2017

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In vitro porcine blastocyst development in three-dimensional alginate hydrogels

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Appropriate embryonic and fetal development significantly impact pregnancy success and, therefore, the efficiency of swine production. The pre-implantation period of porcine pregnancy is characterized by several developmental hallmarks, which are initiated by the dramatic morphological change that occurs as pig blastocysts elongate from spherical to filamentous blastocysts. Deficiencies in blastocyst elongation contribute to approximately 20% of embryonic loss, and have a direct influence on within-litter birth weight variation. Although factors identified within the uterine environment may play a role in blastocyst elongation, little is known about the exact mechanisms by which porcine (or other species') blastocysts initiate and progress through the elongation process. This is partly due to the difficulty of replicating elongation in vitro, which would allow for its study in a controlled environment and in real-time. We developed a three dimensional (3-D) culture system using alginate hydrogel matrices that can encapsulate pig blastocysts, maintain viability and blastocyst architecture, and facilitate reproducible morphological changes with corresponding expression of steroidogenic enzyme transcripts and estrogen production, consistent with the initiation of elongation in vivo. This review highlights key aspects of the pre-implantation period of porcine pregnancy and the difficulty of studying blastocyst elongation in vivo or by using in vitro systems. This review also provides insights on the utility of 3-D hydrogels to study blastocyst elongation continuously and in real-time as a complementary and confirmatory approach to in vivo analysis.

KEYWORDS
alginate hydrogels, blastocyst elongation, in vitro culture models, porcine, tissue engineering

Abbreviations: 2/3D, two/three dimensional; CONT, non-encapsulated control blastocyst; CYP11A1, CytochromeP450 side chain cleavage; CPY19A1, aromatase; ECM, extracellular matrix; ENC±, blastocysts encapsulated in alginate hydrogels [with or without morphological change]; FGF7, Fibroblast growth factor 7; G, α-L-guluronic acid; IL1B, Interleukin1β; M, β-D-mannuronic acid; RGD, Arg-Gly-Asp; SPP1, Secreted phosphoprotein 1; STAR, Steroidogenic acute regulatory protein; TGFβ, Transforming growth factor-β.

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The pre-implantation period of porcine pregnancy is characterized by several developmental hallmarks that are critical for the maintenance of pregnancy, embryo spacing, placental development, fetal growth, uterine capacity, litter size, and ultimately postnatal piglet survival. Between Days 9 and 12 of gestation, the pig blastocyst undergoes dramatic transformation from a spherical structure (~1–2 mm) through transitional ovoid (~4–5 mm) and tubular (~10 mm) morphologies, to end as a long, thin filament (~<10 mm); this process is referred to as blastocyst elongation (Bazer, Geisert, Thatcher, & Roberts, 1982; Geisert, Renegar, Thatcher, Roberts, & Bazer, 1982; Miles, Freking, Blomberg, Vallet, & Zuelke, 2008; Pope & First, 1985). Once initiated, blastocyst elongation is very rapid, with remodeling of the tropheoblast and changes in conceptus length occurring at a rate of 35–40 mm per hour on Day 11–12 (Bazer, Thatcher, Martinat-Botte, & Terqui, 1988); the morphological transition between a tubular and filamentous blastocyst typically occurs within 2–3 hr (Geisert et al., 1982).

Unlike elongation in other domestic animals (i.e., ruminants), rapid elongation of the pig blastocyst is primarily associated with cellular remodeling and differentiation rather than cellular hyperplasia (Geisert & Yelich, 1997). This mechanism is consistent with the reported potential markers of trophectoderm (i.e., Cytokeratin-18 and Plasminogen activator urokinase) and mesoderm (i.e., Vimentin) differentiation identified in the elongating pig blastocyst (Blomberg, Miles, & Zuelke, 2006; Flechon, Degrouard, & Flechon, 2004; Ka, Jaeger, Johnson, Spencer, & Bazer, 2001).

The elongating pig blastocyst produces and secretes estradiol-17β, which serves as the key molecule for maternal recognition of pregnancy that prevents luteolysis (Bazer et al., 1982) and modulates the production and secretion of proteins and growth factors within the uterus (Geisert et al., 2006). Several transcripts involved in steroidogenesis, such as STAR (Steroidogenic acute regulatory protein), CYP11A1 (Cytochrome P450 side chain cleavage), and CYP19A1 (Aromatase), increase in a pattern that is similar to estrogen production during blastocyst elongation (Blomberg & Zuelke, 2005; Yelich, Pomp, & Geisert, 1997). Proper interactions between the blastocyst and receptive uterine endometrium are essential for supporting embryonic development and subsequent implantation (Geisert et al., 2006). These interactions are initiated by the immune responsive cytokine Interleukin-1β (IL1B), which is produced by the blastocyst and uterine endometrium—specifically the luminal and glandular epithelia (Geisert, Lucy, Whyte, Ross, & Mathew, 2014; Ross, Malayer, Ritchey, & Geisert, 2003). IL1B, via its receptor, stimulates signaling that up-regulates uterine-specific factors (e.g., Leukemia inhibitory factor and Prostaglandin-endoperoxide synthase 2) via Nuclear factor-kappa beta activation (Geisert, Fazleabas, Lucy, & Mathew, 2012).

Expression of these transcripts may be used as markers to assess the developmental competence of pre-implantation porcine blastocysts.

An elongated blastocyst begins its initial apposition with the uterine endometrium around Day 13 of gestation (Keys & King, 1990). Completion of blastocyst implantation occurs around Day 18 of gestation, and is characterized by superficial attachment of the trophectoderm and the uterine luminal epithelium (Keys & King, 1989). Pig blastocyst elongation and subsequent implantation are dependent on the proper interaction between embryonic and maternal tissue (Blomberg, Hashizume, & Viebahn, 2008), which trigger uterine endometrial transitions that result in the production and secretion of various proteins and growth factors as well as complex interactions between the glycocalyx of the endometrium and blastocyst that support implantation (Burghardt, Bowen, Newton, & Bazer, 1997; Geisert, Brookbank, Roberts, & Bazer, 1982). As a result, many uterine-specific factors are up-regulated within the uterine milieu as the blastocyst initiates elongation and subsequently transitions to its superficial implantation on the uterine endometrium (Geisert et al., 2012). For instance, Prostaglandin E, Fibroblast growth factor 7 (FGF7), Transforming growth factor-β (TGFβ), Secreted phosphoprotein 1 (SPP1), Fibronectin, and Laminin were suggested to play a role in these conceptus-maternal interactions (Bazer, Kim et al., 2012; Geisert et al., 2012; Jaeger et al., 2003).

During maternal recognition of pregnancy, conceptus estradiol-17β redirects the secretion of Prostaglandin F2α from an endocrine secretion into the uterine vasculature to an exocrine secretion in the uterine lumen, thereby preventing luteolytic effects of Prostaglandin F2α (Bazer, Song et al., 2012). This transition results in extensive accumulation of Prostaglandin F2α and Prostaglandin E in the uterine lumen during early pregnancy (Geisert et al., 2012), which is thought to play an important role in embryo-maternal cross-talk in the pig (Blitek, Morawska, Kiewisz, & Zlecik, 2011).

FGF7 abundance also peaks within the uterine milieu around Day 12 of the estrous cycle and pregnancy (Ka et al., 2007). FGF7 and its receptor are up-regulated in the endometrial epithelium via paracrine signaling between the conceptus (i.e., estrogen) and the uterine stroma (i.e., progesterone) (Ka et al., 2007), and FGF7 can stimulate proliferation and differentiation of a trophectoderm cell line in vitro (Ka et al., 2001). Thus, uterine FGF7 is important for embryonic development during elongation.

During the peri-implantation period of pig pregnancy (Day 10–14), expression of TGFβ1, -B2, and -B3 and their respective receptors increases within the uterine luminal epithelium and on the conceptus (Gupta, Bazer, & Jaeger, 1996; Gupta, Dekaney, Bazer, Madrigal, & Jaeger, 1998). This synchronized up-regulation suggests that TGFβs are involved in embryo-maternal cross-talk (Gupta et al., 1998). TGFβs are secreted from cells as a homodimer in a latent complex that is associated with Latency-associated peptides (LAP), which interact with TGFβ1 and B3 containing the Arg-Gly-Asp (RGD) amino acid sequence that binds specific integrins (Massuto et al., 2010). Interestingly, exogenous intrauterine infusion of recombinant LAP-RGD, beginning at Day 9 of pregnancy, decreases conceptus survival and reduces elongation, possibly due to
competition with endogenous LAP-TGFβ (Massuto et al., 2010). These results illustrate that conceptus elongation is likely regulated by interactions of TGFβs and integrins (Massuto et al., 2010). SPP1, an RGD peptide-containing extracellular matrix (ECM) glycoprotein secreted by uterine epithelial cells, was also shown to play a role in the porcine implantation cascade during pregnancy (Garlow et al., 2002). Previous evidence suggested that the SPP1 RGD peptide binds to Integrins α5 and β3 on the embryonic trophoectoderm, inducing cytoplasmic reorganization during conceptus elongation by stimulating cell–cell adhesion and promoting cell migration (Garlow et al., 2002). Furthermore, SPP1 can enhance development of early porcine embryos to the blastocyst stage in vitro when added exogenously to culture medium (Hao et al., 2008), suggesting the importance of SPP1 on early embryo development.

The surface of the oviduct and uterus contains several other ECM components that may influence embryonic development, morphogenesis, and function by stimulating cell–cell interactions (Adams & Watt, 1993; Swain & Smith, 2011). Two ECM glycoproteins involved in cellular adhesion, Fibronectin and Laminin, were previously shown to increase the hatching rates of cultured human embryos when added to the medium (Turpeenniemi-Hujanen, Feinberg, Kauppila, & Puistola, 1995). A study involving the culture of porcine blastomeres on Fibronectin-coated culture dishes demonstrated that Fibronectin enhanced development to the blastocyst stage compared to control blastomeres (Saito & Niemann, 1991), suggesting its role in early embryonic development. Further investigations of these components are warranted to reveal how each contributes to pig blastocyst elongation.

A significant portion of blastocysts within individual pregnancies fail to undergo proper initiation of elongation, leading directly to embryonic loss as well as delayed elongation, resulting in asynchronous intra-litter embryo development. Deficiencies in blastocyst elongation contribute to approximately 20% of embryonic loss (Pope, 1994), and have a direct impact on intra-litter birth weight variability (Vallet, Miles, & Freking, 2009). Clear evidence indicates that prenatal development has profound effects on birth weight and subsequent postnatal survival and growth performance (Foxcroft et al., 2009), illustrating the importance of adequate embryonic, placental, and fetal development in regards to the pre-weaning survival of piglets (Vallet et al., 2009). Therefore, developing a comprehensive understanding of early embryonic mechanisms—in particular, mechanisms involved in elongation—can lead to interventions that decrease embryonic mortality, reduce within-litter birth weight variability, and improve pre-weaning survival, resulting in an overall increase in the number of healthy piglets weaned.

2 | CHALLENGES TO STUDYING BLASTOCYST ELONGATION

Despite advances in the identification of signaling pathways within the blastocyst and uterine factors present during the pre-implantation period of pregnancy in the pig, little is known about the exact mechanisms by which porcine (or other species') blastocysts initiate and progress through elongation, due to technical and biological challenges that prevent investigations into mechanisms of blastocyst development. Previous studies investigating blastocyst development in vivo utilized blastocysts flushed at various time points during the pre-implantation period, when the developmental competence of individual blastocysts is not entirely known and is interrupted by their removal from the uterine environment (Miles et al., 2008). The dramatic blastocyst diversity observed, particularly during the transitional period (Day 10 and 11 of gestation), can also alter the physiology of developmentally delayed blastocysts (i.e., spherical or ovoid blastocysts) within heterogeneous blastocyst populations (i.e., spherical, ovoid, tubular, and filamentous blastocysts) (Blomberg, Schreier, & Li, 2010; Degrelle, Blomberg, Garrett, Li, & Talbot, 2009), further complicating the identification of components that have specific roles for later developmental competence of individual blastocysts. Thus, effective in vitro culture systems that are capable of replicating the elongation process would be useful to study molecular factors that regulate the initiation of blastocyst elongation as well as allow for real-time monitoring and studying of elongation in a controlled environment.

Traditional studies within the field of developmental biology have cultured blastocysts in two-dimensional (2-D) systems. The developmental potential of the blastocysts in such systems is limited to pre-elongation stages; if longer culture periods are attempted, the blastocysts either remain in spherical form or attach to the bottom of the dish, which disrupts embryo structure and cell-to-cell communication, halting normal embryonic development (Brandao et al., 2004). Such a blockade that has also been reported in 2-D cultures of other tissues and organ systems (e.g., follicle cultures) (Kreeger, Deck, Woodruff, & Shea, 2006; Smits & Cortvriendt, 2002). Cells and tissues often behave very differently when cultured in 2-D, exhibiting major differences in gene expression compared to their in vivo counterparts in their natural, three-dimensional (3-D) environment (Smalley, Lioni, & Herlyn, 2006).

Previous attempts to culture blastocysts at later stages by incorporating a type of supporting structure (i.e., mimicking 3-D cultures) were largely unsuccessful. In cattle, limited elongation of blastocysts in vitro was demonstrated using an agar gel tube system (Brandao et al., 2004; Vajta, Alexopoulos, & Callesen, 2004); however, this system did not provide complete encapsulation or support of the blastocysts. Rather, the tubes served as reservoirs for growth, and the blastocysts filled these tubes, most likely via physical induction—that is, the shape of the tube may have forced growth that appeared to be elongation such that the high percentages of agar used may have restricted optimal embryonic development. The high concentrations of media supplements used in these bovine cultures may have stimulated rapid cell growth, further contributing to the appearance of embryonic elongation. Nevertheless, previous attempts to replicate these results using pig blastocysts were unsuccessful (Vejlsted, Du, Vajta, & Maddox-Hyttel, 2006). Our group has instead turned to tissue-engineering principles to develop 3-D culture systems that maintain blastocyst or tissue architecture and allow for direct physical interaction with the surrounding environment, which better mimics in vivo development. These in vitro tissue-engineering approaches
provide an exciting, more physiological alternative to traditional 2-D systems of development.

3 | TISSUE ENGINEERING APPROACHES FOR IN VITRO CULTURE MODELS

Tissue engineering emerged at the interface of biology and engineering with the initial goal of engineering organs for patients who otherwise were beholden to scarce donors for whole-organ transplantation (Langer & Vacanti, 1993). Since Langer and Vacanti (1993) first defined tissue engineering, it has come to combine three fundamental aspects of biology: cells or tissues, scaffolding, and bioactive signals (Ikada, 2006). Cells and tissues can be collected from many sources, including animals or a patient, and the cells may be fully differentiated or derived from stem cells (Langer & Vacanti, 1993); for the purposes of this review, we will focus on intact blastocysts as the cell source. The scaffolding can be used to mimic the native extracellular matrix or environment surrounding the cells (Ikada, 2006). Finally, the bioactive signals provide the appropriate signals to the cells or tissue within their respective scaffolding (Ikada, 2006).

Within the field of tissue engineering, researchers combine cells, scaffolds, and bioactive signals in an attempt to engineer a variety of tissues for therapeutic applications (e.g., bone, cartilage, skin, nerves, trachea, bladder, and cardiovascular tissues, to name only a small few; see review by Shafiee & Atala [2017]).

While originally developed to produce tissues for transplantation, the principles of tissue engineering (i.e., scaffolds, cells, and provision of bioactive signals) have now been applied to in vitro culture models to produce biologically suitable substitutes for organ systems or tissues, which are then utilized for pharmaceutical, diagnostic, or research purposes (Nam, Smith, Lone, Kwon, & Kim, 2015)—essentially bridging the gap between traditional 2-D cell culture and animal studies (Figure 1). Most tissue-engineered models are developed and constructed for a specific purpose using cells of many different types, scaffolds made of varying materials, and a wide range of bioactive signals. Applications for in vitro culture models include the exploration of various aspects of disease dynamics, drug discovery, diagnostics, and developmental biology.

Recently developed 3-D culture models have already proven to be more physiologically relevant than either cell culture or animal models, and have demonstrated improved responses to controlled stimuli (Antoni, Burckel, Josset, & Noel, 2015). For example, 3-D in vitro culture models are extensively used to study cancer and metastasis, as well as other diseases, and have probed for the response of tissues to therapeutic and pharmacological agents (Burgues et al., 2007; Jaganathan et al., 2014; Song, Park, & Gerecht, 2014; Wang et al., 2014). Models of cancers in the bladder, breast, kidney, lung, ovary, pancreas, and prostate have utilized 3-D in vitro cultures to better understand the development, metastasis, vascularization, and response to therapeutic drugs (Dash et al., 2009; Gilmour, Woolley, Poole-Warren, Thomson, & Green, 2016). Pampaloni, Reynaud, and Stelzer (2007), for instance, used 3-D cultures with reconstituted basement membrane, or Matrigel, plus bioactive signals to show the significant role of the extracellular context in tumorigenesis (Pampaloni et al., 2007). Osteoarthritis has also been studied in vitro to understand the degradation and calcification of articular chondrocytes by treating with factors known to be involved in the disruption of articular cartilage (Cortial et al., 2006). Tissue-engineered 3-D models of gastrointestinal inflammatory disease, rheumatoid arthritis, heart disease, and of many more organ-system disorders helped identify biomarkers, such as gene expression and protein secretion reminiscent of the tissue disease, that their monolayer counterparts were unable to achieve (Cortial et al., 2006; Eschenhagen et al., 1997; Hartman et al., 2014; Smollan et al., 2001).

3-D culture models have utilized a variety of scaffold materials that were carefully chosen to mimic the appropriate biomechanical, biophysical, and molecular interactions that occur in vivo. For example, we chose alginate as the hydrogel scaffold system for studying pig blastocyst elongation (Sargus-Patino et al., 2014). Alginate is used in many tissue-engineering applications—including injectable cell delivery vehicles (Atala, Kim, Paige, Vacanti, & Retik, 1994), wound dressings (Rezvanian, Amin, & Ng, 2016), and matrices for a variety of tissues (e.g., cartilage, bone, pancreatic islets [Lee & Mooney, 2012])—because of its biocompatibility, tunable mechanical properties, and easy modification (Lee & Mooney, 2001), which will be discussed further in the next section.

4 | ALGINATE

4.1 | Structure and properties

Hydrogels—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 (Tibbitt & Anseth, 2009) as well as their high porosity for efficient diffusion of proteins and nutrients (Smidsrod & Skjak-Braek, 1990). Alginate hydrogels, in particular, are widely applied in tissue engineering due to their desirable properties as a biomaterial (Lee & Mooney, 2001). Alginate is a biocompatible, naturally derived, linear polysaccharide produced as an ECM component in brown algae, such as Macrocystis pyrifera and Ascophyllum nodosum, and some bacteria, such as Pseudomonas aeruginosa (Gacesa, 1998; Ueno & Oda, 2014). The polysaccharide is composed of repeating units of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, and forms hydrogels by ionic cross-linking of the G residues in the presence of a divalent cation like calcium (Figure 2) (Andersen, Strand, Formo, Alsberg, & Christensen, 2012; Lee & Mooney, 2001).

An external gelation process 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, which permits the diffusion of proteins and hormones that are essential for cell and tissue growth and development (Gombotz & Wee, 1998; Smidsrod & Skjak-Braek, 1990). Increasing the concentration of alginate in gels decreases the rate of diffusion of proteins through the pores (Gombotz & Wee, 1998). Since only the G residues of alginate participate in intermolecular cross-linking to form the hydrogels
(Figure 2), the M-to-G ratio has a significant impact on the mechanics of cross-linking (Lee & Mooney, 2001). Thus, gels made of alginate with a high G content have the most open-pore structure and allow the greatest rate of protein diffusion (Smidsrod & Skjak-Braek, 1990). In addition to its gentle encapsulation process and diffusion capabilities, alginate exhibits negligible non-specific protein absorption or cell adhesion, leading to minimal interaction between encapsulated cells/tissue and the surrounding alginate hydrogels (Rowley, Madlambayan, & Mooney, 1999). This feature allows alginate to provide a “blank slate,” acting only as a mechanical support system to study the effects of 3-D culture without any other interference; however, varying the concentration of alginate alters the mechanical properties of the matrix, and can be used to evaluate the effect of matrix mechanics on cell and tissue growth (West, Xu, Woodruff, & Shea, 2007).

Degradation of alginate hydrogels does not readily occur under physiological conditions (Kong, Kaigler, Kim, & Mooney, 2004), although unmodified calcium-cross-linked alginate hydrogels slowly and uncontrollably disassemble due to ion exchange (Shoichet, Li, White, & Winn, 1996). Increased rates of degradation requires the presence of chelating agents that accelerate the removal of calcium ions from the matrix, such as ethylenediaminetetraacetic acid (EDTA), lactate, citrate, or phosphate, (Gombotz & Wee, 1998). No known alginate-degrading enzymes exist in mammals, but alginate lyases have been isolated from marine algae and a wide variety of microorganisms (Andersen et al., 2012). Alginate lyases degrade the matrix via β-elimination, in which the glycosidic 1–4 O-linkage between monomers is cleaved (Wong, Preston, & Schiller, 2000). These properties make alginate hydrogels an appropriate material for...
maintaining the structural integrity during extended culture of encapsulated mammalian cells and tissues. Furthermore, alginate lyase or chelating agents can readily be used to retrieve cells or tissue from alginate beads for further assays.

4.2 Modulation of alginate mechanical properties

The ability to control the mechanical properties of biomaterials used for in vitro culture is essential because tissues have a broad range of stiffness. The Young’s moduli (i.e., the stiffness) of alginate hydrogels can be modified by changing the concentration of alginate in solution, the chemical makeup of the alginate molecule, the solvent used to dissolve the alginate, the gelation temperature, and the divalent cation used for cross-linking (Drury, Dennis, & Mooney, 2004; Morch, Donati, Strand, & Skjak-Braek, 2006; West et al., 2007). The compressive modulus for alginate hydrogels can range from less than 1-kPa to over 1,000-kPa, wherein lower moduli can be used to mimic ECM for tissues like smooth muscle while higher moduli can be used in applications for bone tissue engineering. The development of alginate hydrogels for the culture of porcine blastocysts (described later in this review) employed alginate hydrogels with a Young’s modulus ranging from 5- to 20-kPa, which were prepared by varying density (w/v) of medium-viscosity (>200 mPa) alginate (Figure 3). Although the mechanical properties of alginate can be varied with relative ease, chemical modification of the alginate molecule and hydrogels are often required to extend the stability of calcium cross-linked alginate hydrogels, provide controlled release of encapsulated factors, provide greater range of mechanical properties, and promote desirable cellular functions.

4.3 Chemical modifications of alginate

Alginate’s free hydroxyl and carboxyl groups (Figure 2) provide numerous sites that are ideal for chemical modifications. Properties such as degradability, controlled release of factors, and biological characteristics can be altered by modifying available hydroxyl and/or carboxyl groups, or by interfering with carbon-carbon bonds (Yang, Wie, & He, 2011). The addition of oxidation points to the alginate molecule, by using γ-radiation and/or periodate oxidation, lowers the molecular weight of the polysaccharide, thus allowing for strict regulation of alginate hydrogel degradation rate without changing its initial physical properties (Boontheekul, Kong, & Mooney, 2005; Kong et al., 2004). Another useful chemical modification of alginate is sulfation, in which alginate is reacted with chlorosulfuric acid in formamide (Yang et al., 2011). This process gives alginate a structural similarity to heparin, especially mimicking heparin’s high affinity for certain growth factors—a property that was exploited 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 (Freeman, Kedem, & Cohen, 2008).

Mammalian cells do not normally adhere to alginate polymers, which make alginate relatively inert; however, interactions with extracellular ligands are necessary for cell adhesion, which is a basic requirement of cellular survival, migration, proliferation, and differentiation (Price, 1997; Rowley et al., 1999). Cellular surface receptors, such as integrins and syndecans, bind to cell adhesion molecules such as Collagens, Fibronectin, Laminin, and SPP1, resulting in physiological changes that support cell survival, migration, proliferation, and differentiation (Burghardt et al., 2009; Takahashi et al., 2007). These ECM molecules are easily blended within the alginate solution prior to cross-linking, thus incorporating them within the hydrogels and providing physiological changes to cells consistent with in vivo phenotypes (Kreeger et al., 2006). Amidation is another functional modification of alginate that promotes cell-alginate interactions (Yang et al., 2011). This modification utilizes carbodiimide chemistry to form amide linkages between amine-containing molecules and the carboxylic acid functional groups of the alginate polymer backbone (Rowley et al., 1999). This chemistry is useful for covalently linking specific peptides to the alginate backbone to promote cell adhesion interactions (Rowley et al., 1999). The RGD peptide sequence is found in many ECM proteins, such as Fibronectin, Laminin, and SPP1, that is effective and commonly used to promote cell adhesion (Hersel, Dahmen, & Kessler, 2003). When covalently linked to alginate, RGD peptide sites serve as ligands for cell-surface integrins, thus promoting cell adhesion and migration; ligand density can be varied to produce desired degrees of adhesion (Rowley et al., 1999). These unique physical and chemical properties of alginate, along with its innate biocompatibility, underlie its extensive use in industrial food, biomedical, and tissue-engineering applications.

4.4 Applications for alginate hydrogels

Alginate is commonly used as a thickening agent to increase the quality of foods, such as ice cream and dressings, due to its biocompatibility and low toxicity (Andersen et al., 2012). It is also widely used in the pharmaceutical industry as a drug- and protein-delivery agent, whereby alginate hydrogels can release macromolecules in a controlled manner and can be orally administered or injected using a non-invasive approach.

![Figure 3](image-url)  
**FIGURE 3** Young’s modulus as a function of alginate concentration for unmodified (black circles) and RGD-conjugated (red triangle) alginate hydrogels. The plotted lines display the linear, best-fit equations for both alginate conditions. (Unmodified alginate, \( R^2 = 0.9375 \); RGD-alginate, \( R^2 = 0.9981 \) )
(Lee & Mooney, 2001). The extensive application of alginate in tissue engineering is due to its biocompatibility, relatively low cost, and gentle gelation process (Andersen et al., 2012; Lee & Mooney, 2001); alginate hydrogels were previously used for the encapsulation and culture of a variety of cell types for tissue engineering, including articular chondrocytes (Alsberg, Anderson, Albeiruti, Rowley, & Mooney, 2002), skeletal myoblast (Rowley et al., 1999), neural stem cells (Purcell, Singh, & Kipke, 2009), and mouse embryonic stem cells (Candiello, Singh, Task, Kumta, & Banerjee, 2013).

In addition to cellular encapsulation for transplantation, alginate hydrogels have been utilized as a 3-D matrix for the in vitro culture of a variety of organs and embryos (Elsheikh, Takahashi, Hishinuma, Nour, & Kanagawa, 1997; Sargus-Patino et al., 2014; Subramanian et al., 2010; Xu, Kreeger, Shea, & Woodruff, 2006; Zhao et al., 2015). For example, alginate hydrogels were previously used to support the in vitro development of ovarian follicles in mice (Kreeger et al., 2006; West et al., 2007) and non-human primates (Xu et al., 2009); cryopreserved-thawed human cortical follicles (Kedem et al., 2011); and sections of ovaries and oviducts to model specific changes that might lead to ovarian cancer (Xu, West, Shea, & Woodruff, 2006). Alginate hydrogels were also successfully applied for the in vitro development of mouse pronuclear-stage embryos (Elsheikh et al., 1997) and bovine embryos up to the blastocyst stage (Yaniz, Santolaria, & Lopez-Gatius, 2002). Therefore, alginate hydrogels have a proven, significant potential as an artificial 3-D matrix for the in vitro culture of pre-implantation blastocysts. As such, we utilized tissue-engineering principles to develop a 3-D culture system using alginate hydrogels to encapsulate and support in vitro porcine blastocyst elongation (Sargus-Patino et al., 2014). This culture system was recently adapted by other groups to investigate embryonic development in human (Arjmand et al., 2016) and bovine blastocysts (Zhao et al., 2015). The remainder of this review will highlight our alginate culture system and illustrate the utility of 3-D hydrogels to study porcine blastocyst elongation continuously and in real time.

5 | CULTURE OF PORCINE BLASTOCYSTS IN ALGINATE HYROGELS

5.1 | Alginate hydrogel design

The principal purpose of a tissue-engineering scaffold is to provide a framework and space in which tissue growth can occur that mirrors its native environment (Drury & Mooney, 2003). The physical properties of the scaffold are essential to dictate the direction in which tissue growth proceeds and how cell/tissue architecture is maintained. Several methods were investigated to create alginate hydrogels that could support the in vitro culture of pre-implantation porcine blastocysts. Four encapsulation techniques were initially tested to identify the optimal approach for pre-implantation porcine blastocysts.

The first technique was a simple “drop” method, similar to how ovarian follicles were encapsulation (Kreeger et al., 2006; Shikanov, Xu, Woodruff, & Shea, 2011), in which 0.5- to 1-mm porcine blastocysts were placed in a 25-μl drop of alginate solution that was slowly released from the edge of a wide-orifice pipette tip into cross-linking solution (50 mM CaCl₂, 140 mM NaCl) for 3 min. This formed a single bead ~3–4 mm in diameter that contained an individual, encapsulated blastocyst. We tested encapsulation of blastocysts in various concentrations of alginate solution (0.3%, 0.7%, and 1.5%) to investigate the role of scaffold mechanics on development over the next 96 hr. Although blastocyst survival did not vary among the different alginate concentrations, only limited morphological changes were observed in the 0.7% alginate solution (personal observation). One significant problem with this single-drop technique was that a significant percentage of blastocysts (>50%) did not remain encapsulated within the hydrogels after 24 hr of culture, as the majority of the blastocysts were initially encapsulation at the edge of the bead and eventually dissociated from the bead.

Two additional techniques were tested in an attempt to ensure full encapsulation of the blastocysts within the hydrogel. First, an internal gelation method using a calcium slurry (Andersen et al., 2012) was utilized in an attempt to better position the blastocysts within the hydrogels. For this technique, various alginate solutions (0.3%, 0.7%, and 1.5%) were placed in CaSO₄, creating an alginate/calcium slurry prior to inserting blastocysts. Gelation of the hydrogels occurs as the calcium slowly leaks from the alginate solution (Andersen et al., 2012). Although this method allowed for precise positioning into the alginate hydrogels, none of the blastocysts survived the culture period (personal observation). This negative outcome was likely due to the reduction in pH that occurs as calcium is slowly released, and may be harmful to cells within the alginate solution during gelation (Andersen et al., 2012). The next technique used a combination of both the single-drop and calcium slurry techniques wherein the hydrogels were completely cross-linked prior to inserting a blastocyst into the hydrogel using a 14-gauge needle. This approach improved placement of the blastocysts within the hydrogels, thus ensuring that the blastocysts remained encapsulated throughout the culture period. Unfortunately, none of the blastocysts survived the culture period using this precast technique (personal observation), likely because of the trauma they experienced during their placement into the precast hydrogels.

The final encapsulation technique developed (and currently used in our lab today) is an extension of the single-drop encapsulation technique that includes multiple layers of alginate hydrogels (Figure 4), referred to as double encapsulation (Sargus-Patino et al., 2014). The blastocyst is first placed in 25-μl drop of alginate solution. This alginate droplet containing the blastocyst is then dropped into the cross-linking solution (50 mM CaCl₂, 140 mM NaCl), using a wide-orifice pipette tip, for 3 min. The encapsulated blastocyst in the single-layer alginate bead is transferred to a new 45-μl drop of alginate solution, and the cross-linking process is repeated, yielding a blastocyst encapsulated with two layers of alginate hydrogel. This technique resulted in >95% of blastocysts remaining encapsulated throughout the culture period (personal observation). Furthermore, survival and morphological changes were consistently observed using this technique. Furthermore, blastocysts exhibiting morphological changes during culture (i.e., initiation of elongation) would typically migrate out of the hydrogels
As a result, double encapsulation was the primary technique chosen to assess the development of pre-implantation porcine blastocysts in vitro within alginate hydrogels.

5.2 Development of porcine blastocysts in alginate hydrogels

Our first study using the double-encapsulation method evaluated in vitro development of encapsulated porcine blastocysts by characterizing cellular survival, morphological changes, gene expression of steroidogenic enzyme and immune-response transcripts, and estradiol-17β production (Sargus-Patino et al., 2014). In this study, spherical pig blastocysts ~1 mm in diameter recovered at Day 9 of gestation were either encapsulated using the double-encapsulation method (Figure 4), in 0.7% alginate hydrogels (ENC), or remained non-encapsulated as controls (CONT), and then cultured for 96 hr in serum-based medium. Although no differences in cell survival were observed between the ENC and CONT blastocysts, 32% of surviving ENC blastocysts underwent morphological changes, such as tubal formation with subsequent flattening (Figure 5), whereas none of the CONT blastocysts exhibited morphology changes. In addition, the abundance of transcripts for steroidogenic enzymes (STAR, CYP11A, and CYP19A) was higher in blastocysts that were encapsulated and had morphological changes (ENC+) compared to blastocysts encapsulated with no morphological changes observed (ENC−) or non-encapsulated CONT blastocysts. The expression of these steroidogenic enzyme transcripts in ENC+ blastocysts was consistent with observed increases in later-stage in vivo-produced blastocysts (i.e., ovoid and tubular blastocysts) that have initiated the elongation process (Blomberg, Garrett et al., 2006; Sargus-Patino et al., 2014). Similarly, ENC+ blastocysts produced and secreted more estradiol-17β at 72 and 96 hr of culture compared to CONT and ENC− blastocysts. This increased production and secretion of estradiol-17β by ENC+ blastocysts was also similar to what is observed from later-stage in vivo-produced blastocysts (i.e., ovoid and tubular blastocysts) (Miles et al., 2008), and further illustrated that alginate hydrogels can support successful initiation of elongation using only basal-medium conditions (Sargus-Patino et al., 2014).

Proper interactions between the blastocyst and receptive uterine endometrium are essential for supporting embryonic development and subsequent implantation (Ross et al., 2003). These interactions are initiated and regulated by the immune-response cytokine IL1B (Ross et al., 2003). Although the abundance of IL1B mRNA was greater in ENC+ blastocysts compared to CONT blastocysts, its expression was decreased in ENC+ compared to in vivo-produced tubular blastocysts (Sargus-Patino et al., 2014). This difference suggests that direct interaction with the maternal endometrium and specific factors within the maternal milieu are required for the up-regulation of IL1B expression in the blastocyst, whereas up-regulation of steroidogenic enzyme transcripts and estradiol-17β production may be independent of maternal-embryonic crosstalk. These data suggest that additional factors, either specific soluble factors or components of the ECM, may be necessary to elicit the appropriate maternal-embryonic crosstalk required for progression through the elongation process. Therefore, modifications to the alginate hydrogel culture system, such as addition of soluble and ECM factors, could improve the system for evaluating and understanding specific mechanisms of blastocyst elongation.
5.3 Incorporation of soluble factors into the alginate hydrogel culture system

Dramatic changes in protein content within the uterine environment occur during the estrous cycle and early pregnancy in the pig (Roberts & Bazer, 1988). Interestingly, only limited differences in proteins within the uterine milieu are observed between Days 10 and 13 of the estrous cycle and pregnancy, illustrating that many of these proteins are produced and secreted by the endometrium independent of the conceptus (Kayser, Kim, Cerny, & Vallet, 2006). Much of the uterine protein production and secretions are influenced by progesterone levels during early estrous cycle and pregnancy (Vallet, Christenson, Trout, & Klemcke, 1998), which illustrates the importance of early-cycle progesterone priming on uterine receptivity of implantation and corresponding pregnancy success (Bazer, Spencer, Johnson, Burghardt, & Wu, 2009). Asynchronous embryo transfer models in pigs (Wilde, Xie, Day, & Pope, 1988) and cattle (Ledgard, Berg, McMillan, Smolenski, & Peterson, 2012) revealed that exposure of embryos to specific uterine environments (i.e., a 1- to 2-day delayed or an advanced uterine environment in relationship to the embryo) highly influenced subsequent embryonic development and survival. As a result, appropriate timing for the presentation of uterine factors is critical for proper conceptus development and subsequent implantation.

Given these observations, we tested whether cellular survival or morphological changes could be achieved by culturing alginate-encapsulated pig blastocysts (~1 mm) for 96 hr in a serum-free RPMI-1640 medium containing purified uterine proteins (3.5 mg/ml) collected from either Day 9 or 10 pregnant gilts. No difference in blastocyst survival was observed between the CONT blastocysts or those ENC blastocysts cultured in medium containing uterine proteins compared to those cultured in standard serum-based medium (traditional RPMI-1640 medium containing 10% FBS) (Table 1). In addition, no differences were observed for morphological changes between ENC blastocysts cultured with uterine protein from Day 9 or 10 pregnant-uterine flushings or with standard serum-containing medium. As before, CONT blastocyst did not undergo any morphological changes. Thus, culturing encapsulated blastocysts within serum-free medium containing uterine proteins from Day 9 and 10 of gestation is sufficient to maintain blastocyst survival and to facilitate morphological changes in vitro.

TGFβs are also thought to play a key role in porcine blastocyst elongation and subsequent implantation (Gupta et al., 1996, 1998; Massuto et al., 2010). As such, we successfully cultured blastocysts for 96 hr using our alginate hydrogel encapsulation system with the addition of various concentrations of commercial, recombinant TGFβ1 (R&D Systems, Minneapolis, MN) within traditional serum-based culture medium. Although no significant difference in morphological change was observed in this preliminary study (a low sample size of 33 total cultured blastocysts were tested), combining the encapsulated groups treated with various concentrations of TGFβ1 (0, 0.1, or 1.0 ng/ml) demonstrated a tendency for morphological changes in ENC blastocysts compared to CONT blastocysts (Table 2), implying that TGFβ1 created a culture medium environment that facilitated morphological changes, as indicated by the percent of surviving blastocysts that initiated morphological change. Thus, our culture system can be used to specifically test if and how soluble factors affect blastocyst elongation and development.

5.4 Incorporation of ECM factors into the alginate hydrogel culture system

We chemically modified the alginate hydrogels by covalently attaching RGD peptides to the alginate polymer backbone using carbodiimide chemistry, as previously described (Rowley et al., 1999), to ask if the addition of ECM factors into our established alginate hydrogel culture system benefits elongation (Sargus-Patino et al., 2014). We successfully cultured blastocysts for 96 hr encapsulated in unmodified alginate, alginate conjugated with RGD, and with 0.1 μg/ml SPP1 incorporated in the alginate (Laughlin et al., 2017). At the termination of culture, overall survival was greater for blastocysts encapsulated in hydrogels conjugated with RGD and with 0.1 μg/ml SPP1 compared to non-encapsulated controls and those encapsulated in unmodified alginate. Furthermore, the proportion of blastocysts that underwent morphological change was greater for those encapsulated blastocysts.
in hydrogels conjugated with RGD compared to the standard (alginate-only) hydrogels. Cellular proliferation did not differ between these treatments, which supports previous literature reporting that morphological changes during elongation are not primarily driven by hyperplasia, but rather by cellular reorganization (Geisert & Yelich, 1997). Interestingly, only blastocysts encapsulated within RGD-hydrogels exhibited both increased abundance of transcripts for steroidogenic enzymes (i.e., \textit{STAR} and \textit{CYP19}) and immune-response genes (i.e., \textit{IL1B}) and increased estradiol-17β production, consistent with blastocysts undergoing elongation in vivo (Laughlin et al., 2017).

Thus, blastocysts encapsulated in alginate hydrogels conjugated with RGD or supplemented with SPP1 showed improved development in vitro compared to non-encapsulated control blastocysts and to those in a standard alginate hydrogel. Furthermore, the presence of RGD conjugation within the hydrogel improved blastocyst development in terms of the proportion of blastocysts undergoing morphological changes, as well as eliciting appropriate maternal-embryonic crosstalk (i.e., up-regulation of \textit{IL1B}).

### 6 | CONCLUSIONS

Tissue-engineering approaches provide innovative, more-physiological alternatives to traditional 2-D systems for in vitro models of development. We developed a 3-D culture system using alginate hydrogel matrices that can encapsulate pig blastocysts, maintain viability and blastocyst architecture, and facilitate morphological changes that are compatible with the initiation of elongation in vivo, thus allowing us to study the initial stage of blastocyst elongation in real time. This approach should serve as a complementary and confirmatory approach to in vivo analysis, with the benefit of controlled manipulation using exogenous factors. Further investigations with our 3-D culture system will focus on the effects of certain growth factors, hormones, ligands, ECM components, and uterine epithelial cell interactions on the development of preimplantation porcine blastocysts and on developing strategies to improve pregnancy outcomes in the pig by identifying mechanisms to decrease embryonic mortality, reduce within-litter birth weight variability, and improve pre-weaning survival. Our alginate culture system can also be applied to study blastocysts elongation by other ungulate species (Zhao et al., 2015), such as cattle and sheep, to identify and compare potential regulators of normal embryonic development among animals.

### ACKNOWLEDGEMENT

This manuscript was supported, in part, by Agriculture and Food Research Initiative Competitive Grant no. 2017-67015-26458 from the USDA National Institute of Food and Agriculture as part of the ICPR program.

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| Table 1 | Summary of embryo survival and observed morphological changes following 96 hr of culture of either non-encapsulated control porcine embryos or porcine embryos double encapsulated in 0.7% alginate hydrogels and cultured in various concentrations of recombinant TGFB1 (0, 0.1, and 1.0 ng/ml TGFB1)\(^1\)\(^2\). |
| --- | --- | --- | --- | --- |
| CONT | ENC (Day 9) | ENC (Day 10) | ENC in Serum | \(p\)-value |
| Number of embryos | 40 | 43 | 43 | 43 |
| Embryo survival (%)\(^3\) | 52.5 ± 7.9 | 48.8 ± 7.6 | 51.2 ± 7.6 | 51.2 ± 7.6 | 48.8 ± 7.9 | 0.99 |
| Morphological change from all embryos (%) | 0\(^a\) | 11.6 ± 4.9\(^b\) | 14.0 ± 5.3\(^b\) | 12.2 ± 5.1\(^b\) | 0.17 |
| Morphological change from surviving embryos (%) | 0\(^a\) | 23.8 ± 9.3\(^b\) | 27.2 ± 9.5\(^b\) | 25.0 ± 9.7\(^b\) | 0.16 |

\(^1\)Values are reported as least-squares means ± standard error, as determined using GLIMMIX analysis for the main effect of treatment (i.e., control vs. encapsulated embryos in Day-9 uterine protein, Day-10 uterine protein, or 10% serum-based medium).\(^a,b\)Significance (\(p < 0.05\)) for the effect of treatment was determined using chi-square analysis.

\(^2\)Unpublished data.

\(^3\)Assessed by blastocyst fragmentation.

| Table 2 | Summary of embryo survival and observed morphological changes following 96 hr of culture of either non-encapsulated control porcine embryos or porcine embryos double encapsulated in 0.7% alginate hydrogels and cultured in media containing \textit{RMPI}–1640 + day 9 uterine protein (d9), + day 10 uterine proteins (d10), or +10% serum (Ser)\(^1\)\(^2\). |
| --- | --- | --- | --- | --- |
| CONT | ENC (Day 9) | ENC (Day 10) | ENC in Serum | \(p\)-value |
| Number of embryos | 40 | 43 | 43 | 43 |
| Embryo survival (%)\(^3\) | 55.6 ± 16.6 | 75.0 ± 15.3 | 62.5 ± 17.12 | 50.0 ± 17.7 | 0.99 |
| Morphological change from all embryos (%) | 0\(^a\) | 37.5 ± 17.1\(^d\) | 37.5 ± 17.1\(^d\) | 37.5 ± 17.1\(^d\) | 0.37 |
| Morphological change from surviving embryos (%) | 0\(^a\) | 50.0 ± 20.4\(^d\) | 60.0 ± 21.9\(^d\) | 75 ± 21.7\(^d\) | 0.19 |

\(^1\)Values are reported as least-squares means ± standard error, as determined using GLIMMIX analysis for the main effect of treatment (i.e., control vs. encapsulated embryos in Day-9 uterine protein, Day-10 uterine protein, or 10% serum-based medium).\(^a,b\)Significance (\(p < 0.08\)) for the effect of treatment (combining all ENC groups) was determined using chi-square analysis.

\(^2\)Unpublished data.

\(^3\)Assessed by blastocyst fragmentation.
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