EFFECTS OF EXTRACELLULAR MATRICES ON PORCINE UMBILICAL CORD MATRIX STEM CELLS

by

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Abstract

The three transcription factors, Nanog, Oct-4 and Sox-2, are central regulators of pluripotency in embryonic stem cells. Porcine umbilical cord (PUC) matrix stem cells also express these transcription factors. Wharton's jelly is composed of an extracellular matrix high in hyaluronic acid and various collagens and serves as a reservoir for several growth factors and cytokines. We expect that Wharton's jelly includes a stem cell niche that provides a microenvironment that maintains and supports the stem-cell characteristics of PUCs. The mechanisms by which the PUCs remain primitive within the Wharton's jelly are unknown.

We developed methods for producing an extracellular matrix product extracted from porcine Wharton's jelly that we named Pormatrix (PMX). When PMX is incubated at 37°C, it becomes a matrical gel that provides a matrix allowing PUC attachment and growth. Concentrating the protein in PMX by filtration provides a low molecular weight by-product which we refer to as flow through (FT). In Experiment 1, PUCs were seeded on Pormatrix, Matrigel or plastic substrates in the presence or absence of FT. PUCs cultured on Matrigel, Matrigel+FT, Plastic+FT and PMX had higher expression of Nanog compared to PUCs cultured on PMX+FT (P-value <0.05).

In Experiment 2, the PMX and Matrigel were diluted to low protein concentrations (1.2-1.5 mg/ml protein) so that gelling did not occur. Adding FT to PMX, Matrigel and plastic increased gene expression of Nanog 2.78 fold compared to treatments without FT ($P = 0.10$). Sox-2 expression was increased by adding FT to Matrigel but adding FT to the other matrix proteins had no effect resulting in a tendency for a matrix*FT interaction($P=0.10$). The transcription factor Oct-4 remained unchanged regardless of treatment.

To evaluate the effects of in vitro maintenance on Nanog, Oct-4 and Sox-2 we measured the relative gene expression in PUCs over the first six passages in vitro. Nanog, Oct-4 and Sox-2 did not differ over these passages. This may indicate that during

the first six passages in vitro, PUCs remain relatively primitive. In summary, we prepared an extract from Wharton's jelly from porcine umbilical cords. The extract supported PUC attachment and growth and appeared to regulate gene expression. Perhaps with further investigation the interactions of PUCs with their in vivo environment can be elucidated.

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CHAPTER 1 - A Literature Review

Components of the ECM

Mesenchymal cells are embedded in an extracellular matrix that is composed of secreted proteins and polysaccharides. The extracellular matrix (ECM) is responsible for filling in the spaces between those cells as well as binding cells and tissues together. Epithelial cells secrete an extracellular matrix termed basal lamina. Basal lamina can support epithelial cells, adipose cells, muscle cells and peripheral nerves. The types of connective tissues comprised of extracellular matrix include bone, tendon and cartilage (Cooper 2000).The ECM is composed of tough fibrous proteins rooted in a gel-like polysaccharide ground substance. The major structural protein of the ECM is collagen, specifically type I or type IV for the basal lamina. Collagen is the single most abundant protein in animal tissues constituting about 25% of total protein in animals. The structure of collagen includes a triple helix in which three polypeptide chains (alpha chains) are wound tightly around each other. The three amino acids, glycine, proline, hydroxyproline are a combination that stabilize the polypeptide chains to form the triple helix of collagen (Cooper 2000). Proline has a ring structure that stabilizes the helical conformation and glycine is regularly spaced at every third residue throughout the central region of the alpha chain. Glycine is the smallest amino acid and its spatial arrangement on the polypeptide chain allows the three helical chains to pack tightly together into the "super helix" (Cooper 2000). It has been observed that the microfibrils of the super helix have a right handed super-twist that allows the collagen to interdigitate with neighboring microfibrils (Vakonakis 2007). There are at least 25 different members of the collagen family with over 90% of the collagen in the body being types I, II, III and IV with the most abundant being type 1 found within skin and bone.

Glycosaminoglycans, or GAGs, create a gel where fibrous structural proteins reside (Cooper 2000). GAGs are polysaccharides that consist of repeating units of

disaccharides of hexosamines and hexuronic acid (Jones et al., 1982). Keratin sulfate is a GAG that contains glucosamine and galactose rather than hexosamines and hexuronic acid, (Jones et al 1982). All GAGs except hyaluronan include a sulfate group (Alberts et al., 2002). The other GAGs include chondroitin sulfate, dermatan sulfate, heparan sulfate and keratan sulfate. The GAGs are extremely negatively charged and they bind to positively charged ions to trap water molecules for formation of hydrated gels. The hydrated gels that result provide mechanical support to the extracellular matrix (Alberts et al., 2002).

Hyaluronan is the only glycosaminoglycan that does not contain a sulfate group. It occurs as a single polysaccharide chain. The other GAGs are linked to proteins and form proteoglycans. Proteoglycans interact with hyaluronan and this forms large networks within the ECM (Cooper, 2000). Because of the hydrated gel consistency of hyaluronan it is able to resist compressive forces on tissues and joints (Alberts et al., 2002). In embryonic development hyaluronan serves as a space filler to induce a change in shape and structure of the developing embryo. It also serves as a medium for cell migration and is produced in large quantities during wound healing (Alberts et al., 2002).

Proteoglycans are important for cell signaling. For example proteoglycans containing heparan sulfate bind to fibroblast growth factors (FGFs). FGFs stimulate a variety of cell types to proliferate (Alberts et al., 2002). Proteoglycans also have the ability to regulate and bind other proteins and influence the fate of those proteins. Proteoglycans can immobilize protein at the site where they are produced. They can block the activity of proteins, delay the release of the secreted protein, protect proteins from degradation, and alter proteins to make them more effective for presenting to cell surface receptors. For example, proteoglycans that branch from heparan sulfate have the ability to enable FGFs to activate their cell-surface receptors which in turn increases cell proliferation. Transforming growth factor β (TGF-β) will bind to decorin, a matrix proteoglycan, and this action inhibits the activity of other growth factors (Alberts et al., 2002).

The ECM is comprised of glycoproteins that contribute to the organization of the matrix and aid in cell attachment. Glycoproteins tightly bind to collagen, elastin and proteoglycans (Jones et al., 1982). Glycoproteins have binding sites for glycosaminoglycans such as heparin sulfate and can react with specific collagen types (Jones et al. 1982). Fibrillin-1 is a multidomain glycoprotein that forms elastic microfibrils within the ECM (Vakonakis et al., 2007). Together with elastin, fibrillin provides elasticity and resiliency for a variety of tissues (Vakonakis et al., 2007). Fibrillin-1 contains epidermal growth factor-like domains (EGF) as well as TGF-β-binding protein-like domains (Vakonakis et al., 2007).

Fibronectin plays a major role in synthesis and composition of the ECM. Fibronectin is a dimer composed of two subunits joined by disulfide bonds at one end and has binding sites for collagen as well as GAGs to form cross links between these components. One of the important roles of fibronectin is attaching cells to the matrix and in vertebrate embryos it can guide cell migrations (Cooper, 2000). Fibronectin stabilizes and organizes the matrix (Jones et al., 1982). It is composed of three different domain types, FNI, FNII and FNIII (Vakonakis et al., 2007). The interactions exerted by the fibronectin domains and tension exerted through cell-surface receptors are essential (Vakonakis et al., 2007). Fibronectin has mechanical properties allowing living cells to stretch the fibril component by as much a four fold (Vakonakis et al., 2007).

Laminin is another glycoprotein that is closely associated with the basal lamina and has a less defined function when compared to fibronectin. Laminin is a large flexible protein composed of three polypeptide chains held together by disulfide bonds. The three polypeptide chains are arranged in a manner that resembles an asymmetric cross (Cooper, 2000). Laminin is confined to the lamina lucida of the basal lamina and is synthesized by cultured endodermal cells and endothelial cells. Basal laminae provide an active barrier to the movement of cells and molecules. It is involved in tissue regeneration after injury by serving as a scaffold in which regenerating cells can migrate. Only type IV collagen is found in the basal lamina. Type IV collagen has less organization than type I collagen (Jones et al., 1982).

The extracellular matrix is comprised of many different types of macromolecules that can influence cell shape, migration, proliferation, survival, development and function. Extracellular matrices influence many cell types including stem cells. There are many types of extracellular matrices that can be produced *in vitro* and these are useful for examining the potential *in vivo* effects on stem cells (Alberts et al., 2002).

Functions of the ECM

Extracellular matrices provide structural as well as biological support for cells *in vivo*. Specifically, ECMs function structurally by providing a scaffold for cells, tensile strength and cushioning. The ECM provides a molecular filter, a boundary, a storage depot of growth factors and cytokines and can block cryptic sites (Kleinman et al., 2003). Biologically the ECM functions in cell polarity, cell adhesion, morphogenesis and differentiation, migration, proliferation and prevention of apoptosis (Kleinman et al., 2003). The biological responses of the ECM are regulated by cell-surface receptors. The most biologically active ECM molecules include laminins, collagens, thrombospondin as well as fibronectin. The biological importance of the ECM was illustrated in study using knock out mice (Kleinman et al., 2003). The mice lacked individual ECM component genes. Mice without laminin experienced muscular dystrophy as well as skin blistering and the defect is lethal. Loss of fibronectin results in mesodermal and cardiovascular defects and is lethal to the mice as well. The loss of entactin/nidogen found in basal lamina resulted in neurological defects. Lastly, without collagen there are vascular defects, renal failure and ultimately lethality (Kleinman et al., 2003). Therefore the components of the ECM are in a combination that promotes specific biological functions and loss of one ECM component can be life threatening and disrupt phenotypes. The ECM macromolecules interact with each other to stimulate and allow important biological functions to take place.

The basal lamina is the first ECM synthesized in the developing embryo. Laminin is expressed at the two cell stage while the basal lamina makes its first appearance at gastrulation. It should be noted that stem cells first come into contact with basal lamina during development so it would be safe to assume that ECM has a profound impact on the differentiation or maintenance of stem cells. The basal lamina *in vivo* is enriched with laminin, entactin/nidogen and collagen IV. Entactin may promote and enhance laminin-cell receptor binding and is able to provide a regulated cell-matrix interaction (Hagedorn et al., 2001).

Basement membranes have lesser amounts of proteoglycans and growth factors and have the ability to regulate cell polarity (Kleinman et al., 2003). The basement membrane ranges in thickness between about 100 to 300 nm and can be seen with light microscopy. Basal laminae are less than 100 nm in thickness thus can only be seen with transmission electron microscopy. The components of basement membranes include collagen type IV, laminins and heparan sulfate (Hagedorn et al., 2001). Basement membranes create boundaries between different tissue types and have a specialized function in the kidney (Kleinman et al., 2003). Basement membranes reside underneath epithelial cells, around blood vessels and are associated with certain distinct mesenchymal cells (Hagedorn et al., 2001).

ECM and tumors

ECM differs for normal cells and malignant tumors. Malignant tumors are invasive and have the ability to destroy various matrix structures (Hagedorn et al., 2001). Tumors cells with the ability to metastasize secrete different amounts of ECM components and produce enzymes that degrade the ECM components of the basement membrane. This allows them to invade the different tissues of the body (Jones et al., 1982). Basement membranes are usually impenetrable to invasive cells. The basement membrane can be invaded by the action of degrading enzymes secreted by metastazing tumor cells (Jones et al., 1982). The loss of the basement membrane signifies the initial step of tumor metastasis (Hagedorn et al., 2001). A malignant tumor is characterized by the ability of tumor cells to invade host tissues, cross basement membranes and to

metastasize to distant sites in the body (Jones et al., 1982). The destruction of the ECM occurs in the area of tumors cell *in vivo* (Jones et al., 1982). Malignant tumor cells have the ability to degrade ECM components through the use of hydrolases.

Collagenase is commonly expressed by tumor cells and degrades collagen fibers including the collagen IV fibers found in the basal lamina of the basement membrane (Jones et al., 1982). Plasminogen activator is secreted in large amounts by metastatic tumor cells as well, but its role and malignant phenotype has not been clearly defined (Jones et al., 1982). There are other degradative hydrolytic enzymes produced by tumors and tumor cell lines including cathespins, thiol proteinases and proteoglycan-degrading activity (Jones et al., 1982). Breakdown of connective tissue is very important in the invasive process of malignant tumor cells.

Breakdown of basement membrane material is enhanced by expression of major matrix degrading enzymes as well as metalloproteinases (MMPs) (Hagedorn et al., 2001). MMPs help degrade the collagenous matrix (Hagedorn et al., 2001). Different types of MMPS are responsible for degrading certain parts of the extracellular matrix. For example, MMPs 1, 8, 13, 18 are collagenases; Gelatinases, (MMP-2 and MMP-9) degrade collagen IV and V (Hagedorn et al., 2001). Stromelysins are responsible for the degradation of laminin, fibronectin, and can denature collagens (Hagedorn et al., 2001). The molecular alteration of the ECM explains the loss of control of cellular proliferation in carcinomas. Malignant tumor cells destroy components of the ECM and are capable of secreting an excessive amount of glycosaminoglycans.

An extracellular component secreted in large quantities by malignant tumor cells is hyaluronan (Stern 2008). Hyaluronan secreted prior to mitosis functions to promote detachment of cells to allow motility and this may possibly contribute to the movement of metastatic tumor cells (Stern 2008). It has been hypothesized that cancer cells synthesize increased levels of HA in order to detach from their substratum to go through mitosis for division, and continue to divide endlessly. The ability of cancer cells to divide infinitely separates them from normal cells that will re-attach to the substratum and continue normal functions (Stern 2008). The inability of cells to shed their hyaluronan coat, via hyaluronidase activity, may promote malignant cell growth and the development of

cancer (Stern 2008). Normal cells need to lose their hyaluronan coat in order for differentiation to occur. An increase in hyaluronan is correlated with tumor virulence and can be used as a prognostic indicator. Hyaluronan is produced by cancer cells but production is induced by the tumor cells in their surrounding stromal cells (Stern 2008). There are other glycosaminoglycans present in cancer cells at abnormal concentrations. One such GAG includes heparan sulfate. Heparan sulfate proteoglycans such as syndecans, glypicans and perlecan are associated with tumor progression or suppression, and sometimes both (Stern 2008).

Specific ECMs

The ECM has been studied intensively to assess the biological activity of the matrix itself as well as its role in cell differentiation. The ECM can be studied in vitro using three dimensional matrices that are derived from cells or tissues as well as laminin or collagen or peptides in the form of proteolytic or recombinant fragments. Matrigel is a matrix derived from the Engelbreth-Holm Swarm tumor found in mice. Matrigel promotes the differentiation of some cell types and can induce growth in cells from tissue explants. When incubated at 37° C for 30 minutes, Matrigel becomes a hydrated gel and cells can be plated on top of this matrix. Matrigel is considered to provide a three dimensional matrix that mimics the basal lamina *in-vivo*. Stem cells have been cultured on Matrigel (Kleinman and Martin 2005). Three dimensional matrices may mimic the in vivo environment because cells are suspended and grown in a three dimensional environment.

Cartrigel: An In Vitro ECM

Philp et al. (2002) created a matrix from an extract of cartilage (Cartrigel) and compared the effects of Cartrigel and Matrigel pluripotent rhesus monkey embryonic stem (ES) cells grown in a monolayer or in a rotating wall vessel bioreactor (RWV). The results indicate that Matrigel, as a solution or as a gelled substrate, induced cell growth and differentiation. Large tubular-like structures and cell aggregates were observed for cells grown on Matrigel. By day 11, the cells grown on Matrigel continued to form large

organ-like tubular structures. Cells grown in RWV bioreactors using soluble Matrigel became cell aggregates with increased size and also differentiated into highly organized tubular- and glandular-like structures. Cells grown on Cartrigel as a gel or as a solution grew as a monolayer *in vitro*. In the RWV bioreactor Cartrigel, increased the cell number was but had no obvious effects on differentiation.

Matrigel was able to promote epithelial/glandular-like structures while Cartrigel promoted chondrogenesis with round cartilage nodules visible among the undifferentiated stem cells. In the RWV bioreactor, Matrigel produced greater differentiation compared to Cartrigel (Philp et al., 2002).

Humatrix: Myoepithelial Derived ECM

A myoepithelial-derived matrical gel termed Humatrix was prepared by Kedeshian et al. (1998) to observe the relationship between carcinoma cells and the extracellular matrix *in vivo*. Humatrix is prepared from salivary gland and breast myoepithelial tumors. It is physically similar to Matrigel in that gelation occurs at 25-37° C when the protein concentration is greater than or equal to 3 mg/ml. A major difference between the two matrices is that Matrigel extraction yielded 9 mg/ml protein/g of Engelbreth Holm-Swarm (EHS) tumor and Humatrix yielded only 1.5mg protein/g of human matrix secreting xenografts from salivary gland (HMS-X). Protein analysis indicated that fibronectin and type I collagen bands are predominate in Humatrix whereas Matrigel has more laminin and type IV collagen. Both matrices have comparable glycosaminoglycan content but Humatrix has a large amount of chondroitin sulfate and Matrigel has more heparan sulfate. Humatrix contains EGF and IGF-I but lacks TGF-β. Humatrix seeded with metastatic non-myoepithelial cells from a human melanoma line and a breast carcinoma line demonstrated reduced invasion, angiogenesis and metastasis.

Myoepithelial cell lines established from benign human salivary gland and breast myoepithelial tumors resulted in tumorigenicity when seeded on Humatrix. The tumorigenic effect of Humatrix may be due to its specific extracellular matrix synthesized from myoepithelial carcinomas or the composition of growth factors within the matrix itself or both. Humatrix is considered to be a tool that can be used to observe biological

processes of tumor cell growth, angiogenesis, invasion and metastasis (Kedeshian et al., 1998).

Synthetic Matrices

Synthetic matrices have also been studied for their effects on cells. The use of synthetic ECM may aid in stem cell self renewal. Li et al. (2006) created a hydrogel composed of a semi-interpenetrating polymer network (sIPN). The sIPNs consisted of poly (N-isopropylacrylamide-co-acrylic acid) [p (NIPAAm-Co-AAc) which was loosely cross-linked with an acrylated peptide; Gln-Pro-Gln-Gly-Leu-Ala-Lys-NH2. This was interpenetrated by a polyacrylic acid-graft linear polymer chain, [p (AAc)-g-RGD]. The RGD motif present in the polyacrylic acid-graft is an active site in many ECM proteins and a binding domain for cellular integrins. The sIPNs used in this study were hydrophilic and became hydrated in aqueous media.

The ability of the hydrogel to become hydrated mimics *in vivo* ECMs. Important characteristics of the hydrogel are the abilities to manipulate matrix stiffness and to control cell-adhesion ligand density. These two things regulate cell adhesion, migration and differentiation. When human embryonic stem cells (hESCs) were cultured on the sIPN hydrogel they maintained their original phenotype as evidenced by cell morphology. The cells had distinct colony borders surrounded by small tightly packed cells. Oct 4and SSEA-4 (a cell surface antigen also expressed in undifferentiated hESCs) were observed for cells grown on the sIPN on day 5 of culture. The authors also assessed whether ligand concentration had an impact on hESC cell growth on the sIPN. Higher RGD concentrations resulted in morphologies characteristic of undifferentiated hESCs, including distinct colony borders with small tightly packed cells within the colony. Whereas cells grown on sIPNs with lower RGD concentrations had low hESC adhesion (Li et al., 2006)

Levenburg et al. (2003) created a three dimensional, porous, biodegradable polymer scaffold. hESCs were plated on the polymer scaffold or Matrigel. The polymer scaffold was designed to resist compressive stress exerted by the cells. By introducing

different growth factors to the polymer scaffold different structures could be grown. The polymer scaffold was fabricated from a blend of 50% poly (lactic-co-glycolic acid) PLGA and 50% poly (L-lactic-acid) PLLA. PLGA degrades quickly while PLLA gives the scaffold its stiffness. The pore size of the scaffold is between 250-500um to help facilitate the seeding and growth of the cells into the polymer scaffold.

Both Matrigel and the polymer scaffold supported cell attachment, growth, and viability. The polymer scaffolds were supplemented with various growth factors including retinoic acid (RA), activin-A, TGF-β and IGF-1. The scaffold supplemented with IGF-1 resulted in cells forming large open tubular structures that had a cuboidal, columnar epithelial morphology. Supplementing the scaffold with RA increased the cytokertain positive areas and the cellular structures secreted extracellular matrix components. The addition of TGF-β to the scaffold resulted in the secretion of a cartilage-like glycosaminoglycan matrix. Supplementation of either activin-A or IGF resulted in the formation of structures similar to developing liver and cells contained high levels of alpha-fetoprotein and albumin. The ability of the scaffold to induce vascularization was determined using histology and immunohistochemical staining. Capillary like networks were formed within the scaffolds with cells that expressed the endothelial markers CD34 and CD31. Formation of vessel like structures in the Matrigel coated scaffold occurred with higher expression on the control scaffold and the scaffold supplemented with IGF-1. Scaffolds coated with fibronectin had higher levels of endothelial differentiation and vascularization regardless of growth factor supplementation.

The therapeutic potential of the polymer scaffolds was tested in the subcutaneous tissues of severe combined immunodeficiency deficient (SCID) mice. Implanted scaffolds were retrieved 2 weeks later and the cells growing in the constructs were viable, and there were no detected inflammatory responses to the scaffold. The implants were loosely surrounded by connective tissue and had been penetrated with host blood vessels. Red blood cells were within the construct-derived blood vessels suggesting that anastomosis between the construct and the host vessels occurred (Levenburg et al., 2003). Other *in vitro* studies have shown that both Matrigel and collagen gels can induce

capillary-like formation of endothelial cells (Kleinman et al., 2003) as may have been observed here.

It was observed that cells continued to differentiate within the host after transplantation as evidenced by the lack of SSEA-4 and Tra-1-60. Therefore a polymer scaffold seeded with early differentiating hESCs results in the formation of three-dimensional structures of human cells. Important characteristics of the polymer scaffold are its abilities to biodegrade within the host, to support viability of the differentiating cells, and to vascularize (Levenberg et al., 2003).

Stem cell responses to ECM

Stem cells respond differently to the different types of ECM, (see table 1.1). Tissue-specific matrices such as Humatrix may be able to manipulate development of cancer cells in tumors whereas Matrigel influences cells to readily differentiate into organ like structures. Some matrices allow stem cells to self renew and maintain potency while others induce differentiation into different cell lineages.

Potency

Stem cells are primitive cells found in various tissues of the human body and have varying degrees of potency. During embryogenesis, the inner cell mass of the blastocyst is composed of totipotent stem cells. These cells have the ability to become any specialized cell type of the body including extraembryonic membranes. Other potencies include pluripotency or the ability of a stem cell to give rise to the three germ layers, endoderm, ectoderm and mesoderm. Multipotency is the ability to become a specialized cell from a specific tissue layer. For example, mesenchymal stem cells, isolated from post-natal tissue, can give rise to adipose tissue, bone marrow and chondrocytes all of mesodermal lineage. Spermatogonial stem cells are an example of unipotent stem cells because give rise to one specialized cell type, the spermatozoa.

Umbilical cord matrix stem cells

The umbilical cord serves as a link between mother and fetus during gestation. The umbilical cord is composed of separate compartments including: the surface epithelium, perivascular stroma, intervascular stroma (Wharton's jelly), clefts, subamnion, umbilical vein subendothelium and the umbilical cord blood (Can et al., 2007 and Troyer and Weiss 2008). Clefts as described by Nanaev et al. (1997) are within the Wharton's jelly and occupied by a homogenous ground substance, now understood to be collagen. Clefts are devoid of collagen fibrils and basal lamina molecules. Clefts are thought to contribute to the turgor of the cord (Can et al., 2007). The structure of the umbilical cord includes two arteries and one or two veins depending on the species.

The vessels are surrounded by Wharton's jelly. Umbilical arteries carry the lower oxygen fetal blood to the placenta and the vein(s) carry oxygenated, nutrient-enriched blood to the fetus (Rao et al., 1994). Wharton's jelly was first described by Thomas Wharton in 1656 (Sarugaser et al., 2005). Wharton's jelly is an embryonic mucous connective tissue that lies between the umbilical cord vessels and is covered by the amniotic epithelium. The physiological roles of Wharton's jelly are to prevent tearing and bending of the enclosed vessels and serve as an adventitia to bind and encase the umbilical vessels. Wharton's jelly is comprised of specialized fibroblast-like cells and is rich in proteoglycans of which hyaluronic acid is the quantitatively predominant species (Can et al., 2007). The large amount of hyaluronic acid within the umbilical cord keeps the tissue hydrated and the large amount of collagen makes the cord resistant to extension and compression induced by the fetus and by contraction during delivery (Malkowski et al., 2007). Stern et al. (2008) supports the notion that hyaluronan is a major constituent of fetal structures including Wharton's Jelly.

The stromal cells of the umbilical cord are responsible for synthesis of collagen as well as other matrix components. The cells are partially covered by external lamina composed of, laminin, collagen type IV and heparan-sulfated proteoglycan (Can et al., 2007). The external lamina of stromal cells in the umbilical cord is similar to the sarcolemma of smooth muscle cells. The stromal cells within the Wharton's jelly contain subplasmalemmal and intracytoplasmic focal dense plaques typical of smooth muscle cells, but they are defined as myofibroblasts. Myofibroblasts share features with both fibroblasts and smooth muscle cells.

A Stem-Cell Niche in Wharton's Jelly

The cytokines and growth factors that reside in the Wharton's jelly of the umbilical cord may collectively constitute a niche in which umbilical cord matrix stem cells (UCMS cells) are able to communicate and their division, maintenance, and perhaps differentiation are supported and regulated. It may be that the entire Wharton's jelly compartment provides a stem-cell niche or there may be sub-compartments that regulate the cells differently and perhaps even sequentially. Alp Can et al. (2007) demonstrated differences in the differentiation state and other properties of UCMS cells in the perivascular compartment as compared to those deeper in Wharton's jelly. This could indicate a 'production line' with cells deep in Wharton's jelly maintaining and providing stem cells to replace transient amplifying cells near the vasculature. Such a system could provide mesenchymal stem cells to the vasculature or perhaps to the whole fetal-placental unit during pregnancy. Support for this idea would require identifying sub-environments within the umbilical cord that provide signals and support for pluripotency or multipotency.

Regardless, the Wharton's jelly of the umbilical cord, as a site of stem-cell residence, must contain a stem-cell niche according to current concepts. A stem-cell niche is defined as a specialized microenvironment that supports stem cells and varies in nature depending on the tissue type (Li and Williams., 2006; Peerani et al., 2007). Anatomically, the stem cell niche is a space where molecular interactions guide spatial relationships (Scadden et al., 2007). There are several common themes our present understanding identifies for stem cell niches. One theme is that the niche is composed of cells in a special location that functions to maintain stem cells (Li et al., 2006). The special location in the umbilical cord is the Wharton's jelly or a compartment of it, and the cells that maintain stem cells may be other UCMS cells or a subpopulation of them. The stem cell niche in Wharton's jelly constitutes a three dimensional scaffold that

harbors collagens, fibroblasts, immune cells, growth factors and cytokines and gives physical support to the UCMS cells (Troyer and Weiss 2008).

Another theme considered important for a stem-cell niche is that adhesion molecules in the ECM anchor the stem cells to the matrix and allow for niche-stem cell interaction (Li et al., 2006). Collagens are components of the ECM important for the anchoring of cells to the niche. In Wharton's jelly, a variety of collagens constitute the ECM including type 1, IV, V, IV and VII (Franc et al., 1998; Ryynanen et al., 1993). The fibrillar and microfibrillar constituents of Wharton's jelly include 67-nm striated collagen fibrils but fibrils range from 30 to 60 nm (Franc et al., 1998). The 67-nm striated collagen fibril is most abundant within the Wharton's jelly. The striated fibrils have a 100 nm banding pattern characteristic of type VI collagen. Collagen VI may play a role in the anchorage of the cells of the Wharton's jelly or mediate the linkage between the main fibrillar matrix components and hyaluronic acid (Franc et al., 1998).

Another collagen type associated with Wharton's jelly is type VII collagen. Type VII collagen is a homotrimer with the suggested form being an anti-parallel dimer associated through an overlap region between individual molecules. The anti-parallel dimers aggregate laterally to form anchoring fibrils. Type VII collagen has been found in basement membranes of the epithelia of organs such as breast, prostate, bronchi, larynx, esophagus, trachea, and vagina. Type VII collagen is predominately associated with keratinocytes and to a lesser extent, dermal fibroblasts. Ryynanen et al. (1993) examined the localization and spatial orientation of type VII collagen gene expression in the human umbilical cord. The findings demonstrated that cells cultured from the Wharton's jelly have prominent staining for type VII collagen. The endothelial cells from the vein of the umbilical cord had weak, but detectable immunoreaction for Type VII collagen. Northern analyses revealed that cells from Wharton's jelly also express type VII collagen epitopes and high levels of mRNA. Collagens make up the frame of the ECM and may anchor stem cells or supportive cells to the niche to allow interaction between the cells and extrinsic factors.

In the umbilical cord, Wharton's jelly is the ECM and serves an anchorage function and allows for communication between the niche and stem cells. The matrix components of the ECM anchor the cells in the niche environment and could exert influences on the stem cell population that either stimulate or inhibit behaviors (Nurcombe et al., 2007). The stem cells may interpret signals received directly from the components within the matrix. One such component is heparan sulfate (HS). HS is a glycosaminoglycan that can form sugar-protein complexes, known as HS proteoglycans. The three major forms of HS proteoglycans are the syndecans, glypicans and perlecans (Nurcombe et al., 2007). Syndecans are cell membrane-intercalated proteoglycans; glypicans are membrane-associated and perlecans are secreted from the ECM itself. HS regulates a variety of growth factors and adhesive factors within the ECM that act on the surrounding cells. For example, FGF family is able to bind to HS with moderate affinity. Through binding with HS, the FGFs bind to their cell surface tyrosine kinase receptors allowing signal transduction to occur (Nurcombe et al., 2007). A similar scenario occurs between HS and bone morphogenetic proteins (BMPs). HS concentrates growth factors close to the cells, and may protect them from proteases, guides them to the cell surface and facilitates binding to their specific cell receptors (Nurcombe et al., 2007).

A third theme is that extrinsic factors in the stem cell niche are responsible for controlling stem cell number, proliferation, and fate and it is generally believed that these factors are produced locally. Many extrinsic factors have been shown to play a role in stem cell self-renewal and differentiation including hedgehog, Wnts, BMPs, FGFs, and Notch (Li et al. 2006). Hedgehog proteins are important in regulating the development of many tissues and organs and Wnt proteins are essential in embryonic and fetal development. The spatial arrangement of stem cells and support cells within the niche organizes the timing and secretion levels of signal(s) that stem cells receive which in turn directs the fate of the cell (Peerani et al., 2007).

Stem cells undergo two types of cell division. Symmetrical division results in production of two identical daughter cells and asymmetrical division produces one daughter cell that remains a stem cell in the niche but the other daughter is destined for differentiation. The niche controls the balance between symmetrical and asymmetrical

division to provide stem cell self renewal or stimulate differentiation (Li et al., 2006). According to Scadden et al. (2006), polarity of the stem cell to the niche and the orientation of the stem cell niche are critical for determining which type of division stem cells will undergo.

Uncontrolled proliferation of stem cells can lead to tumorigenesis and this is why it is crucial that the niche closely regulates stem cell division. The stem-cell niche regulates stem cell division and communication with the ECM. These niche features are extracellular and result in gene regulation to provide for either stem cell maintenance or differentiation. At the nuclear level, transcription factors form regulatory circuits that control stem cells.

Growth Factors and Cytokines Within the Niche

The extracellular matrix of the Wharton's jelly serves as a reservoir for an array of growth factors and cytokines with known roles in growth and development (see table 1.2). The Wharton's jelly extracellular matrix may be capable of controlling growth factor signaling (Sobolewski et al., 2005). The growth factors within the Wharton's jelly of the umbilical cord are known to control cell proliferation, differentiation and synthesis, and remodeling of the extracellular matrix (Sobolewski et al., 2005). Most of the growth factors characterized within the umbilical cord exert their regulatory end product by autocrine or paracrine action (Sobolewski et al., 2005).

One growth factor found in human Wharton's jelly, that can function by a classical endocrine mechanism is IGF-1. IGF-1regulates metabolic activity and is involved in cell growth and differentiation (Palka et al., 2000). It stimulates collagen and sulphated GAGs biosynthesis and is expressed in most fetal tissues (Palka et al., 2000). IGF-1 is expressed by cells of mesenchymal origin and expression is independent of growth hormone (Palka et al., 2000). IGFs are associated with extracellular binding proteins (IGF-BPs) that regulate IGF tissue activity. IGF-BPs are found in Wharton's jelly (Palka et al., 2000). Palka et al. (2000) and others observed that IGF-1 is expressed

almost 3 times more in the Wharton's jelly compared to umbilical cord serum. Adding IGF-1 to culture media benefits human embryo development by increasing the blastulation rate (and increasing the number of cells in the inner cell mass (Sjoblom et al., 1999).

Transforming-growth factor-β is expressed within the umbilical cord TGF-β signaling is mediated through binding to cell-surface type I and type II receptors containing threonine/serine kinase activity (Peerani et al., 2007). It has been noted that the high expression of TGF-β may result in a reduction in pre-eclampsia. TGF-β binding proteins are found in Wharton's jelly and are structurally similar to fibrillin. The TGF-β binding proteins are responsible for storing TGF-β in the ECM (Sobolewski et al., 2005).

Platelet-derived growth factor (PDGF) is a protein that binds to a secreted protein acidic and rich in cysteine (SPARC) (Sobolewski et al., 2005). SPARC is a pericellular matrix protein and is expressed during development particularly when tissues are undergoing remodeling or repair (Sobolewski et al., 2005). Epidermal growth factor (EGF) is also present in the Wharton's jelly and binds to decorin, a proteoglycan present in the extracellular matrix (Sobolewski et al., 2005). Epidermal growth factor and transforming growth factor-α are present in amniotic fluid as well as fetal blood (Rao et al., 1995).

Rao et al. (1995) has shown that EGF, TGF-α, and their receptor mRNA transcripts can be found in the human umbilical cord. More specifically, the transcripts have been found in the umbilical cord vessels, Wharton's jelly, and umbilical amnion having the highest expression (Rao et al., 1995). EGF is a powerful mitogen and promotes cells to grow, differentiate, and is essential in embryogenesis and important in wound healing. TGF-α binds to the EGF receptor and in doing so stimulates the growth of endothelial cells. The activation of EGF and TGF-α results in a modest increase in COX-1 and COX-2 genes (Rao et al., 1995). Products of these two genes are responsible for catalyzing the formation of PGH2. EGF and TGF-α have also been shown to induce PGE2. PGE2 is of significant importance because it constricts umbilical blood vessels

and may help to maintain a steady supply of blood between mother and fetus (Rao et al., 1995). Likewise, EGF and TGF-α may have the ability to constrict the umbilical cord blood vessels immediately after delivery of the fetus to prevent bleeding from the fetus

Fibroblast growth factors (FGFs) are a group of cytokines important in development, wound healing, hematopoiesis and tumorigenesis (Malkowski et al., 2007). Two types of FGFs have been associated with the umbilical cord, acidic FGF (aFGF) and basic FGF (bFGF). These two factors regulate cellular functions through four distinct membrane receptors that have tyrosine kinase activity (Malkowski et al., 2007; Peerani et al., 2007). FGFR 1 is the most abundant FGF receptor in undifferentiated human ES cells (Liu et al., 2006). Stabilization by cell-surface heparan-sulfate proteoglycans (HSPGs) allows FGF to bind to FGFR (Peerani et al., 2007). FGFs act mainly through paracrine action and the large amount of FGFs present in the Wharton's jelly may result in synthesis of extracellular matrix components and stimulation of cell division (Malkowski et al., 2007). The high concentration of FGFs in Wharton's jelly is crucial in regulating the physiological functions of stem cells (Malkowski et al., 2007). It appears that bFGF signaling is important to human ES cell self-renewal (Liu et al. 2007).

Two other cytokines synthesized within the Wharton's jelly of the umbilical cord are granulocyte macrophage stimulating factors (GM-CSF) and granulocyte colony stimulating factors (G-CSF) (Troyer and Weiss 2008 and Wang et al. 2008). GM-CSF was originally identified as a product of activated T-lymphocytes and is involved in the proliferation and differentiation of myeloid hematopoetic cells (Sjoblom et al., 1999). It is reported that GM-CSF can act as a survival factor for the developing embryo (Sjoblom et al., 1999). Human blastocysts cultured with GM-CSF have a higher number of cells in the inner cell mass (Sjoblom et al., 1999). For mouse blastocysts, GM-CSF promotes the uptake of glucose through binding to trophectoderm cells via the α chain of the GM-CSF receptor (Sjoblom et al., 1999). The increased glucose may stimulate metabolic activity and cell division. An important factor for maintaining undifferentiated spermatogonial stem cells is glial-derived neurotrophic factor (GDNF) and GDNF is secreted by UCMS cells in Wharton's jelly (Troyer and Weiss 2008).

IGF, FGF AND TGF-β Regulate the Stem Cell Niche

A study conducted by Bendall et al. (2007) examined the effects of IGF and FGF production and inhibition on the stem cell niche and their role for the survival and self-renewal of pluripotent hESCs. Using immunocytochemistry, it was observed that IGF1R is expressed exclusively by Oct4 + cells within hESC colonies (Bendall et al., 2007). In contrast, FGFR1 expression was limited to cells surrounding the Oct 4+ stem cell colonies. When Bendall and colleagues blocked the IGF pathway in hESCs cell expansion stopped. A decrease in the number of cells expressing SSEA-3, a stem cell marker, was also observed after blocking the IGF pathway. Blocking the FGF pathway through its receptor resulted in increased differentiation of the hESCs, but did not affect cell number. This suggests that perhaps FGF has an indirect role in the hESCs maintenance. Inhibiting TGF-β signaling increases differentiation, without affecting cell number, apoptosis or cell cycle status regardless if the cells are SSEA3+ or SSEA3-. TGF-β expression is induced by FGF in hESCs cultures. This indicates that FGF may indirectly work with TGF-β to maintain pluripotency in stem cells (Bendall et al., 2007). Because IGF, FGF and TGF-β are in Wharton's jelly they may participate in the stem cell niche for UCMS cells.

Transcription Factors Oct4, Nanog and Sox 2

The three transcription factors demonstrated in pig UCMS cells are Oct 4, Nanog and Sox 2 (Carlin et al., 2006). In ES cells, these transcription factors are responsible for maintaining potency and self renewal. Expression of these transcription factors activates self renewal genes and represses genes responsible for cell differentiation (Liu et al., 2007). Oct 4 is a POU-domain transcription factor and is expressed in the nuclei of all cells during the cleavage stage in embryogenesis (Roberts et al., 2004). In the blastocyst stage, before implantation, expression of Oct 4 is restricted to the inner cell mass. Oct 4 is down-regulated during the blastocyst and gastrulation stages as somatic lineage differentiation begins (Guo et al., 2002). If Oct 4 is down-regulated in the inner cell mass, all the cells of the embryo differentiate into trophectoderm (Roberts et al., 2004).

As stem cells lose their pluripotency, Oct 4 becomes down regulated (Liu et al., 2007). Over expression of Oct 4 can lead to differentiation as well (Pan et al., 2007). Therefore an embryo must regulate expression of Oct 4 to maintain pluripotency of the inner cell mass.

The high mobility group (HMG) factor, Sox 2, is also crucial for maintaining pluripotency in stem cells (Pan et al., 2007). During early development, Sox 2 has a similar expression pattern to Oct 4. Deletion of Sox 2 results in failure of the embryo to undergo implantation but it does not prevent blastocyst formation and differentiation into the trophectoderm (Roberts et al., 2004). Sox 2 together with Oct 4 regulate the production of FGF4 by the epiblast (Roberts et al., 2004). FGF 4 is necessary for trophectoderm proliferation (Roberts et al., 2004). Together the transcription factors Oct 4 and Sox 2 are a heterodimer that regulates downstream genes and also regulates the expression of Oct 4 and Sox 2 genes (Liu et al., 2007).

Nanog, the third transcription factor found in ES and UCMS cells, is also a target of the Oct4/Sox 2 complex (Liu et al., 2007). Nanog is a homeobox-containing factor that is crucial for maintaining pluripotent cells of the inner cell mass and ES cells (Liu et al., 2006). Nanog expression is essential for preventing differentiation of the ICM and ES cells into primitive endoderm (Pan et al., 2007). Nanog is absent in cells that have undergone differentiation (Liu et al., 2007). It is first detected in the cells of the compacted morula in the mouse, but expression is then confined to the inner cell mass and disappears in the trophectoderm in the blastocyst stage (Pan et al., 2007). As Nanog expression is further down-regulated it is restricted to the epiblast and barred from the primitive endoderm (Pan et al., 2007). Nanog is also expressed in primordial germ cells and this expression spans the period of epigenetic erasure and germ-cell commitment (Chambers et al., 2007). During embryogenesis, if Nanog is not expressed, embryonic mice fail to develop after the blastocyst stage and do not contain an epiblast (Pan et al., 2007). Down-regulation of Nanog may predispose cells to differentiate, but cells can remain indefinitely in the pluripotent state in its absence. Perhaps Nanog is needed for

the synthesis of the inner cell mass and construction of germ cells but is not the primary force driving pluripotency (Chambers et al., 2007).

The three transcription factors work synergistically to maintain pluripotency within the inner cell mass of the blastocyst. The three factors co-occupy the promoters of many of the same genes (Pan et al., 2007). For example in the mouse, Sox 2 binds to half of the genes bound by Oct 4 and more than 90% of the promoter region bound by both Oct 4 and Sox 2 are also bound to Nanog. In undifferentiated embryonic stem cells, Oct4, Nanog, and Sox 2 simultaneously bind to 352 genes (Pan et al., 2007). It has also been observed that Oct 4, Nanog and Sox 2 form an interconnected auto-regulatory loop by binding to their own promoters and this further maintains ES cell pluripotency (Pereira et al., 2006, Pan et al., 2007, Cole et al., 2008). The transcription factors that control stem cell pluripotency need to be strictly controlled to balance stem cell self renewal and differentiation into another lineage.

Methods for harvesting UCMS cells

There are several ways to isolate UCMS cells from the Wharton's jelly. Usually the arteries and veins are removed and discarded. Collagenase-containing solutions can be used to digest the umbilical cord and isolate the cell explants for culture (Can et al., 2007). The collagenase solution degrades the matrix ground substance and shortens the time for the isolation process. Carlin et al. (2006) collected term, midgestation, and early gestation porcine umbilical cords rinsed them in PBS and cut them longitudinally into 1-3cm segments. The arteries and veins were removed and discarded and the remaining tissue, Wharton's jelly, was removed using hemostats and surgical scissors. To isolate and culture the porcine UCMS cells, (PUCS) the Wharton's jelly explants were cultured in 6 well plates with DMEM and 20% FBS. After 24-48 hours adherent PUCS were observed. The morphology of PUCS is spindle-shaped and they form colonies of tightly packed round small cells with a high nucleus to cytoplasm ratio. This morphology is characteristic of UCMS cells from other species.

Total RNA was extracted from the PUCS and RT-PCR revealed expression of Nanog, Oct 4, and Sox 2 (Carlin et al., 2006). Quantitative RT-PCR was also conducted to determine the relative expression of the three transcription genes isolated from PUCS. A higher level of mRNA expression of Nanog was present in the PUC cells compared with Oct 4 and Sox-2 (Carlin et al., 2006). UCMS cells from some species do not express Oct 4 but expression of Nanog and Sox 2 appears to be a characteristic of all species studied to date (Can et al., 2007).

UCMS vs. MSCs

UCMS cells are mesenchymal-like stem cells (MSCs). They fit the criteria used to define adult MSCs and have some genetic markers and surface markers that are common to MSCs (Can et al., 2007; Troyer et al., 2008).

The adhesion molecules CD44, CD105 and integrin markers CD29 and CD51 as well as the markers CD10, CD13, CD29, CD44, CD105, CD73, and CD90 characterize MSCs and are consistently found in human MSCs (Can et al. 2007; Troyer et al., 2008; Wang et al., 2004). UCMS cells also express the MSC markers SH2 and SH3 (Wang et al., 2004). MSCs lack hematopoietic stem cell markers such as CD45, CD34, and human leukocyte antigen (HLA)-DR and this is consistent with UCMS cells (Can et al., 2007). Even though stem cells isolated from Wharton's jelly share MSC markers they have faster proliferation and greater in vitro expansion than bone marrow MSCs (BMSCs) (Baksh et al., 2007; Troyer et al., 2008). MSCs were originally isolated from marrow of the bone and have been applied to cell based therapies (Baksh et al., 2007; Wang et al., 2004). UCMS cells express the stem cell factor gene while BMSCs do not (Troyer et al., 2008). CD146 and CD117 have higher levels of expression in UCMS cells than BMSCs (Baksh et al., 2007).

MSCs are multipotent cells that can be derived from bone marrow and fat and can self-renew and differentiate into specialized cells *in vitro* (Troyer et al., 2008). MSCs can also be derived from dental pulp, placenta, umbilical cord blood and fetal tissues such

as spleen, lung, pancreas, kidneys and amniotic fluid from midgestation (Secco et al., 2008). The MSCs are multipotent and can differentiate *in vitro* into bone, muscle, adipose tissue, cartilage and tendon and there is evidence that they can become neural cells as well (Wagner et al., 2007; Troyer et al., 2008). This is also consistent with UCMS cells can differentiate into all the specialized cells listed above and more. In fact, UCMS cells also differentiate into osteocytes faster than BMSCs and generate significantly more adipocytes than BMSCs (Baksh et al., 2007). MSCs are plastic adherent cells that can be maintained in culture conditions (Wagner et al., 2007). The morphology of MSCs is large spindle-shaped cells, flat cells and small subpopulations (Wagner et al., 2007). This is comparable to the morphology of UCMS cells. UCMS cells also adhere to plastic wells and grow to confluency in culture conditions.

For MSCs isolated from BM the cell numbers decrease significantly with age, while UCMS cells can be cultured for longer periods of time (Baksh et al., 2007; Secco et al., 2008). MSC can also be isolated from umbilical cord blood but it has been shown that perhaps the more efficient ways to obtain mesenchymal like stem cells is through the umbilical cord stroma, not the blood (Secco et al., 2008).

Differentiation Potentials of UCMS Cells

Investigators believe that UCMS cells are comparable to MSCs because of their ability to differentiate into osteogenic, adipogenic, chondrogenic and other specialized cells *in vitro* and *in vivo* (Can et al., 2007 and Wang et al., 2004 and Karahuseyinoglu et al., 2007 and Conconi et al., 2006 and Wu et al., 2007).

Based on morphology there are two types of cells isolated from Wharton's jelly; Type 1 and Type 2 (Karahuseyinoglu et al., 2007). Type 1 cells appear flattened and fiber rich while type two cells are more fusiform in shape and have cytoplasmic extensions. Type 1 cells strongly express vimentin and pancytokeratin while type 2 cells express only vimentin, hUMSCs were able to differentiate into chondrocytes, adipocytes, and osteocytes, as well as. Isolated stem cells from the umbilical cord formed spherical

shiny surfaced cell masses within 3 weeks of inducing culture media. The cells stained with toluidine blue which is an indicator for accumulation of mucopolysaccharides, the dominant molecule in chondrocytes (Karahuseyinoglu et al., 2007). With the use of alcine blue staining, Wang et al. (2004) observed differentiation of UCMS cells into chondrocytes.

In the study carried out by Karahuseyinoglu et al. (2007) adipogenic differentiation occurred within 40 days of isolation in inducing medium. The observation of adipocytes was first signaled by the appearance of multi-sized, tiny intracytoplasmic lipid droplets inside the fusiform shaped cells. The cells became more cuboid shape and lipid granules tended to fuse together creating larger granules. Wang et al. (2004) observed positive adipogenic differentiation of the UCMS cells via Oil red-O staining. The cells were also positive for the adipocyte marker PPARγ2.

Osteogenic Potential

Osteogenic differentiation occurred after human UCMS cells were exposed to osteogenic medium for 3-4 weeks. There was observed direct evidence of calcium deposits as the accumulation of cells began to increase. Different proteins, including osteopontin, BSP-2, osteonectin, and osteocalcin were expressed in umbilical cord stem cells that successfully differentiated into osteocytes (Karahuseyinoglu et al., 2007). In another study, after 28 days of culture in osteogenic differentiation medium, UCMS cells formed alkaline phosphatase-positive aggregates and were positive for von Kossa staining (Wang et al., 2004). The von Kossa stain indicates calcium in mineralized tissue. There was also expression of the osteogenic marker osteopontin in the newly differentiated osteocytes.

Neuronal Differentiation

UCMS cells also have the ability to become neuronal-like. Karahuseyinoglu et al. (2007) induced hUCMS to become more neuronal like. The hUCMS had rounding of their cell bodies that gave rise to thin extensions that touched each other to a certain

extent. As the extensions became thinner it was apparent that bipolarization was occurring. After 24 hours they spontaneously de-differentiated back into flat cells. Spontaneous de-differentiation is common in mesenchymal stem cells (Karahuseyinoglu et al., 2007).

In another report, Salvia miltiorrhiza was added into the medium containing hUCMS and within 1-2 hours the morphology of these cells had a marked change (Lian et al., 2005). Eight hours after the cells were in contact with the medium the cells became contracted, smaller and there were bipolar or multipolar prominences (Lian et al., 2005). The cells went from a basic fibroblast like morphology to a spherical, star-like or elongated appearance. Multiple neurites extending from the cell body were observable after 24 hours of induction by Salvia miltorrhiza as well as long axon like processes. In addition to morphological changes in the cells, there was also positive expression for the neuronal markers nestin, β-tubulin III, NF and GFAP. Mitchell et al. (2003) were also able to differentiate umbilical cord stem cells into neuronal like cells and glia. The differentiated cells expressed neuron-specific enolase (NSE), a specific marker for neural stem cells; the control stem cells expressed NSE as well however expression was lower (Mitchell et al., 2003). Glial markers, GFAP and CNPase were observed in the differentiated cells and in the control cells in a lower amount (Mitchell et al., 2003).

Wang et al. (2004) reported hUCMS have the ability to differentiate into cardiomyocytes in addition to chondrocytes, osteocytes and adipocytes. In the study, 5-azacytidine was added to the medium of hUCMS and the drug induced changes the expression of cardiac troponin I and N-cadherin in the differentiated cells as compared to lower expression of these genes in untreated cells (Wang et al., 2004).

Myogenic Differentiation

hUCMS that are CD105 (+) have the ability to differentiate into skeletal muscle cells (Conconi et al., 2006). Within 7 to 14 days after myogenic induction, the cells began to elongate and fused into multinucleated rudiments. Around day-7 to day-16, Myf5 was expressed and at day-16 most of the multinucleated cells were positive for

Myo-D (Conconi et al., 2006). Rats were used to observe the differential potential of CD105+ cells within injured tissue. The tibialis anterior muscle of the rat was damaged using bupivacaine cholridate followed two days later by treatment with a myotoxic drug to induce an inflammatory response resulting in large necrotic areas. The stem cells were injected into the rat tibialis anterior muscle to observe the effects of the cells *in vivo*. In both the control and hUCMS treated animals the necrotic areas disappeared. The animals that received hUCMS showed signs of flogosis but that decreased by day 14. The cells were present in the muscle tissue until day 14 and the cells co-expressed sarcomeric tropomyosine (Conconi et al., 2006). This study reveals that hUCMS have the potential to survive *in vivo* and contribute to the formation of muscle after injury when differentiated into skeletal myocytes.

Endothelial Differentiation

Endothelial differentiation of hUCMS was reported by (Wu et al., 2007). Induction was achieved by culture with VEGF and bFGF on Matrigel coated coverslips. Within 48 hours there was a vascular network. *In vitro*, the ability of DiI-labeled ac-LDL to be taken up by the cell is a specific marker for endothelial cells. About 30-50% of the cells expressed endothelial cell markers such as platelet endothelial cell adhesion molecule (PECAM), and CD34. Endothelial cells are needed for many clinical applications including the vascularization of tissue-engineered grafts, anastomosis and treatment of ischemic tissues. The cultured hUCMS were injected into an ischemic site in the left adductor muscle of nude mice 24 h after ischemia via ligation of the left femoral artery (Wu et al., 2007). The stems cells incorporated into the murine vasculature. The cells were labeled with DiI which detected anti-human CD34 antibody in the injection site and by 28 days after ischemia the hUCMS were differentiated into endothelial lineage cells. The study indicates that hUCMS cells have the ability to vascularize areas of ischemic tissue or injured tissue. In order for tissue-engineered transplants to be successful the tissue has to be able to vascularize so that blood can flow in and out of the area. Therefore the ability of hUCMS cells to differentiate in vitro into

endothelial cells is a major advance in regards to tissue engineering and re-vascularization of injured tissues.

Immunogenicity of UCMS Cells

UCMS cells have a unique immunogenic profile that makes them candidates for tissue repair. Cells may or may not possess major histocompatability complexes (MHC) I or II. MHC antigens are displayed on cell surfaces and are responsible for lymphocyte recognition as well as antigen presentation. The MHC can control the immune response through recognition of "self" and "non-self". PUCS contain low amounts of MHC I and no MHC II (Cho et al., 2008). The significance of this finding is that perhaps PUCS do not elicit an immune response. There is evidence that the PUCS demonstrate a low immunogenic profile. Cho et al., (2008) demonstrated this in his study using miniature swine umbilical cords that were haplotype mismatched. SLA^{cc} or SLA^{ac} animals had an immune response via production of an antibody when injected with SLA^{dd} peripheral blood mononuclear cells (PBMCS). The sensitization occurred 3 weeks after the injection. When animals received an injection with the same dose of PUCs there was no detectable antibody directed toward SLA^{dd} cells. An immune response can be elicited when PUCs are activated by interferon gamma (IFN-γ). This is because PUCs activated by IFN-γ have increased MHC I surface expression and induced expression of MHC II (Cho et al., 2008).

Unactivated PUCs injected in a complete freunds adjuvant (CFA) induced inflammatory lesion and were able to induce an immune response (Cho et al. 2008). An immune response was also induced by injecting the unactivated PUCs into the same site 3 times. Another method that induced an immunogenic response by PUCs was through the use of haplotype mismatched skin grafts. The skin graft donors were SLA^{dd} animals. The graft skin was placed on SLA^{cc} or SLA^{ac} animals that were injected with unactivated PUCs, activated PUCs and unactivated PUCs after CFA injection. Pre-treatment with

unactivated PUCs resulted in rejection of the grafts within 5 to 6 days while all other groups had an accelerated rejection to the graft (Cho et al., 2008).

Therefore a single injection of unactivated PUCs across a full MHC barrier does not elicit a detectable immune response. The reduced immunogenicity seems to come from the lack of MHCII expression and the low MHCI expression. The findings in the study suggest that perhaps precautions must be taken to avoid sensitization of the cell therapy product. Administration of the cells into the same location a repeated amount of times may cause rejection of previously engrafted cells negatively affecting the benefits of the therapy. UCMS cells have a low immunogenic profile, and this makes them a prime candidate for potential treatment of diseases.

Parkinson's Disease Therapy

Dopaminergic neuronal loss results in Parkinson's disease. Both in vitro and in vivo, HUCS have the ability to differentiate into dopaminergic neurons (Weiss et al., 2006 and Fu et al., 2006). When the hUCMS are cultured with inducing medium they become positive for the catecholaminergic rate-limiting enzyme tyrosine hydroxylase (TH). TH converts phenylalanine into dopamine. The hUCMS differentiated into dopamine neurons, low yields of norepinephrine as well as GABAergic neurons (Fu et al., 2006). hUCMS were transplanted into Sprague-Dawley rats that had their dopamine-innervated striatum lesioned. At 20 weeks after the cells were transplanted, cells labeled with bis-Benzimide were found in the striatum. There was positive expression of TH as well. The labeled hUCMS migrated approximately 1.4mm away from the implantation site. It was found that the rats treated with the hUCMS had less amphetamine induced rotation compared to the control group. The significant finding of this study was that hUCMS can differentiate in vitro and positive effects of the cells were observed in vivo in rats with induced Parkinson's disease. The ability of the cells to improve the rats' rotations gives hope for the use of the stem cells in clinical applications in human models.

Myocardial Differentiation

UCMS cells may also prove therapeutically beneficial in the rat myocardial infarction model (Wu et al., 2007). The study aimed at observing whether transplanted hUCMS could improve cardiac function in a rat model. The rats underwent a ligation of the left coronary artery to mimic a myocardial infarction (Wu et al., 2007). Rats with less than 60% left ventricular ejection fraction (LVEF) were randomized into two groups, a control group receiving phosphate buffered saline (PBS) and the hUCMS cell group. At first, there was no difference between the two groups. By 2 weeks after transplantation the LVEF improved with the hUCMS cell treated group. The left ventricular dimensions of the stem cell group were significantly smaller than the control group. The left ventricular posterior wall was thicker in the cell treated group compared to control group, (Wu et al., 2007). The tissue samples from the cell treated group showed hUCMS cells within the infarct region. Clusters of transplanted cells resided within the subepicardial and subendocardial infarct tissue. Many hUCMS cells were found around capillaries and large vessels or arterioles (Wu et al., 2007). Perhaps the hUCMS cells were either homing to the site of injury after transplantation, or possibly differentiating into endothelial type cells to help with revascularization. In fact the hUCMS were positive for cardiac troponin-T, von Willebrand factor and smooth muscle actin suggesting that the hUCMS cells transdifferentiate into cardiomyocytes, smooth muscle cells, and endothelial cells (Wu et al., 2007). The transplanted hUCMS cells also seemed to be secreting VEGF, an angiogenic cytokine. This study suggests that perhaps the hUCMS cells after transplantation differentiate into supporting cells around the injury site to help aid in the healing process.

Treatment of Cerebral Ischemia

Ischemia can be defined as loss of blood and oxygen to an area of the body. Therefore, cerebral global ischemia caused by cardiac arrest can lead to brain damage, specifically neuronal loss (Jomura et al., 2007). A study conducted by Jomura et al. (2007) revealed the therapeutic benefits of using Oct-4 + rat umbilical cord stem cells (RUCMS) in rats with cerebral global ischemic damage. The rats underwent cardiac

arrest (CA) and resuscitation to mimic the effects of real cerebral global ischemia. The rats were put into four groups: Sham (A), 8 minutes CA without treatment (B), 8 minutes CA pretreated with defined media (C), 8 minutes pretreated with Oct-4+ RUCMS (D). The cells were injected into the hippocampus region of the brain. It was observed that the group pretreated with Oct-4 + RUCMS had significantly lower neuronal damage compared to groups B and C. The RUCM cells were observed underneath a fluorescent microscope where it was evident that the transplanted cells survived after the transplantation. The cells actually migrated away from the injection site although no particular pattern of migration was noted in the study, perhaps the cells are homing to sites of injury. The RUCM stem cells seem to differentiate in vivo after transplantation and could have migrated away from the site to aid in angiogenesis or even become neurons themselves.

Retina Therapy

A leading cause of blindness worldwide is tapetoretinal degeneration. This is a group of inherited abnormalities in the retina involving the photoreceptors themselves or the cells that interact with the photoreceptors. In the Royal College of Surgeons (RCS) rat, an animal model of photoreceptor degeneration, the use of human hUCMS, seems to provide a way to reduce the neurodegenerative outcome associated with the loss of photoreceptors (Lund et al., 2007). The researchers compared the efficacy of hUCMS cells as well has human-placental derived cells (hPTC) and human bone marrow mesenchymal stem cells (hMSC). The hPTCs were used as a comparable source to the hUCMS and hMSCs have been found to be biologically active in the retina. The control consisted of human adult dermal fibroblasts (hADF) and media alone (Lund et al., 2007). The different cell sources were injected subretinal into the RCS rats and photoreceptor activities were tested by electroretinogram (ERG) responses at P60 and P90 and optomotor and luminance thresholds at P100.

Rats that received hUCMS had achieved a low-level rescue of photoreceptors as indicated by the ERG a-wave responses, but it was not different from the sham group.

The hUCMS did have significantly better recovery of bipolar cells as indicated by the ERG mixed b-wave responsiveness as compared to sham and untreated rats (Lund et al., 2007). There was significant rescue of the rod and cone b-waves as compared to the shams as well. At P90 the a-wave could still be elicited, but the b-wave amplitude had dropped. The b-wave amplitude still held significance over the sham or untreated eyes. In the cell treated rats, measured acuity thresholds illustrated significant change from control levels during degeneration and after the cells were transplanted (Lund et al., 2007). Luminance threshold was measured as a way to indicate efficacy of cell treatment in local rescue of visual function. The hUCMS treated rats retained normal retinal topographic order but the response sensitivity was not uniform. The area that had the best luminance threshold was that of the retina, were the cells were injected. Upon morphological examination of the retinas treated with hUCMS, there were no inflammatory reactions, pathologies or tumors found. In fact it was found that the retinas injected with hUCMS had extensive photoreceptor rescue, about 30% of the retinal length. The rods and cones were rescued as evident from the positive expression of arrestin, recoverin and mGlu96. The cell numbers declined for about 2 weeks but were still seen at P100 (Lund et al., 2007).

The hMSC treated group had some of the same responses and no significance when compared to the group treated with hUCMS. Morphologically though, the hMSC group had poorer rescue than the hUCMS group. The rescue area for the hMSC group was comparable to that of the sham group. Eyes that were injected with hPTCs responded poorly and had restricted morphological rescue. An ERG response could not be elicited at P90 for the hPTC group. There was photoreceptor rescue at P100, although it was not as nearly as extensive as that of the hUCMS group. Lastly, the hADF group had poor ERG responses and nothing was recordable by P90. Also, no photoreceptor rescue was evident at P100 other than right at the injection site, and the appearance was similar to that of the control group (Lund et al., 2007).

This study reveals that hUCMS can rescue rod and cone photoreceptors in the rodent model of retinal disease. Something to consider is that the donor was human and

patient was rat, and no allogenic response was found. Close observation of the animal model is needed to assure that there is no presence of teratomas and no allogenic reactions, for this can be problematic in a clinical setting. The hUCMS injected into the rat may have neutrophic effects allowing the rescue of the photoreceptors, but not actually becoming one (Lund et al., 2007). This is different than the other studies, where it was clearly observed that the hUCMS were differentiating into the tissue or cell type for which it was injected into. The hUCMS may work via a diffuse mechanism and not a cell to cell contact mechanism.

Umbilical cord stem cells have enormous promise in the area of cellular therapy and for clinical use. The unique properties including, extensive passaging, *in vitro* and *in vivo* differentiation into specialized cell types, and low immunogenic profiles make them a candidate for use in a clinical setting and perhaps for agriculture applications as well.

Agricultural Applications

An agricultural use of UCMS cells is to create stem cell lines in each of the farm animal species. As of now, there are not any established lines of ES cells available in the main farm animal species (Renard et al., 2007). Perhaps the use of embryonic stem cells in farm animals will not be needed if UCMS lines are created. The present interest in ES cells for farm animals is using somatic nuclear transfer for cloning (Renard et al., 2007), but there might be more promise in the use of UCMS, particularly for use as a delivery system for drugs or vaccines. Many avenues can be researched in regards to UCMS cells and farm animals and each would benefit from a better understanding of the UCMS cells and their environment in Wharton's jelly.

Table 1.1 Extracellular matrices that have been used as cell culture tools.

Matrix	Composition	GF&Cytokines	Cells used with	Effects on Cells	References
Matrigel	Engelbreth Holm Swarm tumor	TGF-b, FGF, tissue, plasminogen activator and others w/in EHS	Tissue explants, stem cells, tumor cells	Differentiation into specialized cell types	Kleinman et al 2005
Cartrigel	Calf knee cartilage	Media	Rhesus monkey ES cell line (R366.4)	chondrogenesis	Philp et al 2005
Humatrix	Human salivary and breast xenografts	EGF, IGF-1, uPA	Human myoepithelial tumor cell lines (salivary gland and breast)	Tumorigenesis effect on salivary gland cells and decrease of invasion in breast cell lines	Kedeshian et al 1998
Polymer Scaffold	50/50 blend of PLGA and PLLA	Supplemented with IGF, RA, TGF-B, activin-A	Human ES cell line	Differentiation into 3D structures	Levenberg et al 2003
Hydrogel	sIPNs	Cultured on MEFs	Human ES cell line HSF-6	Remained undifferentiated	Li et al 2006

CHAPTER 2 - Effects of Extracellular Matrices on Porcine Umbilical Cord Matrix Stem Cells

Introduction

Extracellular matrices (ECMs) are ubiquitous structures that serve as scaffolds for cells and are comprised of secreted proteins and polysaccharides. The ECM provides structural support and helps regulate biological functions within tissues. ECM plays a role in cell behavior, survival, development, adhesion, and migration as well as shape and function (Alberts et al., 2002). ECM is the first complex substance to interact with stem cells and plays a role in regulating stem cell differentiation and self renewal (Philp et al., 2005 and Chen et al., 2007).

The discovery of stem cells in the loose connective tissue of the umbilical cord of pigs and other species (Mitchell et al., 2003) has focused attention on the characteristics of these unique cells. Much of the umbilical cord originates from extraembryonic mesoderm during embryonic development. The umbilical cord contains two arteries and one or two veins depending on the species and is surrounded by amniotic epithelium. The central part of the cord is filled with a mucous substance termed Wharton's Jelly that was first described by Thomas Wharton in 1656 (Sarugaser et al., 2005). The jelly is composed of a variety of collagens, proteoglycans, mucopolysaccharides, and hyaluronic acid. Its functions include regulation of blood flow and preventing the bending and closing of vessels (Alp Can et al., 2007; Sarugaser et al., 2005). Wharton's Jelly is a reservoir for a number of cytokines and growth factors including fibroblast growth factors, epidermal growth factor, insulin-like growth factor-1 and transforming growth factor- β (Sobolewski et al., 2005 and Malkowski et al., 2007). The accumulation of growth factors and cytokines within the Wharton's jelly undoubtedly plays a major role in regulating the physiology of the cells within it.

An important concept in stem cell biology is the stem cell niche, a concept first proposed by Schofield (1978) for hematopoietic stem cells. By definition, thestem cell niche in the umbilical cord must include a part or all of the Wharton's jelly because it is the microenvironment in which umbilical cord matrix stem cells reside. The three transcription factors Nanog, Oct-4 and Sox-2 play a major role in embryonic stem cell

self-renewal and have been identified in porcine umbilical cord matrix stem cells (PUCs) (Carlin et al., 2006).

In order to elucidate the mechanisms important for maintaining PUCs within Wharton's jelly we sought to extract products from the jelly and apply them in vitro in ways that might recreate the niche from which the PUCS originated. Specifically, a matrical gel termed Pormatrix (PMX) was prepared from the Wharton's jelly of porcine umbilical cords. We evaluated cell growth, relative gene expression of Nanog, Oct-4 and Sox-2, and the cell morphology and immunostaining for PUCs grown on Pormatrix, the commercial ECM product Matrigel, or plastic. We also evaluated a low molecular weight filtrate of PMX that we refer to as flow through (FT)

Materials and Methods

Isolation of Wharton's jelly

Reagents and supplies were from Fisher Scientific (Fairlawn, NY) unless specified otherwise. During each farrowing cycle, approximately 40 to 50 porcine umbilical cords were obtained from postnatal piglets at the Kansas State University Swine Teaching and Research Center (STRC). The umbilical cords were submerged in phosphate buffered saline (PBS, pH 7.4, Gibco, Grand Island, NY) that was supplemented with antibiotic/antimycotic solution (Sigma, St. Louis, MO) after they were obtained, approximately 30 min to 1 hour after birth and remained in the solution for 1 to 3 days at 4° C. Wharton's jelly harvest began by cutting the cords into 5 cm segments and removing the blood vessels. Mechanically, with the aid of hemostats and surgical scissors, Wharton's jelly was removed from the de-veined cords. The Wharton's jelly was minced and placed in a sterile 50ml polypropylene centrifuge tube with PBS. The tissue was recovered by centrifugation for 20 minutes (800 X g, 4° C). The PBS was removed and the Wharton's jelly weighed.

Preparation of Pormatrix

PMX was prepared from Wharton's jelly by extraction in 6M urea and 2 M guanidinium hydrocholoride (Gdn-HCl). First Wharton's Jelly was homogenized in

2ml/g high salt buffer (3.4M NaCl), 50mM Tris-HCl, 20mM EDTA, and 10mM N-ethyl-maleimide (NEM, Sigma-Aldrich, St. Louis, MO) (pH 7.4) using a Tissue Tearor (Biospec Product, Inc Bartlesville, OK). The homogenate was centrifuged in round bottom polypropylene tubes (Nalgene) for 15 minutes (12,000 X g, 4° C) using a JA-17 motor that had been chilled to 4° C. The supernatant was removed and discarded leaving a well defined pellet. The pellet was extracted overnight by gentle stirring at 4° C in 0.5ml/g of urea/Gdn-HCl extraction buffer, (6 M urea, 2 M Gdn-HCl, 50 mM Tris-HCl, 20 mM EDTA, 10 mM NEM (pH 7.4) with an added 2mM dithiothreitol (DTT). Following protein extraction, the pellet was vortexed and the extract centrifuged (30 minutes at 24,000 X g using a chilled rotor). Tubes were placed on ice and the supernatant poured into a sterile 15ml polypropylene centrifuge tube. The supernatant was placed into Specta/Por (MWcut off: 3,500) dialysis 3 membrane tubing, (Spectrum Laboratories, Inc, Rancho Dominguez, CA) and dialyzed against 3 changes (24 hours/change) of Tris-buffered saline (0.15M NaCl, 50mM Tris-HCl, 20mM EDTA, and 10mM NEM at pH 7.4; 1L/change) at 4° C on a stir plate, followed by sequential dialysis against 0.5% chloroform and cell culture media (DMEM + Glutamax, Gibco). After dialysis the supernatant was placed in a new 15ml polypropylene centrifuge tube and centrifuged at 4000 x g for 15 minutes. The supernatant remaining is PMX. PMX was concentrated to a protein concentration of 2.44 to 3mg/ml using an iCON (Pierce Rockford, IL) concentrator (MW cut off 9,000 daltons) for 20 min at 5000 x g and protein concentration determined by Bradford Assay (Wallac Victor2 Perkin Elmer, Shelton, CT). The filtrate was stored frozen (-20^oC) until used. At room temperature (RT) and 4° C the PMX remained liquid; but after for 1 hr 30 minutes at 37° C PMX underwent gelation.

Experiment 1: Effect of gelling matrices on PUCs

PMX was concentrated to 2.44mg protein/ml and underwent gelation upon incubation at 37^oC. The treatments were plated in triplicate in a 24-well plate (Costar Corning, NY). The treatments were: gelling PMX, gelling PMX+FT, gelling Matrigel (prepared according to manufacturers directions, 9mg/ml), gelling Matrigel+FT, flow-

through (FT), or on plastic as the control. FT is the solution that passes through the iCON concentrator. Cells were seeded into wells of a 24-well plate at a density of 18,000 cells/well in 300µl of DMEM + Glutamax media supplemented with 20% fetal bovine serum (FBS) (Invitrogen), B-mercaptoethanol (55uM/ml, Sigma Aldrich), 25ug Gentamicin Sulfate (Sigma), and 100ug Normocin (InvivoGen, San Diego, California). The cells were incubated at 38.5^{o}C in a 5% CO2 in air atmosphere. For each replicate when cells cultured on plastic reached 85 to 90% confluency (3 to 5d) all cells from each treatment were lifted using 1x trypsin + EDTA (Gibco) or Matrisperse (Becton Dickinson Labware, Bedford, MA) for PUCs grown on Matrigel. The cells were counted for viability using trypan blue exclusion on a hemacytometer counting chamber (Hausser Scientific Partnership, Horsham, PA) and RNA was extracted.

Experiment 2: Effect of non-gelling matrices on PUCs

PMX and Matrigel were diluted to 1.2mg/ml to 1.5mg/ml protein using serum free DMEM + Glutamax. FBS (20%) was added to each ECM supplemented medium and to control medium. PUCs were plated in 24-well plates in each of the three media, with 3 wells/treatment. Each medium was tested with and without FT. Cells were seeded into each well at a density of 18,000 cells/well in 300µl of medium and incubated at 38.5^{o} C and in 5% CO_2 in air. When cells cultured on plastic reached 85-90% confluency (3 to 5d) all cells were lifted (trypsin + EDTA) or Matrisperse for those grown with Matrigel. PUCs from each treatment were counted for viability using the trypan blue exclusion test on a hemacytometer counting chamber and RNA was extracted.

Experiment 3: Effect of passage number on gene expression in PUCS

Sterile porcine umbilical cords were collected from the STRC. The cords were placed in sterile 50ml polypropylene centrifuge tubes containing antibiotic/antimycotic PBS (pH 7.4) and transported to the laboratory. Using sterile technique, the cords were cut into 1-3cm segments, and deveined. The Wharton's jelly was removed using surgical hemostats and scissors and minced into explants. The explants were resuspended in complete media with 20% FBS and plated into flat bottom 6-well plates (Costar, Corning,

NY) and grown on plastic at 38.5^{o} C and in 5% CO_2 in air. After the explants attached and cell growth was established, the explants were removed using trypsin + EDTA and discarded. The remaining cells were re-plated into 6-well plates and passaged when 85 to 90% confluent for 6 passages. At each passage, PUCs were counted using a hemacytometer counting chamber. Cells were re-plated at a density of 100,000 cells/well and remaining cells were used for RNA isolation using the RNeasy Micro Kit (Quiagen, Valencia, California).

Immunocytochemistry

Passage-8 PUCs were cultured on gelling PMX, non-gelling PMX, Matrigel, non-gelling Matrigel, FT and plastic. After 4 days of culture the cells were fixed with 10% formalin for 10 minutes at RT. Using PBS, cells were washed 3 times at RT. Fixed cells were permeabilized with 0.2% triton-x 100 (Sigma) at RT for 10 minutes. Nonspecific binding sites in cells were blocked using 3% goat serum (Sigma) and 1% bovine serum albumin (BSA) (Sigma) in PBS for 30 minutes at RT. The primary antiserum, rabbit anti-Nanog (Millipore, Billerica, MA) was diluted 1:500 in 0.5% blocking buffer (0.5% BSA in PBS pH 7.4) and incubated with the cells for 1 hour at RT. After cells were incubated with primary antiserum they were washed 3 times with 0.05% Tween 20 (Bio Rad Laboratories) in PBS. The secondary antiserum (anti-rabbit IgG-Alexa-488, Invitrogen, Eugene, OR) was diluted 1:500 in PBS and incubated with cells for 1 hour at room temperature. Cells were washed 3 times using 0.05% Tween 20/PBS after incubation with secondary antiserum and were counterstained with 4’, 6-diamindino-2-phenylindole (DAPI, Vector Laboratories, Inc, Burlingame, CA) and examined using fluorescence microscopy. The filter used to observe Nanog stain (Alexa-488) was filter EF-4 FITC/GFP with excitation wavelengths from 490 to 520nm. The filter used to observe the DAPI counterstain was the filter EF-4 UV-2A with excitation wavelengths from 330 to 380nm. Images were captured using Hoffman optics and images recorded with a Nikon diaphot 300 digital camera.

RNA isolation and quantitative RT-PCR

Total RNA was collected from PUCS after each passage from experiment 3 and collected from PUCS in experiment 1 and 2 after cells grown on plastic were 85 to 90% confluent. In brief, PUC cells passaged on plastic or grown on PMX, FT and control were lifted using 1x trypsin (GIBCO) while PUC cells grown on Matrigel were lifted using Matrisperse (Becton Dickinson Labware, Bedford, MA). A viability cell count was performed using the trypan blue exclusion test (Sigma) on a hemacytometer counting chamber. The remaining cells were exposed to a lysis buffer supplied by the kit and transferred to an RNeasy column. A DNase digest (RNase-free DNase Set, Qiagen) was performed to remove genomic DNA. After washing, RNA was eluted from the column using 14 μl of nuclease-free water. The RNA was measured using Nano-Drop (ND-1000 Spectrophotometer, Wilmington, DE). The isolated RNA underwent reverse transcription using Taq Man RT reagents (Applied Biosystems, Branchburg, NJ) in a 20 μl reaction.

The PUC cDNA was subjected to quantitative RT-PCR using Nanog, Sox-2, Oct-4 and 18S ribosomal subunit primers (18S primer sequences were as follows: FWD: 5'-GAGGTTCGAAGACGATCAGA-3'; REV: 5'-TCGCTCCACCAACTAAGAAC-3'; annealing temperature = 55°C). The other primer sequences are as follows: Nanog FWD: 5'-CCCGGGCTTCTATTCCTACCA-3'; REV: 5'-TACCCCACACGGGCAGGTT-3'; Sox-2 FWD 5'-TTCCATGGGCTCAGTGGTCAA-3'; REV: 5'-TGGAGTGGGAAGAAGAGGTAAC-3'; Oct-4 FWD 5'-AGAAGGATGTGGTCCGCGT-3'; REV: 5'-ACTGCTTGATCGTTTGCCC-3', (Invitrogen).The reactions were assembled using 1μl of cDNA, 10μl of power SYBR green master mix (Applied Biosystems, Fostercity, CA), 300 nM each of FWD and REV primers (Invitrogen) to make a 20μl 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.

Statistical Anaylsis

Data were analyzed using the PROC Mixed procedure of SAS (SAS, 2000; SAS Inst. Inc., the Satterthwaite degrees of freedom). The significance was declared at $P<0.05$ and a trend at $P=0.10$ to $P=0.06$ unless noted otherwise. When F-tests were significant, means were compared using Least Significance Difference procedure.

Results

Experiment 1: Effect of Gelling Matrices on PUCS

Morphology and Cell Number.

Morphology was observed using Hoffman optics and images recorded with a Nikon digital camera. Cells cultured on plastic grew in a monolayer, and consisted of elongated fibroblastic cells as well as small colonies (Fig. 1D). Cells cultured on PMX were fibroblastic in shape and formed a monolayer with few colonies observed (Fig. 1A). The colonies were small with tightly packed cells. The Matrigel treatment resulted in cells forming relatively larger colonies of tightly packed cells (Fig. 1B). Cells grown on Matrigel differed from those cultured on other treatments in that they did not form a monolayer or have a fibroblastic appearance. The flow-through treatment resulted in cells with morphology similar to those cultured on plastic (Fig. 1c). Fewer cells were harvested from PMX and Matrigel coated wells compared to plastic but FT did not ($P>0.10$) affect cell proliferation (Table 2.1).

Relative Gene Expression of Nanog, Oct-4 and Sox-2

The type of matrix and whether FT was added to the treatments affected ($P<0.05$) the expression of Nanog. Addition of FT to PMX reduced Nanog expression for PUCs (Table 2.2). PUCs cultured on Matrigel, Matrigel+FT, Plastic+FT and PMX expressed Nanog 4.46, 5.27, 4.28, and 4.43 fold higher, respectively, than PUCs cultured on PMX+FT. PUCs grown on different matrices tended ($P=0.10$) to differ in the expression of Oct-4 but not ($P<0.10$) in expression of Sox-2.

Experiment 2: Effect of Non-gelling Matrices on PUCS

Morphology and Cell Number

Cells cultured on plastic resulted in elongated fibroblastic cells and small colonies of tightly packed cells with well defined borders (Fig. 2D). Compared to PUCs grown on plastic, cells cultured on non-gelling PMX had relatively larger colonies with well defined borders and tightly packed cells (Fig. 2A). In addition to colonies there were elongated fibroblastic cells. PUCS cultured on non-gelling Matrigel formed a monolayer with small colonies (Fig. 2B). FT treated cells were fibroblastic in shape with few colonies (Fig. 2C). Number of cells harvested differed ($P<0.05$) between the matrices because fewer cells were collected from Matrigel wells (Table 2.3).

Relative Gene Expression of Nanog, Oct.4 and Sox 2

Nanog gene expression tended ($P=0.10$) to differ due to the addition of FT (Table 2.4). Oct-4 did not differ ($P>0.10$) among the treatments regardless of matrix type or addition of FT. Addition of FT to Matrigel tended ($P=0.10$) to increase Sox-2 expression and PUCs grown on Matrigel+FT expressed Sox-2 7 fold greater than those grown on PMX+FT (Table 2.5).

Experiment 3: Passaging of PUCS

Relative Gene Expression and Immunocytochemistry

Quantitative RT-PCR was conducted to observe the relative gene expression of Nanog, Oct-4, and Sox-2 of PUCS over time (Table 2.6). Expression of Nanog, Oct-4 and Sox-2 did not ($P>0.10$) differ over passages 0 to 6. PUCs at passage 8 that were cultured on gelling PMX, gelling Matrigel, non-gelling PMX, non-gelling Matrigel, FT, and plastic were all positive for the protein Nanog (fig. 3). Nanog was localized predominantly within the nuclei of the PUCs but there was some cytoplasmic staining

perhaps due to non-specific binding of the primary antibody or perhaps Nanog protein turnover. Cells grown on nongelling Matrigel, that were clumped were more likely to be positive for Nanog than individual cells around the periphery (fig. 3g,h).

Discussion

PUCs are of interest due to their stem cell characteristics and the ease with which large numbers can be harvested and grown in vitro. It has been observed that umbilical cord stem cells can differentiate into various mesenchymal cell types, neuronal cells and endothelial cells (Can et al., 2007). PUCs have been shown to be minimally immunogenic (Cho et al., 2008) suggesting they may have applications as transplants with less concern of donor rejection.

The three transcription factors Nanog, Oct-4 and Sox-2 are expressed at high levels in embryonic stem cells. These transcription factors regulate the expression of other genes during embryonic development and are found at high levels in pluripotent cells of the inner cell mass (Liu et al., 2007). When these transcription factors are down-regulated there is a loss of pluripotency and self-renewal and increased differentiation of the cells. It has been shown that these three transcription factors are expressed in PUCs (Carlin et al., 2006).

PUCs are located within Wharton's jelly of the umbilical cord. The Wharton's jelly is composed mainly of hyaluronic acid and collagens (Can et al., 2007). Many growth factors and cytokines are harbored within Wharton's jelly and many of these growth factors are known to regulate stem cell behavior. It can be suggested that Wharton's jelly serves as a niche or microenvironment in which the PUCs reside but the mechanisms operating in this niche are unknown.

Here we report attempts to re-create the Wharton's jelly environment in vitro with the intention to study Wharton's jelly effects on PUCs. The ability to grow PUCs in an environment more similar to their in vivo milieu could be useful for studying the

physiology of PUCs and the interactions of PUCs with their extracellular matrix. The Wharton's jelly of the porcine umbilical cord was used to create PMX, a product that gels when the protein concentration is at least 2.44mg/ml and the temperature is 37°C. In experiment one, PMX was compared to the gelled matrix Matrigel and plastic for effects on gene expression in PUCs. In addition to these matrices we evaluated the effects of a low molecular weight filtrate collected during production of PMX. We used PUCs at passages 3 to 5 for our studies and evaluation of the PUC isolates we used through passage 6 revealed that expressions of Nanog, Oct-4, and Sox-2 were stable over this interval. Therefore early-passage PUCs may provide an adequate in vitro model for evaluating the effects of Wharton's jelly products on the expression of these genes in PUCs.

We found that the gelled matrices reduced cell proliferation by PUCs and this was particularly apparent for Matrigel. Cells grown on gelling Matrigel tended to form tight colonies similar to those formed by embryonic stem cells. Matrigel also increased Nanog expression. Gelled PMX had no effect on expression of the three genes studied, but when FT was added to gelled PMX the expression of Nanog mRNA was decreased.

Culture in the presence of lower concentrations of the matrices that did not gel revealed no effects of the matrices alone (Exp. 2). However when FT was added to cultures containing non-gelling concentrations of the matrix products or plastic there tended to be an increase in Nanog expression. Sox-2 expression also tended to increase when PUCs were cultured on Matrigel+FT compared to Matrigel alone. These effects of FT could be due to growth factors in FT that potentially regulate gene expression in PUCs. We evaluated only one concentration of FT and only two concentrations of each matrix product in our experiments and we do not know the growth factors that are present in our PMX and FT products. Further research will be required to assess the possible effects of other amounts of these products. Based on literature reports of growth factors in Wharton's jelly we speculate that FT could include TGF-β, IGF-1, bFGF and PDGF. Both TGF-β and IGF-1 are known to regulate embryonic stem cells of the ICM (Bendall

et al., 2007). These proteins might be responsible for the effects of FT on PUCs but other unidentified components should also be evaluated.

In conclusion we have prepared an extract of porcine Wharton's jelly that supports attachment and growth of PUCs and identified a low molecular weight filtrate that stimulates expression of Nanog in vitro. We suggest that further work in this area may provide an increased understanding of the environment provided by Wharton's jelly and lead to improved methods for growing PUCs in vitro.

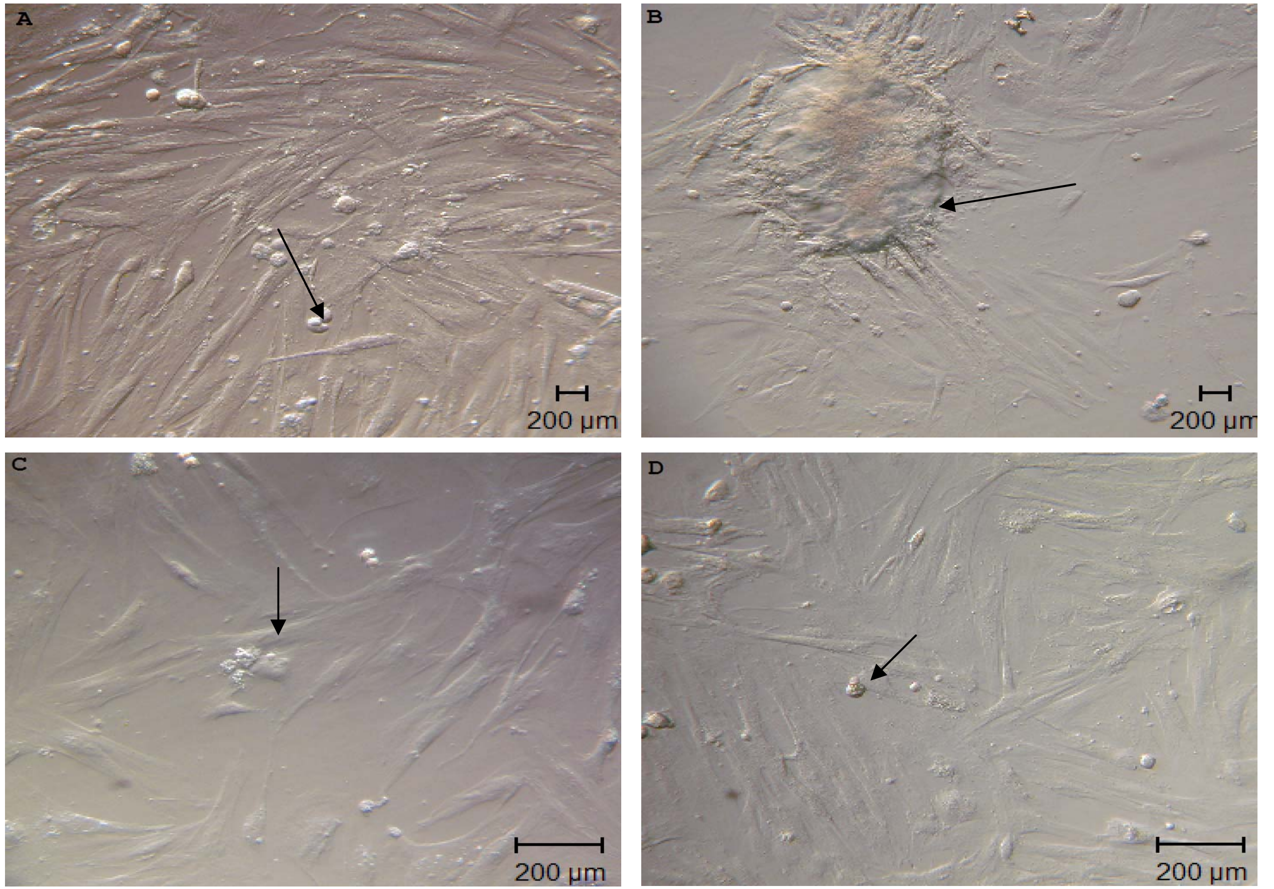

Figure 1. Morphology of PUCS Cultured on Gelling Matrices.

PMX(A), Matrigel(B), FT(C), Plastic(D). Arrows indicate colonies of cells.

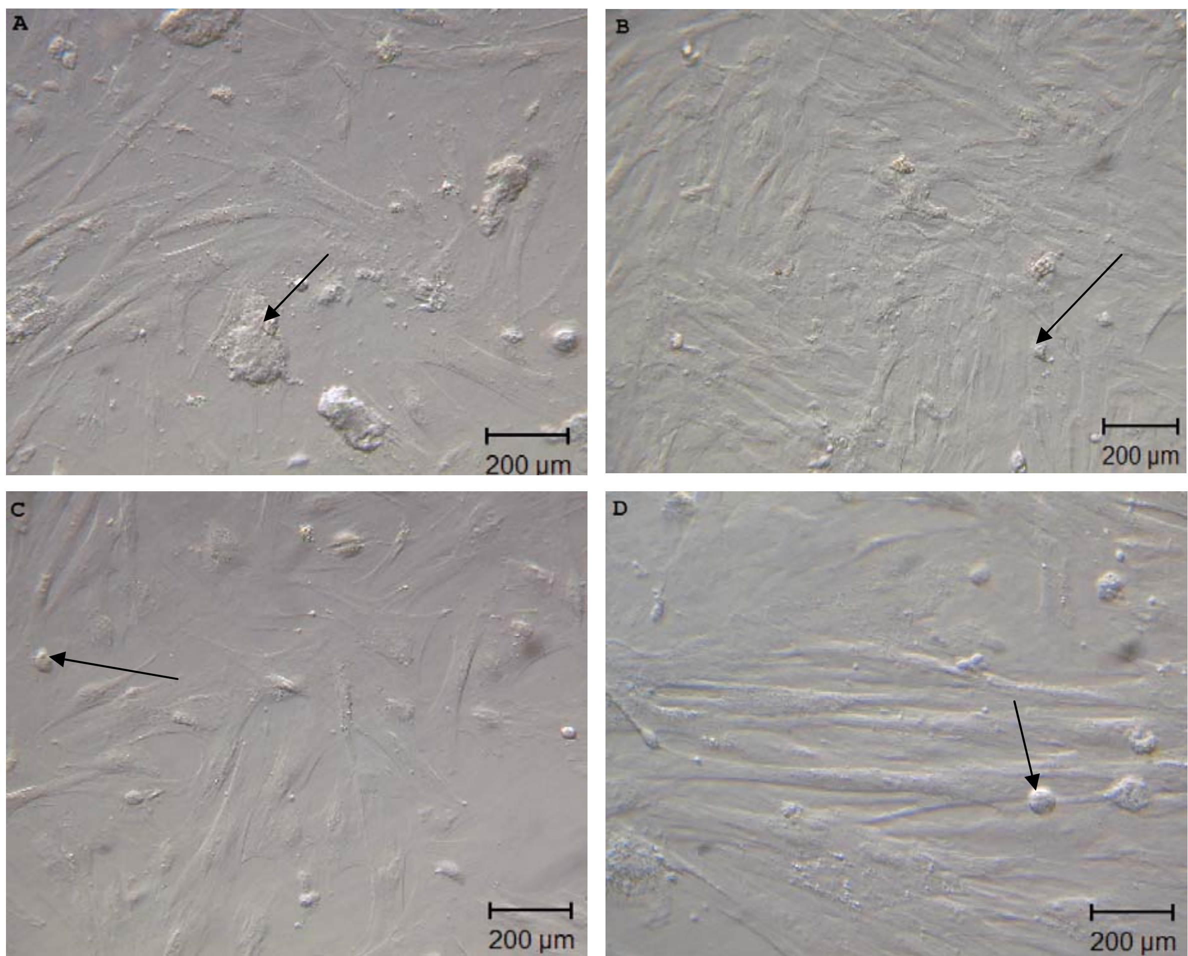

Figure 2. Morphology of PUCs Cultured on Non-Gelling Matrices.

PMX(A), Matrigel(B), FT(C), Plastic(D). Arrows indicate representative colonies of cells.

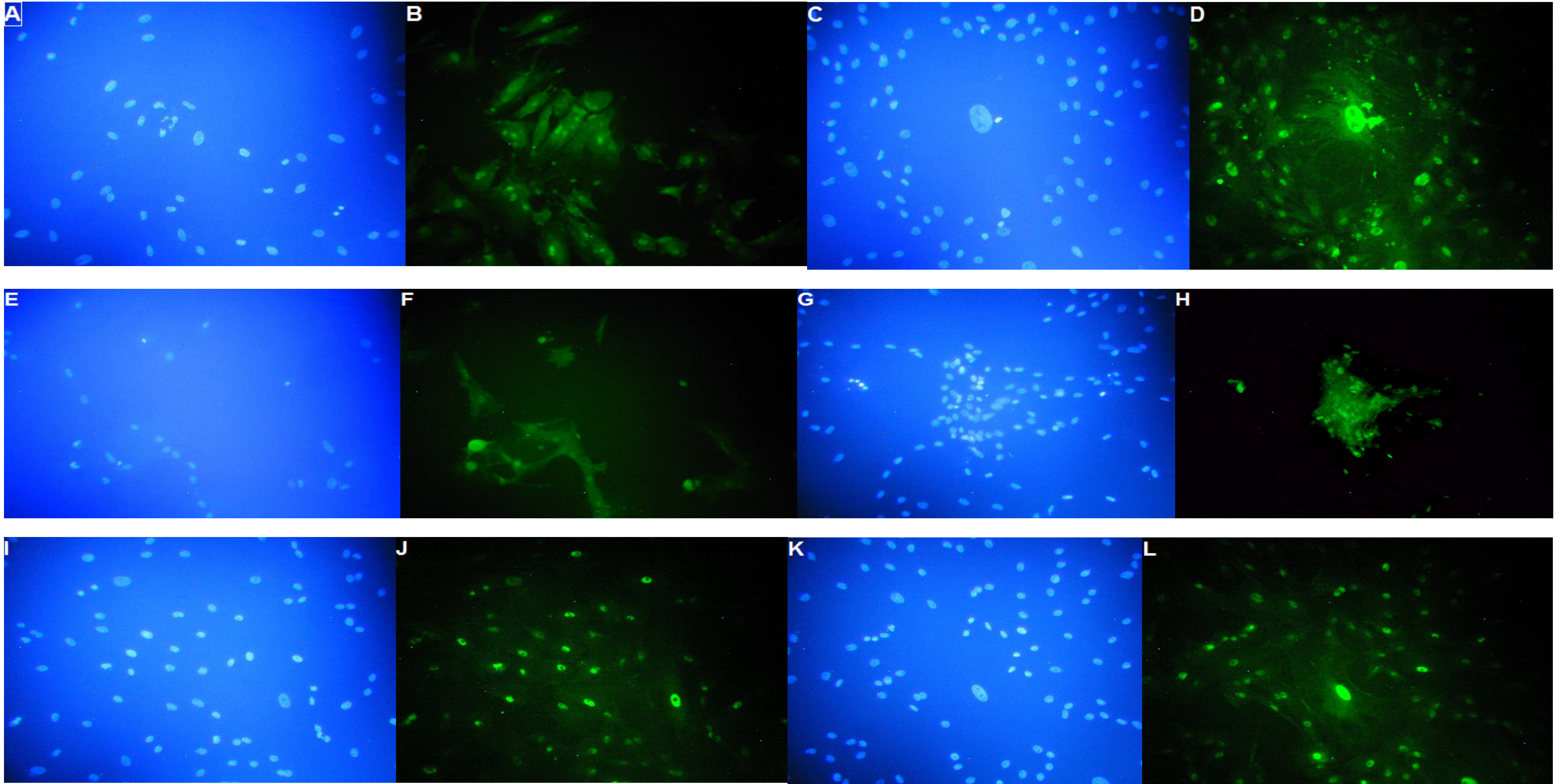

Figure 3. Immunocytochemistry for PUCs grown on different matrices.

DAPI A,C,E,G,J,K (PMX, Non-Gel PMX, Matrigel, Non-gel Matrigel, FT, Plastic respectively) Nanog: B,D,F,H,I,L (PMX,Non-Gel PMX, Matrigel, Non-gel Matrigel, FT, Plastic, respectively)

Table 2.1 Effect of gelled matrices and a low molecular weight filtrate of Wharton's jelly (FT) on proliferation of pig umbilical cord matrix stem cells.

Treatment	Plastic	Matrigel	Pormatrix	SE
n	3	3	3	
Cells[a]	41167^{b}	9458.3^{c}	16083^{c}	10031

[a] Cells/3 wells of a 24-well plate.

[bc]Treatments without a common superscript differ ($P<0.05$).

Table 2.2 Effect of gelled matrices and a low molecular weight filtrate of Wharton's jelly (FT) on gene expression [a] in pig umbilical cord matrix stem cells.

Treatment	**Plastic**		**Matrigel**		**PMX**		**SE**
n	3	3	3	3	3	3	
Gene	-	FT	-	FT	-	FT	
Nanog	17.8^{bc}	17.0^{b}	16.9^{b}	16.7^{b}	16.9^{b}	19.1^{c}	2.54
Oct-4	21.6	21.1	21.9	23.2	20.6	21.4	1.85
Sox-2	23.7	21.9	22.2	20.7	23.9	23.5	1.27

[a] Δ CT= average cycle at threshold (CT) for the 18S housekeeping gene-average CT for the target gene.

[bc] Means with different superscripts differ ($P<0.05$).

Table 2.3 Effect of non-gelled matrices and a low molecular weight filtrate of Wharton's jelly (FT) on cell proliferation of pig umbilical cord matrix stem cell cells.

Treatment	Plastic	Matrigel	Pormatrix	SE
n	3	3	3	
Cells[a]	43250[b]	8833.3[c]	22750[c]	11000

[a] Cells/3 wells of a 24 well plate.

[bc] Treatments without a common superscript differ (P<.05).

Table 2.4 Effect of addition of a low molecular weight filtrate of Wharton's jelly (FT) and non-gelled matrices on Nanog mRNA expression[a] in pig umbilical cord matrix stem cells.

FT	Plastic	Matrigel	PMX	SE
n	3	3	3	
-	21.9	21.9	22.1	22.0$\pm$2.62
+	21.5	20.1	20.8	20.82$\pm$2.62*

[a] ΔCT=Average CT 18S-Average CT for the target gene.

* Addition of FT tended (P= 0.10) to increase Nanog expression.

Table 2.5 Effect of non-gelled matrices and addition of a low molecular weight filtrate of Wharton's jelly (FT) on Sox-2 mRNA expression[a] in pig umbilical cord matrix stem cells[b].

Treatment	Plastic		Matrigel		PMX		SEM
	-	FT	-	FT	-	FT	
n	3	3	3	3	3	3	
	20.8	20.9	21.6	18.7	20.4	21.5	1.29

[a] ΔCT=Average CT 18S-Average CT target gene.

[b] A matrix x ft interaction tended (P=0.10) to affect Sox-2 expression.

Table 2.6 Effect of passage number on gene expression[a] in pig umbilical cord matrix stem cells*.

	Passage							
Gene	P0	P1	P2	P3	P4	P5	P6	SE
Nanog	19.54	19.38	18.75	18.50	19.55	18.64	19.01	1.08
Oct-4	25.14	24.50	24.97	23.90	25.16	24.67	24.21	1.02
Sox-2	22.17	23.30	23.36	24.59	24.01	23.87	23.59	1.25

[a] Δ CT=Average CT 18S-Average CT target gene.

* No significant difference (P>0.05) for transcription factors between passages.

CHAPTER 3 - References

PUBMED: MESH.2007.

PUBMED: Wnt proteins.2006.

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