FBS FREE CULTURE OF PORCINE UMBILICAL CORD MATRIX CELLS

by

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Abstract

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The common choice of medium for culturing pig umbilical cord matrix stem cells (PUCs) is high glucose Dulbecco's Minimum Essential Medium (HG-DMEM) supplemented with fetal bovine serum (FBS). FBS is a chemically undefined supplement that encourages attachment of explants and cells and is useful for long-term proliferation in an undifferentiated state. Removing FBS from the culture medium would decrease the possibility of microbial contamination and might produce more consistent results. A defined medium would facilitate experiments to determine requirements for specific growth factors and nutrients. Starting PUCs in a FBS-free environment proved to be a challenge. The results of 15 experiments testing various media, supplements, and culture conditions indicate that PUCs initially plated in an FBS-free environment do not attach as readily as those in HG-DMEM supplemented with FBS. PUCs were collected using enzyme digestion of the whole cord or by plating explants from the cord in culture medium. In the final experiment PUCs were seeded in 24-well plates ($5.0 * 10^4$ viable cells per well) with a collagen coating and cultured in Knock-out DMEM (KO-DMEM) with basic fibroblast growth factor (5ng/mL) and platelet derived growth factor (5ng/mL) in a low oxygen atmosphere ($5\%O_2$/ 5% CO_2/ 90% N_2). The total non-adherent cell count at passage 1 was $1.78 * 10^5 \pm 3.68 * 10^4$ and the total adherent cells were $2.58 * 10^5 \pm 9.29 * 10^4$. The well confluence during initial cell proliferation appeared similar to cells cultured in the control media with 20% FBS (total adherent cells = $6.40 * 10^5 \pm$ S.E. $1.61 * 10^5$ and total non-adherent cells = $2.88 * 10^5 \pm 7.60 * 10^4$). However the number of adherent cells recovered for passage 2 was considerably less for cultures in FBS-free media than for the control group. Serum may affect attachment by providing attachment factors or it could change expression of integrins or other attachment molecules on the PUCs that enhance attachment to plastic or other substrates. In future studies the requirements for attachment of PUCs should be further evaluated.

Table of Contents

List of Figures

List of Tables

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CHAPTER 1 - General Review of the Literature

Introduction

Today Stem cell research is a hot topic currently and is very controversial in the public's eyes. Stem cells are undifferentiated cells that replicate without differentiating, and in specific environments they can be induced to differentiate into one or more specialized cells. Stem cells are sometimes considered in two main categories according to the tissue source; embryonic stem cells and other stem cells. The latter are often called somatic stem cells, adult stem cells, or just non-embryonic. Embryonic stem cells are derived from the inner cell mass of the developing embryo (ES cells) or from the developing gonads (embryonic germ, EG, cells). The tissue sources of non-embryonic stem cells are many and varied in their properties and stem-cell state.

Another way to describe stem cells is according to their potency (Stem Cell Information. 2009). Totipotent stem cells can produce daughter cell lines of any cell lineage in the organism including the cells of the extra-embryonic membranes. Human embryonic stem cells are totipotent because they can become any of the over 200 different cell types in the human body. Pluripotent stem cells have the same ability as totipotent cells, with the exception of the potential to form trophoblasts. Multipotent stem cells are considered to have the least potential, and some consider their potential to be limited to derivatives of one germ layer (Rosenbaum, Grande, and Dines 2008).

Somatic stem cells cause fewer concerns from the public and offer a viable source of stem cells for therapeutic use. A specific subtype of multipotent stem cells, mesenchymal stem cells (MSCs), is highly researched due to the ease of their isolation from a variety of tissues. The in vivo distribution of MSCs is widespread and includes bone marrow, adipose, periosteum, synovial membrane, skeletal muscle, dermis, pericytes, blood, trabecular bone, umbilical cord,

lung, dental pulp and periodontal ligament (Karahuseyinoglu et al. 2007). MSC's can be obtained from Wharton's jelly of the umbilical cord. This organ is generally discarded after parturition and is a medical waste in humans. The cord offers a rich supply of mesenchymal like stem cells (Romanov et al. 2003). Currently the culture media for in vitro expansion of stem cells is generally supplemented with Fetal Bovine Serum (FBS). Fetal bovine serum is variable between lots and is a possible source of microbial contamination. A chemically defined system for culturing stem cells would overcome these limitations and might also improve efficiency by eliminating variation in cell culture results. A defined medium would also permit more thorough evaluations of the requirements of stem cells in vitro.

The Umbilical Cord

The umbilical cord is the physiological connection between the placenta and the fetus. It develops from the extra-embryonic mesoderm at day 13 of embryonic development in humans (Karahuesyinoglu, Cinar, Kilic et al 2007). The umbilical cord has two arteries and one vein (in most species) and a large amount of embryonic mucous connective tissue. The outer layer is squamous- or cuboidal epithelium and seems to be derived from the amnion. The mucous connective tissue is called Wharton's jelly and one of its' functions is to prevent twisting and bending of the blood vessels so the blood supply of the fetus is not interrupted. Can and Karahuseyinoglu (2007) describe six zones in the umbilical cord; the surface epithelium, the subamniotic stroma, clefts, the intervascular stroma which is identified as the Wharton's jelly, the perivascular stroma, and the vessels.

Characteristics of Mesenchymal Stem Cells

Mesenchymal stem cells have the ability to differentiate into connective tissue lineages, including bone, fat, cartilage, muscle, as well as ectodermal and endodermal lineages. MSCs can

be obtained in relatively large quantities from a variety of sources (Ng, Boucher, Koh, et al. 2008). Wagner and Ho define MSC's as plastic-adherent cells isolated from bone marrow or other tissues that are positive for specific mesenchymal antigens including, CD44, CD105, CD73, and CD90. MSCs must be negative for hematopoietic antigens including CD34 and CD45. They must also be able to differentiate at least into osteoblasts, adipocytes, and chondroblasts under specific in vitro conditions. Bone marrow is currently the primary research source for mesenchymal stem cell (Romanov et al 2003). MSCs from the umbilical cord are a fairly new discovery and have warranted further research as a multipotent somatic stem cell.

Baksh, Yao, and Tuan (2007) compared the proliferative and multi-lineage differentiation potential of human MSCs derived from umbilical cord and bone marrow. Human umbilical cord perivascular cells (HUCPVCs) were collect from consenting parents who underwent full-caesarian sections and bone marrow mesenchymal stromal cells (BMSCs) collected from hips of consenting patients.

HUCPVCs and BMSCs both underwent multi-lineage differentiation. HUCPVCs differentiated osteogenically at a faster rate than BMSCs and displayed greater mineralization than BMSCs. BMSCs and HUCPVCs displayed similar patterns of chondrogenesis and adipogenesis. The authors concluded that HUCPVCs could be an alternative source of cells for MSC-based therapies.

Another recently examined source for MSCs in the umbilical cord is the stroma, also known as Wharton's jelly. Wharton's jelly originates in the extra-embryonic mesoderm and encloses two arteries and one or two veins. It provides a connective tissue stroma that is rich in proteoglycans and mucopolysaccharides (Karahuseyinoglu et al. 2007). These authors described the phenotype and structure of the human umbilical cord MSCs (HUCs) in culture. They

characterized proliferation of HUCs, demonstrated that HUCs differentiate into mesodermal lineages, and compared HUCs with bone marrow MSCs. HUCs tend to have two distinct morphologies when grown in vitro. The first type was extremely flat and stress fiber-rich whereas the second type was more fusiform shape, wider in the middle and tapering toward the ends, and with long cytoplasmic extensions. The second type expressed vimentin which is a mesenchymal marker, but the first type expressed pancytokeratin, which is an ectodermal/endodermal marker. The first type of HUC declined in numbers with increased passages and these cells may not be mesenchymal stem cells. Results from a proliferation comparison between BMSCs and HUCs showed that the umbilical cord stromal cells proliferated at a faster rate. It has also been observed that HUCs are available in larger numbers per collection than MSCs in bone marrow, and are more easily obtained than BMSCs (Karahuseyinoglu, Cinar, Kilic et al. 2007).

Transcription Factors

In 2006 Carlin et al. reported expression of the pluripotency-associated transcription factors Nanog, Oct-4, and Sox-2 in porcine umbilical cord MSCs (PUCs). Nanog, Oct-4, and Sox-2 are central regulators of gene transcription in embryonic stem cells (ESCs). They regulate pluripotency and self renewal properties in mouse and human ESCs. Reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate RNA expression of each transcription factor. PUCs express Nanog at high levels comparable to porcine embryonic disks, but express relatively lower levels of Sox-2 and Oct-4. Carlin et al. (2006) suggest that this pattern of transcription factor expression is probably significant because Oct-4, Sox-2, and Nanog provide a core regulatory network in mouse and human ESCs and mouse embryos. Interpretation of this tissue comparison should also consider that the embryonic disks were evaluated as recovered and

without exposure to an in vitro environment while the PUCs were grown for several days in vitro in the presence of serum and that may have altered their gene expression.

Mesenchymal Stem Cell Markers

MSC phenotype includes expression of certain surface markers and the absence of other markers. Cluster of differentiation (CD) markers CD10, 13, 29, 44, 49, 73, 90, 105, 146, and 166 are associated with MSCs. HUCs are consistently positive for CD105, CD73, and CD90 and do not express cell surface markers CD45 and CD34 which are markers for hematopoietic stem cells (Can, Karahuseyinoglu 2007). It has been shown that HUCs positive for CD105 differentiate in vitro to skeletal myocytes (Baksh, Yao, and Taun 2007). It has also been shown that HUCs express adhesion molecules CD44 and CD105 as well as integrin markers CD29 and CD51. HUCs also express mesenchymal stem cell markers SH2 and SH3 (Wang, Hung, Peng et al. 2004). Expression of surface marker phenotype of HUCs is consistent with their classification as MSCs.

Growth Factors and MSCs

Basic Fibroblast Growth Factor (bFGF)

Basic FGF is thought to help ESCs proliferate in an undifferentiated state. Human ESCs cultured in the presence of bFGF expressed slightly elevated levels of Oct-4 and telomerase reverse transcriptase (hTert) mRNA compared to those not exposed to bFGF (Yeouh, Van Os, Weersing et al. 2006). This may indicate that bFGF is an important regulator in maintaining ESCs in an undifferentiated and proliferating state (Xu, Rosler, Jiang et al. 2005).

Plate-Derived Growth Factor (PDGF)

Ng, Boucher, Koh et al. (2008) found that bFGF and PDGF signaling plays a vital role in regulating differentiation. Undifferentiated MSCs strongly express platelet derived growth factor (PDGF). Inhibition of bFGF and PDGF signaling has been shown to negatively affect chondrogenic and osteogenic differentiation of MSCs (Kourembanas and Faller. 1989). Supplementation of PDGF, unlike bFGF supplementation, does not completely inhibit differentiation of MSCs in culture. PDGF signaling is important in adipogenesis and chondrogenesis (Ng, Boucher, Koh, et al. 2008). These results indicate that bFGF along with PDGF are important for growth, proliferation and reduced differentiation of MSCs.

Epidermal Growth Factor (EGF)

EGF is another protein that functions in maintaining mesenchymal cell proliferation and attachment. When EGF interacts with its receptor, intracellular signaling is activated that promotes attachment, proliferation, and some cell migration (Fan, Au, Tamama et al. 2007). EGF has been shown to promote cell growth in bone marrow mesenchymal cells and may have the same positive effects with mesenchymal stromal cells from the umbilical cord perivascular tissue (Platt, Roman, Wells et al. 2009). Interestingly tethering EGF to a synthetic matrix rendered it more stimulatory to MSCs that soluble EGF (Fan, Au, Tamama et al. 2007).

Umbilical Cord Matrix Cells

Differentiation Lineages

Umbilical cord matrix cells (UCMCs) are obtained from the Wharton's jelly for all species studied to date and have been most completely characterized for humans (HUCs). They attach readily to plastic, are positive for certain cell surface markers associated with MSCs (CD73-, CD29-, CD 105-), while negative for others (CD14-, CD34-, CD45-), and have the

ability to self replicate. UCMCs are multipotent as indicated by their ability to differentiate in vitro to a variety of cells including; bone, fat, cartilage, neural cells as well as several others (Weiss, Anderson, Medicetty et al. 2008).

The primary lineages of differentiation researched for UCMCs are adipogenesis, chondrogenesis, and osteogenesis. In 2003 Romanov, Svintsitkaya, and Smirnov reported HUCs ability to differentiate into these lineages. Umbilical cords were collected postpartum and HUCs were cultured in Dulbecco's modified Eagle's medium (DMEM) with adipogenic or osteogenic treatments and 10% FBS. Adipogenic differentiation was seen after 1 week of incubation in adipogenic conditions. At the end of 2 weeks in culture almost all cells contained Oil-Red-O-positive lipid droplets. Cells treated with osteogenic medium resulted in the majority of cells being alkaline-phosphatase positive, indicating osteogenic differentiation had occurred. The control cells grown in DMEM with 10% FBS did not differentiate and continued to proliferate (Romanov, Svintsitskaya, and Smirnov, 2003).

Mitchell, Weiss, and Mitchell et al. (2003) induced differentiation of UCMS cells to differentiate to neural cells after culture in DMEM with 10% FBS supplemented with dimethylsulfoxide, butylated hydroxyanisole, potassium chloride, valporic acid, forskolin, hydrocortisone, and insulin. This medium for neural growth caused drastic changes in morphology. UCMCs changed from a fusiform or stellate appearance to cells with a single axon-like process, and cells forming multiple neurite extensions. UCMCs induced to form neural cells stained positive for neural-specific enolase (NSE), a marker for neural stem cells. Staining decreased slightly 5 hours after neural induction. NSE was also expressed in untreated UCMCs. The mature neural marker neurofilament M (NFM) was expressed on days 1 and 3 post-induction and TuJ1 (class III β-tubulin), another marker for neuronal differentiation,

increased from day 1 to 10 post-induction. TuJ1 was highly expressed in the cell body and proximal part of the axon-like structures. Differentiation into specific neural type tyrosine hydroxylase (TH), a marker for catecholaminergic neurons, was analyzed. TH was present in neurosphere-like colonies and in fully induced UCMCs but TH was not observed in untreated UCMCs (Mitchell, Weiss, Mitchell et al. 2003). This initial report concluded that UCMSCs have to the ability to differentiate into multiple lineages and therefore the potential for therapeutic use.

Therapeutic Potentials

Rat UCMCs have been transplanted into the brain of Parkinsonian rats (Weiss et al. 2006). These cells continued differentiation after injection to become dopaminergic neurons. The formation of these neurons alleviated the behavioral deficits associated with Parkinson's disease.

Jomura, Uy, and Mitchell et al. (2007) reported that cells from the rat umbilical cord (RUCs) that are positive for Oct 4. RUCs were used to evaluate the possible prevention of brain damage brought on by cerebral global ischemia from induced asphyxia leading to cardiac arrest. Eight minutes after the onset of cardiac arrest, the rat was resuscitated. Rats treated with RUCs 3 days before cardiac arrest showed less brain damage than those that were not pretreated with RUCs. Brain damage was evaluated by the loss of pyramidal neurons of the hippocampus (Jomura, Uy, Mitchell et al. 2007). The therapeutic value of this treatment would rely on the ability to predict that cerebral ischemia (stroke) was going to occur but this area requires more research to determine whether this treatment can be effective after the insult.

In a comparison of human bone marrow derived MSCs and HUCs for cartilage tissue engineering both cell types were initiated in LG-DMEM with 2%FBS. After initial isolation,

cells of both types were seeded in DMEM-LG with 10% FBS on non-woven PGA scaffolds. After passage 4 the media was replaced with HG-DMEM supplemented with 1% non-essential amino acids and 10ng/mL of transforming growth factor beta-1. HUCs achieved a 75% seeding efficiency (percentage ratio of DNA of attached cells versus total DNA of all plated cells) while BMSCs only achieved a seeding efficiency of 62%. HUCs also reached 80% to 90% confluence 2 to 3 days earlier than BMSCs. When exposed to chondrogenic media both cell types produced cartilage-like tissue. HUCs had a more efficient seeding rate and proliferated faster than MSCs, and produced more collagen (Wang, Tran, Seshareddy et al. 2009).

Culture Media

Serum Containing Media

Bone marrow MSCs used for clinical trials are generally grown in a medium supplemented with FBS. Use of UCMSCs in clinical trials has not been reported but in vitro culture is generally maintained in a medium supplement with FBS.

Differentiation Inducing Media

Romanov, Svintsitskaya, and Smirnov (2003) used the umbilical cord vein as an alternative source for human mesenchymal stem cells. The cord vein was canulated on both sides and rinsed with Earl's balanced salt solution (EBBS). Then 0.1% collangenase in Medium 199 was infused. The vessel was washed again with EBBS and massaged to remove endothelial and subendothelial cells. The cells were cultured in DMEM with low glucose (LG-DMEM), 20mM Hepes, 100U/ml of penicillin, 100ug/ml of streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, and 10% FBS. After 2 weeks cells were cultured in the media supplemented with 0.5uM isobutyl-methylxantine, 1uM dexamethasone, 10uM insulin, and 200uM

indomethacin for adipogenesis, or 0.1uM dexamethasone, 10uM B-glycerophospate, and 50uM ascorbate-phosphate for osteogenesis. Cells growing in LG-DMEM without 20% FBS did not spread, migrate, or proliferate. Adipogenic differentiation was observed after 1 week of incubation in adipogenic media. After the second week of incubation in differentiation medium the cells contained several Oil-Red-O-positive lipid droplets, an indicator of adipocytes. When cells were induced with osteogenic media the MSC-like cells became alkaline-phosphate-positive, indicating that osteogenesis had occurred. The cells that were in the LG-DMEM with 20% FBS did not display signs of spontaneous osteogenesis or adipogenesis after 3-4 weeks of cultivation (Romanov, Svintsitskaya, Smirnov 2003). They also showed that FBS promotes proliferation of mesenchymal stromal cells from the umbilical cord vein and allows them to proliferate in an undifferentiated state through multiple passages.

When Mitchell, Weiss, Mitchell and others in 2003 generated neurons and glia from matrix cells of Wharton's jelly, they started cells in HG-DMEM supplemented with 20%FBS. After initial culture the cells were transplanted and culture in HG-DMEM medium with 2% FBS and supplemented for differentiation into neural lineages (Mitchell, Weiss, Mitchell et al. 2003).

It has been shown that UCMSCs can differentiate into cardiomyocytes when cultured in HG-DMEM supplemented with glucose, 5-azacytidine, and 10% FBS. Cells in conditioned media for cardiogenesis (serum-free DMEM with 3μM 5-azacytidine) expressed N-cadherin, a cardiomyocyte marker. Cells in unconditioned media (HG-DMEM with 10% FBS) expressed low levels of N-cadherin also but at lower levels than those in conditioned media. This indicates that FBS provides some differentiating factors but not to the same extent as media conditioned by cardiomyocytes (Wang, Hung, Peng et al. 2004).

Shahdadfar, Fronsdal, Haug and others (2005) compared serum preparation for effects on human bone marrow MSCs ability to differentiate into mesodermal lineages. All cells were cultured on HG-DMEM/F12 supplemented with 20% FBS from Biochrom AG lots 098B and 074EE, FBS from Gibco, human off-the-clot pooled allogeneic serum, and autologous serum. After first passage cells the FBS was reduced to 10%. FBS from Gibco resulted in the highest cell counts through passage 10 and was used for differentiation into the mesodermal lineages instead of FBS from Biochrom AG. Cells cultured in FBS supplements of HG-DMEM/F12 expressed a slower cell doubling time than autologous serum supplemented media, and allogeneic serum supplemented media resulted in proliferation for a short time but cells did not survive beyond passage 1. HG-DMEM/F12 with autologous serum or FBS allowed differentiation into osteocytes, chondroblasts, and adipocytes. While cells cultured in autologous serum proliferated at a faster rate than those in FBS, the differentiation was slower in autologous serum supplemented media. Adipocytes were visible after 3-4 days in FBS supplemented HG-DMEM/F12 compared to 8-12 days in HG-DMEM/F12 supplemented with autologous serum and the same tendency was noted for chondrogenic induced cultures (Shahdadfar, Fronsdal, Haug et al. 2005).

In 2006 Sortiropoulou, Perez, Salagianni and others reported comparisons of proliferation and differentiation of human bone marrow stromal cells cultured in LG-DMEM with L-glutamine, HG-DMEM with L-glutamine, HG-DMEM with Glutamax, LG-DMEM with Glutamax, modified Eagle's medium alpha (α-MEM) with L-glutamine, α-MEM with Glutamax, and Optimem, all supplemented with 10% FBS. α-MEM was determined to be the more suitable

culture medium for isolation and expansion and media supplemented with glutamax compared to L-glutamine expressed greater proliferation (Sortiropoulou, Perez, Salagianni et al. 2006).

Wang, Tran, Seshareddy et al. (2008) also compared culture media for bone-marrow derived MSCs. Initially LG-DMEM, HG-DMEM-HG, F12-DMEM, and Knockout DMEM (KO-DMEM) were all supplemented with 10% FBS. MesenCult, another medium, was supplemented with mesenchymal stem cell stimulatory supplement (Stem Cell Technologies, 05042). MSCs cultured in KO-DMEM and F12-DMEM maintained typical spindle morphology through 25 passages, but population doubling time was significantly less in KO-DMEM. Bone marrow MSC failed to continue to proliferate past passage five in HG-DMEM when cultured in MesenCult. LG-DMEM maintained typical morphology through passage 10 and then declined through passage 25. Since KO-DMEM appeared to be the optimal basal medium a comparison of FBS concentrations experiment was conducted. KO-DMEM with FBS supplement concentrations compared were 2% and 5% FBS and 10% Knockout-serum replacement (KOSR). KO-DMEM with KOSR failed to support growth, while cells supplemented with 2% FBS maintain normal growth in early passages it failed to stimulate growth further than passage five (Pal, Hanwate, Jan, and Totey. 2009). Bone marrow MSC's culture in KO-DMEM with 5% FBS maintained normal growth and morphology past passage 10.

Serum Free Media

FBS has many positive effects on MSCs but it also has draw backs. FBS is an undefined supplement that varies in its effects on cells between lots, and may contain contaminants. FBS is also a source of xenogeneic antigens and has the potential risk of transmitting animal viral, prion, and zoonose contaminants (Shahdadfar, Fronsdal, Haug et al. 2005).

A chemically defined growth medium for MSCs including UCMSCs is needed. When considering such a medium one should evaluate the effects on cell proliferation and the differentiation potential.

FBS may or may not contain factors that cause death or differentiation of stem cells in culture. Serum contains a number of growth factors, nutrients, and inorganic elements that vary between batches of serum causing varied results in cell cultures. There have been efforts to develop serum free supplements for cell culture to reduce the possible contaminates but the efficiency of cell replication remains in question (Petkov and Anderson 2008). Use of these supplements has the potential for standardizing cell expansion and increasing efficiency of replication.

Serum-free culture of other stem cells

Brown, Xu, Dusing et al. (1997) analyzed culture conditions for bone marrow hematopoietic stem cells in serum-free culture. When cells reached 21 days in culture they were evaluated for granulocyte-macrophage colony forming units (GM-CFC), spleen colony forming units (SCF), and cells responsible for short term and long term hematopoietic repopulation. Bone marrow cells were cultured in QBSF-58, a serum free medium designed for hematopoietic cell growth, containing Iscove's modified Dulbecco's medium (IMDM) plus 20% FBS. Mouse bone marrow cells were cultured for up to 28 days in QBSF-58 supplemented with stem cell factor, Granulocyte-Macrophage colony stimulating factor (GM-CSF), or a combination of the two cytokines. Cells cultured in QBSF-58 with either stem cell factor or with GM-CSF showed a slight increase in cell numbers, followed by a decline in cell number after day 21. When QBSF-58 was supplemented with both stem cell factor and GM-CSF it was reported that cell numbers increased 90-fold over initial seeding rate by day 28. This is considerably higher than

the 3.2 fold increase for cells grown in media with 20% FBS. In serum-free media without any cytokines, granulocyte-macrophage colony forming cells rapidly decreased and by day 10 there were almost no GM-CFC to be found. When serum free media treated with SCF or GM-CSF, the GM-CFC were maintained cells for the first 14 days but began to decline by day 14 and 21. Cells cultured in serum-free media with SCF and GM-CSF the colony forming cells increased at approximately 42-fold over the initial seeding. Serum free media treated with GM-CFS and SCF were necessary to maintain spleen colony forming units.

Passier, Ward Van Oostwaard, Snapper, and colleagues (2005) described a marked enhancement of cardiomyocyte differentiation in serum-free medium for human ESCs co-cultured with a visceral endoderm-like cell line, END-2. Initial plating of hESCs was done in HG-DMEM containing insulin, transferin, and selenium (ITS) and with 20% FBS for 6 days. Concentrations of FBS were then varied from 0% -20% in HG-DMEM, to examine serum effects on cardiomyocyte differentiation. During culture the hESCs developed three-dimensional structures with differentiated cells spreading out from the structures and after co-culture for 12 days the edges of cells were more defined in the absence of serum. A 24-fold up regulation of beating areas was present in cultures without FBS compared to those with 20% FBS. The addition of FBS to culture 6 days after being in serum-free culture resulted in a decrease in beating area by 57% and by 2% for those in 20% serum initially and then removal of serum after 6 days, compared to those in serum free culture continuously. A similar decrease in the number of beating areas was observed when KOSR was added rather than serum. The increase in number of beating areas in serum-free conditions suggests that there is a greater efficiency on cardiomyocyte differentiation in serum-free culture. While this is a more defined culture medium, embryoid bodies were not maintained entirely in serum-free conditions, since they were

started in HG-DMEM with 20% FBS a the possibility of contamination from FBS remains (Passier, Ward –van Oostwaard, Snapper et al. 2005).

Petkov and Anderson (2008) investigated the possibility of substituting KOSR for FBS when establishing and maintaining porcine embryonic germ cells. Porcine embryo tissue pieces were cultured in HG-DMEM supplemented with 17% FBS, 17% KOSR, and 17% KOSR with growth factors (LIF, SCF, and bFGF). DMEM supplemented with KOSR produced similar total colony areas and number of colonies in primary culture when compared to HG-DMEM with FBS. Total and average colony areas were significantly higher for KOSR supplemented media compared to media supplemented with KOSR and growth factors. Cell proliferation was considerably slower initially for cells cultured in HG-DMEM with KOSR and growth factors compared to HG-DMEM with FBS and HG-DMEM with KOSR alone. After five to six passages, cell proliferation for those cultured in HG-DMEM with KOSR and growth factors were similar to the other treatments. Cell proliferation continued on average for 10 passages longer when cultured in HG-DMEM with FBS or DMEM with KOSR and growth factors compared to HG-DMEM with KOSR alone. Addition of growth factors to HG-DMEM supplemented with KOSR appears to be essential for maintaining long term cell culture in media without FBS but have actually been shown to cause adverse effects in short term cell culture (Petkov and Anderson 2008).

MSCs in FBS-free culture

A comparison of ex vivo expansion culture conditions of MSCs was conducted by Perez-Ilzarbe, Diez-Campelo, Aranda et al. (2009). Bone marrow mononuclear cells were culture in α-MEM in four different media supplementations. The first was α-MEM with 10%FBS and 1ng/mL bFGF, the second medium consisted of α-MEM with 10% human serum and bFGF. The

third culture media supplement used was 5% platelet lysate (PL) in α-MEM and the final media in this comparison was α-MEM and PL with bFGF. All cultures were maintained until passage 4 and were then terminated. The addition of bFGF to PL did not significantly increase the number of cells obtained in culture and the time it took reach passage 4 was not significantly different among any treatments. All treatments had the same osteogenic, adipogenic, and chodrogenic potential. The authors concluded that further research into PL and human serum is needed for definitive proof that they are an equivalent substitute for FBS in culture.

Adipose derived MSCs have been characterized in serum-free media. Adipose derived stem cells have broad differentiation abilities. They are able to differentiate into not only mesenchymal lineages but also neurogenic and hepatic lineages. In a effort to avoid FBS in cell culture three serum replacement substitutes were evaluated by Lund, Pilgaard, Duroux et al. (2009). DMEM low glucose and high glucose in addition HG-DMEM/F12 and α-DMEM were also used as basal medium and all four were supplemented with either FBS, serum replacement 1 (SR1) (Sigma, S0638), serum replacement 3 (SR3) (Sigma, S2640), or KOSR (Gibco, 10828). Serum replacement 1 and serum replacement 3 are defined serum substitutes for FBS. SR1 is designed as a substitute when evaluating growth factors and SR3 is primarily used for long-term culture of human or other mammalian cell in the absence of FBS. SR1 and SR3 are defined products from Sigma-Aldrich (St. Louis, MO). KOSR is another chemically defined direct substitute for FBS in culture of ES cells. Cells were initiated in F12-DMEM with 10% FBS and changed to culture conditions tested when cells had attached after 6 hours. Culture conditions after cells were attached included; F12-DMEM with 5% or 10% FBS, F12-DMEM 1 x serum replacements SR1 and SR3, and F12-DMEM in 10% KOSR or 20% KOSR. For comparison of basal media 10% FBS was supplemented in F12-DMEM, HG-DMEM, LG-DMEM, F12, or α-

MEM. After 14 days of cell expansion to test basal media, or after 21 days to test serum replacements, the media was changed to induce adipogenesis, osteogenesis, and chondrogenesis.

Adipogenic differentiation was accomplished with adipogenic induction medium consisting of DMEM/F12, 10% FBS, 0.1µM dexamethasone, 0.45µM isobutylmethylsnthine, 170nM insulin, 0.2 µM indomethacin, and 1µM rosiglitazone. Adipogenesis was evaluated by visualization of staining lipid droplets with Oil Red O. Osteogenic differentiation was accomplished by osteogenic medium containing DMEM/F12 with 10% FBS. Evaluation of osteogenesis was done by visualization of calcium deposits with Alizarin Red. Chondrogenesis was induced by chondrogenic medium consisting of DMEM-HG supplemented with ITS. SR1 and SR3 did not support adipocyte stem cells to the same extent as FBS or KOSR. There was no significant difference in cell growth between media supplemented with 5% or 10% FBS, and 10% or 20% KOSR. Media with 10% and 5% FBS and 20% KOSR were the only media effective in adipogenic and chondrogenic differentiation, while cells cultured in 10% FBS, 5% FBS, 10% KOSR, and 20% KOSR stained positive for osteogenesis. Media supplemented with 20% KOSR showed similar results as 10% and 5% FBS for differentiation ability. α-MEM was the most suitable medium for adipocyte expansion. A comparison of composition of the media revealed that α-MEM; DMEM/F12 and F12 contained alanine and proline amino acids, vitamin B12, and ascorbic acid. DMEM/F12 and F12 along with α-DMEM were all identified as the best basal media for adipocyte derived stem cells (Lund, Pilgaard, Duroux et al. 2009). Possibly the presence of all or some of the nutrients alanine, proline, vitamin B12, and ascorbic acid are necessary for the preservation of differentiation potential during cell expansion.

Conclusions

A chemically defined medium free of animal products would be beneficial for UCMS cell culture. Removal of animal products from the medium would eliminate the possibility of zoonose transmission. The ability to culture cells in media that is absent FBS might increase efficiency in replication and increase ease of expansion for acquiring large numbers of cells from one collection. Ideally a serum replacement would be effective for initial cell isolation because cells established in serum-containing medium could be contaminated or their phenotype altered. Bone marrow mesenchymal stem cells are currently used as a therapeutic for some cancers. MSCs can be obtained from the umbilical cord blood and have been proven to aide bone marrow hematopoietic cells in maintaining an undifferentiated state (Hayashi, Takahashi, Abe, and Kashiwakura 2009). This is beneficial in regards to hematopoiesis and improving immune response after bone marrow transplant. MSCs obtained from the umbilical cord or other mesenchymal stem cells like cord blood have a vast potential that has yet to be fully appreciated. A chemically defined culture medium would help further our understanding and treatment of certain illnesses and diseases.

References

Can, A., S. Karahuseyinoglu, 2007. Concise review: Human umbilical cord stroma with regard to the source of fetus-derived stem cells. Stem Cells 25: 2886-2895.

Carlin, R., D. Davis, M. Weiss, B. Schultz, D. Troyer. 2006. Expression of early transcription factors oct-4, sox-2, and Nanog by porcine umbilical cord (PUC) matrix cells. Reproductive Biology and Endocrinology 4: 8.

Baksh, D., R. Yao, R. Tuan. 2007. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 25: 1384-1392.

Brown, R. L., F. Xu, S. Dusing, Q. Li, R. Fischer, M. Patchen. 1997. Serum-free culture conditions for cells capable of producing long-term survival in lethally irradiated mice. Stem Cells 15: 237-245.

Bryan, K. Effects of extracellular matrices on porcine umbilical cord matrix stem cells. http://hdl.handle.net/2097/1081. 2008

Fan, V. H., A. Au, K. Tamama, R. Littrell, L. Richardson, J. Wright, A. Wells, L. Griffith. 2007. Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells. Stem Cells 25: 1241-1251.

Hayashi, N., K. Takahashi, Y. Abe, I. Kashiwakura. 2009. Placental/umbilical cord blood-derived mesenchymal stem cell-like stromal cells support hematopoietic recovery of X-irradiated human CD34+ cells. Life Sciences 84: 598-605.

In Stem Cell Information. http://stemcells.nih.gov/info/glossary.asp. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services. 2009.

Jomura, S., M. Uy, K. Mitchell, R. Dallasen, C. Bode, Y. Xu. 2007. Potential treatment of

cerebral global ischemia with oct-4^+ umbilical cord matrix cells. Stem Cells 25: 98-106.

Karahuseyinoglu, S., O. Cinar, E. Kilic, F. Kara, G. Akay, D. Demiralp, A. Tukun, D. Uckan, A. Can. 2007. Biology of stem cells in human umbilical cord stroma: In situ and in vitro surveys. Stem Cells 25: 319-331.

Kourembanas, S., D. Faller. 1989. Platelet-derived growth factor production by human umbilical vein endothelial cells is regulated by basic fibroblast growth factor. J. Biological Chemistry 264: 4456-4459.

Lund, P., L. Pilgaard, M. Duroux, T. Fink, V. Zachar. 2009. Effect of growth media and serum replacements on the proliferation and differentiation of adipose-derived stem cells. Cytotherapy 11: 189-197.

Mitchell, K. A., M. Weiss, B. Mitchell, P. Martin, D. Davis, L. Morales, B. Helwig, M. Beerenstrauch, K. Abou-Easa, T. Hildreth, D. Troyer. 2003. Matrix cells from wharton's jelly form neurons and gila. Stem Cells 21: 50-60.

Ng. F., S. Boucher, S. Koh, K. Sastry, L. Chase, U. Lakshmipathy, C. Choong, Z. Yang, M. Vemuri, M. Rao, V. Tanavde. 2008. PDGF, TGF, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): Transcriptional profiling can indentify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. Blood 112: 295-307.

Pal, R., M. Hanwate, M. Jan, S. Totey. 2009. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. J Tissue Eng Regen Med 3: 163-174.

Passier, R., D. Ward-van Oostwaard, J. Snapper, J. Kloots, R. Hassink, E. Kuijk, B. Roelen, A. Brutel De la Riviere, C. Mummery. 2005. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free conditions. Stem Cells 23: 772-780.

Perez-Ilzarbe, M., M. Diez-Campelo, P. Aranda, S. Tabera, T. Lopez, C. del Canizo, J. Merino, C. Moreno, E. Andreu, F. Prosper, J. Perez-Simon. 2009. Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy. Transfusion 1537: 1-10.

Petkov, S. G., G. Anderson. 2008. Culture of porcine embryonic germ cells in serum-supplemented and serum-free conditions: The effects of serum and growth factors on primary and long-term culture. Cloning and Stem Cells 10: 263-275.

Platt, M.O., A. Roman, A. Wells, D. Lauffenburger, L. Griffith. 2009. Sustained epidermal growth factor receptor levels and activation by tethered ligand binding enhances osteogenic differentiation of multi-potent marrow stromal cells. J. Cell Physiol. 221: 306-317

Romanov, Y., V. Svintsitskaya, V. Smirnov. 2003. Searching for alternative sources of postnatal human mesenchymal stem cells: Candidate MSC-like cells from umbilical cord. Stem Cells 21: 105-110.

Rosenbaum, A. J., D. Grande, J. Dines. 2008. The use of mesenchymal stem cells in tissue engineering. Organogenesis 4: 23-27.

Shahdadfar, A., K. Fronsdal, T. Haug, F. Reinholt, J. Brinchmann. 2005. In vitro expansion of human mesenchymal stem cells: Choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells 23: 1357-1366.

Sotiropoulou, P. A., S. Perez, M. Salagianni, C. Baxevanis, M. Papamichail. 2006. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 24: 462-471.

Wang, H., S. Hung, S. Peng, C. Huang, H. Wei, Y. Guo, Y. Fu, M. Lai, C. Chen. 2004. Mesenchymal stem cells in the wharton's jelly of the human umbilical cord. Stem Cells 22: 1330-1337.

Wang, L., I. Tran, K. Seshareddy, M. Weiss, M. Detamore. 2009. A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived mesenchymal stromal cells for cartilage tissue engineering. Tissue Engineering 15: 1-8.

Weiss, M. L., C. Anderson, S. Medicetty, K. Seshareddy, R. Weiss, I. Vanderwereff, D. Troyer, K. McIntosh. 2008. Immune properties of human umbilical cord wharton's jelly-derived cells. Stem Cells 26: 2865-2874.

Weiss, M. L., S. Medicetty, A. Bledsoe, R. Rachakatla, M. Choi, S. Merchav, Y. Luo, M. Rao, G. Velagaleti, D. Troyer. 2006. Human umbilical cord matrix stem cells: Preliminary characterization and effects of transplantation in a rodent model of parkinson's disease. Stem Cells 24: 781-792.

Xu, C., E. Rosler, J. Jiang, J. Lebkowski, J. Gold, C. O'Sullivan, K. Delavan-Boorsma, M. Mok, A. Bronstein, M. Carpenter. 2005. Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 23: 315-323.

Yeoh, J.S,G., R. Van Os, E. Weersing, A. Ausema, B. Dontje, E. Vellenga, G. De Haan. 2006. Fibroblast growth factor-1 and -2 preserve long-term repopulating ability of hematopoietic stem cells in serum free cultures. Stem Cells 24: 1564-1572.

CHAPTER 2 - FBS-Free Culture of Porcine Umbilical Cord Matrix Cells

Introduction

Currently culture medium for in vitro expansion of stem cells is generally supplemented with Fetal Bovine Serum (FBS). Fetal bovine serum contains unidentified components making it undefined, it is variable between lots, and is a possible source of microbial contamination. A chemically defined system for culturing stem cells could overcome these limitations and might also improve consistency of results by reducing variation between experiments using different lots of FBS. The goal of the research presented here is to grow porcine umbilical cord matrix stem cells (PUCs) in a chemically defined medium. There have been reports of other mesenchymal stem cells cultured without the supplementation with FBS (Perez-Ilzarbe, Diez-Campelo, Aranda et al. 2009 and Lund, Pilgaard, Duroux et al. 2009), but there are no published data for the culture of PUCs without FBS.

In our experiments the attachment of explants and cells, the ability of cells to proliferate, and the survival of the cultures were evaluated for FBS-free cultures of PUCS. The experiments evaluated the basal media, addition of growth factors, supplementation with serum substitutes, matrix coating of the wells, and oxygen concentrations for their ability to promote attachment and growth for multiple passages.

Materials and Methods

Collection of Cords

Umbilical cords were collected as piglets were delivered by sows and gilts during normal parturition. Care was taken that the cords did not contact any non-sterile surface after exiting the

vulva. The cord was tied with sterile umbilical cord tape and excised with sterile surgical scissors. Individual cords were placed in 50mL polypropylene centrifuge tubes containing 25mL of sterile phosphate buffered saline (PBS) (Gibco 20012) supplemented with 2% antibiotic/antimycotic (anti/anti) (Sigma Aldrich A5955). Anti/anti is an antimicrobial for gram negative and gram positive bacteria, fungi, and yeast.

Cord Processing

Cords were removed from PBS within 48 hours after collection and processed individually in Fisherbrand petri dishes (Fisherbrand Pittsburgh, PA) containing PBS and anti/anti. Each cord was opened longitudinally with scissors and the arteries and vein were removed. Wharton's jelly was removed in pieces that were placed in a Petri dish with PBS and anti/ anti and were minced into approximately 2 mm pieces and transferred to cell plates for culture.

Plating

Serum-free treatments were cultured in 6-well or 24-well tissue culture plates designed for cell culture (Corning Inc. Corning, NY). Control cultures containing 20% FBS were included in each experiment. Explants and cells were cultured on plastic, plastic coated with porcine gelatin or plastic coated with bovine collagen. Porcine gelatin (Sigma G1890) is a collagen based protein that forms a matrix for attachment and is obtained from acid-cured porcine skin tissue. Gelatin solution (0.10g/100mL ultrapure water) was added to each well to provide a complete covering of the surface. The gelatin was incubated for one hour the gelatin solution aspirated. Bovine collagen (Gibco A10644-01) is obtained from bone, tendons and other connective tissues. Bovine collagen was suspended in 0.02 mM acetic acid (5ng/mL), and

was placed in each well to provide an even coating. The collagen was incubated for one hour before being aspirated and the well was rinsed three times PBS to remove residual acetic acid.

Each well in a 24-well plate received 5-10 explants, while the well of 6-well plates received approximately 10-20 explants. Plates were placed in a controlled atmosphere incubator with either low oxygen (5% CO_2, 5% O_2, 90% N_2) or standard atmosphere gas (5% CO_2 in air, ~20% O_2). Cultures were at 38.8 ˚C which is near pig body temperature. The explants were removed when media was aspirated during the second media change. Media changing was performed every 3-4 days. A pan of water was kept in the incubator to maintain humidity and slow evaporation of the media.

Media

The media treatments for each experiment are in table 1. The first medium used was porcine zygote medium-3 (PZM-3), which was designed to support growth of porcine zygotes (Yoshioka et al. 2002). Other media evaluated were Dulbelco's Modified Eagles Medium (DMEM) formulations of low glucose DMEM (LG-DMEM) (Gibco 10567), high glucose DMEM (HG-DMEM) (Gibco 10564), Minimum Essential Medium alpha (α-MEM) and knockout DMEM (KO-DMEM) (Gibco, 10829). DMEM is a standard cell culture medium that is intended to be used with a serum product, usually fetal bovine serum. All media were supplemented with Normocin-O 0.1mg/mL (Sigma, Ant-nr-1), Gentamicin 25µg/mL (Gibco, 15750-060), and beta-mercaptoethanol 2.86mM/L (Sigma, M-7522). After the second experiment, basic fibroblast growth factor 5ng/mL (bFGF, Gibco, 13256-029) was added to all media. The effects of supplementing media with Knockout Serum Replacement (KOSR) (Gibco, 10828) were also tested and media supplemented with FBS (Gibco 26140) were included in all experiments as a positive control. A formulation of each medium is in Table 2.

Cell Passaging

Passaging of cells was performed when the cells achieved approximately 90% (unless noted otherwise) confluence by visual assessment. At time of passage the medium was removed, the wells were rinsed three times with Hank's solution (Gibco, 14175) and then either trypsin (Gibco, 15400) or Accutase (Sigma, A6964) was added to lift the cells. The trypsin was inactivated by adding an equal volume of medium containing FBS or by dilution with PBS for the serum-free treatments. Trypsin needs to be inactivated or highly diluted to prevent damage to the cells. When working with FBS-free media, trypsin was diluted 1:3 with PBS. Trypsin inhibitor (Gibco, R-007-100) was also used to inactivate trypsin in FBS-free cultures in some experiments. Cells in suspensions of trypsin or Accutase were transferred to a 1mL micro-centrifuge tube and centrifuged (800 x G for 10 min). The supernatant was aspirated and the required medium added to the cell pellet which was resuspended and transferred to a well in a new plate that contained the proper medium. The wells in 24-well tissue culture plates were seeded with 20,000-25,000 viable cells and each well in 6-well plates received approximately 100,000 viable cells.

Cell Counting

All cell counts were obtained using flow cytometry with the Guava EasyCyte plus with cytosoft (Guava Technologies. Hayward, CA). Cells were counted at each passage in all experiments and the non-adherent cells during media changes and rinses for passage were also counted in some experiments. Cells counted for passage or media changing were resuspended in 1mL of PBS and diluted with via-count reagent. After cell counting, the cells were re-centrifuged and the cell pellet was resuspended in the appropriate medium. The media from

three wells of cells in the same treatment and from the same cord were removed and counted together. Once the non-adherent cell counts were obtained the non-adherent cells were discarded.

Exp. 1

Two umbilical cords were collected from sow O-118 were collected. Explants from each cord were plated (three wells) in either PZM-3 or PZM-3 supplemented with 20% FBS. The explants originally plated for cell growth were removed when media were changed.

Exp. 2

Two umbilical cords collected form sow O-192 provided explants (3wells/treatment). The trial media were; PZM-3 supplemented with 20% FBS, PZM-3 supplement with basic fibroblast growth factor 5ng/mL (bFGF), and PZM-3 supplemented with bFGF and 19% flow through (0.75mg/mL) (Bryan 2008). Flow through is a by-product of concentrating the protein from Pormatrix, an extracted extra-cellular matrix from Porcine Wharton's jelly. The cultures were incubated in 6-well tissue culture plates under 5% CO_2 in air.

Exp. 3

One male umbilical cord was collected from sow W-6 and the explants collected were plated in serum-free media after coating the well with gelatin 0.1mg/ml. In this trial there were three basal media used for cell culture on plastic: PZM-3 supplemented with 20% FBS, PZM-3 supplemented with bFGF, and PZM-3 supplemented with bFGF and flow through. The plate with gelatin covering for explant attachment was also cultured in PZM-3 supplemented with bFGF and flow through. This experiment was conducted in 6-well tissue culture plates and incubated under 5% CO_2 in air.

Exp. 4

For this experiment we used frozen PUCs (passage 42) that were originally cultured in HG-DMEM with 20% FBS and 20,000 cells were plated in each well. Cells were thawed as described in recovery cell culture freezing medium (Gibco, 1264B). HG-DMEM with 20% FBS and PZM-3 with 20% FBS were used as controls. HG-DMEM and PZM-3 were used with or without flow through and with flow through on gelatin or not on gelatin. This trial was conducted in 24-well tissue culture plates in 5% CO_2 in air.

Exp. 5

To assist in holding explants to the surface for better attachment, a sterile piece from a microscope slide was used to hold down the explants in the 24-well plates. These glass pieces were cut and fire polished to smooth the edges to minimize cells adhering to the glass. Then the glass pieces were tissue culture washed and autoclaved for sterilization. The glass slide pieces were placed over the explants in wells of 24-well tissue culture plate and media added. Explants were plated on plastic or gelatin with or a without slide piece in PZM-3 or HG-DMEM. HG-DMEM and PZM-3 supplemented with 20% FBS were controls. All plates were incubated in 5% CO_2 in air.

Exp. 6

Experiment 5 was repeated with two cords collected from sow Y-84 and Amphotericin (Fungizone, 0.025µg/ml) (Sigma, 15290) was added to the media to reduce the risk of mold growth. All plates were incubated 5%CO_2 in air.

Exp. 7

For this experiment, 3 cords were collected from sow Y-73. Three basal media were used in this trial and explants were cultured with or without the addition of a slide piece on

plastic or in wells with a gelatin coating. This trial consisted of three groups, those with a serum substitute, those with slide pieces in each well to hold the explants to the surface of the well, and those without the addition of slide pieces. The serum substitute was knock-out serum replacement (KOSR), which was designed to replace FBS in cultures of embryonic stem cells. The experiment was a factorial that tested the main effects of HG-DMEM versus PZM-3, KOSR (0 or 10%) and gelatin or plastic for the cell attachment. We also evaluated knockout DMEM supplemented with 5ng/mL bFGF. HG-DMEM and PZM-3 supplemented with 20% FBS were controls. This experiment was conducted in 24-well tissue culture plates and incubated in 5% CO_2 in air.

Exp. 8

In this experiment we tested the hypothesis that lowering oxygen concentrations would increase the attachment and growth of PUCs when grown in serum-free conditions. PZM-3 and HG-DMEM supplemented with bFGF were positive controls and were cultured on gelatin in either in (90%N_2, 5% O_2, 5% CO_2) or (95% air and 5% CO_2). There were four main effects tested for serum-free media: PZM-3 or HG-DMEM, incubation in low or atmospheric oxygen, with or without slide pieces and with or without the addition of KOSR (10%). This experiment was conducted in 24-well tissue culture plates.

Exp. 9

Exp. 9 was a repeat of Exp. 8.

Exp. 10

PZM-3 was not included in Exp. 10 or later experiments. In this experiment low oxygen was compared as were the addition of slide pieces and the supplementation of KOSR (10%). HG-DMEM supplemented with 20% FBS in 5% CO_2 in air was the positive control. Cells

were passaged with Accutase upon achieving 90% confluence by visual assessment. This trial was conducted in 24-well culture plates.

Exp. 11

We repeated the culture treatments in Exp. 10. We also evaluated enzyme digestion to provide PUCs individually and avoid the problems encountered with explants not attaching to the dish. The cord for enzyme digestion was acquired as described for explant culture and stored in PBS with anti/anti. The umbilical cord was then placed in a 50mL sterile conical with 15mL of enzymatic solution (100mL Hanks Buffered Salt Solution, 0.10g Type I Collagenase (345 units/mg), 0.01g DNAse (552 kunitz/mg), and 0.10g Hyaluronidase (1320 units/ mg)) for digesting off the amnion and to allow for the matrix cells to be released into the enzyme solution. Next the cord was incubated in enzyme solution in 5% CO_2 in air for one hour. After digestion, the enzyme solution is diluted with PBS (15ml) and then filtered (60um). The cells are then centrifuged and the enzyme solution discarded. A cell count was acquired by flow cytometry and cells were cultured on a 24-well tissue culture plate in 3 wells coated with gelatin and 50,000 viable cells were added to each well. Cells were passaged with Accutase upon achieving 90% confluence by visual assessment. This trial was conducted in 24-well tissue culture plates and incubated in 5% O_2, 5%CO_2, and 90%N_2.

Exp. 12

We evaluated the effect of a lower glucose concentration (LG-DMEM). HG-DMEM used in Exp. 1 to 11 contains 4,500mg/L of glucose and LG-DMEM contains 1,000mg/L glucose. HG-DMEM and LG-DMEM were supplemented with either 10% KOSR or 20% KOSR and PDGF (5ng/ml) or 10ul of HCl buffer (vehicle) per 100mL of media in a factorial design. An extra cord was collected for another enzyme digest and was processed as described

in Exp. 11. All treatments were incubated in low oxygen and all wells were coated with gelatin. HG-DMEM with 20% FBS cultured on either plastic or plastic coated with gelatin was the control medium for this experiment. Cells were passaged with trypsin and cultured in 24-well tissue culture plates in 5% O2 in air.

Exp. 13

Adipose derived stem cells are reported to prefer Alpha-Minimum Essential Medium (α-MEM) (Lund, Pilgaard, Duroux et al. 2009). Adipose derived stem cells also have mesenchymal stem cell properties so α-MEM might facilitate growth of PUCs. Media compared in Exp. 13 were α-MEM and HG-DMEM and supplemented with 10% KOSR, bFGF and PDGF. Additional supplements were either none, bovine serum albumin (BSA, 5mg/ml) (Sigma, A-8022), or polyvinyl alcohol (PVA, 5 mg/ml) (Sigma, P8132). HG-DMEM with 20% FBS was the control. Cells were passaged with trypsin and cultured on 24-well tissue culture plates in low oxygen.

Exp. 14

In this experiment epidermal growth factor (EGF) (Sigma, E4127) was evaluated as a possible growth and attachment promoting factor. Two basal media, HG-DMEM and KO-DMEM were compared as was the addition of EGF (5ng/mL), along with an evaluation of coating the plastic with either gelatin or collagen. For establishing cultures, cells were released from the Wharton's jelly by mechanically aided enzymatic digestion. The cord was placed in 15mL of stock enzyme solution in a small whirl pack bag used for mechanically aided digestion in the stomacher (Seward 400). The veins and arteries were removed. Each bag contained one cord with enzyme solution and was stomached for 15 minutes. Upon completion of mechanical digestion, the bag containing the cord was incubated at 38˚C in 5% CO_2 in air for 1 hour. After

incubation, the supernatant containing the PUCs along with red blood cells, macrophages and other debris, was filtered using vacuum (60µm pore size). The filtrate was collected and centrifuged (10 minutes at 800 x g). A cell count was obtained and 50,000 cells/cm^2 was initially plated to account for red blood cells and other cells that were included in the cell count. The first media change was within 48 hours to remove the non-adherent cells and debris that can be cytotoxic. HG-DMEM with the addition of 20% FBS acted as the control for this experiment. This trial was conducted in 24-well tissue culture plates in low oxygen.

Exp. 15

EGF (10 ng/ml) was evaluated as a supplement to KO-DMEM and DMEM and all serum-free media contained 10% KOSR and PDGF and cells were grown on collagen coating. Three cords provided cells for this experiment. For processing the cord, mechanical digestion was omitted and only enzyme treatment was performed. The veins and artery were removed from the cord prior to placing the cord in 15 mL of enzyme to digest for one hour at 38˚C in 5% CO_2 in air. After incubation the supernatant, containing the PUCs along with red blood cells, macrophages, and other debris, was filtered and processed as described for Exp. 14. After the initial medium change, media were changed every 3-4 days. Wells were passaged with trypsin upon achieving 90% confluence by visual assessment. In this trial trypsin inhibitor (Gibco, R-007-100) was used to inactivate trypsin. . HG-DMEM with the addition of 20% FBS was the control. This trial was conducted in 24-well tissue culture plates in low oxygen.

Results

Exp. 1

Explants that were plated in PZM-3 without FBS displayed some ability to attach and produce cell outgrowths. Growth of cells in serum free PZM-3 was extremely slow and sporadic when compared to those cultured in PZM-3 with 20% FBS. The explants cultured in PZM-3 with FBS attached within 48 hours of plating and cells began to proliferate around explants that had attached to the plate. Cells cultured in PZM-3 with FBS became 80-90% confluent 5 days after plating. After a fourth passage of the control wells, all plates were discarded since culture in PZM-3 without FBS did not result in 80-90% confluence. Approximately 35% confluence was obtained after 30 days in culture without serum. In the absence of serum, explants did not to attach well. The few explants that did attach produced only limited cell growth around the explant and cells did not continue to proliferate at a noticeable rate after 20 days in culture.

Exp. 2

An extract of Wharton's jelly (flow through) was available from previous work (Bryan 2008) and was added to PZM-3 without FBS. Flow through was investigated as an additive because it is obtained from the Wharton's jelly. Attachment of explants cultured in PZM-3 with or without flow through was minimal when compared to PZM-3 with FBS. Explants that did attach in the serum-free media produced a limited number of outgrowing cells that proliferated at a much slower rate than those cultured in PZM-3 with 20% FBS. Control wells were passaged three times before being discarded but the cells from the explants in serum free PZM-3 and serum free PZM-3 with flow through did not reach confluence during 30 days of culture.

Exp. 3

Because explants attached less frequently without serum we evaluated the effects of gelatin coating of the wells to provide a matrix for attachment. Explants cultured on gelatin with PZM-3, and with or without flow, had moderate rates of explant attachment but growth of cells around the explants was limited. Explants cultured in serum-free PZM-3 alone failed to produce significant numbers of attached explants and only scattered cells could be seen in the plates. The control wells reached initial confluence 6 days after plating of explants. Control wells were passaged three times and all serum-free cultures failed to reach confluence by the time the experiments was terminated at 26 days after plating of explants.

Exp. 4

Cells that had been established in medium with FBS and frozen after passage 42 were thawed for culture in Exp. 4. Cells plated in PZM-3 with flow through (33% by volume, 0.75mg protein/ml) on gelatin and in DMEM with flow through on gelatin showed good cell growth. Cells grown in serum-free media with bFGF and without flow through on gelatin were the best among treatments observed to this time but still considerably less than cells in the control medium. All serum-free cultures expressed considerably less attachment of explants and cells compared to control wells. Cells growing in PZM-3 with flow through appeared to be free floating or loosely attached to the plastic, as did cells in HG-DMEM on plastic and with PZM-3 on plastic. After the control wells reached confluence for a third time and no trials were ready for passage, all were discarded. By this time cells in all serum-free media appeared to have died or had quit growing. Cells cultured in HG-DMEM with flow through on gelatin and those in HG-DMEM on gelatin all had quit growing before reaching a confluent state. These cells were

few in number and usually in small groups of 5-10 cells. After 28 days in culture the experiment was terminated.

Exp. 5

After 10 days in culture cells in HG-DMEM with a slide piece on gelatin, HG-DMEM with a slide piece on plastic, and HG-DMEM without a slide piece on gelatin all showed good cell growth. After 19 days of culture the wells were 80% confluent ready for their first passage. Pictures were taken of cells ready for passage (Figures 1 and 2). The cells in each well were lifted with trypsin, 250µl of HG-DMEM with 20% FBS was added to each well to inactivate trypsin and cells were replated onto new plates with their appropriate serum free-medium but without slide pieces. Treatment designation refers to the initial presence or absence of slide pieces. Cells replated and cultured in HG-DMEM without slide pieces on gelatin displayed good reattachment and growth as did PZM-3 without slide pieces on gelatin. Cells established with PZM-3 and slide pieces on gelatin displayed moderate attachment and growth in one well but not in the other two for that treatment. After being in culture for 12 days after initial passage, cells in PZM-3 without a slide piece on gelatin, HG-DMEM without a slide piece on gelatin, and HG-DMEM initially with a slide piece on gelatin achieved 75% confluence and were passaged. After passage all wells had mold two days after the second passage. All other serum-free cultures failed to grow to confluence after 5 weeks in culture. The control cells were passage six times before discarding.

Exp. 6

This experiment was a repeat of Exp. 5 but with fungizone added to all media. HG-DMEM with a slide piece on gelatin resulted in explant attachment and good cell growth. The cells were more round shaped than elongated and were in small clusters. PZM-3 with a slide

piece on gelatin also encouraged explant attachment. Cells found in these wells were also in clusters and were round or elongated in about equal numbers. Explants cultured in HG-DMEM without slide pieces and on gelatin also attached well with moderate outgrowth of cells. Cells were mainly found in small groups and were round in shape. There also were isolated elongated cells. Explants cultured in PZM-3 with a slide piece on plastic along with all the other treatments displayed poor explant attachment and minimal cell growth. Only a few cells were in these wells. Cells cultured in HG-DMEM with a slide piece on gelatin, PZM-3 with a slide piece on gelatin, and HG-DMEM without a slide piece on gelatin were lifted with Accutase and passaged after 19 days in culture at 80% confluence. Three days after cells were replated there were no visible cells in the wells. Apparently cells were lost during initial passage. All other treatments failed to reach confluence and were discarded. By 19 days in culture the control wells had been passaged three times and were also discarded.

Exp. 7

Explants cultured in KO-DMEM on plastic or on gelatin failed to attach and cells did not grow. HG-DMEM plus KOSR resulted in explant attachment and growth of round and elongated cells, while PZM-3 plus KOSR produced similar results but provided fewer cells. HG-DMEM with slide pieces on gelatin displayed the best attachment of explants and growth rate of cells. HG-DMEM with slide pieces on gelatin was very similar to that on HG-DMEM and PZM-3 plus KOSR on gelatin. Over all HG-DMEM appeared to yield slightly more cells than PZM-3 and explants attached more readily on gelatin than on uncoated plastic. The use of slide pieces did not appear to improve explant attachment and cell growth. After 20 days in culture all wells with KOSR except those in KO-DMEM were ready for passage (Figures 3 and 4). Three days after passaging visible cells were detected in HG-DMEM plus KOSR on gelatin. The cells were

separated and few in numbers but did survive the passage with Accutase. These cells survived 10 days after passage and at this time there were no cells in other KOSR treatments. After 22 days in culture, cells grown in HG-DMEM with a slide piece on gelatin, PZM-3 with a slide piece on gelatin, HG-DMEM without a slide piece on gelatin, and HG-DMEM with a slide piece on plastic reached 75% confluence and were initially passaged. Some cells attached and grew from explants started in HG-DMEM with a slide piece and without a slide piece on gelatin, however most cells disappeared. The cells that did reattach were no longer present 7 days after passaging. The addition of KOSR to PZM-3 and HG-DMEM seemed to result in better attachment of explants and more cells outgrowing from the explants compared to explants without KOSR. KO-DMEM appeared to have a negative effect on explant attachment compared to other serum-free treatments. Control groups with 20% FBS were passaged four times before being discarded during this experiment.

Exp. 8

Explants cultured in HG-DMEM seemed to attach and grow better without the slide piece but when explants were started in PZM-3 the establishment of attached cells appeared to be better with a slide piece holding down the explants. HG-DMEM plus KOSR without a slide piece in low O_2 had the most explants attached and displayed the best cell outgrowth. Explants cultured in low O_2 with slide pieces and in HG-DMEM plus KOSR or PZM-3 had good attachment of explants and outgrowth of cells but fewer cells were present than for HG-DMEM plus KOSR without a slide piece. Explants that were cultured in media without KOSR were slower to attach and displayed fewer cells outgrowing than those in media with KOSR. Cultures in HG-DMEM were 80% confluent and ready for passage after 16 days of incubation. Four days after cell cultured in HG-DMEM went through passage one; they were observed to be

contaminated with mold and were discarded. Cells cultured in PZM-3 were discarded when they grew mold after 20 days in culture.

Exp. 9

Cultures in HG-DMEM plus KOSR in low O_2 with or without a slide pieces resulted in the best attachment and outgrowth of cells. HG-DMEM plus KOSR in 5% CO_2 in air and a slide piece also resulted in attachment of explants and cell outgrowth. PZM-3 supported attachment of explants with limited cell outgrowth. After 16 days, cells cultured with HG-DMEM plus KOSR in low O_2 and 5% CO_2 in air were 80% confluent and were passaged for the first time. All cells in HG-DMEM plus KOSR were lost during passage, possibly when rinsing the cells. PZM-3 plus KOSR in low oxygen and in 5% CO_2 in air, with or without a slide piece appeared to have a higher instance of cell lysis than cultures with HG-DMEM plus KOSR under any conditions with or without a slide piece. Low O_2 appeared to be best for HG-DMEM plus KOSR with or without a slide piece. The addition of a slide piece did not seem to affect the ability of explants to attach in HG-DMEM plus KOSR in low O_2 or in atmospheric conditions. HG-DMEM plus 10% KOSR without a slide piece on gelatin produced the most cells, estimated visually.

Exp. 10

After 24 days of incubation cells in low O_2 and in air cultured in HG-DMEM plus 10% KOSR with and without a slide piece, the cells were 80% confluent and were passage. Numbers of attached cells recovered at passage 1 are in table 3. Other serum-free treatments did not have a visible cell pellet after centrifugation during passage. Twenty eight days after the initial passage cells in HG-DMEM plus KOSR and a slide piece in low O_2 and atmospheric conditions were ready for a second passage and cell counts are in table 3. After the second passage all cells

failed to reattach and soon there was only debris in the wells. Explants cultured in HG-DMEM without KOSR exhibited weak attachment and extremely slow cell growth compared to the control groups. The control group was passaged 8 times and cell counts for each passage are recorded (table 3) before being discarded.

Exp. 11

The enzymatically digested cord failed to provide any cells that attached and grew. Fifteen days after plating 50,000 cells from the digested cord in HG-DMEM plus KOSR in low O_2 and on gelatin there were no visible cells. Similar results were observed for explants cultured in HG-DMEM with or without 10% KOSR with or without a slide piece and for those cultured in low O_2 or 5% CO_2 in air. HG-DMEM plus 10% KOSR with or without slide pieces resulted in good explant attachment a rapid cell growth when compared to all other trials, but is still considerably slower than the control group. After 18 days in culture all plates exhibited mold growth and cells were lost.

Exp. 12

The enzyme released cells did not appear to attach and grow in HG-DMEM plus 10% KOSR and PDGF. In general, explants cultured in HG-DMEM were more likely to attach and produce outgrowths than explants cultured in LG-DMEM. Addition of PDGF appeared to increase the probability that outgrowths of cells would be produced. After 9 days of culture, cells from cord 1, 2, 3, 5, and 6 in HG-DMEM or PDGF plus 10% KOSR, and HG-DMEM plus 20% KOSR were ready for passage (Figure 4). HG-DMEM plus 10% and 20% KOSR without PDGF LG-DMEM with 10% KOSR with or without PDGF and LG-DMEM with 20% KOSR with or without PDGF (Figure 5) were approximately 60% confluent but were not ready for passage at the same time. The cell counts for each cord and treatment are in table 4. The control

wells were also ready for their initial passage at the same time and their cell counts are in table 4. Six days after initial passage cells from cords 1, 3, and 6 cultured in HG-DMEM with 10% KOSR and PDGF were ready for passage two. Cells from cords 1, 2, 3, and 6 cultured in HG-DMEM with 20% KOSR and PDGF were also ready for passage two as well as cells from cord 6 cultured in HG-DMEM with either 10% or 20% KOSR without PDGF. Cell counts for passage two are listed in table 4. Cells from cord 2 and 5 cultured in HG-DMEM with either 10% KOSR or 20% KOSR with PDGF reattached to the well and began proliferating, like those that went through a second passage. Then the cells stopped growing and appeared to have entered a quiescent stage. Cells from cord 6 cultured in LG-DMEM plus 10% KOSR and with or without PDGF and LG-DMEM plus 20% KOSR and with or without PDGF also entered the apparent quiescent stage. The cells that went into a quiescent stage had all died after 31 days in culture. After 16 days of incubation, cells from cord 2 cultured in LG-DMEM plus 10% KOSR and with or without PDGF were ready for passage and the cell counts are in table 4. After passage, these cells failed to reattach and grow. After 30 days of culture cells grown in LG-DMEM plus 10% KOSR and with or without PDGF were passaged at 70% confluent. These cells that were slow growing failed to survive passaging with Accutase. The control wells became contaminated after passageand were discarded. The cell counts obtained from the only passage of control wells for this experiment are in table 4.

Exp. 13

Explants in HG-DMEM were generally more likely to produce cell outgrowths and approached confluence (table 5). After 7 days of culture cells from cords 1, 2, 3, and 4 grown in HG-DMEM plus PDGF and 10% KOSR, HG-DMEM plus PDGF, 10% KOSR, and BSA, and HG-DMEM plus PDGF, 10% KOSR, and PVA were ready for an initial passage as were cells

from cord 5 cultured in HG-DMEM plus PDGF and 10% KOSR. Cell from cord 2 cultured in α-MEM plus PDGF and 10% KOSR, α-MEM plus PDGF, 10% KOSR, and BSA, and α-MEM plus PDGF, 10% KOSR, and PVA were ready for passage at 80% confluent after 9 days in culture, as were cells from cord 3 and 4 cultured in α-MEM plus PDGF and 10% KOSR. The cell counts for passage one with experiment 13 are in table 5. Addition of BSA or PVA did not seem to affect the cells harvested for the initial passage. The cell counts for control wells are in table 6.

Exp. 14

In this trial there were three variables evaluated for their contribution to culturing PUCs in an animal product free environment. KO-DMEM was compared to HG-DMEM plus 10% KOSR, and the addition of EGF to cell culture media was also investigated. In addition to supplementing or not supplementing each media with EGF, a reexamination of the use of coating each plate with gelatin or collagen was performed. After the first media change to remove the non-adherent cells from the enzyme digest, wells in HG-DMEM plus 10% KOSR, bFGF, PDGF, and EGF, all wells in HG-DMEM plus 10% KOSR, bFGF, and PDGF, all wells in KO-DMEM plus bFGF, PDGF, and EGF, and KO-DMEM plus bFGF, and PDGF from cord 6 plated on collagen were contaminated and were discarded . The control wells for comparing the trial wells were 90% confluent after 7 days in culture with FBS. After the second passage of the control wells the cell counts began to decline. Cords 1, 2, 4, and 5 cultured in HG-DMEM plus 10% KOSR, bFGF, PDGF, and EGF on collagen initially appeared to have good attachment but were lost through the multiple media changes. The number of non-adherent cells collected during each media change shows that these cells were not attached tightly enough to remain attached during media changes.

During the third passage, 18 days after initial plating, cords 5 and 6 in control media provided enough cells to plate one well of each serum-free media group on collagen and on gelatin. This was done to evaluate whether cells established using serum would proliferate and attach in the serum free media. Cells did attach and started to proliferate. Media were changed based upon visual detection of orange color. The established cells of Cord 5 and 6 cultured in HG-DMEM with 10% KOSR, bFGF, PDGF, and EGF on collagen and gelatin achieved confluence for one passage, but the cell counts were lower than expected. Cord 6 cells also made confluence (80%) for passage when culture in HG-DMEM plus 10% KOSR, bFGF, and PDGF, as well as KO-DMEM plus bFGF, PDGF, and EGF. The cells appeared to have reattached after passage but failed to proliferate. The cells that did reattach presented normal morphology but were slow in proliferation after their first passage, and entered a stage of quiescence.

After the established cells stopped proliferating, the media were replaced with HG-DMEM plus 20% FBS to assess whether the cells were responsive to serum after proliferation stopped. Cell proliferation did not resume after the addition of serum containing medium. These cells were terminated after 30 days after the replacement of serum free media with a medium containing serum. After a fourth media change cells in Cord 6 in HG-DMEM plus 10% KOSR, bFGF, PDGF, and those with and without EGF on gelatin became contaminated with mold and were discarded. After a sixth media change of serum-free treatments and a fourth passage of the control group all trial wells were contaminated with mold and discarded.

Counts of the non-adherent cell were obtained through the sixth media change (table 7), but after that there were no visible cells in the wells. It appears that the cells began proliferating

after the initial plating, because the total non-adherent cell counts were considerably more than the total number of cells initially plated.

Exp. 15

This is a repeat of Exp. 14 except that gelatin was not evaluated and all serum-free treatments were plated on collagen. Three cords were collected for 3 replicates of the four treatments. After two days of culture and the initial media change preformed to remove RBC's and other non-desirable cells, wells from cord 2 were devoid of cells. Apparently cells did not attach and were lost during the first media change. After three days of culture, cells from cord 3 in HG-DMEM with 10% KOSR, bFGF, PDGF, and EGF displayed strong attachment and high proliferation rate, as did cells from cord three in HG-DMEM with 10% KOSR, bFGF, and PDGF, KO-DMEM with bFGF, PDGF, and EGF, and KO-DMEM with bFGF and PDGF. Serum-free grown cells from cord 3 also had small colonies with the typical elongated appearance. Cells from cord 1 also appeared to have good attachment of cells early, as indicated by the non-adherent cell counts obtained during media changes. Serum-free grown cells from cord 1 did not form colonies. Cells in KO-DMEM with bFGF and PDGF from cord one appeared to have the strongest attachment and highest proliferation rate at passage one. Cord 3 cells grown in KO-DMEM with bFGF and PDGF had the highest viable cell count at passage 1. Nine days after initial plating all treatment groups from cord one and three had achieved 80-90% confluence and were passaged (Figures 6 and 7). After passage one, all serum-free wells appeared to reattach and continued proliferating. Cells counts for cells in HG-DMEM with 10% KOSR, bFGF, PDGF, and EGF, HG-DMEM with 10% KOSR, bFGF, and PDGF, and KO-DMEM with bFGF, PDGF, and EGF from cord one were considerably lower than expected. Cells from 3 wells resulted in enough cells to replate one well. The cell count from three wells

of KO-DMEM with bFGF, and PDGF for cord one resulted in enough cells to replate three wells. The cell count for this group was lower than those of cord 3. Cells from cords 1 and 3 were cultured for an additional 9 days after the first passage before being passaged again (60% and 75% confluent). The cell counts from passage two indicated that the cells that were replated attached to the collagen coated plastic but did not begin to proliferate again after passage. The cell counts of non-adherent and adherent cells approximately equal the number of cells plated during passage one. Addition of EGF to each basal medium resulted in fewer viable adherent cells but appeared to show no major effect on the total non-adherent cell. The cell counts for adherent and non-adherent cells for all treatment groups are compared in figure 8 (passage 1) and figure 9 (passage 2).

Discussion

In these experiments different culture media were evaluated for the ability to promote attachment and proliferation of PUCs in the absence of FBS. Because FBS is obtained from the blood of fetal calves there is the potential of xenogeneic antigen and microbial contamination. FBS is not a chemically defined supplement and differences between lots of serum for their ability to support cells in vitro are reported (Petkov and Anderson 2008). Culture in defined, serum-free media would allow more consistent media composition and would be useful for cells to be certified under good manufacturing procedures.

Initiating PUCs in serum free culture has proven difficult. In the early experiments using explants, attachment of the explants was a significant problem as was slow cell proliferation for the outgrowing cells. The use of a slide piece to hold the explants it did not appear to enhance attachment of explants. Providing gelatin as a matrix for attachment improved attachment of explants in FBS-free media. The addition of bFGF to media also appeared to help with explant

attachment and cell proliferation. Atmospheric conditions during incubation were also evaluated in Exp. 8 to 12. Air and 5% CO_2 is not a close approximation of in vivo oxygen levels in vivo. Tissue oxygen concentrations are approximately 5% compared to approximately 20% oxygen in air. Because the in vivo concentrations of O_2 are considerably lower cells were cultured in two atmospheric conditions (90%N_2, 5%CO_2, 5%O_2 or 5% CO_2 in air). There were no differences noted for cell growth or attachment of explants between the two gas phases. After experiment 12, cells were cultured in low oxygen (5% O_2), because it is a closer to the physiological O_2 concentration.

Because UCMCs have similar properties of embryonic cells, KOSR (10% by volume) was tested as a substitute for FBS. When comparing serum-free media for explants, HG-DMEM supplemented with 10% KOSR and bFGF resulted in better attachment of explants and cell proliferation than other media used in the early trials with explants. There were no noticeable differences in attachment or cell outgrowth when KOSR concentration was increased from 10% to 20%. Although cell outgrowths using HG-DMEM with 10% KOSR and bFGF occurred, the time to confluence was considerably more than for HG-DMEM supplemented with 20% FBS. PUCs grown in serum-containing media have elongated fibroblast-like cell morphology and the addition of bFGF helped produce this morphology.

PUCs collected from enzyme digestion of umbilical cords were cultured on either gelatin or collagen while in serum free media. Collagen seemed to encourage stronger attachment of PUCs than gelatin however cell counts of washes revealed that many cells were lost during the wash steps. PUCs cultured in experiment 15 readily proliferated through passage one but the number of PUCs that attached to the collagen coating was considerably less than those in the control medium. There were a larger number of non-adherent cells than adherent cells. While

proliferation of PUCs through passage one showed promise, after passage one the PUCs reattached but failed to continue to proliferate. When the cells were passaged for the second time the total adherent plus non-adherent cells approximately equaled the total number of cells plated. Therefore attachment to the matrix was much weaker for the serum-free cultures. In the course of these experiments, KO-DMEM supplemented with bFGF, and PDGF resulted in the most cells and provided similar numbers of cells to those in serum-supplemented media at passage 1 (Exp. 15). However in this medium the percent of viable attached cells was only 14.1% for cord 1 and 45.1% for cord 3. PDGF facilitates growth of cells in an undifferentiated state and is useful in promoting attachment of cells (Ng, Boucher, Koh et al. 2008). EGF has been shown to promote cell growth in bone marrow mesenchymal cells (Fan, Au, Tamama et al. 2007). The addition of epidermal growth factor to HG-DMEM plus KOSR or KO-DMEM did not noticeably affect attachment of cells or their proliferation. In experiment 15, cells cultured in HG-DMEM or KO-DMEM with EGF resulted in similar number of attached cells but higher total non-adherent cells compared to cells cultured in DMEM or KO-DMEM without EGF.

No FBS-free medium in these experiments supported long term culture of PUCs, but most media provided some attachment and proliferation before passage one. PUCs cultured in KO-DMEM supplemented with bFGF and PDGF and grown on collagen in low oxygen resulted in the highest cell counts at passage one but cell proliferation failed to continue passage one. The culture of PUCs in FBS-free culture has proven difficult. Serum may affect attachment by providing attachment factors or it could change expression of integrins or other attachment molecules on the PUCs that enhance attachment to plastic or other substrates.

References

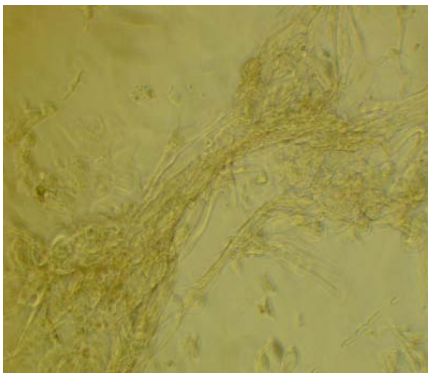
Fan, V. H., A. Au, K. Tamama, R. Littrell, L. Richardson, J. Wright, A. Wells, L. Griffith. 2007. Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells. Stem Cells 25: 1241-1251.

Lund, P., L. Pilgaard, M. Duroux, T. Fink, V. Zachar. 2009. Effect of growth media and serum replacements on the proliferation and differentiation of adipose-derived stem cells. Cytotherapy 11: 189-197.

Ng. F., S. Boucher, S. Koh, K. Sastry, L. Chase, U. Lakshmipathy, C. Choong, Z. Yang, M. Vemuri, M. Rao, V. Tanavde. 2008. PDGF, TGF, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): Transcriptional profiling can indentify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. Blood 112: 295-307.

Perez-Ilzarbe, M., M. Diez-Campelo, P. Aranda, S. Tabera, T. Lopez, C. del Canizo, J. Merino, C. Moreno, E. Andreu, F. Prosper, J. Perez-Simon. 2009. Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy. Transfusion 1537: 1-10

Yoshioka, K., C. Suzuki, A. Tanaka, I. Anas, S. Iwamura. 2002. Birth of piglets derived for zygotes cultured in a chemically defined medium. Biology of Reproduction 66: 112-119



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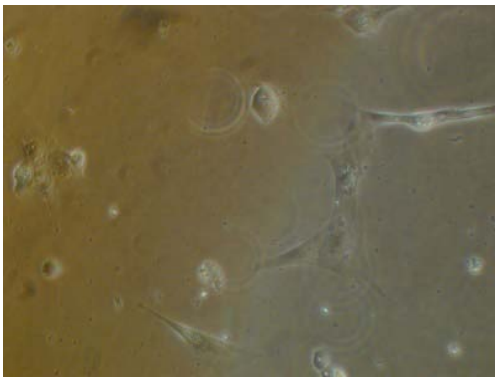

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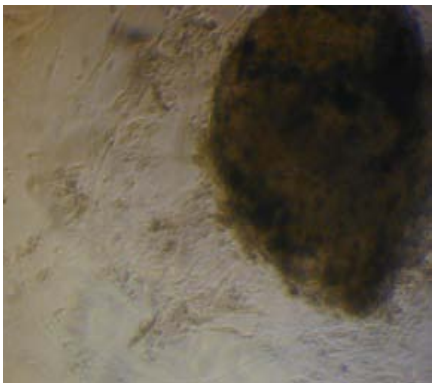

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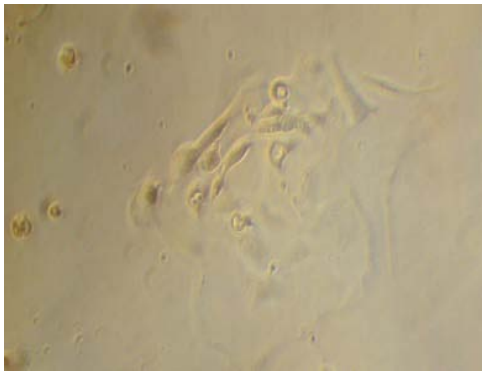


D

Figure 1. Cell morphologies observed during culture in PZM-3 (Exp. 5). **A**. PZM-3 with a slide piece on gelatin, **B**. PZM-3 with a slide piece on plastic, **C**. PZM-3 no slide piece on gelatin, and **D**. PZM-3 no slide piece on plastic.

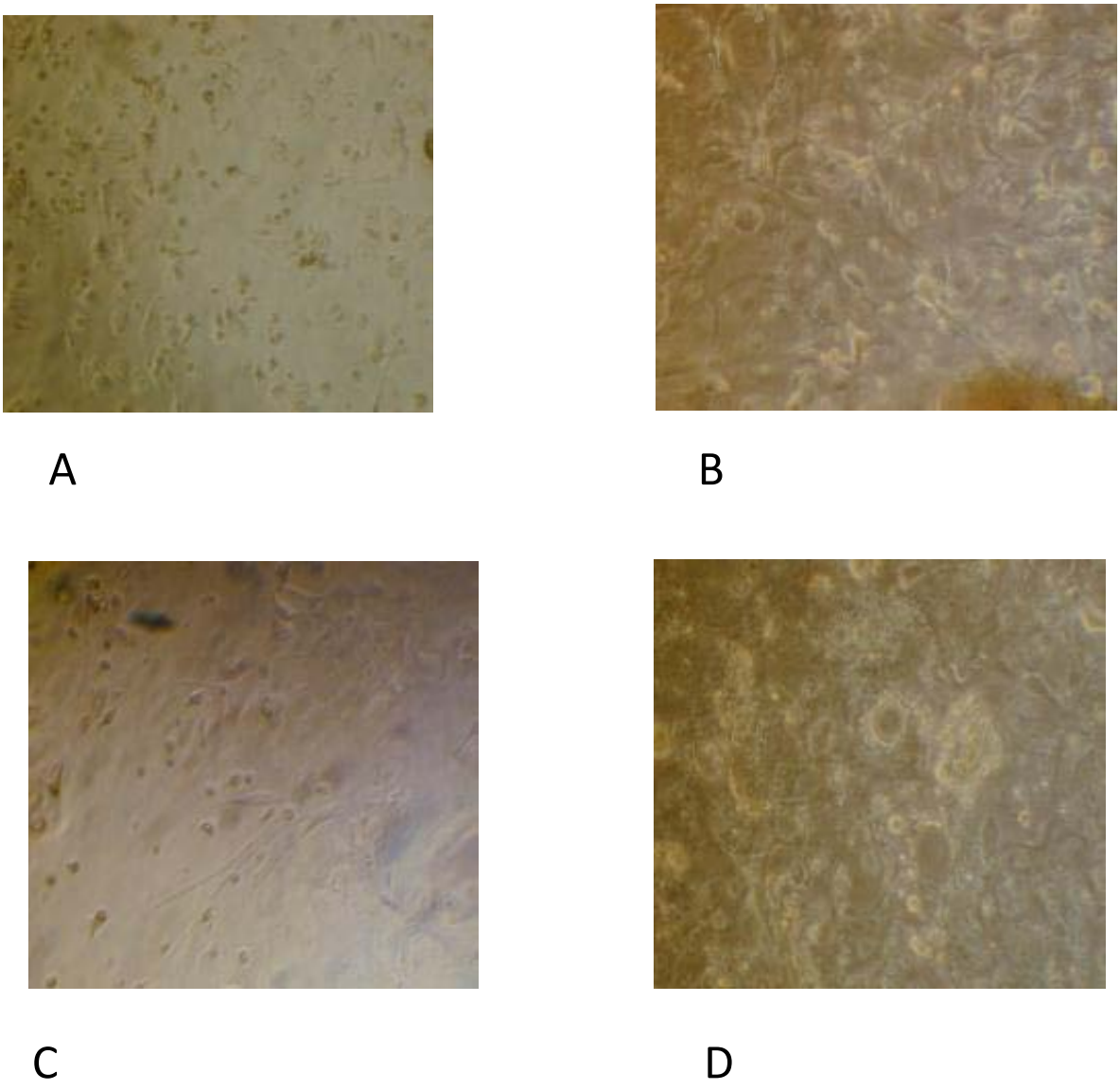


Figure 2. Cell morphologies observed during culture in DMEM (Exp. 5). **A**. DMEM with a slide piece on gelatin, **B**. DMEM with a slide piece on plastic, **C**. DMEM no slide piece on gelatin, **D**. DMEM no slide piece on plastic.

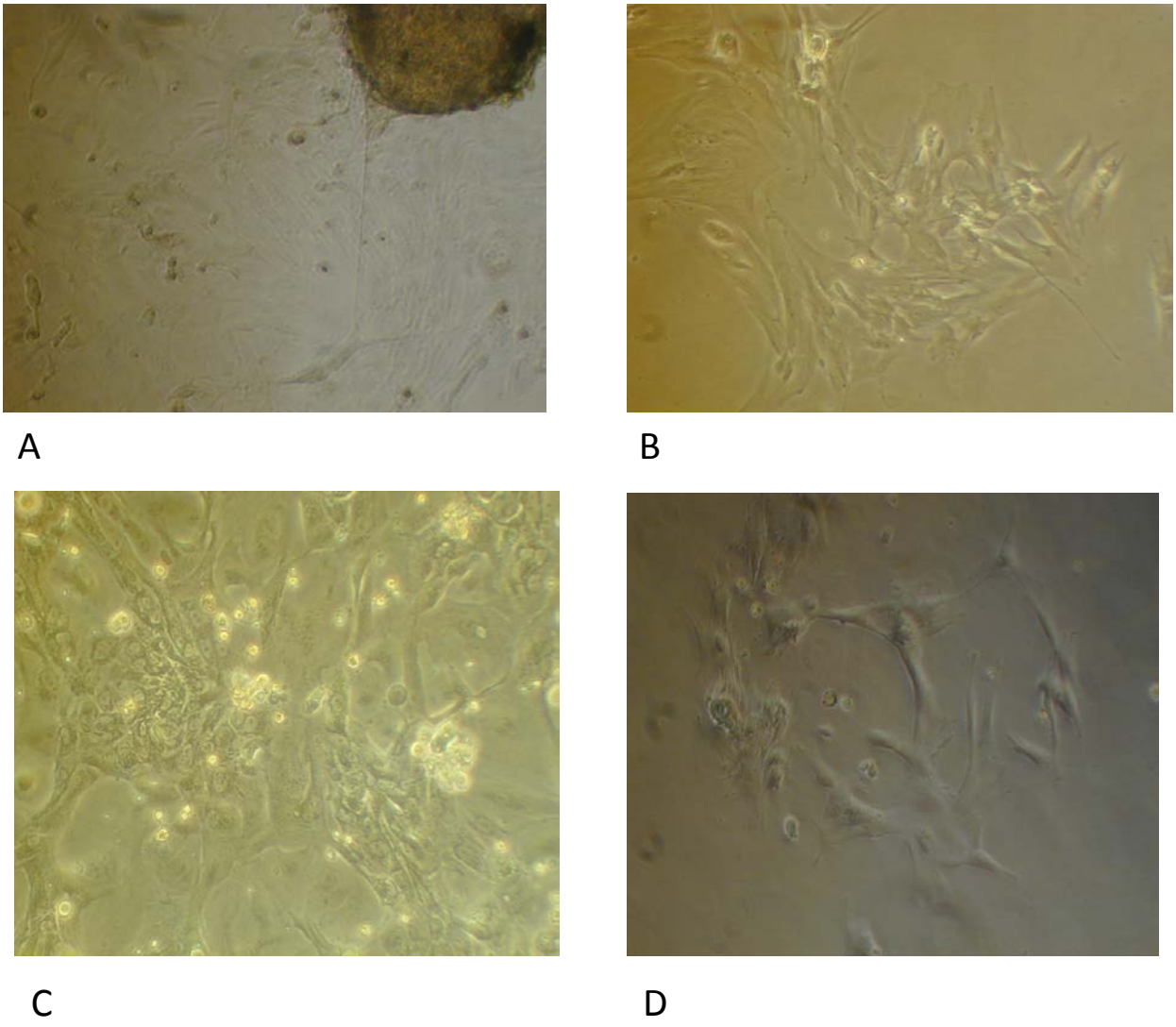
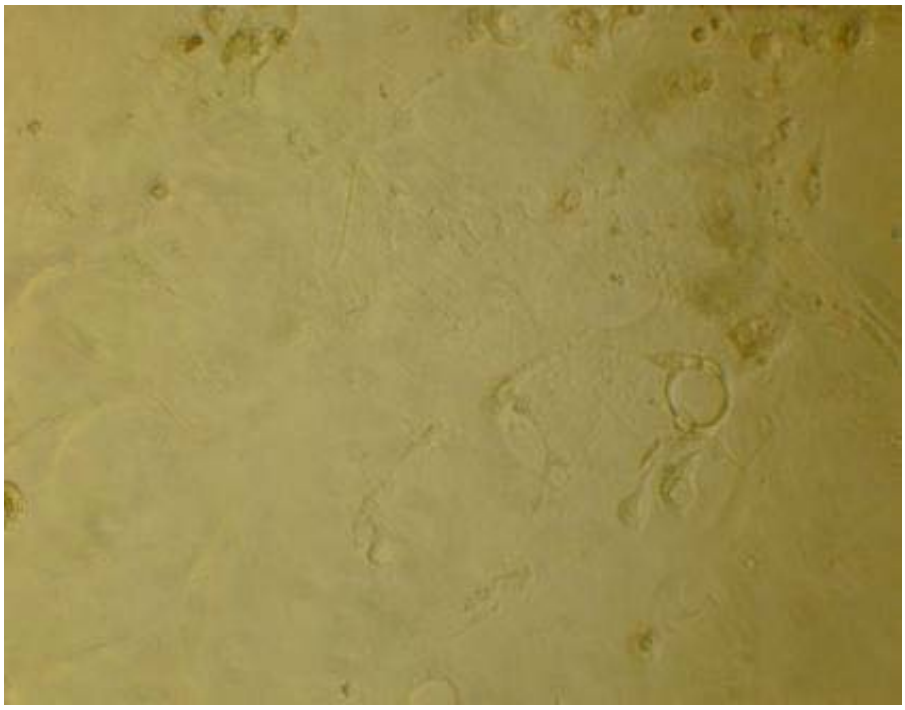


Figure 3. DMEM versus PZM-3 with KOSR on gelatin or not on gelatin (Exp. 7). **A**. DMEM with 10% KOSR on gelatin, **B**. PZM-3 with 10% KOSR on gelatin, **C**. DMEM with 10% KOSR on plastic, **D**. PZM-3 with 10% KOSR on plastic.

A



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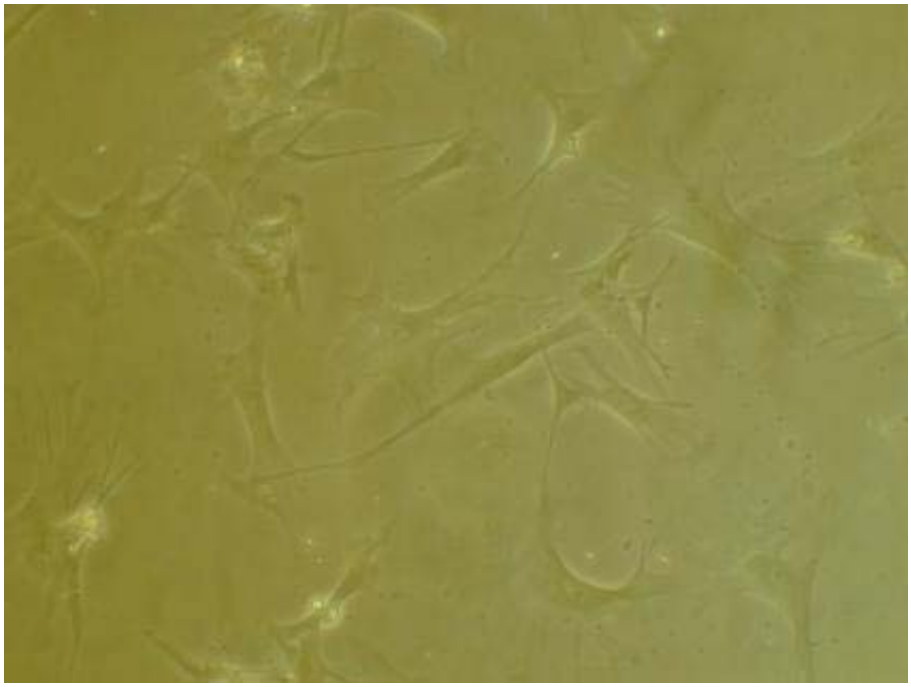
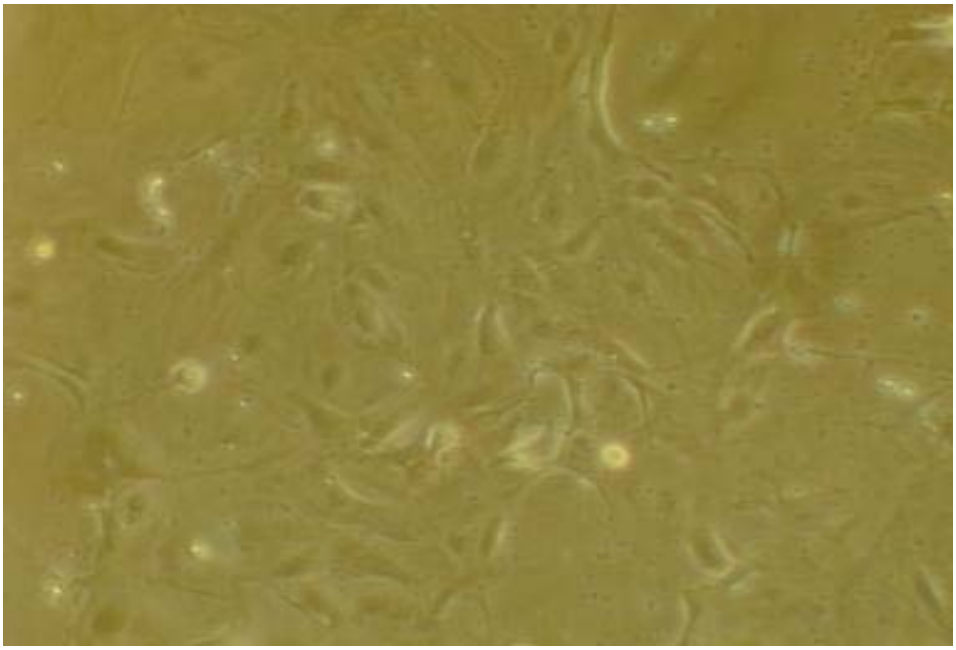


Figure 4. Effects of KOSR concentration in HG-DMEM on gelatin (Exp. 12). **A**. HG-DMEM with PDGF and 10% KOSR, **B**.HG-DMEM with PDGF and 20% KOSR.

A



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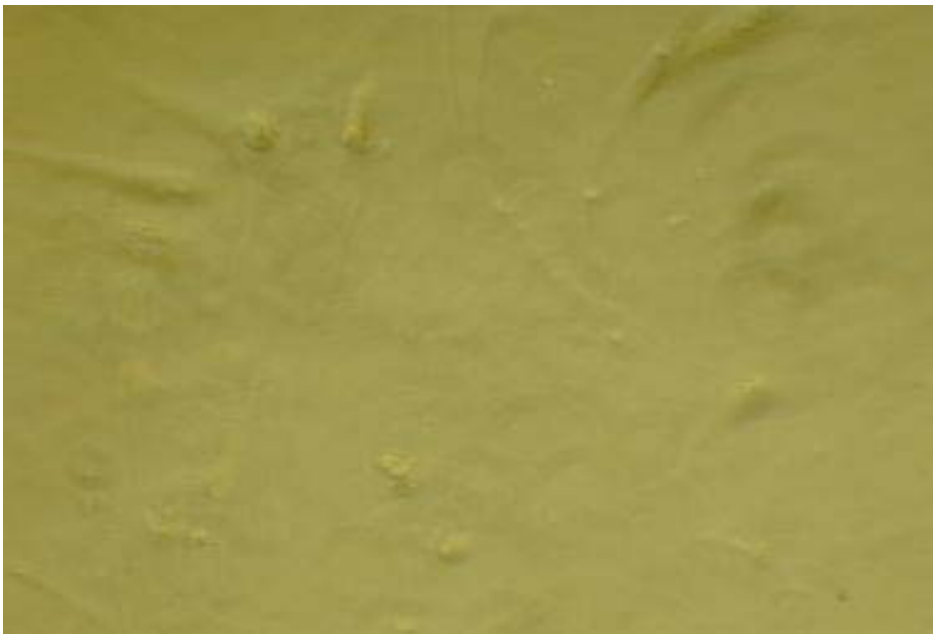
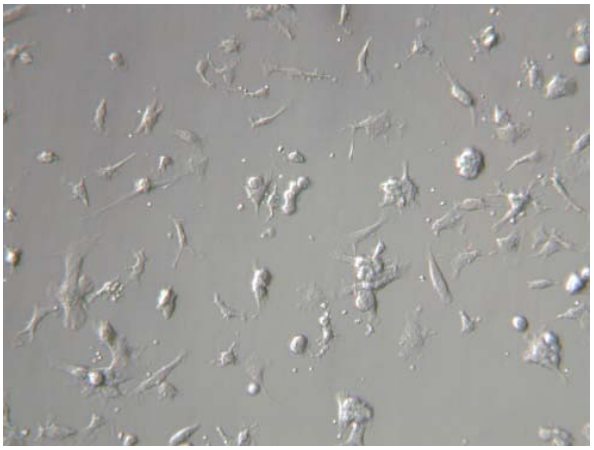


Figure 5. Effects of KOSR concentration in LG-DMEM on gelatin (Exp. 12). **A**.LG-DMEM with PDGF and 10% KOSR, **B**. LG-DMEM with PDGF and 20% KOSR.

Cord 1 DMEM with 10% KOSR, bFGF, PDGF, EGF on Collagen. 40X Figure 6.A

Cord 1 DMEM with 10% KOSR, bFGF, PDGF, EGF on Collagen. 100X Figure 6.B

Cord 1 DMEM with 10% KOSR, bFGF, PDGF on Collagen. 40X Figure 6.C



Cord 1 DMEM with 10% KOSR, bFGF, PDGF on Collagen. 100X Figure 6.D

Figure 6. Cord 1 treatment groups at second media change before passage 1 (Exp. 15).

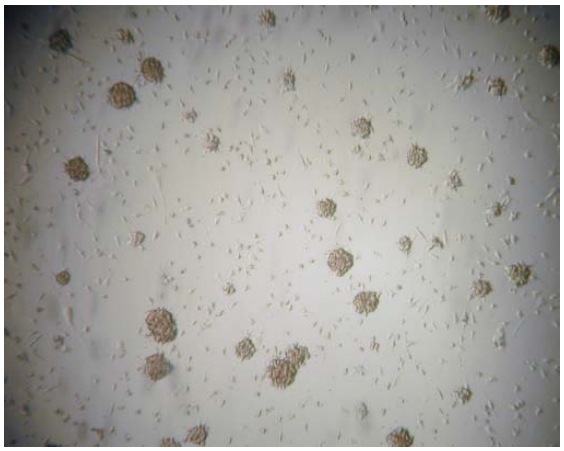
Cord 1
KO-DMEM with bFGF, PDGF, EGF on Collagen
40X Figure 6.E

Cord 1
KO-DMEM with bFGF, PDGF, EGF on Collagen.
100X Figure 6.F

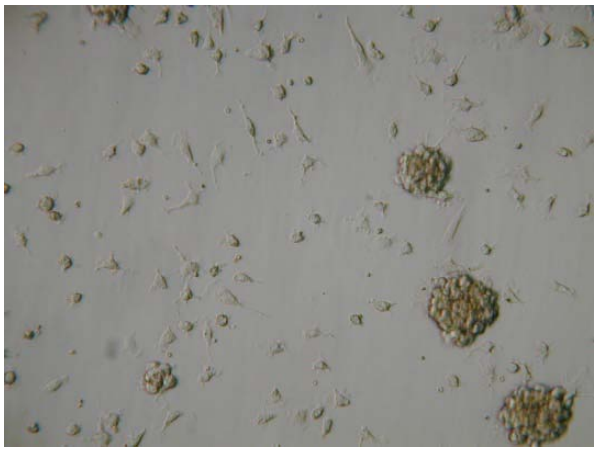
Cord 1
KO-DMEM with bFGF, PDGF on Collagen.
40X Figure 6.G

Cord 1
KO-DMEM with bFGF, PDGF on Collagen.
100X Figure 6.H

Figure 6. Cord 1 treatment groups at second media change before passage 1 (Exp. 15).

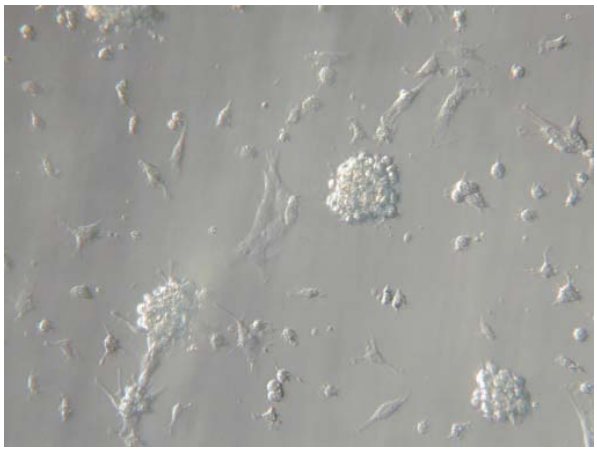


Cord 3
DMEM with 10% KOSR, bFGF, PDGF, EGF on Collagen.
40X Figure 7.A



Cord 3
DMEM with 10% KOSR, bFGF, PDGF, EGF on Collagen.
100X Figure 7.B

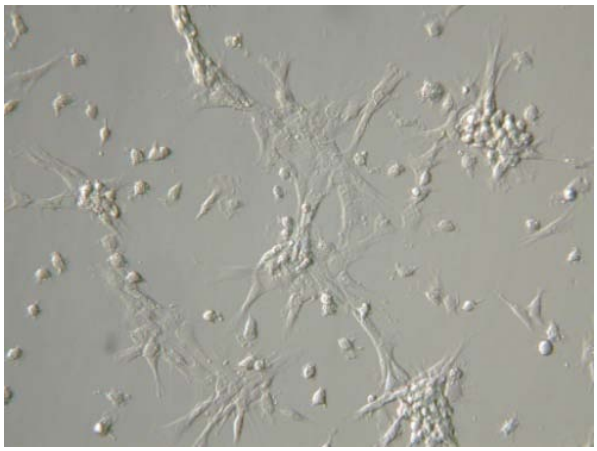
Cord 3
DMEM with 10% KOSR, bFGF, PDGF on Collagen
40X Figure 7.C



Cord 3
DMEM with 10% KOSR, bFGF, PDGF on Collagen.
100X Figure 7.D

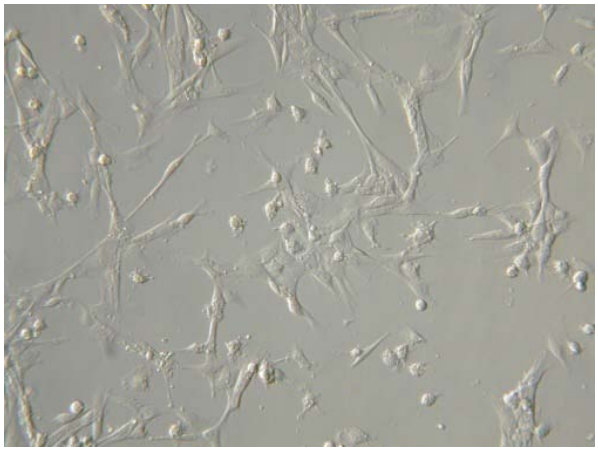
Figure 7. Cord 3 treatment groups at second media change prior to passage 1 (Exp. 15).

Cord 3
KO-DMEM with bFGF, PDGF, EGF on Collagen.
40X Figure 7.E



Cord 3
KO-DMEM with bFGF, PDGF, EGF on Collagen.
100X Figure 7.F

Cord 3
KO-DMEM with bFGF, PDGF on Collagen.
40X Figure 7.G



Cord 3
KO-DMEM with bFGF, PDGF on Collagen.
100X Figure 7.H

Figure 7. Cord 3 treatment groups at second media change prior to passage 1 (Exp. 15).

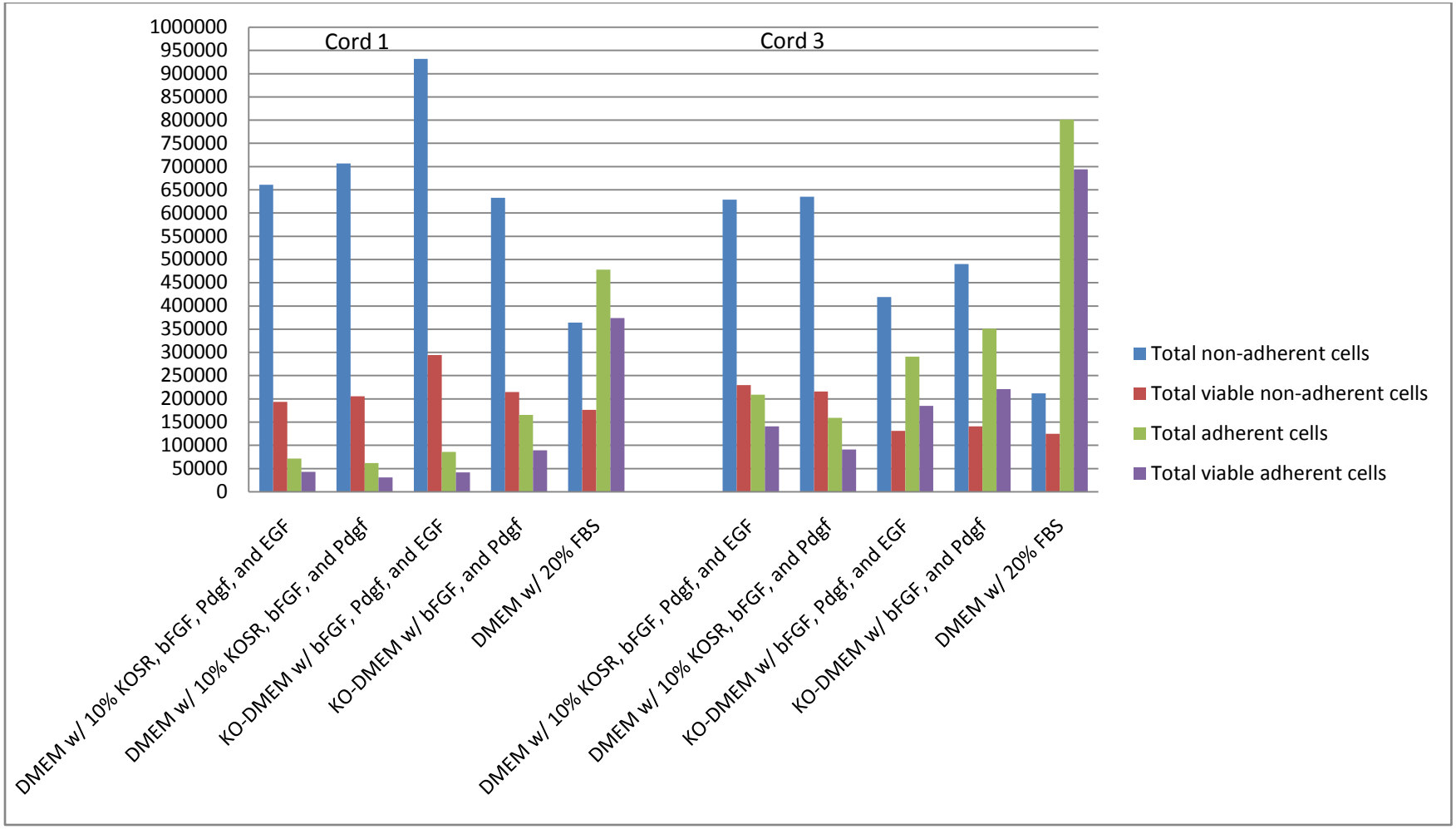


Figure 8 (Exp. 15). Total adherent versus total non-adherent cell counts for cord 1 and cord 3 at passage 1. Each count is representative of 6 wells. The non-adherent cells are from two media changes from the trial groups and the one media change of the control group, the media removed from the well before trypsinizing the well and also includes the PBS applied to each well while rinsing the well prior to adding trypsin. The adherent cell counts are the total cells lifted from the plate during passage with trypsin.

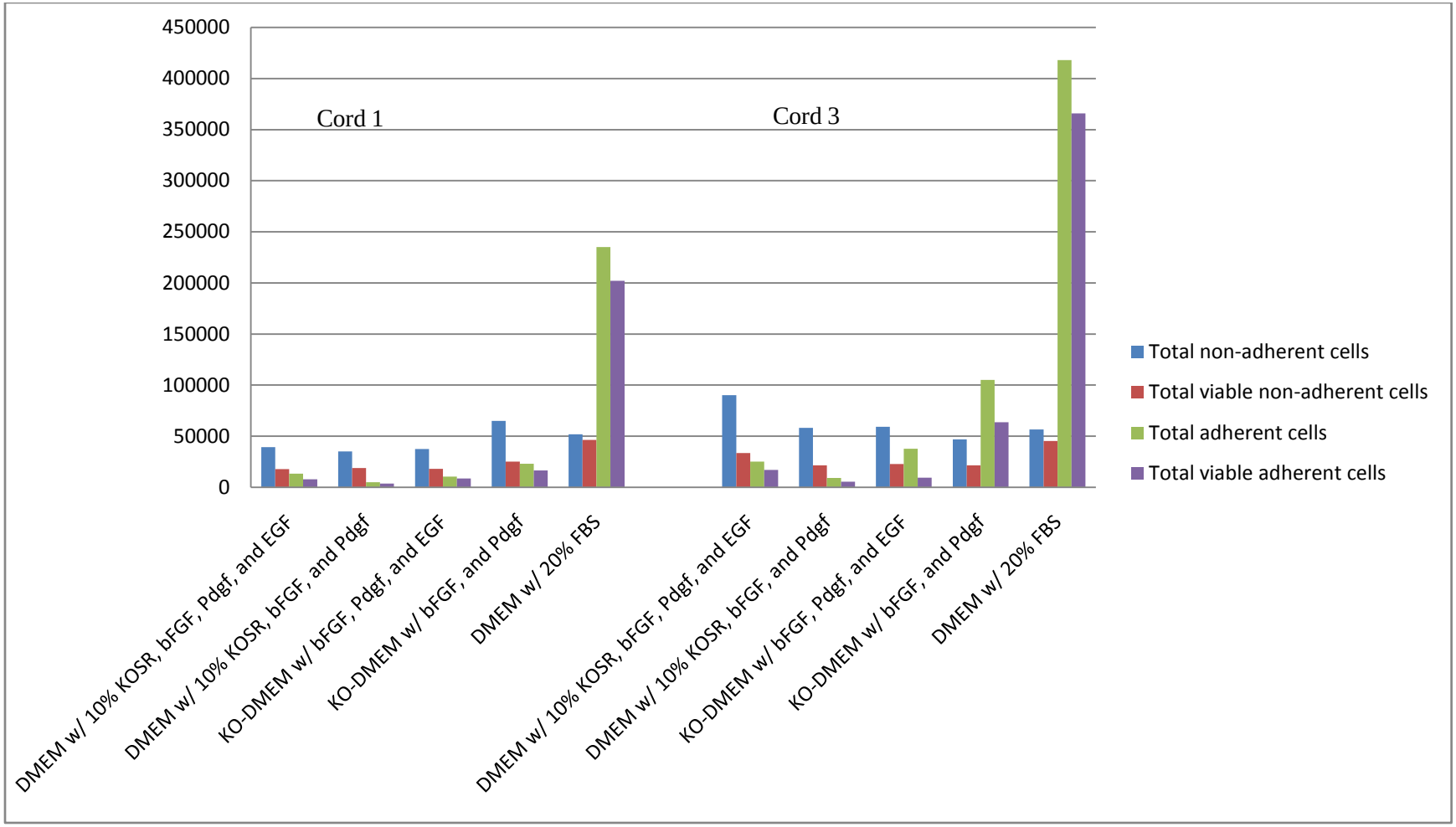


Figure 9 (Exp. 15). Total adherent versus total non-adherent cell counts for cord 1 and cord 3 at passage 2. Each count is representative of two wells. The non-adherent cells are from two media changes, the media removed from the well before trypsinizing, and also the PBS applied to each well while rinsing three times prior to adding trypsin. The adherent cell counts are the total cells lifted from the plate during passage with trypsin.

Table 1. Media used in each experiment.

Exp.	PZM-3[a]															
1	20% FBS[b]								None							
2	20% FBS[b]					bFGF[c]						Flow Through				
	PZM-3 [a] + bFGF															
3	20% FBS[b]					Flow Through						Flow Through and Gelatin				
	PZM-3 [a] + bFGF[c]								**HG-DMEM[d] + bFGF[c]**							**KO-DMEM[e] + bFGF[c]**
4	20% FBS[b]	Flow Through		Flow Through Gelatin		None			20% FBS[b]	Flow Through		Flow Through Gelatin		None		
5	20% FBS[b]	Slide Piece	Gelatin	Slide Piece Gelatin		None			20% FBS[b]	Slide Piece	Gelatin	Slide Piece Gelatin		None		
6	20% FBS[b]	Slide Piece	Gelatin	Slide Piece Gelatin		None			20% FBS[b]	Slide Piece	Gelatin	Slide Piece Gelatin		None		
7		10% KOSR[f] Gelatin	Slide Piece Gelatin		Gelatin					10% KOSR[f] Gelatin	Slide Piece Gelatin		Gelatin		Gelatin	
		10% KOSR[f]	Slide Piece		None					10% KOSR[f]	Slide Piece		None		None	
	PZM-3 [a] + bFGF[c] on Gelatin								**HG-DMEM[d] + bFGF[c] on Gelatin**							
	5% CO_2 / 95% air			**5% CO_2/5% O_2/90% N_2**					**5% CO_2 / 95% air**			**5% CO_2/5% O_2/90% N_2**				
8	20% FBS[b]	10% KOSR[f] Slide Piece	Slide Piece	10% KOSR[f] Slide Piece	Slide Piece				20% FBS[b]	10% KOSR[f] Slide Piece	Slide Piece	10% KOSR[f] Slide Piece	Slide Piece			
		10% KOSR[f]	None	10% KOSR[f]	None					10% KOSR[f]	None	10% KOSR[f]	None			
9	20% FBS[b]	10% KOSR[f] Slide Piece	Slide Piece	10% KOSR[f] Slide Piece	Slide Piece				20% FBS[b]	10% KOSR[f] Slide Piece	Slide Piece	10% KOSR[f] Slide Piece	Slide Piece			
		10% KOSR[f]	None	10% KOSR[f]	None					10% KOSR[f]	None	10% KOSR[f]	None			

Table 1. Continued

Exp.	HG-DMEM[d] + bFGF[c] on Gelatin					LG-DMEM[g] + bFGF[c] on Gelatin					
	5% CO_2 / 95% air			5% CO_2/5% O_2/90% N_2		5% CO_2 / 95% air			5% CO_2/5% O_2/90% N_2		
10	20% FBS[b]	10% KOSR[f] Slide Piece	Slide Piece	10% KOSR[f] Slide Piece	Slide Piece						
		10% KOSR[f]	None	10% KOSR[f]	None						
11	20% FBS[b] Gelatin	10% KOSR[f] Slide Piece	Slide Piece	10% KOSR[f] Slide Piece	Slide Piece						
	20% FBS[b]	10% KOSR[f]	None	10% KOSR[f]	None						
12				20% KOSR[f] PDGF[h]	10% KOSR[f] PDGF				20% FBS[b]	20% KOSR[f] PDGF[h]	10% KOSR[f] PDGF[h]
				20% KOSR[f]	10% KOSR[f]					20% KOSR[f]	10% KOSR[f]
13	HG-DMEM[d] + bFGF[c] + PDGF[h] + 10% KOSR[f] on Gelatin					α-DMEM[i] + bFGF[c] + PDGF[h] + 10% KOSR[f] on Gelatin					
	5% CO_2/5% O_2/90% N_2										
	20% FBS[b]	None	BSA	PVA			None	BSA	PVA		
	20% FBS[b] Gelatin										
	HG-DMEM[d] + bFGF[c] + PDGF[h] + 10% KOSR[f]					KO-DMEM[e] + bFGF[c] + PDGF[h]					
	5% CO_2/5% O_2/90% N_2										
14	20% FBS[b]	EGF[j] Collagen	Collagen	EGF[j] Gelatin	Gelatin		EGF[j] Collagen	Collagen	EGF[j] Gelatin	Gelatin	
15	20%FBS[b]	EGF[j] Collagen	Collagen				EGF[j] Collagen	Collagen			

[a] Porcine Zygote Media-3. [b] Fetal Bovine Serum. [c] Basic Fibroblast Growth Factor. [d] High Glucose Dulbecco's Modified Eagle's Medium. [e] Knock Out Dulbecco's Modified Eagle's Medium. [f] Knock Out Serum Replacement. [g] Low Glucose Dulbecco's Modified Eagle's Medium. [h] Platelet Derived Growth Factor. [i] Alpha-Minimum Essential Medium. [j] Epidermal Growth Factor.

Table 2. Media formulations.

Porcine Zygote Medium-3	g/100mL	High Glucose Dulbecco's Modified Eagles Medium	g/L	Low Glucose Dulbecco's Modified Eagles Medium	g/L	Minimum Essential Medium Alpha Medium	mg/L
NaCl	0.6313	$CaCl_2$ - $2H_2O$	0.265	$CaCl_2$ - $2H_2O$	0.265	Glycine	50
KCl	0.0746	$Fe(NO_3)_3$ - $9H_2O$	0.0001	$Fe(NO_3)_3$ - $9H_2O$	0.0001	L-Alanine	25
KH_2PO_4	0.0048	$MgSO_4$	0.09767	$MgSO_4$	0.09767	L-Alanyl ~ L-Glutamine	406
$MgSO_4$ -$7H_2O$	0.0099	KCl	0.4	KCl	0.4	L-Arginine	105
NaHCO3	0.2106	$NaHCO_3$	3.7	$NaHCO_3$	3.7	L-Asparagine - H_2O	50
Na-Pyruvate	0.0022	NaCl	6.4	NaCl	6.4	L-Aspartic Acid	30
$Ca(Lactate)_2$ - 5H2O	0.0617	NaH_2PO_4	0.109	NaH_2PO_4	0.109	L-Cysteine hydrochloride	100
L-Gutamine	0.0146	L-Arginine - HCl	0.084	L-Arginine - HCl	0.084	L-Cystine	31
Hypotaurine	0.0546	L-Cystine - 2HCl	0.0626	L-Cystine - 2HCl	0.0626	L-Gluatamic Acid	75
Basal Medium Eagle Amino Acids	2mL	L-Glutamine	0.584	L-Glutamine	0.584	L-Histidine	31
Minimum Essential Medium, Non-Essential Amino Acids	1mL	Glycine	0.03	Glycine	0.03	L-Isoleucine	52.4
Gentamicin	50ul	L-Histidine - HCl- H_2O	0.042	L-Histidine - HCl- H_2O	0.042	L-Leucine	52.4
BSA	.5g	L-Isoleucine	0.105	L-Isoleucine	0.105	L-Lysine	58
Phenol Red	250ul	L-Leucine	0.105	L-Leucine	0.105	L-Methionine	15
pH	7.3 ±0.02	L-Lysine - HCl	0.146	L-Lysine - HCl	0.146	L-Phenylalanine	32
Normocin	200µL	L-Methionine	0.03	L-Methionine	0.03	L-Proline	40
b-FGF	500µL	L-Phenylalanine	0.066	L-Phenylalanine	0.066	L-Serine	25
		L-Serine	0.042	L-Serine	0.042	L-Threonine	48
		L-Threonine	0.095	L-Threonine	0.095	L-Tryptophan	10
		L-Tryptophan	0.016	L-Tryptophan	0.016	L-Tyrosine	36
		L-Tyrosine - 2Na - $2H_2$	0.10379	L-Tyrosine - 2Na - $2H_2$	0.10379	L-Valine	46
		L-Valine	0.094	L-Valine	0.094	Asordic Acid	50
		Choline Chloride	0.004	Choline Chloride	0.004	Biotin	0.1
		Folic Acid	0.004	Folic Acid	0.004	Choline Chloride	1
		myo-Inositol	0.0072	myo-Inositol	0.0072	D-Calcium pantothenate	1
		Niacimamide	0.004	Niacimamide	0.004	Folic Acid	1
		D-Pantothenic Acid - ½	0.004	D-Pantothenic Acid - ½	0.004	Niacinamide	1
		Pyridoxine - HCl	0.004	Pyridoxine - HCl	0.004	Pyridoxal Hydrochloride	1
		Riboflavin	0.0004	Riboflavin	0.0004	Riboflavin	0.1
		Thiamine	0.004	Thiamine	0.004	Thiamine Hydrochloride	1
		D-Glucose	4.5	D-Glucose	4.5	Vitamin B12	1.36
		Phenol Red - Na	0.0159	Phenol Red - Na	0.0159	i-Inositol	2
						$CaCl_2$ - $2H_2O$	264
						$MgSO_4$ - $7H_2O$	200
						KCl	400
						$NaHCO_3$	2200
						NaCl	6800
						NaH_2PO_4 - $2H_2O$	158
						D-Glucose	1000
						Lipoic Acid	0.2
						Phenol Red	10
						Sodium Pyruvate	110

Table 3. Effects of atmosphere, slide piece, and knock out serum replacement (KOSR) on the number of cells harvested (Exp. 10)[a].

Cord			DMEM - 5% CO_2/95% air				DMEM - 5% O_2/5% CO_2/09% N_2			
					Slide Pieces				Slide Pieces	
	FBS	FBS, Gelatin	None, Gelatin	KOSR, Gelatin	None, Gelatin	KOSR, Gelatin	None, Gelatin	KOSR, Gelatin	None, Gelatin	KOSR, Gelatin
1	51800	62900		40900		56400				28000
2	55200	181000		36300						
3	110000	65000								
1	150000	249000								12500
2	102000	220000		8230						
3	116000	185000								
Passage 3										
1	66400	110000								
2	82100	115000								
3	81900	44400[a]								
Passage 4										
1	[b]	401000								
2	303000	310000								
3	314000	339000								
1	[b]	223000								
2	97500	182000								
3	102000	87900								
1	[b]	256000								
2	38800	181000								
3	72300	176000								

1	[a]	351000								
2	164000	260000								
3	158000	138000								
1	[a]	59800								
2	15000	48500								
3	17300	57400								

[a] All counts are from three wells of a 24 well plate. [b] Lost well to contamination.

Table 4. Comparison of Glucose Concentration and KOSR Concentration (Exp. 12)[a].

Cord	HG-DMEM on Gelatin					LG-DMEM on Gelatin			
	FBS	10% KOSR		20% KOSR		10% KOSR		20% KOSR	
	None	PDGF	None	PDGF	None	PDGF	None	PDGF	None
1	121000	3260		8660					
2	147000	10600		13100		4970	8230		
3	72800	81200		19300					
4	67900					1920	3190		
5	76100	3690		7300					
6	42700	16500	24700	33900	19400	9510	9090	13300	11600
1		1700		1560					
2									
3		7670		1280					
4									
5									
6		2840	3260	6250	1700				

[a] All counts are from three wells of a 24 well plate.

Table 5. Cells harvested after growth in HG-DMEM or α-MEM and with or without the addition of BSA or PVA (Exp. 13)[a].

Cord	HG-DMEM on Gelatin			α-MEM on Gelatin		
	PDGF, KOSR	PDGF, BSA, KOSR	PDGF, PVA, KOSR	PDGF, KOSR	PDGF, BSA, KOSR	PDGF, PVA, KOSR
1	15900	9950	33600	5800	3980	
2	23000	24800	29500	8150	22700	19800
3	55000	14500	40100	23500		
4	26900	42500	16100	7150		
5	13200					
1	6200[b]					

[a] Passage 1 is three wells combined to make each viable adherent cell count listed. [b] Only one well was able to be replated after the first passage. This is a viable adherent cell count from one well.

Table 6. Total viable adherent cells for serum-containing treatments (Exp. 13)[a].

Cord	FBS	FBS on Gelatin
7	113000	150000
7	108000	142000
7	103000	210000
8	95300	182000
8	130000	78600
8	64900	115000
7	323000	204000
7	375000	521000
7	757000	676000
8	298000	361000
8	269000	898000
8	344000	360000
7	178000	189000
7	190000	188000
7	178000	199000
8	190000	181000
8	230000	179000
8	169000	165000
7	326000	276000
7	379000	344000
7	227000	288000

8	301000	407000
8	320000	277000
8	312000	277000

[a] Three wells combined make a single count of total viable adherent cells at each passage.

Table 7. Total non-adherent versus total plated cells (Exp. 14)[a].

Cord	DMEM + 10%KOSR + bFGF + PDGF		DMEM + 10% KOSR + bFGF + PDGF + EGF		KO-DMEM + bFGF + PDGF		KO-DMEM + bFGF + PDGF + EGF		Total Cells Initially Plated
	Total Non-adherent								
	Collagen	Gelatin	Collagen	Gelatin	Collagen	Gelatin	Collagen	Gelatin	
1	194,700	198,300	264,000	257,000	212,600	231,000	268,000	271,000	496,000
2	184,200	210,000	189,000	248,000	178,500	218,000	210,000	255,000	448,000
3	479,000	460,000	517,000	457,000	402,000	307,000	449,000	397,000	672,000
4	294,000	241,000	344,000	312,000	228,000	208,000	354,000	288,000	490,000
5	878,000	920,000	829,000	811,000	111,3000	837,000	755,000[b]	995,000	972,000
6	380,000[c]	571,000[d]	311,000[c]	620,000[d]	473,000[c]	591,000[d]	458,000[c]	509,000[d]	666,000
Mean	401,650	433,383	409,000	450,833	434,516	398,666	415,666	452,500	624,000
SE	105,682	115,270	95,099	92,441	143,796	105,614	78,731	115,410	79,684

[a] Counts are from 6 wells of a 24 well tissue culture plate in low oxygen environment. Counts of non-adherent cells are from 6 media changes. The total cells plated is the total number of cells that were placed in all 6 wells after enzyme digest. [b] Contaminated with mold, count is total for 5 media changes of 6 wells and the 6th media change is for 5 wells. [c] Contaminated with mold, counts are from 1 media change. [d] Contaminated with mold, counts are from 4 media changes.