of pregnancy, in order to identify potential regulators of normal embryonic development in a variety of species.
CHAPTER 4

The effect of parathyroid hormone and RGD peptide ligands on the \textit{in vitro} behavior of growth plate chondrocytes within 3-D alginate matrices

4.1. Introduction

Osteochondrodysplasias, a group of disorders characterized by abnormal growth and remodeling of bone and cartilage, occur with over 200 different phenotypes, with a prevalence estimated to be between 1 in 3000 to 1 in 5000 births [88]. Mutations responsible for these dysplasias may cause defects in the synthesis of extracellular matrix proteins, specific growth factors, receptors, and transcription factors, leading to short stature and/or cranial defects [89]. Furthermore, skeletal growth can be affected by defects in growth plate function due to radiation and chemotherapy as well as cartilage injury from sports and high impact activities [134, 135]. In addition to affecting quality of life, growth plate damage due to injury or disease is often associated with long-term intensive health care needs due to impact on the joints, the central nervous system, and the ocular system [90, 91]. As cartilage does not heal, strategies to replace damaged or diseased cartilage through tissue engineering or regenerative approaches are of great interest.

Within growth plate cartilage, chondrocytes differentiate in a spatially regulated manner that results in morphologically and functionally distinct zones of cells, forming a continuum from progenitor cells (resting chondrocytes) to terminally differentiated, hypertrophic chondrocytes [83, 85] (Fig. 4-1). The middle proliferative zone is distinguished by a columnar arrangement of flattened chondrocytes regulated by an indirect, functional feedback loop between the secreted proteins parathyroid hormone
related protein (PTHrP) and Indian hedgehog (Ihh), produced by resting and early hypertrophic chondrocytes, respectively [83-85]. Prehypertrophic chondrocytes exiting the proliferative columns express Ihh, which stimulates the production of PTHrP by resting cells and perichondral cells located at the bone ends [83, 85].

![Schematic illustration of zonal arrangement of chondrocytes and Ihh/PTHrP feedback loop within the growth plate.](image)

**Fig. 4-1.** Schematic illustration of zonal arrangement of chondrocytes and Ihh/PTHrP feedback loop within the growth plate.

PTHrP receptors are expressed at low levels in chondrocytes of the proliferative zone and high levels in the pre-hypertrophic chondrocytes as they transition from the proliferative to hypertrophic state [83, 136]. PTHrP primarily acts to keep these cells in the proliferative pool, delaying further differentiation [83, 137]. As chondrocytes in the proliferative columns become too distant from the PTHrP-producing cells located at the bone ends, PTHrP concentration becomes too low to stimulate them; this lack of stimulation causes the chondrocytes to stop proliferating, enter a pre-hypertrophic maturation phase, and begin to synthesize Ihh, thereby forming a feedback loop that controls the length of the proliferative columns and site of hypertrophic differentiation.
[83, 137]. The chondrocytes of the resting and proliferating zones are also characterized by the secretion of structural proteins, predominately type II collagen, while hypertrophic cells primarily synthesize type X collagen and begin to remodel the cartilage matrix into a calcifying matrix [83-85].

Although significant advances have been made in discovering signaling pathways and gradients that regulate growth plate function, it remains unclear whether these factors alone are sufficient to induce normal growth plate structure and function. Previous attempts to engineer growth plate cartilage have failed to produce native cartilage architecture, in particular a distinct zone of proliferating columnar chondrocytes, suggesting that these factors are not enough to replicate proper structure in vitro. Therefore, an effective in vitro model of the growth plate could serve as the necessary tool for further evaluating specific mechanisms that regulate proper growth plate structure and function. Understanding these mechanisms by studying the generation of growth plate architecture in vitro is critical for enhancing tissue engineering strategies in order to provide optimal replacement tissue for abnormal or damaged growth plates.

We hypothesize that a three dimensional (3-D) matrix with appropriate biomechanics, extracellular matrix (ECM) factors, and signaling gradients can establish an in vitro model of native growth plate cartilage. The objective of the current study was to first investigate the effect of parathyroid hormone (PTH) on isolated mouse growth plate chondrocytes in vitro, encapsulated within alginate hydrogels as the 3-D artificial matrix. Alginate is a linear polysaccharide composed of repeating units of β-D-mannuronic acid (M) and α-L-guluronic acid (G) [9] that forms a hydrogel by ionic cross-linking of the G residues in the presence of a divalent cation [10]. In addition to its gentle
gelation process and high porosity for diffusion of nutrients and growth factors [7, 13], alginate promotes negligible non-specific protein adsorption and cell adhesion [14], allowing alginate to act as a “blank slate” into which specific cell-matrix interaction factors can be added. The arginine-glycine-aspartic acid (RGD) peptide sequence, which is found in extracellular matrix proteins of cartilage such as collagen, fibronectin, laminin, is the most effective and commonly used peptide sequence to promote cell adhesion [43]. When covalently linked to alginate, RGD peptide sites serve as ligands to cell integrins to mediate cell migration and adhesion. Therefore, RGD peptide conjugation to alginate was the second factor investigated in this study for its effect on in vitro growth plate chondrocyte behavior. In vitro chondrocyte behavior within unmodified and RGD-conjugated alginate gels, with and without PTH treatment, was evaluated by characterizing cellular proliferation and analyzing gene expression of transcripts involved in chondrocyte hypertrophy.

4.2. Methods

4.2.1. Preparation of alginate solutions

For the unmodified alginate condition, sodium alginate (Pronova UP MVG, >60% guluronic acid, 200,000 – 300,000 g/mol, NovaMatrix, Norway) was dissolved in sterile 1X phosphate buffered saline (1X PBS) to a final concentration of 1.5% (w/v). For the RGD peptide treatment, alginate was covalently modified with a GRGDSP peptide (Anaspec, Fremont, CA) to a concentration of 4 μmol/g alginate using carbodiimide chemistry, as previously described [14]. Briefly, two grams of sodium alginate were
added to 200 mL of MES buffer (0.1M MES, 0.3M NaCl, pH 6.5) and the alginate was allowed to dissolve for 7-8 hours on a stir plate. 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC; Sigma Aldrich, St. Louis, MO) was added to activate 5% of the uronic acids of the alginate polymer backbone in order to form amide linkages with the peptide. To stabilize the reactive EDC intermediate against a competing hydrolysis reaction, N-hydroxy-sulfosuccinimide (sulfo-NHS; Pierce, Rockford, IL) was also added to the solution at a 2:1 EDC to NHS ratio. Lastly, the GRGDSP peptide was incorporated into the solution, conjugating to the alginate backbone via the terminal amine of the peptide. After 20 hours of reacting, the conjugation reaction was quenched through the addition of hydroxylamine hydrochloride (Sigma Aldrich). The alginate solution was then dialyzed against decreasing salt solutions and finally ddH$_2$O over the course of 3 days. The final dialyzed solution was lyophilized to dryness and dissolved in 1X PBS to a final concentration of 1.5% (w/v).

4.2.2. Isolation of growth plate chondrocytes

Growth plate cartilage was dissected from the distal growth plate of the femur and the proximal growth plate of the tibia from three- and four-day-old neonatal Swiss Webster mice. The growth plate cartilage was then incubated in 1 mL of 0.5% collagenase in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) for 3-4 hours at 37°C and 8% CO$_2$, resulting in a suspension of chondrocytes. Next, 10 mL of DMEM with 10% FBS was added to the suspension of chondrocytes and centrifuged for 5 minutes at 125 x g. After aspirating the
supernatant, the cell pellet was resuspended in 10 mL of DMEM with 10% FBS and cells were counted with a hemacytometer.

4.2.3. Chondrocyte encapsulation and culture

Chondrocytes were cultured in four different treatment groups, as shown in Table 4-1: unmodified alginate gels without the addition of PTH (Unmodified/no PTH), unmodified alginate gels treated with 1 µM PTH (Unmodified + PTH), GRGDSP-conjugated alginate gels without the addition of PTH (RGD/no PTH), and GRGDSP-conjugated alginate gels treated with 1 µM PTH (RGD + PTH). Prior to encapsulation, chondrocytes were once again pelleted by centrifugation, resuspended in a volume of culture media equal to 10% of the volume of alginate, and then mixed into the alginate and RGD-alginate solutions to obtain a concentration of 8x10^6 cells/mL alginate. A separate batch of cell/alginate suspension was prepared for each condition. To encapsulate the chondrocytes, 10-µL drops of the alginate/cell mixture were placed one at a time into crosslinking solution (50 mM CaCl₂/140 mM NaCl) for 2 minutes, forming cell-embedded alginate hydrogel beads. The beads were then transferred to wells of rinse media to leach out excess calcium ions before being placed into fresh wells of cartilage culture media (MEM alpha medium without Phenol Red [Invitrogen] supplemented with 50 µg/mL Penicillin-Streptomycin-Glutamine [Invitrogen], 10 mM β-glycerophosphate, and 50 µg/mL ascorbic acid). For the PTH conditions, PTH (Sigma Aldrich) was added to the culture media to obtain a final concentration of 1 µM. A total of 9 beads were made per condition so that a subset of 3 beads could be analyzed at each time point (Table 4-1). Additionally, 4 extra beads were made for the RGD/no PTH condition to perform
live/dead staining, which will be described below. The beads were cultured for 3 days, with the media replaced every 24 hours. Endpoint analyses were performed on Day 1, 2, and 3 of culture as described in the following sections.

**Table 4-1.** Overview of number of beads per culture condition and per endpoint analysis. Total number of beads per condition were divided up between the three time points (Days 1, 2, and 3) so that a subset could be taken on each day for analysis. Live/dead staining for the RGD + PTH condition was performed on Day 1 for the first and last beads made for that condition, and on a representative bead at Days 2 and 3.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified/no PTH</td>
<td>3</td>
<td>6</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Unmodified + PTH</td>
<td>3</td>
<td>6</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>RGD/no PTH</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>RGD + PTH</td>
<td>3</td>
<td>6</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total no. beads in culture:</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>40</strong></td>
</tr>
</tbody>
</table>

4.2.4. **Characterization of cell viability and proliferation**

Because beads were made sequentially after blending the cells into the alginate solution for each condition, some cells were required to remain in alginate solution longer than others prior to crosslinking. To determine if this extra time spent in alginate solution lowered cell viability, the first bead prepared from the batch of alginate/cell suspension for the RGD/no PTH condition, along with the last bead prepared from this batch, were live/dead stained after Day 1 of culture. Additionally, one representative bead from the same condition (RGD/no PTH) was removed from culture on Day 2 and Day 3 for live/dead staining to evaluate cell viability over the course of the culture period. These beads were rinsed twice with 1X tris-buffered saline with 1.8 mM CaCl$_2$ (1X TBS + 1.8 mM CaCl$_2$) and then placed into 1xTBS with 2 µM calcein AM and 4 µM ethidium
homodimer-1 for 30 minutes to stain live and dead cells, respectively. After another rinse in 1X TBS + 1.8 mM CaCl$_2$, the beads were placed on a glass coverslip and imaged using an epifluorescence microscope (Leica DMI6000B, Wetzlar, Germany).

For analysis of cell proliferation on Days 1, 2 and 3 of culture, a subset of beads, one from each condition, was analyzed using the Click-iT EdU Imaging kit (Invitrogen, Carlsbad, CA), following manufacturer’s instructions. EdU (5-ethyl-2’-deoxyuridine) is a nucleoside analog of thymidine that becomes integrated into DNA during active DNA synthesis, serving as a marker for cellular proliferation when covalently bound to a fluorescent probe. Briefly, the encapsulated cells were incubated with EdU for 1 hour and then fixed in 4% paraformaldehyde with 1.8 mM CaCl$_2$. After rinsing in 1xTBS +1.8 mM CaCl$_2$, the beads were embedded in 6% agarose and then sectioned into 300-µm sections within a 1xTBS + 1.8 mM CaCl$_2$ bath using a vibratome (Leica). Sections were then incubated in the Click-iT reaction cocktail consisting of an Alexa Fluor azide to detect EdU incorporation. Finally, cellular DNA was stained with Hoechst 33342 to identify nuclei. The cells were then imaged using an epifluorescence microscope (Leica DMI6000B). The Volocity software (Perkin Elmer, Waltham, MA) was used to perform deconvolution of z-stacks to remove background fluorescence and then used to count total number of cells in each section (one section per condition), as well as cells that stained positively for EdU, to determine percent proliferation.

4.2.5. Transcript expression analysis

Real-time PCR (RT-PCR) was used to analyze expression of collagen 10a1 (ColX), Indian hedgehog (Ihh), and Sox9 (Sox9; marker for chondrocyte differentiation)
transcripts. At the specified time points, a subset of alginate beads from each condition was taken from culture and dissolved in 50 mM EDTA for 10 minutes to release the cells for RNA extraction. Total RNA was extracted using TRIzol reagent following the manufacturer’s instructions (Invitrogen). Total RNA was then treated with DNase I (Promega, Madison, WI) to remove any genomic DNA and reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and random primers (Promega) combined with oligoDT<sub>20</sub> primers (Invitrogen). Real-time PCR was performed using ABsolute Blue Sybr Green ROX mix (Thermo Scientific, Waltham, MA) and a StepOnePlus System (Applied Biosystems, Carlsbad, CA). Gene specific primers designed for the genes specified above are shown in Table 4-2. Data were normalized to an endogenous control, actin, using the primers shown in Table 4-2 for β-actin.

### Table 4-2. Description of the designed primers.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen 10a1</td>
<td>TTCTGCTGCTAATGTTTCTTGACC</td>
<td>GGGATGAAGTATTGTGTCTTTGGG</td>
</tr>
<tr>
<td>Ihh</td>
<td>CCCCAAACTACAATCCCGACA</td>
<td>TCATGAGGCGGTCGGC</td>
</tr>
<tr>
<td>Sox9</td>
<td>ACGGCTCCAGCAAGAACAAG</td>
<td>TTGTGCAGATGCAGGTACTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAATCGTGCCTGACATCAAAGA</td>
<td>GCCATCTCTCCTGCTGAGTC</td>
</tr>
</tbody>
</table>

4.2.6. **Statistical analysis**

For RT-PCR results, RQ values are reported as mean ± standard deviation. After normalizing to the endogenous control, data within each day were normalized to the values for the unmodified/no PTH condition to observe treatment effects. One-way analysis of variance (ANOVA) was used to determine if means were statistically
different. If ANOVA analysis showed a significant difference among the means, Tukey’s multiple comparison test was then used to perform pairwise comparisons.

4.3. Results

4.3.1. Evaluation of chondrocyte viability and proliferation

After 24 hours of culture, chondrocytes encapsulated in the first and last bead prepared from the alginate/cell suspension for the RGD/no PTH condition were live/dead stained and compared in order to assess whether extra time spent in alginate solution prior to crosslinking affected cellular survival. No considerable differences in cellular survival or death were observed between the first and last bead that were made for this condition (Fig. 4-2A and 4-2B). In addition, no substantial differences in cell viability were observed between Days 1, 2, and 3 of culture within the RGD-alginate gels (Fig. 4-2C and 4-2D).
To identify the number of proliferating chondrocytes in each treatment group (Table 4-1), 300-µm sections (one section per condition) were treated with EdU and stained for EdU detection. Cellular DNA was then stained to evaluate total number of cells in order to calculate the percentage of cells that were positive for EdU staining. Representative images are shown in Figure 4-3 and calculated results are presented in Table 4-3, with some treatments missing due to loss of sections. No substantial effects of RGD ligands or PTH on percent proliferation were observed, as the percentages for each treatment group only differed by a few percent at most (Table 4-3). However, despite the low percentages for all conditions, the RGD-alginate condition had an approximately 2.5-fold higher percent proliferation by Day 3 compared to the corresponding unmodified condition.
alginate condition (Table 4-3), and the PTH treatment groups exhibited a 1.3- to 1.5-fold higher percent proliferation by Days 2 and 3 compared to the corresponding gel conditions without the addition of PTH (Table 4-3).

![Representative images of nuclear (blue) and EdU (green) staining of chondrocytes cultured in unmodified alginate gels without PTH (Unmodified/no PTH), unmodified gels with 1 µM PTH (Unmodified + PTH), RGD-alginate gels without PTH (RGD/no PTH), and RGD-alginate gels with 1 µM PTH (RGD + PTH). No considerable differences in percent proliferation (i.e. percent EdU positive) were observed.]

**Fig. 4-3.**
4.3.2. Transcript expression levels

On Day 1 of culture, expression of ColX (Fig. 4-4A) was statistically greater (p<0.05) in chondrocytes encapsulated in RGD-alginate compared to chondrocytes encapsulated in unmodified alginate, when no PTH was added to either condition; however, no differences existed between these two gel conditions when PTH was added. After two days in culture, ColX expression was significantly greater (p<0.05) in chondrocytes encapsulated in the unmodified alginate gels compared to those cultured in RGD-alginate, when no PTH was added to either gel condition. However, no difference was observed between the Unmodified + PTH and RGD + PTH conditions on Day 2. For both gel conditions, PTH treatment resulted in significantly reduced (p<0.05) expression of ColX in encapsulated chondrocytes by Day 2 compared to the corresponding gel conditions without PTH treatment. On Day 3 of culture, chondrocytes in the

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>EdU Positive</th>
<th>Cell Count</th>
<th>% EdU Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>RGD/no PTH</td>
<td>92</td>
<td>2114</td>
<td>4.35%</td>
</tr>
<tr>
<td>Day 1</td>
<td>RGD + PTH</td>
<td>29</td>
<td>2793</td>
<td>1.04%</td>
</tr>
<tr>
<td>Day 1</td>
<td>Unmodified/no PTH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 1</td>
<td>Unmodified + PTH</td>
<td>81</td>
<td>2539</td>
<td>3.19%</td>
</tr>
<tr>
<td>Day 2</td>
<td>RGD/no PTH</td>
<td>61</td>
<td>4003</td>
<td>1.52%</td>
</tr>
<tr>
<td>Day 2</td>
<td>RGD + PTH</td>
<td>42</td>
<td>2080</td>
<td>2.02%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Unmodified/no PTH</td>
<td>7</td>
<td>588</td>
<td>1.19%</td>
</tr>
<tr>
<td>Day 2</td>
<td>Unmodified + PTH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 3</td>
<td>RGD/no PTH</td>
<td>58</td>
<td>2848</td>
<td>2.04%</td>
</tr>
<tr>
<td>Day 3</td>
<td>RGD + PTH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 3</td>
<td>Unmodified/no PTH</td>
<td>33</td>
<td>3988</td>
<td>0.83%</td>
</tr>
<tr>
<td>Day 3</td>
<td>Unmodified + PTH</td>
<td>16</td>
<td>1315</td>
<td>1.22%</td>
</tr>
</tbody>
</table>
Unmodified/no PTH group still exhibited the greatest expression of ColX (p<0.05) compared to the RGD-alginate conditions. Furthermore, expression of ColX was significantly lower (p<0.05) in chondrocytes cultured in RGD-gels treated with PTH, compared to those in RGD-gels without the addition of PTH. Results for the Unmodified + PTH treatment group were undetermined for Day 3.

Likewise, expression of Ihh (Fig. 4-4B) on Day 1 of culture was significantly greater (p<0.05) in the RGD-alginate treatment group compared to the unmodified alginate treatment group, when no PTH was added to either condition. PTH treatment led to significantly lower Ihh expression in chondrocytes cultured in RGD-alginate gels, while no difference was observed between the Unmodified/no PTH and Unmodified + PTH groups. On Day 2 of culture, Ihh expression was significantly highest (p<0.05) in the Unmodified/no PTH treatment group. Furthermore, PTH-treated groups of both unmodified and RGD-alginate gels exhibited significantly lower (p<0.05) expression of Ihh compared to the corresponding gel condition without PTH. Although expression levels were significantly different between unmodified alginate and RGD-alginate conditions when no PTH was added, no difference was observed between the two gel conditions when PTH was incorporated into the culture media. On the third day of culture, no differences in Ihh expression existed between the unmodified alginate and RGD-alginate gel conditions, with or without the addition of PTH. However, Ihh expression was significantly reduced (p<0.05) in both gel conditions treated with PTH throughout the culture period.
On Day 1 and Day 2 of culture, no statistical differences in $Sox9$ expression (Fig. 4-4C) existed between treatment groups. On Day 3, however, $Sox9$ expression in chondrocytes cultured in the RGD + PTH condition was significantly greater ($p<0.05$) than those cultured in the unmodified alginate conditions, with and without the addition
of PTH. When no PTH was added to either gel condition, no statistical differences were observed between the unmodified alginate and RGD-alginate groups. Furthermore, PTH addition did not significantly effect Sox9 expression on Day 3 in either the unmodified or RGD-alginate gel conditions.

4.4. Discussion

In the present study, unmodified and RGD-conjugated alginate hydrogel gels were used as an artificial 3-D matrix for growth plate chondrocyte culture over the course of 3 days, with and without the exogenous addition of soluble PTH. Previous studies have employed unmodified alginate gels for the in vitro culture of growth plate chondrocytes to evaluate the effects of 3-D culture on the expression of differentiation markers [92], as well as the effects of gelation conditions on chondrocyte formation in vitro [12], demonstrating that alginate gels are able to maintain chondrocyte genotype and phenotype in vitro compared to monolayer culture. Furthermore, RGD-conjugated alginate gels, in particular, have previously been investigated in several studies for their effects on chondrocyte behavior and chondrogenesis, including in vivo articular cartilage growth [24], in vitro chondrogenesis of bone marrow stromal cells [138], and calcium signaling response of articular chondrocytes to fluid flow [139]. However, the present study is the first to use an in vitro alginate model to test the effects of RGD ligands and soluble PTH on the behavior and gene expression of encapsulated growth plate chondrocytes.

The alginate encapsulation method utilized in this study was able to maintain chondrocyte viability throughout three days of culture, as demonstrated by live/dead
staining of a representative bead on each day. Furthermore, cell viability appeared to be unaffected by extra time spent in alginate solution prior to crosslinking, which can be concluded from the live/dead staining images of the first and last beads that were prepared from the alginate/cell suspension for the RGD/no PTH condition. These results confirm alginate’s gentle encapsulation process and its favorable use as a 3-D artificial matrix. No clear trends in cell proliferation could be concluded from EdU staining of the chondrocytes, despite some small differences between groups. Although the RGD peptide ligands did increase percent proliferation by Day 3 compared to the corresponding unmodified alginate condition, a larger proliferative effect was expected due to the peptide’s known ability to increase proliferation in other cell types cultured in RGD-alginate gels [14, 27, 140]. These results, however, could be explained by the RGD ligand density chosen for this study (4 µmol/g), which may have been too low to induce substantial proliferation effects in the growth plate chondrocytes cultured in our system.

Similarly, PTH treatment did not have a substantial effect on chondrocyte proliferation, although it did promote a slightly higher percentage of proliferating chondrocytes by Days 2 and 3 of culture. Unlike the results for RGD effect on proliferation, these PTH results were not surprising; the results of the present study support previous reports that revealed that Ihh is the driving force of proliferation in the growth plate and is independent of PTHrP signaling [141]; that is, changes in PTHrP-related parameters interact specifically with hypertrophy, as opposed to affecting rate of proliferation [86, 142].

In growth plate cartilage \textit{in vivo}, PTHrP binds to its PTH/PTHrP receptor expressed by proliferating chondrocytes and suppresses chondrocyte hypertrophy,
retaining the chondrocytes in the proliferative zone [83, 85]. In areas where PTHrP concentration drops below a critical level, chondrocytes stop proliferating, begin hypertrophy, and start to produce Ihh and type X collagen [84]. Hence, PTHrP, and therefore PTH, acts to delay chondrocyte hypertrophy. According to the results of the present study, our alginate system was able to mimic this occurrence in vitro by Day 2 and Day 3 of culture, which was indicated by the significantly lower expression of both ColX and Ihh as a result of PTH addition to chondrocytes cultured in unmodified and RGD-alginate gels. The Day 1 results, which did not follow this trend, can most likely be explained by the chondrocytes recovering from the dissection and encapsulation process and adjusting to the in vitro environment during this time; hence, 24 hours was not a long enough time for real changes in maturation state to occur. The fact that proliferation rates were not affected by PTH treatment despite greater Ihh expression in encapsulated chondrocytes that were not treated with PTH suggests that Ihh production, enabled by a lack of PTH, did not yet have time to stimulate chondrocyte proliferation by Day 3. Longer culture times could be evaluated in future studies to reveal this effect.

Interestingly, RGD conjugation to alginate also suppressed the expression of hypertrophic markers in our in vitro system by days 2 and 3 compared to unmodified alginate gels. However, the addition of PTH to the culture media of unmodified and RGD-conjugated alginate gels eliminated the differences in hypertrophic gene expression that existed between the two gel conditions without the PTH treatment; therefore, PTH treatment, at the 1 µM concentration we used, overrides the hypertrophic effect of unmodified alginate on encapsulated chondrocytes when comparing to the same treatment in the RGD-alginate condition. The suppression of hypertrophy by RGD
peptide conjugation to alginate supports results from previous studies that investigated the effect of RGD ligands on chondrogenic differentiation \textit{in vitro} [143, 144]. In a previous study involving the culture of growth plate chondrocytes seeded on fibronectin, an ECM protein that contains the RGD peptide as its active site, exogenously added RGD to the media resulted in increased alkaline phosphatase levels, implying terminal differentiation [143]. The terminal differentiation could be explained by the act of the exogenously added RGD blocking the link between cellular integrins and the RGD-containing fibronectin protein, suggesting that interactions of chondrocytes with RGD ligands suppress terminal differentiation [143]. This effect was also demonstrated in the current study utilizing RGD-conjugated alginate; that is, higher \textit{ColX} and \textit{Ihh} expression levels in the unmodified gel condition compared to the RGD-conjugated gel condition indicated that hypertrophic effects were increased in the absence of RGD ligands.

Finally, Sox9 is a known transcription factor that acts at every stage of chondrocyte differentiation in the growth plate, from the formation of condensations by mesenchymal cells to the transformation of proliferating chondrocytes into hypertrophic chondrocytes [83, 145]. As Sox9 is essential for chondrocyte formation, Sox9 expression is often used as a marker for chondrocyte differentiation in \textit{in vitro} studies to detect or confirm chondrogenic lineage. In the present study, Sox9 expression levels were evaluated to ensure that chondrocytes from each treatment group were not dedifferentiating during culture with respect to the other treatment groups. Overall, Sox9 expression was similar in all treatment groups on each day, confirming that differences in \textit{Ihh} and \textit{ColX} expression levels between groups were not a result of dedifferentiation effects.
In conclusion, this report reveals that PTH treatment and RGD peptide ligands suppress hypertrophy after Day 1 in chondrocytes cultured in alginate hydrogels, demonstrating the ability of these factors to regulate growth plate chondrocyte behavior within our 3-D culture system. This study was the first step utilizing this alginate culture model to evaluate factors necessary for inducing native growth plate architecture and function in vitro. Future studies with our 3-D in vitro culture system can focus on the effects of treatment with other signaling factors, such as Ihh, and the combination of signaling factors to produce feedback loops as seen in vivo. Furthermore, dose-dependent effects of PTH and other factors, along with variations in RGD ligand density and gel mechanics, can be investigated with our alginate culture system to identify optimal conditions for in vitro chondrocyte culture. Knowledge of the effects of these factors can be used to establish zonal arrangement of growth plate chondrocytes in vitro through the use of signaling gradients, which would have a significant impact on tissue engineering strategies for the replacement of growth plate cartilage damaged due to injury or disease.
CHAPTER 5
Future studies and conclusions

5.1. Introduction

In this thesis, alginate hydrogel was first designed and characterized for its use as a 3-D matrix for in vitro models of tissue development. After demonstrating its ability to be manipulated in shape, chemical structure, and mechanical properties, alginate gels were employed as an artificial extracellular matrix (ECM) for pre-implantation pig embryo culture in vitro. This study validated the potential of the alginate culture system to serve as a matrix for an in vitro model of pig embryo development, in particular elongation during the pre-implantation stage of pregnancy. Next, unmodified and GRGDSP-conjugated alginate gels were used for the encapsulation and culture of mouse growth plate chondrocytes. This culture system was used as a model to study the effects of parathyroid hormone (PTH) and GRGDSP peptide conjugation on chondrocyte behavior, demonstrating that both PTH and RGD adhesion ligands decrease expression of collagen X and Indian hedgehog (Ihh) transcripts, two markers for hypertrophic chondrocytes, relative to control treatments. Together, the results presented in this thesis indicate alginate’s promising use as a 3-D matrix for in vitro models of pig embryo development as well as growth plate development. Future studies are needed to further investigate factors and mechanisms involved in development of these tissues, utilizing our alginate hydrogel system as the in vitro research tool. Recommended studies for both projects are described in detail below.
5.2. Future directions for the *in vitro* pig embryo model

In Chapter 3, an *in vitro* culture system was established using a 3-D alginate hydrogel matrix that can support native embryo architecture and facilitate morphological changes of porcine embryos at the onset of elongation, in base serum media without the addition of special growth factors [50]. Furthermore, this system was shown to induce a corresponding increase in estrogen production and steroidogenic transcript expression, consistent with *in vivo* elongation [50]. With a base system established, the *in vitro* alginate culture system can now be used as a model for evaluating the effect of specific and undefined uterine factors on pre-implantation embryo development. Therefore, future studies involving the alginate culture model for pig embryo elongation should investigate the molecular and cellular aspects of pre-implantation embryo development in response to components added to the culture media or alginate gel that are known to exist in the uterus during elongation. The following three-tiered approach is recommended: 1) evaluate the effect of culturing encapsulated embryos within uterine flushes collected between days 9 through 12 of gestation; 2) evaluate the effect of specific uterine factors added to basal media or incorporated within the alginate on the development of encapsulated embryos; and 3) investigate the effect of co-culturing the encapsulated embryos with uterine lumen and glandular epithelial cells. Each of these approaches is described in detail below.

The uterine environment prepares for embryo elongation and implantation by releasing nutrients, metabolites, and hormones in a time-dependent manner relative to paracrine signals from the embryo (i.e., estrogen) and endocrine/paracrine signals within the endometrium (i.e., progesterone) [69, 70, 146]. It has been shown that total protein
increases in the uterine lumen from day 7 to 10 in both cyclic and pregnant gilts followed by a more significant increase from day 10 to 13 [147]. Therefore, an evaluation of uterine milieu between days 9 through 12 of gestation should be made by adding uterine flushes from these pregnancies to basal culture media to determine the effect of undefined uterine components on embryo development within our alginate culture system. Preliminary studies were conducted to investigate whether uterine flushes from day 9 or day 10 of gestation were capable of supporting in vitro embryo development of in vivo-derived day 9 porcine embryos using the 3-D alginate culture system. Embryos collected on day 9 of gestation were assigned to be cultured in pooled day 9 flushes, day 10 flushes, or base serum media for 96 hours within 0.7% alginate gels. Our morphological results revealed that encapsulated embryos cultured in base serum media (as used for the system in Chapter 3) exhibited the greatest percentage of morphological changes (22.22%) compared to the encapsulated embryos cultured in the day 9 (12.5%) or day 10 flushes (16.67%), as shown in Table 5-1. Furthermore, the trend was similar when comparing the percentage of surviving embryos with morphological changes across the media treatment groups (Table 5-1).

In addition to observing embryonic survival and morphological changes, real-time PCR was used to analyze steroidogenic transcript expression at 96 h in the encapsulated embryos cultured in the uterine flushes compared to those cultured in base serum media. Overall, expression of steroidogenic transcripts STAR, CYP11A1 and CYP19A1 was greater in encapsulated embryos cultured in base serum media (Enc serum) compared to embryos cultured in the pooled day 9 (Enc d9) or day 10 flushes (Enc d10) (Fig. 5-1). Expression of STAR, CYP11A1 and CYP19A1 are known to be increased in a similar
pattern as estrogen production during embryo elongation \cite{130, 131}; therefore, greater expression of these transcripts in the Enc serum group correlates with the higher percentage of morphological changes that occurred compared to the Enc d9 and Enc d10 groups.

**Table 5-1.** Summary of embryo survival and morphological changes observed following 96 h culture of day 9 porcine embryos encapsulated in 0.7% alginate gels. Encapsulated embryos were assigned to be cultured in day 9 uterine flushes (D 9), day 10 uterine flushes (D 10), or in base serum media (Serum).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>D 9</th>
<th>D 9 Cont</th>
<th>D 10</th>
<th>D 10 Cont</th>
<th>Serum</th>
<th>Serum Cont</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of embryos</td>
<td>24</td>
<td>6</td>
<td>24</td>
<td>6</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Embryo survival (n)</td>
<td>8</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Embryo survival (%)</td>
<td>33.33%</td>
<td>66.67%</td>
<td>41.67%</td>
<td>66.67%</td>
<td>38.89%</td>
<td>50.00%</td>
</tr>
<tr>
<td>Morph change (n)</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Morph change from all embryos (%)</td>
<td>12.50%</td>
<td>0.00%</td>
<td>16.67%</td>
<td>0.00%</td>
<td>22.22%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Morph change from surviving embryos (%)</td>
<td>37.50%</td>
<td>0.00%</td>
<td>40.00%</td>
<td>0.00%</td>
<td>57.14%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

**Fig. 5-1.** Expression levels of *CYP11A1* (blue), *CYP19A1* (red), and *STAR* (green) mRNA measured by qPCR from Enc d 9 (encapsulated embryos cultured in d9 flushes), Enc d 10 (encapsulated embryos cultured in d10 flushes) and Enc serum (encapsulated embryos cultured in serum media). Data were log-transformed before analysis and back-transformed to observable values. Least-squares means \(\pm\) SEM values are expressed as a relative quality (RQ).
These preliminary results indicate that soluble factors present in uterine flushes at day 9 or day 10 of gestation do not support *in vitro* embryo development for 96 hours within our 3-D alginate culture system as efficiently as base serum media. These results are likely due to asynchrony issues between the embryos and their corresponding environment, which has previously been suggested to be one of the causes of early embryonic loss during the elongation stage of embryo development [148]. For example, an early increase in estrogen in the uterine environment of pregnant pigs (demonstrated by *in utero* injections 20-30 hours prior to elongation) stimulated a pre-mature increase of uterine secretions, resulting in increased embryo loss that was most likely due to the asynchrony of the embryos with the uterine environment [148]. Therefore, to overcome this issue, future studies with uterine flushes should involve a sequential uterine flush media system (i.e. sequential culture in day 9 through day 12 uterine flushes) to achieve appropriate synchrony between the embryo and the corresponding environment, thus establishing the most effective system to mimic *in vivo* elongation. Mass spectrometry can then be used to determine the specific proteins and growth factors within the pools of uterine flushes to determine differential concentrations of each factor in the flushes from days 9 through 12 of gestation, in order to correlate these factors to embryo development.

In addition to undefined factors in uterine flushes, specific components up-regulated within the uterine milieu during early pregnancy (e.g., TGFBs, FGF7, and OPN) should also be evaluated to explore biological pathways regulated during the pre-implantation stage of embryo development. Adding these components to a basal media or within the alginate gels can be used to identify specific regulatory pathways that drive embryo elongation prior to implantation. Osteopontin (OPN), in particular, is an ECM
glycoprotein secreted by uterine epithelial cells that plays a role in the porcine implantation cascade during pregnancy [71]. OPN production by uterine epithelial cells is induced by estrogens secreted by the embryo during elongation and has been reported to stimulate cell-cell adhesion, increase cell-ECM interactions, and promote cell migration [149, 150]. Previous evidence suggests that the arginine-glycine-aspartic acid (RGD) peptide of OPN binds to integrin ανβ6 on the embryonic trophoderm, inducing cytoplasmic reorganization during embryo elongation by stimulating cell-cell adhesion and promoting cell migration [151]. In previous in vitro studies, OPN has been shown to enhance development of early porcine embryos to the blastocyst stage when added exogenously to culture media [72]. Furthermore, Erikson and colleagues demonstrated that OPN stimulates migration of porcine trophoderm cells via haptotaxis, supporting the hypothesis that OPN mediates cell migration during elongation [151]. Their results also indicated that the RGD sequence of OPN is required to stimulate haptotaxis of the trophoderm cells, as no migration was observed with the OPN that contained a mutated binding sequence [151]. Given the potential involvement of OPN and other uterine factors during early embryonic development in the pig, further investigations are warranted to elicit the exact mechanisms by which these factors regulate pig embryo elongation. The alginate hydrogel system described in Chapter 3 was able to facilitate embryonic growth without any adhesion between the embryo and the matrix; this in vitro system can now serve as a “blank slate” for evaluating these mechanisms by incorporating factors that promote cellular adhesion and migration.

Preliminary studies have been performed to evaluate the effect of OPN and, more specifically, an RGD peptide sequence on embryo elongation within the in vitro alginate
hydrogel system. Day 9 pig embryos were encapsulated in one of the following treatment groups, using the same method as described in Chapter 3: unmodified alginate gels (Alg), alginate gels incorporating 0.1 µg/mL OPN (Alg + OPN), or alginate gels conjugated with GRGDSP peptide (RGD-Alg; prepared as described in Chapter 2). Non-encapsulated embryos were used as a control group (Control). Results from these studies indicated that RGD-Alg promoted the greatest percentage of embryo survival (~68%) compared to the Alg and Alg + OPN treatment groups (Table 5-2). Furthermore, RGD-Alg induced the greatest percentage of embryo morphological changes from all embryos (~43%) and from surviving embryos (~63%). The Alg + OPN treatment promoted intermediate percentages of embryo morphological changes from all embryos (~31%) and from surviving embryos (~62%). Representative images of embryos from each treatment group are pictured in Fig. 5-2. These results suggest the necessary role of OPN, and more specifically its RGD peptide, for proper embryo elongation. Additional studies are warranted for investigating a dose-effect of OPN and various RGD ligand densities on embryo development, along with the evaluation of triggered signaling pathways, to determine optimal levels for embryo elongation and mechanisms by which elongation is promoted by these factors.
Many of the factors within the uterine milieu that are described above are secreted by maternal epithelial cells via embryo-maternal signaling [68]. Because embryo-maternal communication is vital for proper embryo development during the pre-implantation stage of pregnancy, future studies should involve the co-culture of uterine lumen and glandular epithelial cells with the encapsulated embryos to determine if this embryo-maternal cross-talk can be mimicked in our alginate hydrogel system. The co-

### Table 5-2. Summary of embryo survival and morphological changes observed following 96 h culture of day 9 porcine embryos encapsulated in unmodified alginate (Alg), alginate blended with 1 µg/mL osteopontin (OPN), alginate conjugated with GRGDSP, and non-encapsulated control embryos (Control).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Alg</th>
<th>Alg + OPN</th>
<th>RGD-Alg</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of embryos</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Embryo survival (n)</td>
<td>8</td>
<td>8</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Embryo survival (%)</td>
<td>50.00%</td>
<td>50.00%</td>
<td>68.75%</td>
<td>37.50%</td>
</tr>
<tr>
<td>Morph change (n)</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Morph change from all embryos (%)</td>
<td>18.75%</td>
<td>31.25%</td>
<td>43.75%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Morph change from surviving embryos (%)</td>
<td>37.50%</td>
<td>62.50%</td>
<td>63.64%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

**Fig. 5-2.** Representative images of Alg, RGD-Alg, and Alg + OPN embryos that changed morphologically by 96 hours of culture, along with a control embryo that did not change morphologically, other than swelling.
culture model could lead to better understanding of specific embryo-uterine interactions during the elongation process. For co-culture studies, reproductive tracts can be collected from pregnant gilts to isolate uterine epithelial cells. Luminal and glandular epithelial cells can then be isolated and prepared for culture following a previously reported procedure [152]. After establishing a monolayer of these cells in culture, two different approaches are recommended for co-culturing the uterine cells with encapsulated pre-implantation pig embryos. First, the uterine cells can be mixed in with the alginate prior to encapsulation, resulting in a cell-embedded gel surrounding the embryo (Fig. 5-3A). With this approach, the uterine epithelial cells would be surrounding the embryo in all directions, allowing for a more direct exposure to secretory factors. The second recommended approach involves culturing the alginate-encapsulated embryo on a monolayer of uterine epithelial cells (Fig. 5-3B). This approach would better mimic the uterine lumen environment, but would prevent direct contact between the embryo and uterine cells. Therefore, future studies employing both of these approaches would be attractive for establishing and evaluating maternal-embryo crosstalk in our in vitro alginate culture system. Overall, studies involving undefined uterine flushes, defined uterine components, and co-culture studies can be used together to identify and explore biological pathways regulated during the pre-implantation stage of pig embryo development.
Once uterine factors are evaluated and optimized to promote proper embryo elongation in vitro, the alginate culture system can be used to investigate biomechanical effects on elongation by culturing cells in various concentrations of alginate hydrogels, i.e. gels varying in stiffness (as demonstrated by mechanical testing in Chapter 2). Previous studies have found evidence that stiffness is important during embryogenesis in vivo. For example, during gastrulation in *xenopus laevis*, stiffening of the involuting marginal zone occurs to prevent the embryonic tissue from collapsing [153]. However, it remains unclear whether these changes in tissue stiffness occur solely for the purpose of providing mechanical stability or also occur in order to regulate other cellular processes via mechanotransduction [96]. Due to the complicated task of evaluating mechanical forces in vivo, an in vitro embryo model is necessary for understanding complex developmental behaviors that are regulated by mechanical forces. Preliminary studies involving the culture of day 9 pig embryos in 0.375%, 0.7%, and 1.5% alginate gels in base serum media demonstrated that 0.7% alginate promoted the greatest extent of embryonic morphological changes (data not shown); however, because mechanical

![Fig. 5-3. Schematic illustration of the approaches for co-culturing uterine cells with encapsulated pig embryos. A) Uterine epithelial cells can be mixed in with the alginate solution prior to encapsulation, resulting in the cells surrounding the embryo in all directions. B) Encapsulated embryo can be cultured on a monolayer of uterine epithelial cells.](image)
properties may play a role in biochemical signaling, future studies should revisit the investigation of alginate biomechanics on embryo development in conjunction with optimal uterine factors and/or co-cultures. Furthermore, future studies with this model could investigate the effect of a mechanical gradient on embryo elongation, in which outer gel layers are composed of increasing concentrations of alginate. This approach would allow for the embryo to reside in a more permissive environment until it elongates and reaches the stiffer outer layers, which may help to prevent the embryo from extruding from the gel. The cylindrical and tubular alginate gels as well as the alginate wells described in Chapter 2 could also be used to investigate the effect of alginate biomechanics on embryo development; these methods would provide a greater amount of space for the embryo to elongate compared to the bead method, which resulted in embryos growing out of the gel by the end of culture in the studies described in Chapter 3. Utilizing each of the aforementioned methods to investigate the influence of matrix biomechanics on pig embryo elongation could lead to the understanding of mechanical stability that may be required for proper elongation to occur.

In addition to using our alginate system for studying molecular, cellular, and mechanical factors that promote elongation, as described above, future studies should encompass a more extended evaluation of embryo development in order to better correlate the in vitro results to in vivo occurrences. Unlike elongation in other domestic animals, rapid elongation of the pig embryo has been primarily associated with cellular remodeling and differentiation rather than cellular hyperplasia [61]. As a result, potential markers for differentiation of the trophectoderm (i.e., cytokeratin-18 [KRT18]) and mesoderm (i.e., vimentin [VIM]) have been identified in the elongating pig blastocyst
Therefore, future studies should assess cellular differentiation and proliferation in embryos that undergo a morphological change within the alginate hydrogels to determine if the growth is occurring via differentiation. One recommended method is to measure the expression of KRT18 and VIM transcripts in the encapsulated embryos and compare the expression levels to those of in vivo control embryos. Another recommended method involves a measure for the proliferation status of a cell population. Cellular proliferation can be quantified by determining mitotic index, i.e. the ratio of cells undergoing mitosis (condensed DNA) to the total number of visible cells. To calculate this ratio, nuclei in the embryos can be stained with DAPI and imaged with confocal microscopy. Cells in which the chromosomes are visible instead of an intact nucleus can be counted as undergoing mitosis. Preliminary staining of nuclei and actin on a day 9 in vivo-derived embryo was performed as a test, and representative confocal images are shown in Fig. 5-4 (white arrows indicate cells undergoing mitosis). Future investigations of mitotic index of embryos cultured in the alginate hydrogel system will determine if our encapsulated embryos change morphologically via similar mechanisms as in vivo elongating embryos, which will aid in demonstrating alginate's ability to serve as a 3-D matrix for a developmental model of embryo elongation.
5.3. Future directions for the in vitro growth plate model

In Chapter 4, 3-D alginate hydrogels were used as an in vitro extracellular matrix for growth plate chondrocytes to investigate the effect of RGD peptide ligands as well as exogenously added parathyroid hormone (PTH) on chondrocyte behavior in vitro. With this alginate culture system, we were able to determine that alginate conjugated with an RGD peptide suppressed chondrocyte hypertrophy compared to unmodified alginate, demonstrating the effect of this extracellular matrix adhesion peptide on chondrocyte behavior in vitro. Furthermore, the addition of PTH to the culture media of both RGD-alginate and unmodified alginate cultures also repressed hypertrophy, as concluded by the lower expression of collagen X and Ihh transcripts in cells cultured with PTH compared to those cultured without PTH; these results demonstrated that the effect of PTH on chondrocyte hypertrophy in the growth plate can be mimicked to an extent within our in vitro model.
in vitro alginate culture system. This study was the first step in using the alginate culture model to evaluate factors necessary for inducing native growth plate architecture and function for potential tissue engineering applications. The recommended next step is to evaluate PTH effect in a dose-dependent manner by varying PTH concentration. Future studies are then warranted for the investigation of signaling feedback loops, signaling gradients, mechanical gradients, and co-culture studies on in vitro chondrocyte behavior, utilizing alginate as the 3-D culture matrix. Recommended studies are described in detail below.

Together, the interactions of parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh), a member of the hedgehog family of secreted ligands, form a feedback loop that regulates the length of the proliferative columns in growth plate cartilage and defines individual zones of chondrocyte maturation [83-85]. Studying factors independently and observing their isolated effects can lead to greater insight into the performance of a system as a whole. Therefore, in addition to observing PTH effects on chondrocyte behavior in vitro as described in Chapter 4, it is recommended that Indian hedgehog (Ihh) also be investigated independently for its effects on the in vitro behavior of growth plate chondrocytes. Sonic hedgehog (Shh) binds to the same receptors as Ihh, leading to activation of the same signaling pathways; hence, Shh can be used to represent the effects of Ihh. A preliminary study was conducted to observe the effects of 0.2 ng/mL and 0.5 ng/mL Shh on neonatal mouse growth plate chondrocytes encapsulated within GRGDSP-conjugated alginate gels. RT-PCR was performed to determine gene expression of transcripts involved in growth plate development and function. Expression levels of collagen X (Fig. 5-5A) and PTHrP (Fig. 5-5B) are reported here as an example
of transcript expression results obtained from this study. A dose-dependent effect of Shh was difficult to determine from the gene expression results of this preliminary study, suggesting that the concentrations of Shh used (0.2 ng/mL and 0.5 ng/mL) were too low for our model. Due to hindered diffusion to the cells through the alginate gel, recommended concentrations for future Shh experiments should be an order of magnitude higher to observe clear effects.

Once PTH and Shh are evaluated independently in the alginate model, their cooperative effects should then be evaluated by delivering both PTH and Shh to alginate-encapsulated growth plate chondrocytes during the same experiment. Dose-dependent effects should be tested again for these cooperative studies as the optimal concentrations of each factor may change when added to culture media together.

Another variable that should be tested in future studies is the incorporation of PTH and/or Shh into the alginate solution prior to crosslinking, in contrast to adding the factors to the culture media after beads are crosslinked, as was done in the study described above and in Chapter 4. This approach would be advantageous for maintaining
an efficient localized concentration of signaling factors around the cellular environment throughout the duration of culture. Preliminary release studies demonstrated alginate’s ability to retain PTH within the beads for a duration of 5 days, with very little release during this time (data not shown). If longer release studies show that a more sustained release is needed for longer culture times, recommended studies should investigate the incorporation of heparin into the alginate beads; the heparin should bind to the signaling factors and aid in preventing release. Another option to promote sustained release (and therefore inhibit burst release) from alginate hydrogels would be to chemically modify alginate with sulfate groups. Sulfation of alginate mimics heparin’s affinity for certain growth factors, including PTH and Ihh/Shh, and has previously been used to provide protection and sustained release of heparin-binding growth factors [42]. However, published methods for sulfation of alginate involve the use of harsh solvents, specifically the reaction of alginate with chlorosulfuric acid in formamide [39]. Future studies involving the investigation of signaling factors within the alginate model should explore the possibility of a safer, non-toxic method to achieve alginate sulfation. In vivo, Ihh diffusion from hypertrophic cells to perichondral cells is regulated by Ihh binding to heparan sulfate in the extracellular matrix [86]. Therefore, the incorporation of heparin or conjugation of sulfate groups would allow the alginate system to better mimic the in vivo extracellular matrix of the growth plate.

In addition to investigating the effects of soluble factors added to our chondrocyte culture system, RGD ligand density should be varied and studied for its effect on chondrocyte behavior. The methods described in Chapter 4 involved the conjugation of GRGDSP peptide to alginate at only one concentration, and this particular concentration
(estimated to be approximately 4 µmol/g alginate [14]) significantly decreased the expression of collagen X in encapsulated chondrocytes compared to unmodified alginate, suggesting a decrease in the amount of hypertrophic cells. Varying the concentration of GRGDSP on the alginate backbone will vary the amount of cellular adhesion sites, and may therefore further affect proliferation and/or hypertrophy of encapsulated chondrocytes [27]. Peptide conjugation, however, occurs on the carboxylic acid groups of the alginate polymer backbone, which are the same groups that participate in ionic crosslinking with calcium for hydrogel formation [39]. As a result, increasing ligand density may decrease the stiffness of resulting gels. Young’s moduli of gels with various RGD peptide densities should therefore be tested to determine if mechanics differ significantly with increased ligand density.

Finally, mechanical and chemical gradients should be established with these alginate hydrogels and investigated for their effects on in vitro growth plate chondrocyte behavior. The cylindrical gel method (Fig. 5-6A and 5-6B) or layered bead method (Fig. 5-6C and 5-6D), both described in Chapter 2, can be used to establish these gradients. With the cylindrical gel method, chondrocytes can be encapsulated within the middle area of the cylinder to represent the proliferative zone. Ihh can then be incorporated at a high concentration at one end of the cylinder, representing the hypertrophic zone of Ihh-expressing chondrocytes, with decreasing concentrations towards the other end of the cylinder. At the opposite end, PTH can be incorporated at high concentrations, representing the resting zone, with decreasing concentrations towards the Ihh-concentrated end. This method is depicted in Fig. 5-6A. A co-culture study involving transfected HEK293T cells that express Ihh or PTH could also be used to establish
signaling gradients in place of the factors themselves (Fig. 5-6B). The layered bead method uses a similar approach in that chondrocytes can be encapsulated in a middle layer, with PTH and Ihh or PTH- and Ihh-expressing cells incorporated into the inner and outer gel layers (Fig. 5-6C and 5-6D). Preliminary studies involving the encapsulation of rat chondrosarcoma (RCS) cells with Ihh-expressing HEK293T cells within alginate beads demonstrated that RCS cells were successfully encapsulated within an outer gel layer surrounding an inner bead of HEK293T cells (Fig. 5-7).

**Fig. 5-6.** Schematic illustration of the encapsulation of chondrocytes and formation of chemical gradients within alginate hydrogels via incorporated signaling factors (A,C) or co-culture with factor-expressing cells (B,D). Gradients can be established in a cylindrical gel formation (A,B) or with a layered bead method (C,D).
For both the cylindrical gel method and layered bead method, different concentrations of alginate can be used in each layer to establish a gradient of mechanical properties to determine biomechanical effects on growth plate chondrocyte behavior. A recent approach to the manipulation of transcription factors that have been discovered to play important roles in cartilage and bone formation is to consider the mechanical influences on their regulation [84]. For example, studies have revealed that biomechanical stimuli influence the regulation of NF-κβ transcription factors, which generate intracellular signals that induce or suppress pro-inflammatory and reparative genes in chondrocytes [154, 155]. Furthermore, a study involving chitosan substrates of varying stiffness properties revealed that increased stiffness promoted articular chondrocyte growth and proliferation [156], linking the biomechanical environment to cartilage development and function. Therefore, future studies involving mechanical gradients within our *in vitro* growth plate model could reveal biomechanical mechanisms.

**Fig. 5.7.** 1.5% RGD-alginate bead with two gel layers, consisting of Ihh-expressing HEK293T cells (HEK) encapsulated within the inner bead and rat chondrosarcoma cells (RCS) encapsulated within the outer layer. Cells were live/dead stained prior to imaging with a fluorescence microscope.
that underlie spatial organization of chondrocytes in the growth plate. Taken together, the optimal combination of appropriate biomechanics, ECM factors, and signaling gradients are hypothesized to generate native cartilage architecture \textit{in vitro}, in particular a distinct zone of proliferating columnar chondrocytes as seen \textit{in vivo}.

5.4. Conclusions

This thesis describes the establishment of \textit{in vitro} culture systems for pre-implantation pig embryos and growth plate chondrocytes via a tissue engineering approach, utilizing alginate hydrogels as a 3-D scaffold. Alginate, a linear polysaccharide derived from brown algae, demonstrated tunable physical and chemical properties, making it an ideal matrix for \textit{in vitro} culture models. In regards to pig embryo culture, embryos encapsulated within alginate hydrogels exhibited greater survival and morphological change than non-encapsulated control embryos, along with increased expression of steroidogenic transcripts and estrogen production, consistent with \textit{in vivo}-elongation. However, this system consisted of only a base serum media; therefore, future studies should use this alginate hydrogel system as a tool for evaluating specific uterine and/or biomechanical factors that play a role in embryo elongation, with further endpoint evaluations to characterize elongation. These factors can then be targeted to improve pregnancy outcomes, thereby increasing sow productivity and the profitability of swine production. In addition, alginate hydrogels were used for the encapsulation of growth plate chondrocytes \textit{in vitro} to study the effects of soluble PTH, a signaling factor that regulates growth plate structure and function, along with the effects of an RGD peptide conjugated to alginate. This study demonstrated that both PTH and RGD peptide ligands
play a role in the regulation of chondrocyte hypertrophy in our in vitro alginate system with similar effects as seen in vivo, as indicated by the decrease in collagen X and Ihh transcript expression. However, this study was only the first step in the evaluation of factors that effect growth plate chondrocyte behavior to determine necessary factors for inducing native growth plate architecture in vitro. Future studies using this alginate system should involve the investigation of dose-dependent effects of signaling factors and RGD peptide ligands, as well as mechanical and/or chemical gradients on chondrocyte structure and function. Knowledge of microenvironmental parameters that induce cartilage architecture can then be used in tissue engineering strategies to regenerate native cartilage to address critical health issues associated with growth plate injury and disease. Overall, information gained from utilizing these in vitro models as research tools can be used to advance the field of developmental biology and enhance tissue engineering therapies for the treatment of degenerative diseases.
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