EFFECTS OF PORCINE JELLY MATRIX (JMX) ON GENE EXPRESSION OF PORCINE UMBILICAL CORD (PUC) STEM CELLS

by

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Abstract

Culturing stem cells is usually done on tissue-culture treated plastic. Over time the cells change their gene expression and start to differentiate. Porcine umbilical cord (PUC) stem cells express the embryonic transcription factors Oct4, Nanog and Sox2 and changes in their expression may be useful for to evaluating culture-induced changes in the cells.

We developed an extract of porcine Wharton's jelly matrix (JMX) that may provide some characteristics of the stem cell niche located in the umbilical cord. Our extract used whole cords and enzyme digestion to simplify preparation of the product. We compare cells cultured on plastic to those grown on thin and thick gels of JMX in four experiments.

In Exp 1a and b, growing PUCs on a thick JMX coating for 3(1a) or 4(1b) d increased the number of cells at the end of culture ($P \leq 0.05$) with minimal effects on gene expression. In Exp 2 we compared PUCs grown on thin and thick layered JMX with added collagen (+C) and to control cells. The JMX layers caused the cells to adopt a small, round shape and to form clumps or colonies during culture. No differences ($P > 0.10$) were seen between thin10 +C and control wells for viable and total cell counts but thick layered +C resulted in decreased numbers of viable cells compared to thin + C ($P < 0.10$) and control wells ($P < 0.05$). In a follow up experiment (Exp. 3) growing the PUCs mixed within, rather than plating on top of, a thick layer of JMX + C caused marked morphological changes with dense 3-dimensional structures formed.

Exp 4 compared JMX allowed to gel for 10 (Thin10 +C) or 60 (Thin60 +C) min before the non-gelled fraction was removed. There were no effects on cell numbers at the end of culture ($P > 0.10$) but Sox2 expression was increased in Thin60 +C compared to controls on plastic ($P < 0.05$) and Thin10 +C ($P < 0.10$).

In summary, JMX extracts change cell morphology and in some formats increased cell proliferation and may increase Sox2 expression. Further investigation is needed to fully understand the effects of JMX on PUCs.

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Chapter 1 - General Review of the Literature

Potency of stem cells receives considerable attention, particularly since the 1980s and the large amount of scientific literature on embryonic stem (ES) cells. ES cells are considered pluripotent, because they are able to develop into any cell or tissue in the body. Mesenchymal stromal (stromal) cells (MSCs) are another cell with stem properties. MSCs are widely distributed and perhaps are found in all organs where they have a central role in homeostasis by providing for replenishment of specific cells needed for the organ to function. MSCs have a more restricted potential than ES cells. This restricted potential limits their use as general tools for regenerative medicine but also makes MSCs an ideal candidate for many cell-based therapies including regenerative medicine because they are not tumorigenic. In addition, many MSCs can be easily isolated with no or limited ethical issues (Le Blanc and Pittenger 2005).

PUCs are MSCs, but also have some characteristics similar to primitive pluripotent stem cells (Carlin et al., 2006) and they can develop into multiple tissue and cell types. PUCs are derived from the Wharton's Jelly of the umbilical cord and are collected at birth or as early as day 20 of pregnancy (Carlin et al., 2006). *In vitro* these cells can be stored cryogenically and expanded after thawing (Carlin et al., 2006). These characteristics make them a viable research tool. This review will describe current knowledge of mesenchymal stromal cells, the stem cell niche and culturing systems that can influence expression of Oct4, Sox2, and Nanog in MSCs.

Stem Cells

A wide variety of cells are considered stem cells because they have the ability to self-renew by making identical copies themselves, and are able to differentiate into one or more types of differentiated cells (Lukaszewicz et al., 2010). There are approximately 270 cell types in the body (Le Blanc and Pittenger 2005). The potency of the cell refers to the number of types of

cells the stem cell can become. Four general terms are used to describe potency of stem cells: totipotent, pluripotent, multipotent, and unipotent (Mohammad and Baylin 2010).

Totipotent

Without human manipulation, totipotency is only found in cells of very young embryos that can become all cell types including the extraembryonic membranes of the conceptus. Totipotent cells are found at the zygote and early cleavage stages. To prepare ES cells, the embryo must be taken apart, thus removing the potential for it to become a fetus. The ethical baggage for ES cells is that many in society view this as destroying a life.

Pluripotent

Pluripotency usually refers cells of the inner cell mass of blastocysts or to ES cells. ES cells are collected from the inner cell mass of a blastocyst stage embryo (Lukaszewicz et al., 2010). Another example of pluripotent stem cells is embryonic germ cells, which are derived from the gonadal ridge early in embryonic development (Henningson et al., 2003). Pluripotent cells can give rise to the entire embryo, but not the extraembryonic membranes. When culturing pluripotent stem cells, the use of murine embryonic fibroblast (MEF), MEF-conditioned media, leukemia inhibitory factor (LIF), other cytokines, or growth factors are needed to keep the cells undifferentiated (Carlin et al., 2006, Miyabayashi et al., 2007, Xu et al., 2008, and Miki et al., 2011). These culture conditions prevent differentiation and promote self-renewal but the mechanisms are not completely understood (Miki et al., 2011). Regenerative medicine is interested in using pluripotent stem cells as a treatment. However, pluripotent stem cells when transplanted will often develop into a teratoma (Lukaszewicz et al., 2010). Teratomas are tumors that have cells from all three cell lineages: endoderm, ectoderm, and mesoderm. Teratoma

formation is one reason that utilization of pluripotent stem cells as cell based medicine is risky currently.

A point about pluripotency to consider is that by the terminology described above hematopoietic stem cells are multipotent. However there is another terminology that considers hematopoietic stem cells to be pluripotent in that they provide all the myeloid (monocytes, neutrophils, basophils, eosinophils, erythrocytes, platelets, dendritic cells), and lymphoid (T-cells, B-cells, NK-cells) cells. Therefore care should be taken to understand which terminology is being applied to hematopoietic lineages.

Multipotent

Mesenchymal stem cells are also called mesenchymal stromal cells and multipotent stromal cells. These are all commonly abbreviated MSC. MSCs are usually collected after parturition or from an adult and these cells are considered to have less differentiation potential when compared to pluripotent cells. They may be more easily collected in larger numbers but this varies greatly with tissue source. Some sources of MSCs, in addition to Wharton's jelly are: bone marrow, umbilical cord blood, perivascular space, placental tissue, amniotic fluid, adipose tissue, dental pulp and skin. MSCs are generally easy to culture. When compared to ES cells, there is a reduction seen in the plasticity of multipotent cells (Guilak et al., 2009).

Even though MSCs have more restricted differentiation potential than pluripotent and totipotent cells they will generally differentiate into tissues of at least two and possibly all three germ layers. Differentiation potential is often evaluated in vitro and under appropriate conditions MSCs are expected to differentiate into adipocytes, chondrocytes, myoblasts and osteoblasts (Ling et al., 2009).

Unipotent

As a stem cell that can reproduce itself, but is limited to becoming one type of differentiated cell is considered unipotent. Found in limited areas of the body, unipotent stem cells help maintain homeostasis of tissue (Visvader and Lindeman 2011). Mammary tissue epithelium and testes contain unipotent stem cells (Ko et al., 2010, Van Keymeulen 2011 and Visvader and Lindman 2011). To distinguish unipotent stem cells from progenitor cells, mammary unipotent stem cells are able to self-replicate extensively through morphogenesis, adult life and during multiple pregnancies (Van Keymeulen 2011). Confusion sets in when stem cells are referred to as progenitor cells when there is doubt to the true nature of the cell (Tajbakhsh 2009).

Progenitor Cells

Progenitor cells are similar to stem cells in their ability to self-renew, but their life span and number of divisions are finite (Seaberg and van der Kooy 2003 and Gilbert 2010). Progenitor cells may fit the description of transit-amplifying cells (TAC) for they divide during migration away from the stem cell niche (Gilbert 2010). Progenitor cells are much more lineage-restricted compared to stem cells. Known progenitor cells become blood cells, sperm cells and cells of the nervous system (Seaberg and van der Kooy 2003; Gilbert 2010). The unspecified "progenitor" cell can generate multiple cell types as in the central nervous system (Tajbakhsh 2009).

Transit-amplifying Cells

Strictly defined, TAC are the committed progenitors between stem cells and completely differentiated daughter cells (Diaz-Flores Jr et al., 2006). They have a finite lifespan and arise from stem cells (Tajbakhsh 2009). TACs are found cohabitated with adult stem cells in niches,

such as intestinal crypts and gastric glands (Diaz-Flores Jr et al., 2006). Some stem cells do not transition into TACs before differentiating in skin cell populations (Tajbakhsh 2009). There is leeway in applications of the terms stem, progenitor and transit-amplifying and this creates challenges in interpreting their meaning when they are not strictly defined in publications and presentations (Tajbakhsh 2009).

Embryonic Transcription Factors

Core transcription factors are found in all mammalian pluripotent cells (Carlin et al., 2006, Maucksch et al., 2013, Rizzo 2013, Saunders et al., 2013). High concentrations are only found to be expressed in primitive cells (Carlin et al., 2006 and Saunders et al., 2013). Expression of these transcription factors can provide an indication of cell potency or stemness but the potential must be demonstrated, ideally *in vivo*.

The most influential transcription factor has been shown through reprogramming experiments to be Oct4. Using this single transcription factor, Wang and others added lithium to a culture of differentiated cells to successfully reprogram the cells to a pluripotent status (Wang et al., 2011). The core transcription factors have major overlap of target genes. Chromatin immunoprecipation (ChIP)-chip binding of the core transcription factors found 350 to 600 genes are bound by Oct4, Nanog and Sox2. This could explain how reprogramming of cells by a single gene can occur (Maucksch et al., 2013 and Rizzo 2013). Reprogramming has the promise of taking somatic cells from someone and turning them pluripotent for cell based medicine.

The three core transcription factors have the ability to up regulate their own expression and the expression of the other two core transcription factors and this creates a feed-forward regulation to keep the cells in a self-renewing, undifferentiated state. As human MSCs increase in passage number, a gradual decrease in mRNA expression of the core transcription factors is

observed (Yoon et al., 2011). Therefore lower passage cells would be better suited for some applications due to their greater expression of Oct4, Nanog, and Sox2.

Mesenchymal Stromal Cells (also called Mesenchymal Stem Cells)

MSCs are an easily obtained cell population from adults, newborns or from extraembryonic tissues of the conceptus. These populations are the most widely utilized adult derived stem cell in research (Lukaszewicz et al. 2009). MSCs were first identified by Owen and Friedenstein in 1988. They found the cells in the stromal cell compartment of bone marrow (Ling et al., 2009). Over time, researchers have found many other locations that provide MSCs. They are found in most if not all tissues and as a part of the niche for many other stem cells. Niches are an area where a population of stem cells can be found such as bone marrow or the umbilical cord. The stem cell niche is the microenvironment that provides homeostasis for the stem cells while controlling proliferation activity and the total cell population (Via et al., 2012).

In culture, MSCs readily adhere to plastic and exhibit a fibroblast-like morphology (Ling et al., 2009 and Kollmer et al., 2012). These characteristics alone don't definitively confirm that the cells are MSCs. Through the use of appropriate culture conditions, MSCs readily differentiate into adipocytes, chondrocytes, myoblasts and osteoblasts (Ling et al., 2009). Surface antigens are a third factor needed to determine, if in fact, the cells in question can be considered MSCs. MSC surface antigens may not be universally present in all species and tissues, so researchers need to determine what surface antigens are specific to their species (Dominici et al., 2006).

Medical benefits of MSCs

Therapeutic uses for MSCs have been gaining interest due to the availability and potential beneficial effects. Unlike embryonic stem cells, MSCs do not form teratomas when

injected *in vivo*, but instead engraft in multiple organs and are seen to have site specific differentiation (Le Blanc and Pittenger 2005). Wound healing is another area for MSCs use. Healing rates increase when MSCs are placed at the wound site. The mechanism of increasing tissue repair rates is through the production of cytokines and growth factors, which in turn influence cell survival in the damaged tissue and increase tissue growth (Le Blanc and Pittenger 2005).

Studies have evaluated the effects of injecting MSCs, especially those from Wharton's jelly, into sites of damaged organs that have an overabundance of collagen. Human umbilical cord stem cells have decreased collagen deposition in repair sites in the lung. Lung fibroblast cells did not have the same effect on the damaged tissue (Moodley et al., 2009). MSCs influence the cytokine environment in this situation, perhaps due to their inhibition of transforming growth factor beta (TGF-β) (Moodley et al., 2009). Tissue inhibitors of matrix metalloproteinases (TIMP) are also decreased in lung tissue injected with MSCs compared to lung-fibroblast injections and matrix metalloproteinase (MMP) is up regulated (Moodley et al., 2009). The same effects were seen in research done in liver fibrosis by injecting MSCs (Lin et al., 2010).

Survival of cells in the areas of fibrosis and wounds is influenced by injecting MSCs. Anti-apoptic molecules are secreted by MSCs to stop cell death in the areas of ischemia and scar formation (Caplan and Correa 2011). MSCs have low engraftment levels when injected into the body. Less than 1% engraftment of donor MSCs are usually found even though therapeutic benefits are observed (Phinney and Prockop 2007). Regeneration of damaged blood vessels can be enhanced by MSCs. MSC secretion of VEGF promotes angiogenesis and stabilize new vessel development (Caplan and Correa 2011). Proteins secreted by MSCs modulate endothelial cell9

migration. Angiogenesis and endothelial cell migration lead to vessel remodeling, which is essential for bone growth (Phinney and Prockop 2007).

Immunological effects are mediated by MSCs. MSCs produce anti-inflammatory and anti-apoptic cytokines and a spectrum of growth factors such as vascular endothelial growth factor, hepatic growth factor and IGF-1 (Morigi et al., 2008). They also have immunomodulatory properties, shown by inhibition of T lymphocyte proliferation. Tolerogenic properties because MSCs produce human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I and HLA class II and are not rejected by T cells when transplanted into MHC mismatched recipients (Le Blanc et al., 2003, Morigi et al., 2008 and Corrao et al., 2013). MSCs express proteins for improved immunity and defense and even promote neural activities found through immunodepleated mice (Phinney and Prockop 2007).

Table 1.A MSCs characteristics.

Effects	**Differentiation Lineages**
Wound healing Reduction of fibrosis, anti-scaring	Neural differentiation Differentiate into Mesenchymal lineages
Homing to wound or infection sites Regulate immune cells (HLA-G,-E,-F) Self-renewal, mitotic	Differentiate into tissues other than their tissue of origin
Anti-apoptotic Anti-microbial Angiogenic Sites of large, readily available populations Anti-cancer Immune tolerance instauration during pregnancy	

Porcine Umbilical Cord Stromal Cells

Porcine umbilical cords stromal cells (PUCs) were the first umbilical cord matrix MSCs isolated and they were obtained from pig conceptuses and neonatal pigs (Mitchell et al., 2003). Further characteristics were reported by Carlin et al., (2006) and Packthongsuk (2013). At parturition, cords can be aseptically collected with relative ease and large numbers of cells obtained. The umbilical cord has a jelly matrix, Wharton's Jelly (WJ), protects the blood vessels. WJ is a gelatinous connective tissue comprised of collagen fibers and proteoglycans along with cells (Mitchell et al., 2003). Research is still ongoing to determine the potency of these cells. Carlin and others found that Oct4, Nanog, and Sox2 are expressed in PUCs, but in relatively lower amounts when compared to early porcine embryos. The cells in WJ have been considered to be more primitive than adult MSCs, based on population doubling time and length of expansion time before the cells undergo senescence (Troyer and Weiss 2008). PUCs are expected to be useful in cell based therapies and as a model for human medicine.

Extracellular Matrix

Components

Extracellular matrix (ECM) is found throughout the body, one of its many roles is to participate in stem cell niches. The composition of the ECM is important for its interaction with cells. The ECM composition creates both complexity and flexibility. The components of the ECM are collagen, laminin, fibronectin, proteoglycans, biglycan, decorin, hyaluronate, glycoproteins, glycosaminoglycans, elastin, versican and perlecan (Malkowski et al., 2008, Chen 2010, Lai et al., 2010, Muiznieks and Keeley 2012, Rutnam et al., 2012). Some of these components have been used to make *in vitro* 3-D culture systems that are similar to the ECM.

Collagen is the most abundant protein in the body with at least 28 types (Wikipedia). Types I to V are the most common. The numerous types of collagen fibers are arranged as three polypeptide strands with a left-handed helix that are twisted to form a right-handed coil. This triple helix is stabilized by lots of hydrogen bonds and this gives collagen and as a result ECM the strength to withstand tensile forces (Muiznieks and Keeley 2012). Collagen is very important for the ECM in umbilical cords where it helps the blood supply with stand compression and extension during gestation and the forces exerted on the fetus by contractions of the uterus during parturition (Malkowski et al., 2008). Amount of collagen in ECM can have different effects on the surrounding cells. In *in vitro* cultures, collagen coated plastic can cause the MSCs to undergo differentiation (Lin et al., 2012). When plating MSCs on collagen, the cells are more likely to undergo matrix calcification and osteogenesis (Lin et al., 2012). This is important because making artificial ECMs is hard to replicate and mechanisms are not completely known that keep stem cells in an undifferentiated state. Researchers have been utilizing individual components of the ECM to study the effects on MSCs in culture.

Elastin is another component of the ECM that is extremely important for vertebrates. It gives the matrix the ability to revert to its original form after a force has been removed (Muiznieks and Keeley 2012). This component of ECM is especially important in amniotes where elastin is needed in the vascular walls of the umbilical blood vessels for structural and functional properties, along with other molecules for proper function of the cord (Gogiel et al. 2012). The turnover rate of elastin is extremely slow, so most vertebrates use the elastin formed during fetal development their entire lifespan (Muiznieks and Keeley 2012). Stability of elastin fibers is based on individual proteins bonding with adjacent proteins. Early in fetal development, aggregation of elastin proteins occurs in random order when proteins get within 10 μm of each

other. This forms short, irregular fibrils that are increasingly stable with higher protein aggregation (Muiznieks and Keeley 2012).

ECM needs to be remodeled over time to maintain its complex structure. Enzymes, such as matrix metalloproteinases (MMPs) help with remodeling during development (Lu et al., 2012). The addition of extracellular modifying enzymes gives flexibility (Schambony 2004) but if not highly regulated, the enzymes could damage the ECM and possibly the whole organism. Enzyme regulation is at transcription, translation, and post translation (Lu et al., 2012). The enzymes can become deregulated due to age or disease (Lu et al., 2012). This is seen during cancer development and general aging. Unregulated remodeling enzymes can change characteristics of the ECM: composition, amount, and topography (Lu et al., 2012). This can lead to behavioral changes in the cells located in the ECM.

ECM effects Development

ECM affects homeostatic events in different tissues, affects development in the lung when branching occurs, functions when in the mammary gland at the beginning of lactation and in pathological situations such as tumerigenesis (Butcher et al., 2009). The changes in the matrix causes the cells to adapt, if not, they will undergo apoptosis.

Environmental changes in the stem cell niche can bring the cells out of their primitive state and allow them to undergo differentiation. Initially genetic and molecular mediators were evaluated for their influenced stem cell activity, but more recent work considers the physical environmental provided by the ECM (Guilak et al., 2009). Through interactions with the components of the ECM, stem cells can be guided into a specific linage. The influence comes from the binding of ECM ligands to cell surface receptors and the rigidity of the ECM. The

stiffness of the ECM can influence stem cell behavior, causing stem cells to migrate, proliferate, differentiate, or undergo apoptosis (Guilak et al., 2009).

The properties of ECM that influence stem cells appear to participate during disease and development (Lu et al., 2012). An important function of ECM is how the cell perceives its surroundings and how it reactions to signals from its environment. The elasticity of the ECM and the force that it exerted on the cell determines cell behavior (Lu et al., 2012). Cell fate, differentiation and tissue function are regulated in part by the ECM (Lu et al., 2012).

ECM effects on disease and development result from multiple properties and the effects of these properties ECM are not independent of each other (Lu et al., 2012). An individual property can't independently influence and change cell behavior. When collagen bundles stiffen in the ECM, cells will be unable to migrate through, so MMPs are activated to counteract the cross-linked material and this allows cells to migrate (Lu et al., 2012). ECM is especially important in the heart where crosslinking of collagen in the muscle can influence cardiac function (Butcher et al., 2009). The dynamics needed for proper development and interaction of ECM have two alternative explanations. ECM continues to be modified at various developing and postnatal time points within different tissues in the body (Lu et al., 2012). This shows that remodeling to prevent degradation is more important. The alterative hypothesis is that the dynamic ECM includes how the individual components come together to form the complex matrix (Lu et al., 2012). Their positioning in the matrix is what makes the ECM components so essential to the cells and tissues of the body. The interaction between cells and the ECM also can function in a feed forward loop. Cells act on the ECM by breaking it down and rearranging it components and this in turn affects the cells by inducing them to change their behavior (Lu et al., 2012).

Stem Cell Niche

Environment

The niche is the location where stem cells are maintained in organs and it includes ECM and other non-cellular material that can regulate the cells (Scadden 2006). Stem cells need direct contact with ECM to maintain or regain stem cell-like properties (Lu et al., 2012). The cell population size depends on where the niche is located. The Wharton's Jelly niche has a large cell population within a large ECM component. More than one cell type resides in certain niches. Niches can contain multiple stem cells and the physical structure of the niche depends on its surroundings (Chen et al., 2013).

Information is available for the niches in mammals for hematopoietic stem cells, neural stem cells, and satellite cells in skeletal muscle. Complexity of niches seems to increase with the complexity of the species by amount of somatic cells types that help create the niche (Chen et al., 2013). A mammal's stem cell niches generally are more complex compared to *Drosophila* and *C. elegans* (Chen et al., 2013). Mammal hematopoietic stem cell niche contains at least four cell types: mesenchymal stem cells, neuron-Schwann cells, osteoblasts and vascular cells (Chen et al., 2013). The complexity of the niche itself leads to these characteristics for multiple stem cells in one niche, different cell types in niches throughout the organism or the same cell type in different organisms (Chen et al., 2013). Drosophila testes stem cell niche contains both germline stem cell (GSCs) and cyst stem cells (CySCs). There is evidence that interactions within the niche controls stem cell survival, potency, and cell division. In mammalian and non-mammalian systems adhesion molecules are important for these processes.

Adhesion Molecules

The signals from the niche do not transcend over long distances, so cells have to stay adhered to ECM to undergo self-renewal (Kollmer et al., 2012 and Chen et al., 2013). This way as the daughter cells move away from the ECM, they are able to differentiate. Cadherins and integrins are the most common adhesion molecules for cell attachment (Chen et al., 2013). Cadherins are for cell to cell adhesion in invertebrates and vertebrates. Through homophilic interactions, adjacent cells can adhere through utilizing the extracellular domain of cadherins. The intercellular domain connects to cytoskeleton proteins, which in turn influence catenins in the cell (Chen et al., 2013). Integrins facilitate where cells interact and adhere to ECM. The integrin's extracellular domain binds directly with ECM components such as collagen, fibronectin, and laminin (Chen et al., 2013). In addition to initial protein attachment, integrin signaling affects long term cell viability (Kollmer et al., 2012).

Adhesion molecules contribute more than just attachment of cells and ECM. Cell polarity and signaling are regulated (Chen et al., 2013). The niche can influence longevity of stem cells through its adhesion molecules and the strength of attachment between the cells and the niche influences stem cell self-renewal (Chen et al., 2013). During aging, the attachment strength is gradually lost so over time cells will lose the direct contact with paracrine factors and undergo differentiation. When two or more stem cell types are found in a niche, competition ensues to help control populations. By having to balance out multiple populations, integrins are used to keep cells adhered to the niche and others that are not, differentiate (Chen et al., 2013). This is seen in *Drosophila* GSCs. E-cadherin is the mechanism used in the ovary for GSCs to compete in the niche cells and with higher expression of E-cadherin cells will be more likely to have long term self-renewal (Chen et al., 2013). In the testis, GSCs compete in the niche with CySCs (Chen et al., 2013). To have multiple stem cell populations in a single niche, signaling pathways are

important to segregate and control each individual population. The JAK-STAT pathway controls both GSCs and CySCs, but E-cadherin expression is needed for GSCs while CySCs are controlled by BMPs (Chen et al., 2013). Integrins mediate the cell competition in the niche between the two populations in the testis. It is possible that integrins also function in a quality control mechanism (Chen et al., 2013).

Culturing Systems

2D cultures

In common cell culture techniques the cells grow on tissue-culture treated plastic; this is considered to be a 2-demensional (2D) system because most cells quit growing once confluent due to contact inhibition. Media with growth factors or feeder layers provide factors needed to grow cells. Human MSCs (hMSCs) need serum for exponential growth on plastic (Kollmer et al., 2012). This does not mimic a cell's natural environment. On tissue culture plastic, MSCs lose their ability to self-renew, therefore undergoing senescence or differentiate. A higher cell passage number correlates with an increased probability for the stem cell population to be diluted among committed and differentiated cells (Chen 2010). *In vivo*, MSCs are sensitive to their environment and take cues from growth factors, cytokines, other cells and physical cues from ECM, all which create a complex 3D environment that regulate MSCs activity (Saleh et al., 2012). There is interest in alternative culturing systems because the 2D does not recreate the natural environment and could possibly hinder cell potential (Chen 2010 and Saleh et al., 2012). In monolayers, stem cells bind either to the plastic or to other cells. MSCs secrete adhesion molecules and collagen (Kollmer et al., 2012). Long term cultures are needed to expand large populations of MSCs and prolonged culture can alter the cells. MSCs are known to lose their

ability to undergo self-renewal and lose their stemness in long term cultures on plastic (Chen 2010 and Lai et al., 2010).

3D cultures

The ECM affects cell shape that is associated with self-renewal or specific linage differentiation (Guilak et al., 2009). Stiffness of the ECM also is a part of this signaling. A goal would be to utilize an artificial or reconstituted stem cell niche to produce a large population of cells with stem-cell properties. Soluble signals, cell to cell contact, cell to ECM contact and physical factors of the ECM all contribute to the 3D environment and gives the cells the opportunity to interact with their environment through chemical and physical signals (Saleh et al., 2012).

Some researchers have synthesized porous gels to approximate the niche environment *in vitro*. When poly(ethylene glycol) diacrylate (PEGDA) hydrogels are not porous, viability of cells drops to 15% in the first week (Kollmer et al., 2012). The hMSCs were unable to spread out in a nonporous gel and were found to have a rounded morphology and undergo apoptosis (Kollmer et al., 2012). Modifications to make gels porous allowed the cells will stay viable even after 21 days, but cell proliferation was minimal for hMSCs (Kollmer et al., 2012). This is similar to the expected behavior in the niche where it is expected the cells will be dividing slowly (Kollmer et al., 2012). While encapsulated in porous gels, hMSCs are able to react to their surroundings by secreting ECM as they would in the stem cell niche (Kollmer et al., 2012).

The addition of serum is not needed for cells in 3D culture. Removing animal serum from hMSCs culture increases the likely hood of utilizing these cells for therapeutic use (Kollmer et al., 2012). Compatibility is one of the major issues of hydrogels. Even though hydrogels have some physical characteristics to the stem cell niche, polymer based products lack

biocompatibility and this may limit their use for delivery and therapeutic growth of stem cells (Lin et al., 2012). Polymer based products are not intrinsic, so the ability to maintain MSCs proliferation and biological functions on or in these gels is limited *in vivo* (Lin et al., 2012). Also synthesized gels or pure single proteins of ECM do not have the same effects as ECM on MSCs. ECM regulates MSC exposure to unique cues of cytokines, growth factors and influences the formation of adhesion structures on cells (Chen 2010).

Another way to make a 3D gel is to use decellularized ECM. Theoretically a de-cellularized ECM based culture would provide an ideal environment for MSCs (Lai et al., 2010). Lai and colleagues plated mouse MSCs on mouse marrow stromal derived ECM and saw increase proliferation, preservation of stem like qualities and helped into skeleton-genesis (Lai et al., 2010). The interaction between 3D cultures and mechanical forces placed on MSCs influenced renewal or differentiation. Limited research has been done so far on how decellularized ECM influences stem cell fate through gel rigidity (Her et al., 2013). However all the components of the ECM that influence cellular behavior are not known.

The important bio-factor(s) that keep MSCs in a self-renewal state during population expansion are not yet completely determined (Lin et al., 2012). Decellularized ECM is able to keep MSCs in an undifferentiated state (Lai et al., 2010). This method could be feasible to produce large scale expansion of populations of MSCs for therapeutic use (Lai et al., 2010). Also transplanting the de-cellularized ECM with MSCs could be an effective vehicle delivery route for cellular therapy (Lin et al., 2012). Reduction in cell manipulation and the addition of exogenous growth factors could lead to easier Federal Drug Administration approval.

Chapter 2 - Effects of Porcine Jelly Matrix on gene expression of PUCS

Introduction

Mesenchymal stem cells (MSCs) have great promise for regenerative medicine and other stem cell based therapies. For most applications it will be necessary to expand the number of MSCs harvested by culture. MSCs attach to tissue culture plastic ware and grow in several standard culture media. However there is a concern that cell phenotype is affected by the *in vitro* environment (Chen 2010 and Saleh et al., 2012). Growing on plastic tissue culture flasks and plates, over time MSCs can lose their potential to divide and/or to differentiate into other cell types. A better understanding of MSC response to their *in vivo* environment may provide useful information. The pre-harvest environment is often called the niche and it is considered that extracellular matrix and associated molecules are key components of a niche (Scadden 2006, Lu et al., 2012 and Chen et al., 2013). Culture methods have included hydrogels and individual ECM components. These can cause MSCs to differentiate into specific lineages. Extracts made from ECM potentially have the biofactors found in the stem cell niche and could be a feasible plating method to grow cells *in vitro* (Chen 2010 and Lai et al., 2010).

We prepared and tested an extract of porcine Wharton's jelly (JMX). We used JMX to coat plastic dishes with a thin coating or a thick gel and in some experiments we added collagen to improve gelling. Previously our lab prepared an extract of porcine Wharton's jelly by mechanically harvesting the matrix from umbilical cords (Bryan, 2008). The harvest was laborious and time consuming so we have developed an enzyme-aided procedure for bulk harvest of the matrix. The experiments reported here evaluated the response of PUCs to growth

in and on JMX. We evaluated the effects of JMX on 1) cell morphology, 2) expression of the transcription factors Oct 4, Nanog and Sox2 and 3) growth of PUCs.

Materials and Methods

Umbilical Cord Collection and Processing for Cell Harvest

Umbilical cords were collected aseptically at birth from pigs with birth weights ≥3 pounds at the Swine Teaching and Research Unit (Kansas State University). Individual cords were placed into polypropylene conical tubes containing phosphate buffered saline, pH 7.2 (PBS, Invitrogen) and antibiotic/antimycotic solution (penicillin, 400 u/ml, streptomycin, 400 mg/ml, amphotericin B, 1µg/ml: Gibco).

Cords were kept at 4ºC and processed within 24 h. For cell harvest the cords were placed in a sterile 150 cm^2 petri dish (TPP Cultureware, MidSci St. Louis, MO) and opened longitudinally with two hemostats. The blood vessels were removed from the cords and WJ was removed by scraping with a surgical blade. The WJ was minced into explants (approx. 1 mm^3) with scissors, rinsed in PBS and transferred to a new petri dish. Explants were cultured in growth medium (25ml) consisting of high glucose Dulbecco's Minimum Essential Medium (DMEM, Invitrogen) with 20% Fetal Bovine Serum (FBS, Gibco) and antibiotic/antimycotic (penicillin, 1000 u/ml, streptomycin, 1000 mg/ml, amphotericin B, .25µg/ml: Gibco), Normocin-O (100µg/ml Invitrogen), Gentamicin (25µg/ml Invitrogen) and β-mercaptoethanol (55µmol/ml Sigma). Two to four petri dishes were prepared from each cord and the cells isolated from each cord were grown separately.

Explant and Cell Culture

The dishes were placed in an incubator (38.5 ºC, 5% CO_2, 95% air atmosphere). After 3 d, 10ml of DMEM 20% was added and after 5 d in culture, half the medium was removed and 10ml of complete medium added. At d 7, complete medium was replaced without disturbing the explants. On d 10, medium and loosely attached explants were removed and fresh medium added. Cells were passaged when dishes were 80% confluent or at 14 d after explant plating. For passaging, cells were lifted using 0.025% trypsin EDTA (1ml/25cm^2 Gibco), explants removed by vacuum filtration (Steriflip 60µm Millipore) and viable cells counted using a microcapillary flow cytometer (Millipore Guava Viacount software and reagent). Cells were replated at 10,000 viable cells/cm^2 in 150cm^2 tissue culture flasks. When cells reached 80-85% confluence, they were lifted, counted and aliquotted into Recovery-Cell Culture Freezing Medium (Gibco) cryovials (Nalgene, 2ml) at a concentration of 2 x 10^6 cells/ml. Cells were frozen -80 º C before transferred to liquid N_2 over the vapor.

Before each experiment, frozen aliquots of PUCs were thawed (37 º C) and transferred to a 15ml conical tube. Warm growth medium was added drop wise. Cells were pelleted by centrifugation (500xg for 10 min). Supernatant was removed and the pellet resuspended in fresh medium and cells replated. When cells reached 80-85% confluence, they were lifted and aliqouted to wells containing the treatment matrix or to untreated control wells.

JMX extraction protocol

JMX was prepared from WJ by extraction in 6M urea and 2M guanidinium hydrocholoride (Gdn-HCl). Porcine umbilical cords were collected and stored in the same manner as for cell except they were stored together in gallon sized bags instead of individually with 20 to 30 cords per bag. Cords were weighed; each end was tied with monofilament and

rinsed in fresh PBS with antibiotic/antimycotic. Cords were placed in trypsin solution (0.25% Gibco) (1ml/g tissue) and incubated (37ºC) for 2h with slight agitation, rinsed with PBS and stored at 4ºC overnight. The next day umbilical cords were placed in collagenase (*Clostridium Histolyticum,* Types I and IV, Sigma) solution (0.5ml/g tissue) and incubated (37ºC) for 30 min with gentle agitation. Cords were rinsed twice with PBS, blood vessels removed and the tissue minced with scissors. Tissue was placed in 50ml conical tubes with PBS and washed by centrifugation (4400 x g for 20 min). WJ was homogenized in 2ml/g high salt buffer (3.4M NaCl), 50mM EDTA, and 10mM N-ethyl-maleimide (NEM pH 7.4 Sigma-Aldrich) using a Tissue Tearor (Biospec Product, Inc). The homogenate was centrifuged in round bottom polypropylene tubes (Nalgene) for 15 minutes (12,000 x g, 4ºC). The supernatant was removed and discarded leaving a well-defined pellet. The pellet was extracted for 48 h by gentle stirring at 4ºC in 0.5ml/g of urea/Gdn-HCl extraction buffer, (6M urea, 2M Gdn-HCl, 50mM Tris-HCl, 20mM EDTA, 10mM NEM (pH 7.4) and 2mM dithiothretiol (DTT). Following extraction, the pellet was vortexed and the extract centrifuged (30 min, 24,000 x g using a chilled rotor). Tubes were placed on ice and the supernatant poured into a sterile 15 ml polypropylene centrifuge tube. The supernatant was dialyzed (MW cutoff 3,500; Fisher Scientific) against three changes (24 h each) of Tris-buffered saline (0.15M NaCl, 50mM Tris-HCl, 20mM EDTA, and 10mM NEM at pH 7.4; 1L) at 4ºC on a stir plate protected from light, followed by sequential dialysis against 0.5% chloroform and cell culture medium (DMEM + Glutamax without phenol red, Gibco). After dialysis, the supernatant was place in a new 15ml polypropylene tube and centrifuged (4000 x g for 15 min). The remaining supernatant was filtered through 20 µm (Steriflip Millipore) and 5µm (syringe filters, Millex Millipore) and concentrated to a protein concentration of approximately 4mg/ml using an Amicon-Ultra (Millipore) concentrator (MW

cut off 10,000 Daltons) by centrifugation (20 min 5000 x g). The initial filtrate of the concentration was retained (frozen) for use in future experiments. Protein concentrations were determined using the Pierce 660nm Protein Assay with NanoDrop. Aliquots were frozen and stored (-20ºC) until use.

RNA extraction

RNA extraction was performed using Qiagen RNeasy Micro kit, according to the manufacturer's instructions. A DNase digest (RNase-free DNase Set, Qiagen) was performed to remove genomic DNA. Columns were eluted twice with 20µl 50º C nuclease-free water for a final volume of 16µl. RNA quantity was analyzed with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific), and RNA quality was checked using the Agilent 2100 Bioanalyzer with the Agilent 6000 Nano kit at the Kansas State University's Center of Biomedical Research Excellence (COBRE).

Quantitative Real Time PCR (rtPCR)

RNA underwent reverse transcription (10ng/µl of RNA) using Applied Biosystems TaqMan RT reagents in a total reaction volume of 30µl and cDNA stored (-20 º C) until analysis.

The PUC cDNA was subjected to quantitative RT-PCR using Nanog, Sox-2, Oct-4 and 18S ribosomal subunit primers. Primer sequences (Invitrogen) were as follows: Oct4 FWD: 5'-AGAAGGATGTGGTCCGCGT-3'. Oct4 REV: 5'- ACTGCTTGATCGTTTGCCC-3'. Sox2 FWD: 5'- TTCCATGGGCTCAGTGGTCAA-3'. Sox2 REV: 5'-TGGAGTGGGAAGAAGAGGTAAC-3'. Nanog FWD: 5'-CCCGGGCTTCTATTCCTACCA-3'. Nanog REV: 5'-TACCCCACACGGGCAGGTT-3'. The housekeeping gene used for all experiments was 18s. The primer sequences for 18s were FWD: 5'-GAGGTTCGAAGACGATCAGA-3'. 18s REV: 5'- TCGCTCCACCAACTAAGAAC-3'.

The reactions were assembled using 2µl of cDNA, 18µl of power SYBR green master mix (Applied Biosystems, Rostercity, CA), 100nM each of FWD and REV primers to make a 20 µl reaction per well in a 96 well plate. Relative expressions of Nanog, Oct-4, and Sox-2 were normalized to the 18s mRNA endogenous control.

Each of the 12 real time plates included swine testes cDNA to evaluate assay variation. The within experiment CV for the three genes were: Oct4 4.76%, Nanog 6.8% and Sox2 6.4%.

For experiment 1a rtPCR was scanned using 7500 fast Applied Biosystems. Experiments 1b and 4 were analyzed with an Eppendorf Realplex. Threshold values per gene varied among plates, due to the variation of all values, averages per gene were made to allow comparisons between rtPCR plates. Samples that had a standard deviation higher than 0.5 between triplicates were re-run with newly transcribed cDNA.

Exp. 1: Effects of a thin coating on JMX on PUCs cultured for 3d (1a) or 4d (1b)

Cells from three lines at passage 3 were plated on (12-well plates/cell line). One plate provided cells for counting and the other for RNA harvest. RNA plates had 3 control wells with no coating and 6 wells (2 wells/replicate) with thin JMX coating. The plate for cell counting included 2 control and 3 JMX wells. For the thin coating was prepared by pipetting 100µl of JMX (batch 07/2012) evenly over the well and incubated at 38.5 ºC to allow gelling. After 1h nongelled liquid was removed and 40 x 10^3 viable cells added in 1ml of growth medium and cultured (38.5°C/5% CO_2).

After 3 d (Exp 1a) or 4 d (Exp 1b) cells and RNA were harvested. Cell numbers were determined using the Viacount Flex reagent (Millipore) and the Guava Viacount procedure. Morphology was evaluated by using Hoffman optics.

For RNA extraction the plate was placed on ice, and culture medium replaced with 1ml of ice cold PBS. Cells in each well were lysed by removing the PBS and adding 350µl of Buffer RLT (Qiagen) with β-mercaptoethanol (10µl/ml, Sigma-Aldrich). Cells were pipetted up and down and placed in a microcentrifuge tube, vortexed and placed on ice. Lysed samples were frozen (-80 ºC) until RNA was extracted.

Preliminary evaluation of adding collagen to JMX

Batches of JMX differed in their gelling characteristics. JMX extracted in February and April 2013 was concentrated to a protein concentration of greater than 12mg/ml, but gelling was minimal. To enhance gelling we investigated the effects of adding bovine collagen type I (Gibco; 0.5mg/ml) at a ratio 1:5 to JMX (collagen:JMX). Initially a PUC isolate was cultured with both JMX batches. In a 24 well plate, each of four treatments and a control was plated in a single well (Table 1). Treatments 1 and 2 evaluated a thick coating with the cells either plated on top of the matrix or mixed within the matrix before it gelled. Treatments 3 and 4 considered a thin coating of the well (100 µl of JMX) that was allowed to gel for 60min (trt3) or 10min (trt4) before the cells were plated. For the control the PUCs were grown on plastic. For trt 1 and 2, the serum in the culture medium was doubled on the theory that the entire well contents constituted the culture volume and the JMX contributed half the volume. All wells were plated with 20×10^3 viable cells.

Table 2.A Preliminary evaluation of JMX with added collagen.

Trt	Description	Medium volume/ serum%	Preparation of the culture well
1	Thick layered +C	250 ul/40%	250 ul JMX with collagen incubated for 1 h (air bubbles removed with a needle), cells plated in 40% growth medium on the JMX + collagen
2	Thick mixed +C	250 ul/ 40%	Cells added to the JMX + collagen, incubated 1 h then growth medium with 40% serum was added
3	Thin60 +C	900 ul/ 20%	100 ul JMX +collagen incubated 1 h and cells added
4	Thin10 +C	900 ul/ 20%	100 ul JMX +collagen incubated 1h at rt/non-gelled removed, cells plated
5	Control	1,000 ul/ 20%	None

On d4, cells from all treatments with April 2013 JMX and February JMX treatment 4 wells were lifted and counted. February JMX treatments 1, 2 and 3 were cultured an additional day to determine optimal growth for isolates. A modified method was used to harvest the cells in Treatments 1, 2 and 3 to disperse JMX. In these wells the JMX and medium was pipetted out of the wells, centrifuged, medium removed and the JMX digested in collagenase/hyaluronidase solution [collagenase (25u/mg Type 1, Worthington), hyaluronidase (373u/ml from sheep testes, Sigma-Alrich) in Dulbecco's Phosphate Buffered Saline 1X (DPBS) with $CaCl_2$ and $MgCl_2$]. Digestion was for 20 min in 1ml of enzyme solution at room temperature on a GyroMini Nutating mixer (Labnet International, Inc). Some cells remained attached in the culture wells and they were removed with trypsin solution and added to those digested from the JMX. All single cell suspensions were resuspended in a total volume of 200 µl in PBS and counted in a 96 well plate at a 1:10 dilution on Guava Viacount.

Exp. 2: Growth of PUCs on JMX Thin10 + C and Thick layered + C

After reviewing the preliminary experiment we selected the thin10 +C and thick layered +C for further evaluation. Exp. 3 evaluated cell responses to JMX when added collagen (0.5 mg/ml) was added to JMX because it consistently provided a firm gel. The JMX (batch 02/2013) collagen mixture (1:5 collagen to JMX) was added as a 100 µl drop to wells of 12 well plates and a pipet tip used to spread the mixture over the bottom of the wells so the surface was completely covered. Plates were incubated at room temperature for 10 min. before PUCs were added (10^4 viable cells/cm^2) in 1 ml of growth medium. Three wells of Thin10 + C were plated for Guava Viacount determination of cell number and 6 wells for RNA extraction. For RNA extraction the cells from two wells were pooled, giving 3 determinations for RT-PCR estimates of gene expression.

The thick layered + C, JMX was prepared as a batch (14mL) and 500µL was pipetted into each well. After one hour incubation (38.5°C), cells were added by gentle pipetting (40 x 10^3 viable cells in approximately 15µL of growth medium) and an additional 500 µL of growth medium containing 40% FBS was added. The serum concentration was increased to compensate for the total volume of medium and JMX. After culture the gels were digested for cell harvest using the protocol described in the preliminary trial. Cell counts for Thick layered + C used Guava Easy Fit counts to determine viable and total cells in the Viacount system.

At the beginning of the experiment an aliquot of cells (80 x10^3 viable) from each isolate was lysed and assayed to provide a measurement of gene expression at the beginning of the experiment and this was used to evaluate the real-time PCR results. Control wells (n=5) were plated with no additions to the wells and 2 wells were used for determination of cell numbers and three wells for RNA extraction. The cultures were continued for 4d.

Exp. 3: PUCs cultured on Thick mixed + C

Exp. 3 evaluated the thick mixed +C treatment from our prelimary research compared to cells grown on plastic (control) and used the same cell isolate used in Exp. 2. Cells for each well were mixed by pipetting with JMX and Collagen (500 µl total) in individual microcentrifuge tubes. After mixing in the cells the Thick mixed + C it was pipetted gently into the wells to reduce the formation of air bubbles. Both control and Thick mixed + C were plated at twice the cell density (20 x 10^3 cells/cm^2) used in previous experiments. Following 1h incubation time at 38.5 ° C to allow the gel to set, 500 µl of 40% complete medium was added to each thick mixed +C well.

Cells were allowed to grow for 3d at which time controls were 100% confluent. Gels were digested in a collagenase/hyaluronidase solution at 37° C with trituration until the gel was no longer visible. This required < 20 min. Cells were also found attached to the plastic in these wells. Trypsin solution was used to harvest cells in treatment and control wells. Guava Viacount reagent (1:10 dilution) was used for cell counts. Gel for RNA extraction was enzymatically dissolved as described above before Buffer RLT was added to lyse the cells.

Exp 4: Evaluation of PUCs cultured on Thin10 + C and Thin60 + C

Exp. 4 compared a thin coating (Thin10 + C) and a thin gel (Thin60 + C). JMX (batch April 2013) plus collagen was pipetted into all JMX wells (200µl). Thin60 + C wells were incubated 1h at 38.5°C and thin10 + C was incubated for 10min at room temperature and then non-gelled JMX was aspirated, leaving a thin coating. Cells were plated at 40 x 10^3 viable cells per well (10 x10^3 per cm^2) and growth medium (1000 µL) was added to each well.

Plates were incubated 4d at which time control wells were 100% confluent. Control and Thin10 + C were lifted for cell counts with trypsin solution. For Thin60 + C, the gel removed

from the well and digested in the manner described for Exp. 2 and 3, then cells left in the well were harvested with trypsin solution. Cells were counted using Guava Viacount diluted 1:10 with Viacount reagent. Control cells were used to set thresholds.

Wells grown for RNA were placed on ice and media aspirated, 1 ml ice cold PBS was added to wash cells free and 350 µl of Buffer RLT with β-mercaptoethanol added. The Thin60 + C gel required trituration to break down the gel. All samples were vortexed and stored -80 °C until RNA extraction.

Statistical Analysis

Cell count data was analyzed using the GLIMMIX Procedure and gene expression data was analyzed by Mixed Procedure of SAS (SAS, 2000 and 2006; SAS Inst. Inc., the Satterthwaite degrees of freedom). Significance was declared at $P < 0.05$. Means were compared by using Least Squares Differences procedure.

Results

Experiment 1a

Growth and cell morphology were similar between cells grown on the thin coating of non-gelling JMX and control wells. Cells had a fibroblast-like morphology for both treatments. After 3 days of culture the cells were counted in single cell suspension on Guava using Viacount. Wells containing JMX had more ($P = 0.05$) viable and tended ($P < 0.07$) to have more total cells compared to control wells (Table 2.1).

Transcription factors Oct4, Nanog and Sox2 mRNAs were expressed relative to the housekeeping gene and the expression before the experiment as determined by the expression in an aliquot of each cell isolate prior to culture delta delta CT (ΔΔCT). Nanog expression

decreased in JMX and significantly different between cells plated on JMX and plastic ($P < 0.05$). Oct4 and Sox2 expression was not ($P > 0.10$) affected by culture treatment (Table 2.2).

Experiment 1b

Exp. 1b evaluated the treatments after an additional day of growth as compared to Exp 1a. Again, fibroblast-like morphology was seen with both treatments. An increase ($P < 0.05$) in both viable and total cells was observed for the JMX treated wells (Table 2.3). Expression of the transcription factors was not ($P > 0.10$) affected by treatments (Table 2.4)

Experiment 2

We conducted preliminary trials in which we added collagen to JMX (results in the appendix A.1-A.3). These results indicated that growth rates were increased by the JMX +collagen (+C) products. In particular adding collagen and allowing 60 min for gelling before aspirating the non-gelled fraction (Thin60 +C) resulted in over double the number of viable and total cells at the end of culture compared to cells grown on plastic. A thick layered JMX produced a similar amount of viable cells as the control, but had a higher total cell count. An interesting result was that mixing cells with the thick JMX (Thick mixed) there were 0.35×10^4 viable cells, but 1.54×10^4 total cells (Table A.1). Therefore the percent viable was less that 25%.

In Exp. 2 we evaluated a thin coating allowed to gel for 10min (Thin10 +C) and a thick coating of JMX compared to control wells on plastic only. Morphology (Figure 1) and cell growth (Table 2.5) differences were seen for the Thick layered + C and Thin 10 + C treatments compared to the control cells. Cells grown Thick layered + C formed clumps with extensions. Thin 10 + C treatment wells had cells clump together and had long, thin appendages forming which were longer than individual PUCs.

The protocol for experiment 2, called for a five day growth period, but our controls grew too quickly for this and had wells with 100% confluence. Because all isolates needed to be lifted on the same day of growth, 2 of the isolates weren't as confluent. This did reflect with their cell counts in Viacount (Table 2.5).

No ($P > 0.10$) differences in cells per well at the end of culture were observed between control and Thin10 +C cells. For total cells, there was a trend with Thick layered +C tending ($P < 0.10$) to have fewer cells at the end of culture than control and Thin10 +C wells. For viable cells, a decrease ($P < 0.05$) was observed for wells with Thick layered + C compared to controls and there was a trend ($P < 0.10$) for Thin10+ C wells to have more cells than Thick layered + C. No significant differences were seen in viable cell counts between control and Thin10 + C cells ($P>0.10$).

When extracting RNA from our lysed cells there was a yellow tint tin the thick layered + C tubes. RNA extracted was less than 20 ng/µl. We tried adding more time for elution and heating the columns (50 °C) as recommended by the manufacturer but the RNA we obtained was completely degraded.

Experiment 3

The Thick mixed + C gels shrank to a very small disc over the 3 days of culture. Some of the PUCs in Thick mixed +C cells wells were attached to the plastic had fibroblast like shape while those in the gel grew in clumps with thin appendages similar to thin coating gels (Figure 2C). Cells found in the gels were hard to see individually because they were in a close proximity to each other and the surrounding gel inhibited visualization. Viable and total cells in Thick mixed +C wells were numerically but not significantly ($P > 0.10$) less than control wells (Table 2.6).

Experiment 3 treatment groups were seeded with 20,000 cells/cm^2: double what was plated in earlier experiments. The cells reached 100% confluence after 3 days. The gels in the Thick mixed +C wells contracted and were very small and the cells were difficult to harvest (Figure 2B). Attempts to harvest RNA resulted in a lyses buffer RLT that was tinted yellow and had low RNA content; therefore, only morphology and cell count data were collected.

Experiment 4

This experiment considered two thin coatings and morphology differences between Thin10 + C and Thin60 + C were seen on the periphery of the Thin60 + C wells. Cells on Thin60 + C developed a thick string- like appearance in part of the well. Similarly, the two treatments had cell clumps and thin appendages protruding from the cell clumps (Figure 3).

Neither viable nor total cell counts (Table 2.7) were affected by treatment ($P > 0.10$). Oct4 and Nanog expression was not ($P > 0.10$) affected by the treatments (Table 2.8). However, Sox2 expression was increased in Thin60 + C compared to control cells ($P < 0.05$) and there was a trend in expression of Sox2 in Thin10 + C wells to be less than Thin60 + C wells ($P < 0.10$). No ($P > 0.10$) differences of gene expression were seen between control and Thin10 + C treatment for Sox2.

Discussion

PUCs and WJ cells from other species are MSCs that are easy to collect, provide large populations and in humans have no ethical implications (Mitchell et al., 2003, Le Blanc and Pittenger 2005; Carlin et al., 2006). MSCs expand readily after isolation when grown on standard cell-culture plastic surfaces but there is concern that undifferentiated MSCs are diluted by cells undergoing spontaneous differentiation as passage number increases (Chen et al., 2010). The aim

of the present work was to evaluate the effects of the matrix components of the umbilical cord on some aspects of PUCs soon after isolation.

There are reasons to consider MSCs derived from WJ to be more undifferentiated than those derived from adult tissues (Fong et al., 2011). Importantly WJ-MSCs express markers identifies in early embryos including Oct-4, Nanog, and Sox2 (Carlin et al., 2006, La Rocca et al., 2009, Karahuseyinoglu et al., 2007, Kim et al., 2011). Therefore we evaluated the effects of JMX on expression of these transcription factors in PUCs. Here we report evaluation of components retained by filtration concentrators that have a nominal MW cutoff of 9,000 Daltons. While it is likely there are smaller molecules bound to the matrix components retained by the filter it is expected that many smaller molecules were filtered and this material has been retained for studies in progress to evaluate its effects.

We designed out studies to evaluate mRNA expression and plated cells at a density over twice that routinely used for cell expansion to assure adequate harvest of adequate RNA. We expected that these higher seeding rates would limit the potential for expanding cell numbers but we report cell numbers at the end of culture in case the different culture environments have effects on cell growth at these higher densities.

Earlier work in our lab evaluated an extract made from WJ (Bryan, 2008). Here we have developed a bulk WJ harvesting method using enzymatic digestion to replace the manual separation of WJ from the serosal covering of the cord. Since all biomolecules of the niche are not know, it seems that an extract of WJ could have unique effects on cells compared to hydrogels or single component coating methods often used with other cells (Lin et al., 2012).

For experiments in this thesis we encountered problems getting the JMX to form a firm gel. In more recent studies we deduced this was because the pore size (5μm) of the filter used in

the later stages of preparing JMX was retaining some collagen that was assembled in higher order structures. Therefore in Exp. 1a and 1b, JMX did not gel, even though it was concentrated to a protein content of 4mg/ml, the protein concentration usually considered adequate for gelling in our earlier work (Bryan, 2008).

In experiment 1a, the growth period was 3 days and cell number at the end of culture was not affected by culture environment. However, Nanog expression was decreased by JMX. Gene expression differences were not observed in experiment 1b which extended the culture period to 4 days. Plating on JMX resulted in an increase in both viable and total cells compared to the controls.

Experiments 2-4 used batches of JMX (February and April 2013) that had greater than 12 mg/ml protein. Still this product did not create a thick gel in culture. Gelling was obtained by the addition of 0.5 mg/ml of collagen. The thick layers of this product resulted in changes in the morphology of the cell populations with clumping in areas that what we believe to be multiple cells congregated together. However we often were unable to get cells out of these gels and the cells we did harvest were mostly dead. Perhaps this was due to damage in attempts to remove them from the JMX.

For these reasons we focused on producing thin coatings for growing the PUCs. A problem encountered with Thin60 +C gels was that it produced a thick gel around the edges and the thin gel in the center of the well perhaps producing a heterogeneous environment for the cells. The Thin10 +C was perhaps the most consistent culture environment to produce. Thin60 + C had significantly greater gene expression of Sox2 compared to the control group, while viable and total cell counts were not affected by the treatments.

In summary, we evaluated culturing PUCs on and within JMX. An increase in cell growth was observed when using a 4 day growth period with JMX and Sox2 expression was increased when cells are cultured on Thin60 +C. At the beginning of this work we hypothesized that a JMX would provide an environment similar to that provided by WJ in the umbilical cord and that this environment would keep cells less differentiated. We further hypothesized that the more primitive cells would express more of the embryonic transcription factors and their morphology would be changed. It might also be anticipated that if the JMX provides an environment like the stem cell niche in other tissues the more primitive cells would have a slower rate of cell division. These hypotheses were not supported by our results, which although not entirely consistent suggest that JMX increase cell proliferation, at least in the thin coatings, and only inconsistently affected the transcription factors.

The studies reported here are beginning attempts to develop an approximation of the WJ environment in the umbilical cord. We suggest further research with JMX products for which the final filtration is eliminated would produce gelling products that might more similarly approximate the *in vitro* environment provided by WJ. It may also be important to add back the filtrate from the initial cell concentration as it likely contains smaller proteins and peptides that are a part of the *in vivo* environment.

Table 2.B Viable and total cells for porcine umbilical cord matrix stem cells grown 3d on a thin coating of non-gelling jelly matrix (JMX) or plastic (Exp. La).

Viable cells			
Treatment	**No.** [a]	**SE**	**P <**
Control	**1.39**	**0.23**	**0.05**
JMX	**1.83**		
Total cells			
Control	**1.58**	**0.22**	**0.07**
JMX	**1.98**		

[a] Data are number of cells x 10^4 as determined by Guava Viacount.

Table 2.C Gene expression by porcine umbilical cord matrix stem cells grown 3d on a thin coating of non-gelling jelly matrix (JMX) or plastic (Exp. la).

Gene	Treatment	ΔΔ CT[a]	SE	P <
Oct-4	Control	-1.15	1.03	0.16
	JMX	-0.67		
Nanog	Control	-0.73	0.59	0.03
	JMX	0.34		
Sox2	Control	0.53	1.33	0.96
	JMX	0.56		

[a] Data are real-time PCR. Delta delta CT was calculated by dividing by the gene of interest by the 18s and then by the delta CT for RNA harvested from each isolate of cells before culture.

Table 2.D Viable and total cells after 4d culture of porcine umbilical cord matrix stem cells on a thin coating ofnon-gelling jelly matrix (JMX) or plastic (Exp. 1b).

Viable cells			
Treatment	**No.** [a]	**SE**	**P <**
Control	**1.82**	**0.39**	**0.02**
JMX	**2.06**		
Total cells			
Control	**1.91**	**0.40**	**0.02**
JMX	**2.18**		

[a] Data are number of cells x 10^4 as determined by Guava Viacount.

Table 2.E Gene expression for porcine umbilical cord matrix stem cells grown on a thin coating of non-gelling jelly matrix (JMX) or plastic (Exp. 1b).

Gene	Treatment	ΔΔ CT[a]	SE	P <
Oct-4	Control	-0.13	0.67	0.40
	JMX	-1.02		
Nanog	Control	-0.61	0.92	0.97
	JMX	-0.60		
Sox2	Control	-0.25	0.75	0.39
	JMX	-0.54		

[a] Data are real-time PCR. Delta delta CT was calculated by dividing by the gene of interest by the 18s and then by the delta CT for RNA harvested from each isolate of cells before culture.

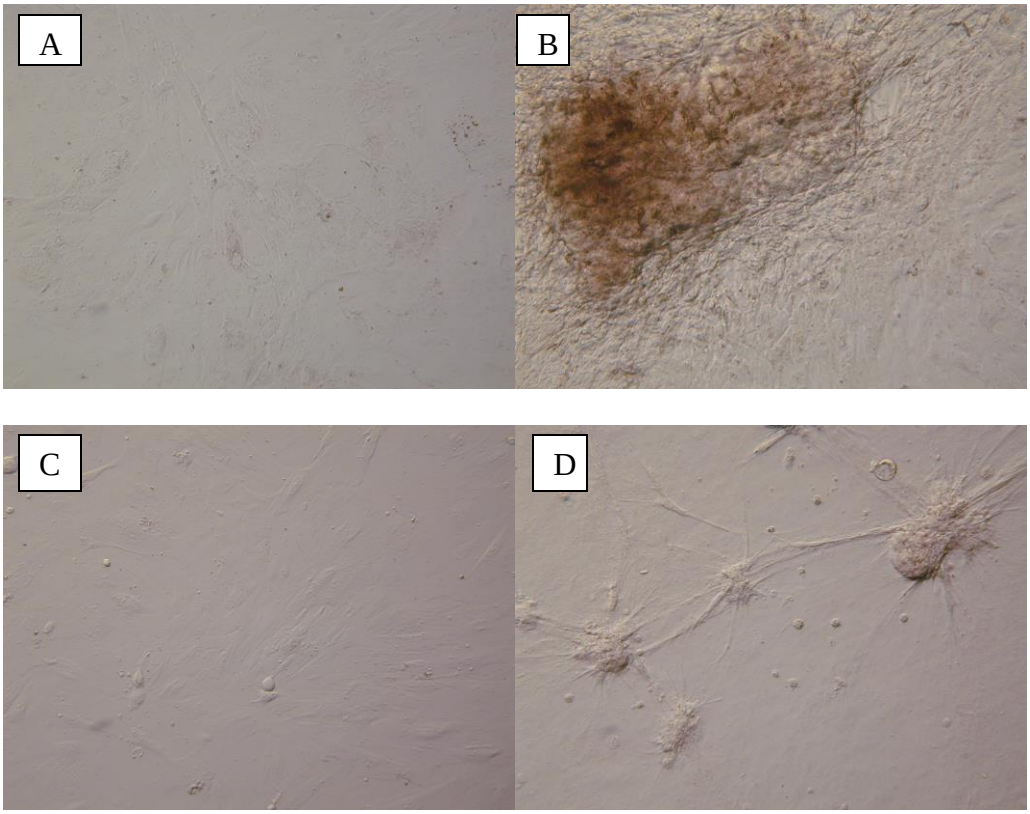

Figure 2.1. Morphology of PUCs grown on different treatments (Exp. 2). Control fibroblast-like morphology (A), Thin10 +C cell colonies (B). Control fibroblast-like morphology (C), Thick layered +C cell colonies with cell extensions (D).

Table 2.F. Viable and total cell counts for porcine umbilical cord matrix stem cells grown on jelly matrix (JMX) Thin10 +C, Thick layered +C or plastic (Exp. 2).

Viable Cells			P <		
Treatment	No. [a]	SE	Con. vs Thin10	Con. Vs Thick layered	Thin10 vs. Thick layered
Con. [b]	1.90	0.33			
Thin10	1.63		0.59		
Thick Layered	0.52			0.04	0.07
Total Cells			**P <**		
Con.	2.00	0.29			
Thin10	1.91		0.84		
Thick Layered	0.87			0.05	0.07

[a] Data are number of cells x 10^4 as determined by Guava Viacount and Easy fit.

[b] Control.

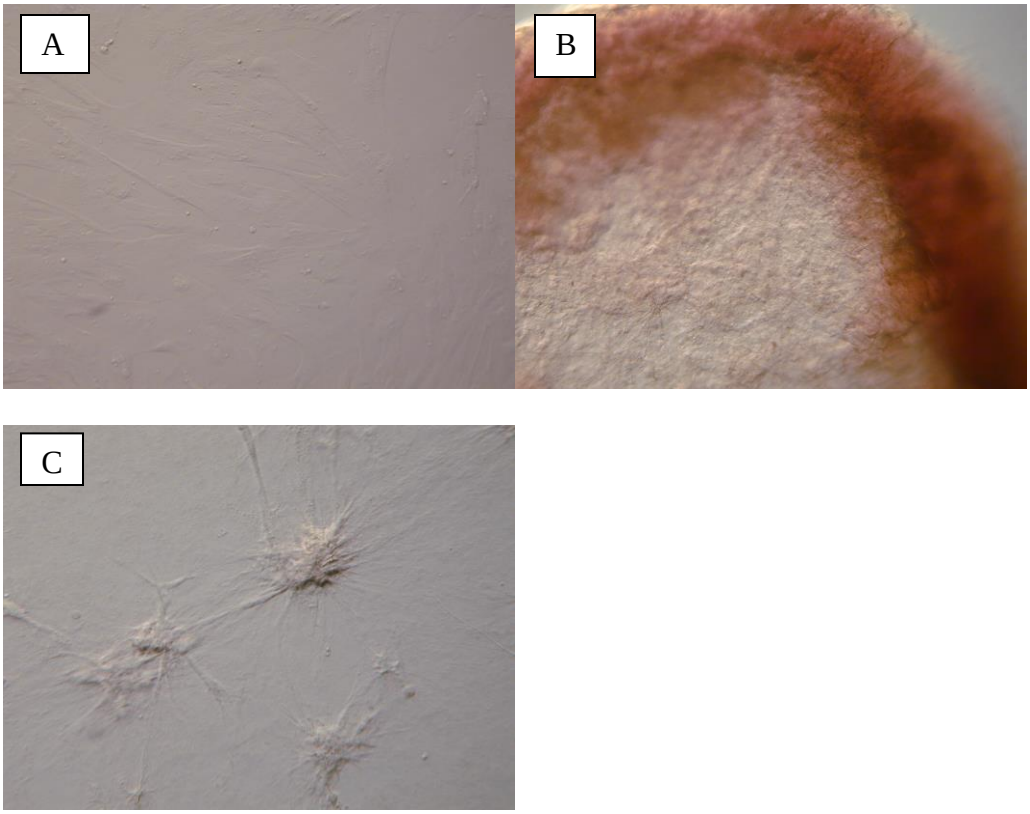

Figure 2.2 . Experiment 3 morphology. Control fibroblast-like morphology (A), Thick mixed +C gel edge (B) and Thick mixed +C PUCs on plastic cell colonies with cell extensions in between (C).

Table 2.G. Viable and total cells after 4d culture of porcine umbilical cord matrix stem cells grown on Thick Mixed +C or plastic (Exp. 3).

Viable cells			
Treatment	**No.** [a]	**SE**	**P <**
Control	**2.14**	**0.36**	**0.32**
Thick Mixed	**1.49**		
Total cells			
Control	**2.18**	**0.34**	**0.56**
Thick Mixed	**1.88**		

[a] Data are number of cells x 10^4 as determined by Guava Viacount and Guava Easy fit.

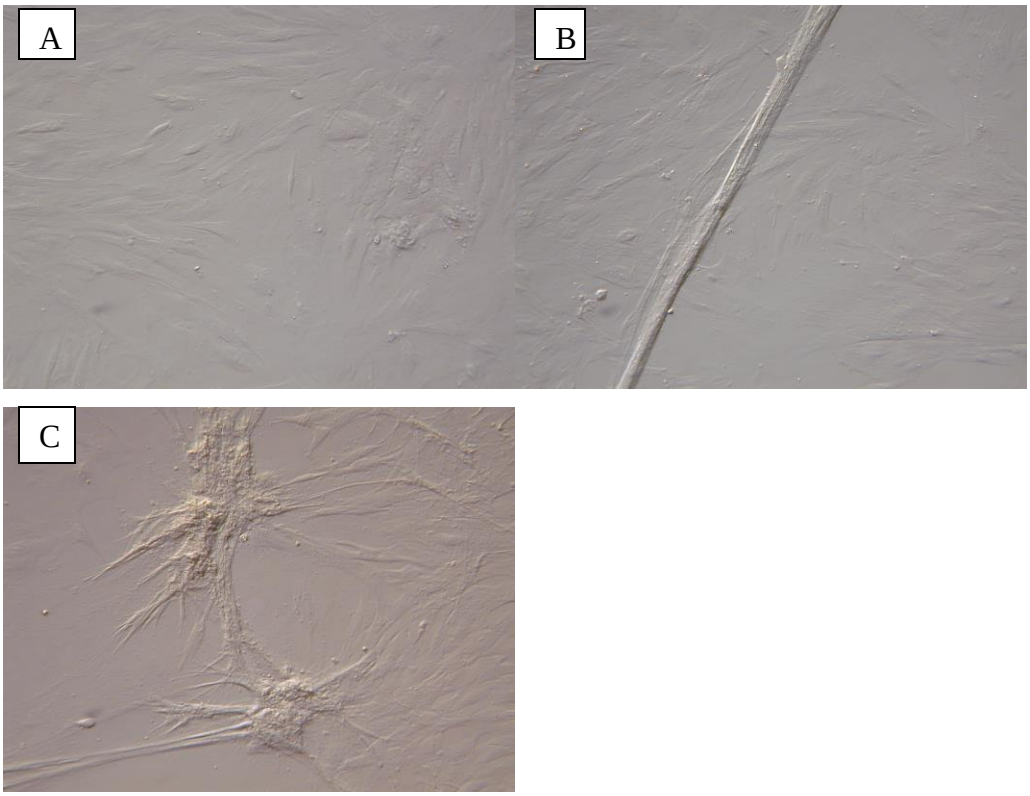

Figure 2.3. Morphology of cells in experiment 4. Control fibroblast-like morphology (A), Thin10 +C thin cell extensions (B) and Thin60 +C cell colonies around edge of well that have cell extensions (C).

Table 2.H. Viable and total cells after 4d culture of porcine umbilical cord matrix stem cells grown on Thin10 +C, Thin60 +C or plastic (Exp. 4).

Viable Cells			P <		
Treatment	No. [a]	SE	Con. vs Thin10	Con. Vs Thin60	Thin10 vs. Thin60
Con. [b]	2.15	0.29			
Thin10	2.04		0.69		
Thin60	1.97			0.51	0.79
Total Cells			P <		
Con.	2.31	0.30			
Thin10	2.33		0.95		
Thin60	2.17			0.59	0.55

[a] Data are number of cells x 10^4 as determined by Guava Viacount.

[b] Control.

Table 2.I Gene expression for porcine umbilical cord matrix stem cells grown on Thin10 +C, Thin60 +C or plastic (Exp. 4).

Gene	Treatment	ΔΔ CT[a]	SE	Thin10 vs Con. P<	Thin60 Vs Con. P<	Thin10 vs Thin60 P<
Oct-4	Con.[b]	0.21	0.36			
	Thin10	0.72		0.37		
	Thin60	1.02			0.18	0.57
Nanog	Con.	0.01	0.50			
	Thin10	0.14		0.87		
	Thin60	1.25			0.15	0.19
Sox2	Con.	-1.15	0.56			
	Thin10	-1.75		0.24		
	Thin60	-2.78			0.02	0.07

[a] Data are real-time PCR. Delta delta CT was calculated by dividing by the gene of interest by the 18s and then by the delta CT for RNA harvested from each isolate of cells before culture.

[b] Control.

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Appendix A - Preliminary Counts and Cell Isolates

Table A. 1 Viacount Results of April JMX 3 day.

Treatment	Viable cells	Total Cells
Control	1.42×10^4	1.63×10^4
Thin60 +C	2.88×10^4	3.44×10^4
Thick Layered +C	1.33×10^4	1.61×10^4
Thick Mixed +C	1.27×10^4	1.58×10^4
Thin10 +C	3.02×10^4	3.44×10^4

Table A. 2 Viacount Results of February JMX 3 day, Thin10 +C and 4 day, all other treatments.

Treatment	Viable cells	Total Cells
Control	2.42×10^4	2.7×10^4
Thin60 +C	5.4×10^4	5.7×10^4
Thick Layered +C	2.6×10^4	3.26×10^4
Thick Mixed +C	0.35×10^4	1.54×10^4
Thin10 +C	2.76×10^4	3.06×10^4

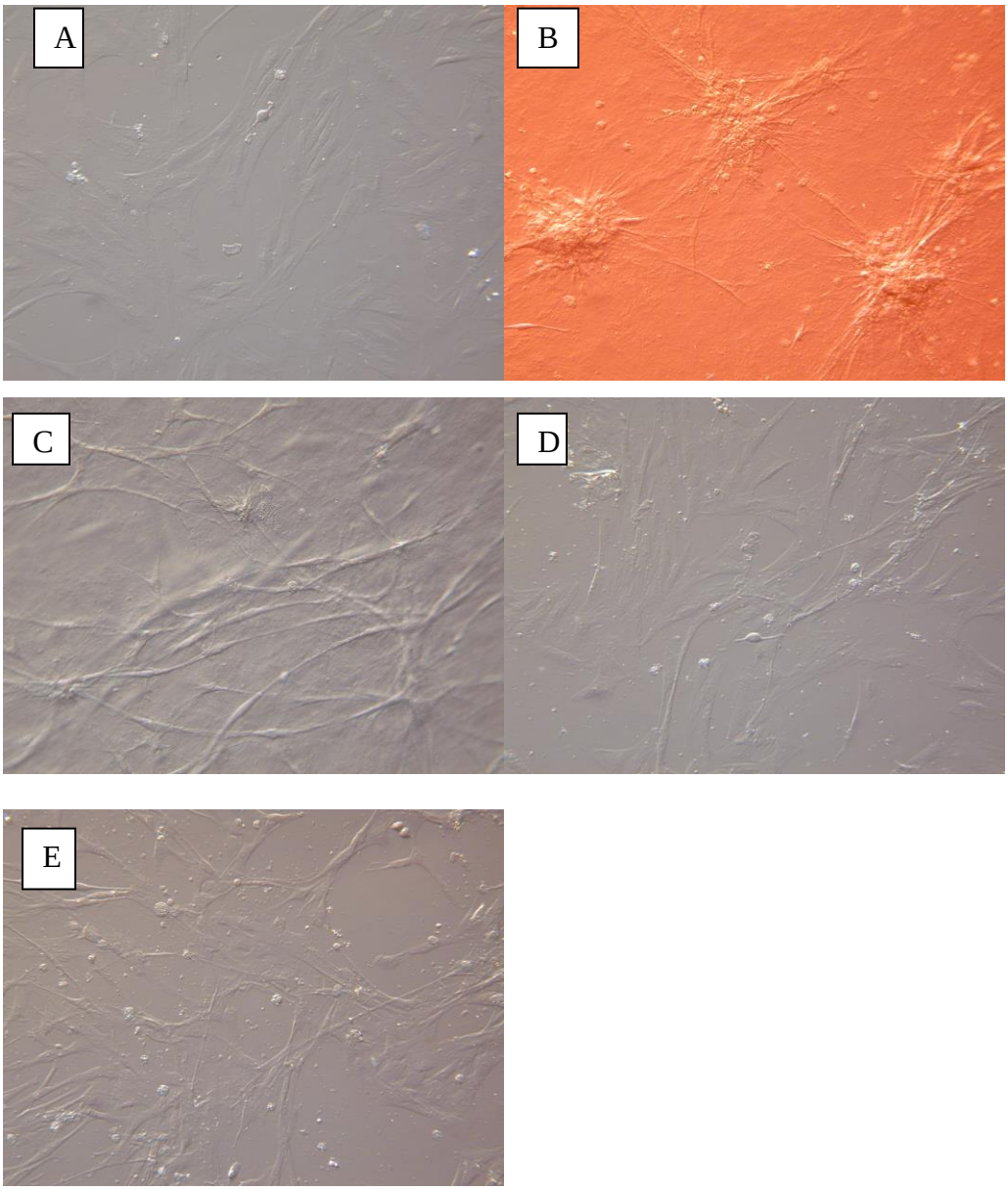

Figure A. 1 Morphology of Cells on JMX in Prelimary experiment. Control fibroblast-like morphology (A), Thick Mixed colonies with cell extensions (B), Thick layered cell extensions (C), Thin10 fibroblast-like with cell extensions (D) and Thin60 fibroblast-like with cell extensions (E).

Table A. 3Cell Isolates used for experiments 1a and 1b.

Sow ID	Sex	Passage Number	Date Processed
Y62	Female	3	09/15/12
Y188	Female	3	10/19/12
Pur155	Male	3	12/29/12

Table A. 4 Cell Isolates used for experiments 2-4

Sow ID	Sex	Passage Number	Date Processed
Pur51	Female	3	06/16/11
Y166	Female	3	08/26/11
Pur2	Male	3	08/26/11
Pur87	Male	3	12/29/12