EFFECTS OF ISOLATION METHODS ON PROLIFERATION AND GD2 EXPRESSION BY PORCINE UMBILICAL CORDS STEM CELLS

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Abstract

Cell isolation method may have effects on the characteristics of the cells isolated from porcine umbilical cords. As stem cells age or approach senescence, it is hypothesized that their properties change. We expect that isolation method and age of cells will have effects on the phenotype of porcine umbilical cord (PUC) cells during in vitro expansion.

We investigated the effects of three isolation methods on PUC population doublings, ability to produce colony forming units (CFU), and amount of ganglioside GD2 (GD2) expression over eleven passages. Isolation methods were explant (Exp) in which the Wharton's Jelly was removed from cords, minced and plated, enzyme digest (Dig), and stomacher assisted enzyme digestion (Stom). Cell isolates were analyzed for GD2 expression, CFU, and population doublings at early (3), middle (7), and late (11) passage. The Exp method produced greater ($P<0.05$) population doublings and more ($P<0.05$) CFU at passage 7. Explant isolates also were numerically more likely to survive to passage 11 (9/9 isolates vs 5/9 for Dig and 7/9 for Stom). In contrast, the percent cells expressing GD2 was greater ($P<0.05$) for Stom isolates than Exp isolates at passage 11. There were no trends for increased passage number to decreased population doubling, CFU formation, or percent GD2 positive cells.

In summary, our results indicate that the Exp isolation method produced the greatest number of population doublings over 11 passages and there were minimal effects of isolation method on CFU and GD2 expression. Although Exp may be more difficult to scale up to isolate all of the PUCs in a cord, it provided greater in vitro expansion than the enzyme methods in our experiment and may provide the most cells for biotechnological and biomedical applications.

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Chapter 1 - Literature Review

What is a mesnchymal stem cell

Mesenchymal stem cells may be described as "clonogenic, self-renewing progenitor cells that can generate one or more specialized cell types" (Anderson et al., 2001, Potten and Loeffler, 1990). These cells are plastic adherent, fibroblastic in appearance and express mesenchymal cell markers but do not express hematapoetic markers. Mesenchymal stem cells were first described as mononuclear cells isolated from bone marrow that were adherent colony forming unit fibroblasts when cultured (Friedenstein et al., 1974). They were originally termed fibroblastoid colony forming units, then as mesenchymal stem cells, and most recently mesenchymal stromal cells (Pochampally 2008) reflecting a changing understanding of their properties. Mesenchymal stem cells are thought to be the precursor of mesenchymal stromal cells (Majumdaret al., 1998). It is assumed they are derived from mesoderm. Stromal describes the structural location of the cell s in the tissues. The term "multipotent stromal cell" captures both the location and the stem potential. The entry "Mesenchymal Stem Cell," in Wikipedia: The Free Encyclopedia provides a good summary of the developing terminology.

Mesenchymal stem cells are defined as multipotent, rather than pluripotent. In general there are three possible fates for dividing stem cells; the cells may divide to produce two identical daughter stem cells with an increase in mesenchymal stem cell number, or one stem cell and one progenitor with no net increase in mesenchymal stem cell number, or two committed progenitors with a net decrease in mesenchymal stem cell number (Chen, 2010). Beyond passage 6 or 7 the stem cell population may be diluted by the last two types of division and the

resulting increase in committed, transiently amplifying and differentiated cells (Chen, 2010). By passage 50-55 a morphological change results in broad band-like cells that are often observed to be undergoing degeneration (Fong et al., 2007).

Tissue sources of mesenchymal stem cells include to mesodermal bone marrow, trabecular bone, synovium, cartilage, fat, muscle, tonsil, thymus, skin, hair follicle, dura mater, dental pulp, umbilical cord, umbilical cord blood, amnion, amniotic fluid and placenta (Kuhn and Tuan, 2009).

Wharton's Jelly (WJ) Cells

Wharton's jelly is a cushioning matrix within the umbilical cord. It surrounds the vein and arteries. First described by the English physician and anatomist Thomas Wharton, this substance is comprised of mucopolysaccharides and is derived from extra embryonic mesoderm ("Wharton's Jelly," Wikipedia: The Free Encyclopedia). This area is rich in cells with mesenchymal morphology. The WJ cells have been isolated from three regions: the perivascular zone, the intervascular zone, and the subamnion. WJ cells share several properties with mesenchymal cells isolated from other areas. They display mesenchymal cell surface markers, have stromal support properties, produce cytokines similar to those of bone marrow mesenchymal cells. WJ cells also resemble pluripotent cells in that they express Oct4, Sox2, and Nanog, and are alkaline phosphatase positive. In vitro WJ cells can be induced to become several cell types. These include bone, cartilage, adipose, cardiac, skeletal muscle, and endothelial cells (Troyer and Weiss, 2008).

Methods for Isolating WJ cells

Two methods have been described for obtaining WJ cells. The simplest is explanting small tissue fragments which attach to plastic culture vessels, and the mesenchymal cells grow out in a few days. This is the explant method used by Mitchell et al., (2003) in the initial description of the stem cell properties of WJ cells. To prepare the explants, Mitchell et al., removed the vein and arteries from umbilical cords and diced the tissue into small fragments that were placed in a culture plate containing DMEM supplemented with fetal bovine serum (FBS). The explant method was described further by Carlin et al. (2006) who prepared tissue fragments (2-5 mm^3) and allowed them to adhere for 24-48 h when the explants were removed the adhering cells were dissociated with trypsin/EDTA and replated for subsequent growth.

Cells have also been isolated directly from WJ by enzyme treatment. Wang et al. (2004) removed of the blood vessels and scraped off tissue from the Wharton's Jelly with a scalpel. This tissue was centrifuged, and the pellet treated with a solution of collagenase and trypsin for 30 minutes before plating (Wang et al., 2004). Karahuseyinoglu et al., (2007) removed the arteries and veins, chopped the cord into sections that were placed in media containing collagenase (1µg/ml), and agitated the digest on an orbital shaker for 4 hours. The enzyme solution was centrifuged and the cell pellet was plated. In a report by Fong et al . (2007) each cord was cut in 2cm pieces that were split longitudinally and the inner surfaces placed face down into an enzyme solution of collagenase types I (2mg/ml) and IV (2mg/ml) , and hyaluronidase (100IU/ml). After 45 minutes incubation the cord pieces were removed, the WJ pieces were detached keeping the vein and arteries intact, and placed in DMEM which was used to plate after centrifugation (Fong et al., 2007).

Sarugaser et al. (2005) isolated cells around the blood vessels. They removed the vessels from the umbilical cords, which were then sutured into loops and placed in an enzyme solution containing collagenase. The digest was then centrifuged and the recovered cells were plated.

Ishge et al. (2009) point out that enzyme digestion can result in decreased cell viability. In their report they found that explants were superior to enzyme digest because they yielded higher cell viability.

Comparison of WJ cells to other sources of mesenchymal cells

WJ cells express mRNA for granulocyte colony stimulating factor and granulocyte macrophage colony stimulating factor but bone marrow cells do not express these cytokines (Lu et al., 2006). The isolation frequency of colony forming units from bone marrow mesenchymal cells is in the range of 1-10 CFU per 10^6 mononuclear cells, whereas WJ cells were reported to have a higher frequency and a faster doubling time (Campagnoli et al., 2001) .

Mesenchymal stem cell differentiation

Mesenchymal stem cells have been shown to differentiate into bone, muscle, adipose tissue, cartilage, tendon and neural cells (Troyer and Weiss 2008). There have been models proposed of two distinct compartments necessary for regulation of mesenchymal stem cell differentiation. In the first compartment, mesenchymal stem cells undergo transcriptional modification generating precursor cells without changes in phenotype and self-renewal capacity. Progression of mesenchymal stem cells to precursor cells is considered the first step in stem cell commitment. There is then a transition from the stem cell compartment to the committed compartment, where precursor cells divide symmetrically while gaining lineage specific

properties. This commitment involves changes in transcription factors, cytokines, growth factors, and the extracellular matrix. Genes involved in this commitment have been identified using DNA microarray technology. Although there are genes that have been identified to control differentiation into specific lineages, there have yet to be identified genes that regulate several differentiation lineages. There are 8 genes identified by Baksh et al. (2004) whose expression was increased during differentiation into osteogenic, adipogenic, and chondrogenic differentiation. These genes may function in all three lineages, and may be master control genes. They are PER1, NEBL, NRCAM, FK506, FKBP5, IL1R2, ZNF145, TIMP4, and A2. The functions of these genes products include cell adhesion, protein folding, organization of the actin cytoskeleton, and inflammatory responses. Commitment of stem cells may require many interactions between molecules and signaling pathways (Baksh et al., 2004). It has also been shown that MSC derived and fully differentiated osteoblasts, adipocytes, and chondrocytes can switch their phenotypes in response to external stimuli (Song and Tuan 2004).

Transplantation and Therapeutic potential of Wharton's Jelly Cells

When transplanted into Parkinsonian rats, human WJ cells reduced apomorphine-induced behavioral defects (Weiss et al., 2006). When WJ cells were transplanted into the brains of rats with global cerebral ischemia they reduced the loss of neurons (Jomura et al., 2007). WJ cells rescued photoreceptors when injected into the eyes of rodents in a model of retinal disease (Lund et al., 2007). WJ cells engineered to express human interferon beta were injected intravenously into mice with lung tumors and the size of the tumors was reduced (Rachakatla et al., 2007). WJ cells were not immunogenic when injected into purposely selected MHC mismatched pigs. However when injected into inflamed skin MHC were upregulated and the cells were

immunogenic (Cho et al., 2007). Following injection of umbilical cord tissue derived cells activated by IFNγ in vitro, alloantibodies were detected within a week and skin graft rejection occured (Cho et al., 2007). Therefore the specifics of transplantation determine the immune response to WJ cells.

Stemness

Stemness has been described as a "pattern of gene expression amongst all stem cells" (Pyle et al., 2004) as well as the "maintenance of stem cells in their undifferentiated and multipotent" state (Sonoyama et al., 2005). It would be useful to identify the intrinsic and extrinsic factors that initiate and maintain stem cell self-renewal, differentiation, and stemness for WJ cells and other stem cells. These mechanisms could be used to develop improved methods for expansion of cell numbers and for therapeutic applications (Kuhn and Tuan, 2009). Small interfering RNA gene knockdown technology might be used to study the regulatory pathways (Song et al., 2006). It is also a concern that culture conditions may induce or modify regulatory pathways in unexpected ways and if so, those effects would need to be taken into account. Furthermore the cell isolates may be a mixed population and that could result in the variable between laboratory results describing stem cell markers (Kuhn and Tuan, 2009).

In addition in vitro adaptation of cell isolates is expected to occur. Research by Song et al. (2006) indicated that there may be 11 common stemness genes expressed in undifferentiated mesenchymal stem cells. The proteins were grouped according to function as regulating metabolism, differentiation, proliferation, extracellular matrix, cytoskeleton, cell adhesion, signal transduction, protein synthesis and cell stress responses. Interleukin-6 is important for mesenchymal stem cell maintenance as it enhanced proliferation, inhibited apoptosis induced by

serum starvation, and suppressed chondrogenic and adipogenic differentiation (Pricola et al., 2009 and Song et al., 2006).

Kuhn and Tuan (2009) report that including α4-laminin in the extracellular matrix regulated stemness, enhanced proliferation and inhibited differentiation of mesenchymal stem cells into osteoblasts, chondrocytes, and adipocytes. Altering in the extracellular matrix affected cell shape and stem cell fate and was important for maintaining stemness. (Engler et al., 2006).

The stem cell niche

The concept that stem cells reside in a niche was first developed in hematapoeitic stem cell biology. The niche is a special environment that houses hematopoietic stem cells and ensures maintenance of the population (Schofield, 1978). The hematopoietic and mesenchymal cell compartments occupy adjacent areas of the marrow and some suggest that they may occupy the same niche. However, it is hypothesized that the signals that maintain hematopoietic and mesenchymal stem cells are different (Baksh et al., 2004) and the details of shared or adjacent niches are not described.

The niche environment is thought to include, supporting cells and their secretory products that maintain and direct stem cell behavior. Within the niche the stem cells are probably quiescent until stimulated by signals to proliferate and/or differentiate (Kuhn and Tuan 2006). Supporting cells in the niche could function through direct contact. They also indirectly control the niche by secretion of growth factors, cytokines, and extracellular matrix proteins (Jones and Wagers, 2008).

Transcription factors and stemness

Oct4, also known as octamer 4 or POU5F1 (POU class 5 homeobox 1), is expressed by all pluripotent cells and by the inner cell mass during embryogenesis. It is called "OCT" because it binds to the octamer DNA sequence 5'-ATGCAAAT-3'. Oct4 is considered to be a marker for pluripotent embryonic stem cells. Q-RT-PCR analyses show that it is downregulated in differentiated cells, and upregulated in undifferentiated cells. In terms of embryogenesis, it has been shown to be involved in preventing trophectoderm and somatic-cell differentiation from the inner cell mass, as well as maintaining pluripotency. In mouse embryonic stem cells the manipulation of Oct4 expression by transgenesis indicates that over and under expressing the gene alters the phenotype, therefore the amount of Oct4 protein is an important determinant of cell fate (Boiani and Schöler 2005).

Sox2 is a high mobility group protein and also is known as sex determining region Y box 2. The HMG domain DNA protein-binding family helps regulate chromatin structure and affects gene expression. Sox2 works with Oct4 on the enhancer DNA sequence of Fgf4 (Boiani and Schöler 2005). Sox2 is expressed by uncommitted, dividing stem and precursor cells in the developing central nervous system and has been used as a marker to isolate these cells (Li et al., 1998; Zappone et al., 2000).

Nanog is a transcription factor that contains a homeodomain and is dependent upon leukemia inhibitory factor to direct cell renewal and pluripotency in mouse embryonic stem cells (Boiani and Schöler 2005). It seems that the presence of Nanog delays, rather than blocks, the differentiation of embryonic stem cells. The amount of Nanog per cell is essential for maintenance of an undifferentiated cell, even in the presence of leukemia inhibitory factor. It has been proposed that, in part, Nanog may regulate differentiation through transcriptional repression of genes that promote differentiation (Boiani and Schöler 2005).

The combination of Oct4 and Nanog provides specific signals. Oct4 is required for Nanog mediated self-renewal in embryonic stem cells (Chambers et al., 2003). The function of Oct4 and Nanog may be the repression of differentiation, and their combined signals lead to renewal and pluripotency (Boiani and Schöler 2005).

Carlin et al. (2006) investigated the expression of these factors in porcine umbilical cord matrix cells. RT-PCR indicated that mRNA for all three transcription factor genes were present in porcine umbilical cord cells and at higher amounts than in porcine fibroblasts. Immunocytochemistry revealed positive labeling for Nanog and Oct4, in the nuclei of virtually every cell.

Cell Surface Marker Characterization

Cluster of differentiation (CD) marker are widely used to identify cell surface molecules present on white blood cells and can act as receptors or ligands and help identify cell lineages. The International Society for Cellular Therapy states that when stimulated with interferon gamme, flow cytometric analysis of mesenchymal cells must have greater than or equal to 95% of the population expressing CD73 (ecto-5'-nucleotidase), CD90 (Thy-1), and CD105 (endoglin), and no more than 2% of these cells may express CD34 (hematopoietic progenitor and endothelial cell marker), CD45 (pan-leukocyte marker), CD11b or CD14 (monocyte and macrophage markers), CD19 or CD79α (B cell markers), and HLA-DR (marker of MSCs) (Dominici et al., 2006). In addition to those characterized by the International Society for Cell Therapy (ISCT), other markers have been reported. These include CD49a (integrin alpha subunit), CD271 (low affinity nerve growth factor), CD200 (OX-2) (myeloid cell activity) and CD146 (melanoma cell adhesion molecule) (Kuhn and Tuan 2006). An additional 3 markers

have been suggested for use as true stem cell markers GD2, SSEA-4, and nucleostemin (Troyer and Weiss, 2008).

Colony forming unit assay

One assay for assessing mesenchymal stem cells is the colony forming unit (CFU) assay which was devised by Friedenstein et al. (1974). Each mesenchymal stem cell colony is a clone, produced by proliferation of a single precursor cell. In steady state conditions in vivo these cells are in the G0 stage of the cell cycle, and enter into S phase after plating (Kuznetsov et al., 1997). Cells are plated at low densities and discrete colonies are counted after several days. When used to assess human cell growth, each colony is generated by a single cell. However, when used in mouse or rat assays, single cells can generate more than a single colony because cells can detach as they expand and re-seed the plate. CFU assays are useful in estimating the proportion of progenitors in different preparations of mesenchymal stem cells (Pochampally, 2008). However clonal expansion is not necessarily synonymous with self-renewal because cells can proliferate without maintenance of their initial multipotency (Kuhn and Tuan, 2006).

Culture conditions

Sotiropoloulu et al. (2006) concluded that culture media based on αMEM were more suitable for isolation and expansion of multipotent cells. Lower glucose concentration in DMEM based media and Glutamax, instead of L-glutamine, consistently supported mesenchymal stem cell growth. Greater proliferation with cultures containing Glutamax, may be attributed to stability of the dipeptide L-alanyl-L-glutamine, as opposed to L-glutamine which is chemically unstable. Sotiropoloulu et al. also found that plating at low densities was beneficial to

mesenchymal stem cell growth. Their report indicates that plating at a density of 5,000 to 10,000 cells/cm^2 results in larger adherent mesenchymal stem cell populations.

The most common growth factor used in mesenchymal stem cell culture is basic fibroblast growth factor (bFGF), and this study confirmed the effectiveness of this additive (Sotiropoulou, 2006). Cultures with specific growth factor mixtures, such as FGF, and leukemia inhibitory factors, have previously failed as an additive in media to preserve the properties of mesenchymal stem cells because they favor a particular lineage and cause loss of self-renewal capacity and mulipotentiality (Jiang et al., 2002; Bianchi et al., 2003; Sotiropoulou et al., 2006). Several attempts to prevent spontaneous MSC differentiation have involved culture under low oxygen, low seeding density and low serum with the presence of a growth factor. These conditions permitted up to 60 population doublings of human and mouse mesenchymal stem cells, however, the full differentiation potential of these expanded populations is not established (Chen, 2010).

Neural disialoganglioside GD2 (GD2)

GD2 is expressed on freshly isolated bone marrow mesenchymal stem cells, as well as these same cells expanded in culture. It was hypothesized that mesenchymal stem cells within the bone marrow were the only cells that expressed this marker (Martinez et al., 2007). This group found that immunocytochemical staining of human bone-marrow derived mesenchymal stem cells produced significant expression of GD2. Flow cytometric analysis also confirmed the presence of GD2. Reverse-transcription PCR assays showed that bone marrow derived mesenchymal stem cells expressed the mRNA for GD2 synthase, which is an essential enzyme for GD2 synthesis. Martinez et al. (2007) tested adipose derived mesenchymal cells expression

of GD2 and found that these cells also showed significant levels of GD2 expression. However foreskin fibroblasts did not express GD2. Further analysis showed that GD2 expression persisted at similar levels through 8 passages. Martinez et al. (2007) concluded that GD2 was the first surface marker to be expressed on all mesenchymal cell populations tested whether freshly isolated or expanded ex vivo and that it may be beneficial for mesenchymal stem cell isolation and identification (Martinez et al., 2007).

Recently Xu et al. (2009) evaluated GD2 in mesenchymal stem cells in the umbilical cord. This group compared GD2+ sorted to GD2- sorted cells in order to test the possibility of GD2 being a marker for primitive precursor cells. Human umbilical cord derived mesenchymal stem cells, and human umbilical cord vein endothelial cells were isolated from the umbilical cord, along with adult and fetal bone marrow mesenchymal cells, and foreskin fibroblasts for controls and comparison. Immunocytochemical analysis showed that, bone marrow and umbilical cord mesenchymal cells expressed GD2 while the umbilical cord vein endothelial cells did not. Flow cytomteric analysis revealed that GD2+ cells continued to express GD2 throughout 10 passages, while GD2- cells did not express GD2 until passage 10. Using immunocytochemistry and immunofluorscence they found that there were GD2 positive cells distributed throughout the cord including the subamnion, intervascular, and perivascular regions. It was shown that GD2+ cells displayed significantly more colony forming units, while GD2- cells showed no colony forming activity. Further flow cytometric analysis showed that the GD2+ cells had significantly increased levels of SSEA-4, Oct-4, Sox-2 and Nanog. Xu et al. (2009) concluded that GD2 could be an important tool for identifying primitive umbilical cord mesenchymal stem cells.

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Chapter 2 - Effects of Isolation Methods on Porcine Umbilical Cord Stem Cells (PUC) GD2 Expression, Colony Forming Units, and Proliferation

Introduction

Stem cells are getting lots of attention for use in regenerative medicine and as tools for biotechnology and biomedicine. It appears that stem cells from different tissues have site-specific characteristics that may lend advantages or disadvantages for certain applications. Because almost all uses of stem cells envisioned require harvest and expansion ex vivo, it is also important to consider the effects of these procedures on the cells.

Here we have considered 3 methods of harvesting cells from Wharton's Jelly (WJ) of the porcine umbilical cord. Two of the methods are in common use and described in the literature. These are enzymatic release and plating explants of intact tissue. The third method is a modification suggested by Dr. Mark Weiss (personal communication) that adds mechanical massaging to the enzymatic method by using a stomacher, a device commonly used in food science to disperse food systems for microbiological evaluations.

To assess effects of these cell isolation methods we considered viable cell numbers after the first passage, persistence in culture through passage 11, survival and expansion after frozen storage, colony forming unit (CFU) potential and expression of ganglioside GD2 (GD2).

Pig umbilical cords provide a good test system to evaluate effects of in vitro methods on WJ cells because pigs are important biological models for human physiology and are an important agricultural species. Furthermore pigs may provide xenografts for tissue and cell

replacements at some time in the future. Individual pig umbilical cords can provide more than a million WJ cells (unpublished observations) and litters average more than 10 pigs. Therefore pig umbilical cord matrix stem cells (PUCs) are available in significant numbers from even small herds.

In addition to measures of cell doubling and CFU we evaluated the percent of cells expressing neural ganglioside GD2 (GD2). GD2 may be a useful marker for mesenchymal stem cells GD2 positive cells are present in both freshly isolated bone marrow and expanded mesenchymal stem cells. GD2 is also expressed by human umbilical cord derived mesenchymal cells and the GD2 positive cells express Oct-4, Nanog, and Sox-2, indicating that they may be a primitive cell population (Xu et al., 2009).

Materials and Methods

Collection of cords

Umbilical cords were collected from pigs at birth at the Kansas State University Swine Teaching and Research Center. Individual cords were placed in conical tubes containing sterile phosphate buffered saline (PBS, 50ml, pH 7.2, Invitrogen Grand Island, NY) with antibiotic antimycotic (2ml, penicillin 400u/ml, streptomycin 400mg/ml, amphoterricin 5µg/ml) (Sigma).

Upon returning to the lab, each cord was rinsed in fresh PBS containing antibiotic antimicotic (Sigma) and refrigerated (4ºC). Within 24 hours the cords were weighed and their length measured. Umbilical cords were placed in Petri dishes, opened longitudinally the arteries and vein removed using hemostats. Each cord was cut longitudinally to yield three pieces of approximately equal size. WJ explants were removed from each of the three sections then each section was transversely and the two halves assigned randomly to either the stomacher aided enzyme digest (Stom) or the conventional enzyme digest (Dig).

Explant method

After removal of the blood vessels, the WJ was cut into approximately $1mm^3$ pieces that were placed in Petri dishes containing PBS and minced with scissors. The PBS was removed and the tissue transferred to culture plates containing 2ml of high-glucose Dulbecco's Minimum Essential Medium (DMEM, Invitrogen) supplemented with Fetal Bovine Serum (20% FBS, Invitrogen), Gentamicin Reagent Solution (25µg/ml Gentamicin, Invitrogen), Normocin (100µg/ml Invivogen, San Diego, CA), and 2-mercaptoethanol (55µmol/ml Sigma) that was used for in vitro expansion (growth medium). Fungizone (2.5 µl/ml) was included for the first two media changes in all methods.

Stomacher method

After removal of the vessels the cord was placed into a 15ml enzyme solution in a 7oz Whirl-pak sample plastic bag (Nasco, Fort Atkinson WI) with a 330 micron filter. The enzyme solution was prepared in 100 ml batches in Hanks Balanced Salt Solution (HBSS, Invitrogen) and contained 274µg/ml collagenase (from *clostridium histolyticum* Type I Collagenase, Sigma-Aldrich, St Louis MO), 68.3units/ml deoxyribonuclease I (from bovine pancreas , Sigma-Aldrich), 373ug/ml hyaluronidase (from sheep testes, Sigma-Aldrich) . The bag was heat sealed and placed in a stomacher (Tekimar, Cincinnati OH) for 15 minutes on normal speed. After stomaching, the bag was rolled down and placed in an incubator (37.5ºC) for 1 hour. Next the enzyme solution was pipetted into a conical tube (50ml), the bag rinsed with PBS (15ml), and the combined digest and rinse was filtered (60 micron Steriflip vacuum filter, Millipore). The filtrate was centrifuged (10 min, 800xg). After removing the supernatant the pellet was resuspended in 10ml of PBS and re-centrifuged the pellet resuspended in PBS (1ml). An aliquot was removed for counting using Guava Viacount® (Millipore, Billerica MA). Next the cell

suspension was centrifuged the PBS and the pellet was resuspended in 1ml of growth medium. The cells were plated on a Corning 6-well culture cluster tissue culture plate at 7500 cells/cm^2 with 2 ml Growth Medium.

Enzyme digestion method

After removal of the vessels the cord was placed into a 7 oz Whirl-Pak sample plastic bag (Nasco) containing a 330 micron filter, the same enzyme solution as used in the stomacher aided enzyme digestion method (15ml) was added. The bag was heat sealed, rolled down, and placed in a plastic beaker on a heated shaker for 1 hour. After removal from the shaker, the enzyme solution was pipetted into a conical tube (50ml) and the bag rinsed with PBS (15ml). The enzyme digestion + rinse was process as described for the stomacher assisted digestion.

Culture Procedures

Culture medium was replaced with Growth Medium every two days and Fungizone (final concentrarion 2.5µg/ml) included in the first two media changes. Cells were harvested (trypsin) when 80-90% confluent and replated for a total of 11 passages. Intially Easyfit (Millipore) software was used to estimate viable cells but it was discovered that for some samples the cell number was underestimated. Retrospectively each samples flow cytometry data was re-gated for viable cells. Because of this some cultures in passages 3 to 11 were plated at greater than 7500 cells/cm^2. Covariance analysis revealed that this had minimal effect on cell doubling, but did affect doubling time consequently we did not evaluate the effects of isolation or passage on cell doubling time. Cells were harvested by adding 500µl of .05% Trypsin-EDTA (Gibco) to each well and incubating at 37ºC for 10 minutes with agitation every 3 minutes. The lifted cells were transferred to a conical tube (15ml) and centrifuged (10 min, 800xg). The cell pellet was resuspended in PBS and the cell concentration determined using Guava Viacount®. At passages

3, 7, and 11 an aliquot of the cells was resuspended in 1ml of Cell Culture Freezing Medium (Gibco, Grand Island, NY) and frozen in liquid nitrogen for later analysis GD2. RNA was extracted from another aliquotfor use in future research. The remaining cells were replated for expansion and a subaliquot was plated for the CFU assay.

CFU Assay

The CFU plates were prepared with about 100 cells per 100 mm^2 and incubated for 10-14 days at 37.5ºC. The wells were washed with PBS and stained with 0.5% crystal violet in methanol for 5-10 minutes at room temperature. The plates were washed twice with PBS and the colonies visible (100x) were counted (Pochampally, 2008).

GD2 staining

Cells frozen at passages 3, 7, and 11 were thawed and plated and grown to ~80% confluence. The cells were lifted with trypsin/EDTA solution as described earlier, centrifuged the cell pellet was resuspended in 1ml of blocking buffer (3% goat serum, and 1% bovine serum albumin (Sigma) in PBS) to minimize non-specific binding, and incubated for 30 minutes at room temperature (21ºC). The sample was divided equally into two, 2ml microcentrifuge tubes. To one tube 250µl of 1µg/ml purified mouse anti-human disialoganlioside GD2 (.5mg/ml BD Biosciences Franklin Lanes, NJ) prepared in blocking buffer was added, vortexed, and incubated for 30 minutes at room temperature (21ºC), 250µl of blocking buffer was added to the other tube. Samples were centrifuged (5min, 800 x g) of PBS 1.25 ml was added the pellet resuspended, and centrifuged (5 min, 800 x g) to wash off non-attached primary antibody. The supernatant was aspirated and replaced of PBS (100µl). Secondary antibody 1µl Alexa Fluor 488 goat anti-mouse IgG (H+L), 2mg/ml Invitrogen) was added to all tubes, the samples were vortexed then incubated for 30 minutes at room temperature. Cells were labeled with ViaCount® reagent

(Millipore, 1:2 dilution) and incubated at room temperature for 5 min. Fluorescence was measured using a microcapillary cytometer (Guava EasyCyte Plus, Millipore). An elliptical gate was set on the side scatter/forward scatter dot plot to eliminate debris, and this gate ("cells") was applied to the histograms for green, yellow, and red fluorescence. Cells were further gated to select those that were both viable (non-yellow) and GD2+ (green). The number and percent positive cells was genereated by Cytosoft^{TM} software (Millipore)

Population doubling was calculated at the end of each passage using the formula Log(2)(cells harvested/cells plated).

RNA extraction

RNA was extracted and stored for future studies of gene expression. Adherent cells were rinsed three times with PBS to remove media and buffer RLT added to lyse the cells. The samples were frozen (-80ºC) for later extraction of RNA to evaluated selected gene expression (RNeasy Micro Kit Qiagen, Valencia CA). RNA analysis is not a part of this thesis.

Statistical Analysis

Data were analyzed using PROC Mixed procedure of SAS (SAS, 2000; SAS Inst. Inc., the Satterthwaite degrees of freedom). The significance was declared at $P<0.05$. Means were compared using the Least Significant Difference procedure.

Results

Population Doubling

Explants attached within approximately 4 hours and cells grew to approximately 80% confluence in 8 to 13 days. Cells isolated with both Stom and Dig methods attached within

24hrs, and grew to 80% confluence in 8 to 11 days. Table 2.1 shows the number of isolates that continued to proliferate to 3, 7, and 11 passages.

At each passage the number of viable cells was determined using flow cytometry (Fig 2). Between early (P3), mid (P7), and late passage (P11) among all isolation methods, the average population doubling was within a range of 0.1 to 2.3 doublings.

The cumulative population doublings to passages 3, 7, and 11 was greater ($P<0.05$) for the Exp isolation method than for the Dig and Stom methods (Table 2.2). At passage 11 Exp isolates had exhibited more than 3x the population doublings exhibited but the Stom and Dig isolates.

The population doublings at passages 3, 7, and 11 also was affected by isolation method. At early, mid, and late passage the Exp isolates had more ($P<0.05$) doublings than the Dig and the Stom isolates (Table2.3). There was no difference in population doublings at passages 3, 7, and 11 with increasing passage in either the Exp, Stom of Dig method (data not shown).

To evaluate the possible effects of isolation method on survival of frozen storage the population doubling after thawing (at the time of the GD2 analysis) was determined. The population doubling after thawing cells frozen at passage 3, but not at passage 7 and 11 was greater ($P<0.05$) for Exp isolates than for the Dig and Stom isolates (Table2.4).

Colony Forming Units (CFU)

Cells were observed using Hoffman optics and images recorded with a Nikon digital camera. Cells formed colonies of 30 or more tightly packed cells (Fig.1). For some CFU wells the colonies converged making identification of single colonies impossible (Fig. 1) and most of these wells were from the Exp isolation method (Table2.5). In these cases the number of CFUs was recorded as 160, the highest number of colonies counted in the experiment. Number of CFU

did not differ between isolation methods for passage 3 (Table 2.6). For passage 7 the Exp isolation method produced more ($P<0.05$) CFUs than the Dig or Stom methods but the number of CFU were similar for the Stom assisted and Dig methods in passage 7. The number of CFUs was not affected ($P<0.05$) by isolation methods in passage 11.

Because several Stom and Dig isolates did not persist to passages 7 and 11 it was only possible to evaluate the effect of passage on CFU within isolation method. Within the Exp isolation method there was no ($P<0.05$) effect of passage on the number of CFU between passages 3 and 7, and between passages 7 and 11. However the CFU did increase significantly from passage 3 to passage 11. Within the Stom and Dig methods there was no ($P<0.05$) effect of passage on the number of CFU.

GD2 Expression

GD2 expression was on the surface of the positive cells as determined by immunocytochemistry (Fig 3) and the percent viable cells expressing GD2 was determined by flow cytometry (Fig 4). One Dig isolate at passage 3 and two Stom isolates at passage 11 were lost during storage and not available for analysis. One isolate from the Stom method did not attach and grow after re-plating at passage 7, and at passage 11 one isolate from each isolation method did not attach and grow after thawing. Cells in the surviving isolates were subjected to flow cytometry and were gated according to viable GD2+ cells. For each sample the histogram was gated using a negative control. There was no difference ($P<0.05$) in the percent cells expressing GD2 at early and mid passage among the three isolation methods (Table 2.7). However in passage 11 the percentage GD2 cells for the Exp isolates was less than for the Stom isolates. The percent GD2 cells in the Exp isolates decreased ($P<0.05$) with increasing passage but the Stom and Dig did not change %GD2 with increasing passage (Table 2.7).

Discussion

Two methods have been described for isolation of cells from WJ; explanting and enzyme treatment. The explant method involves dicing the tissue into small fragments and waiting for cell adhesion to a culture plate (Mitchell et al., 2003). The enzyme method involves digestion of the surrounding tissue in order to release the Wharton's Jelly cells, which are plated for establishing the culture (Wang et al., 2004). However it has been suggested that the enzyme treatment method decreases cell viability therefore rendering explants method of choice for optimal cell growth (Ishge et al., 2009).

WJ is a cushioning matrix within the umbilical cord that provides the stem cell niche (Mitchell et al., 2003). The cells in this region share several properties with mesenchymal stem cells from other sources in the body and resemble pluripotent cells (Troyer and Weiss 2008).

Here we report the effects of isolation method on the GD2 content and proliferation capabilities by assessing the colony forming units as well as the CFU and population doubling through 11 passages.

Isolation method had a clear effect on population doubling but effect on CFU and GD2 were small and inconsistent. Exp yielded higher population doublings for individual passages and the cumulative doublings over 11 passages. Our data may indicate that the Exp isolates were less damaged by isolation and were therefore 'healthy' enough to complete more population doublings than the Dig and Stom methods. Perhaps the enzyme methods in the latter two methods damages the cells or alternatively removed critical elements from the endogenous niche. It may be that more of the environment provided by the WJ was present for initial cell growth and this could have allowed the WJ cells to adapt more effectively to their in vitro environment.

CFU assays reveal the ability of individual cells to give rise to colonies and is a recognized indicator of the quality of the cell isolate (Ponchampally 2008).The Exp method yielded the most CFU at only passage 7. However there were numerically more more colonies for Exp isolates at passage 3 and 11 also and the relatively high number of plates that were overgrown and could not provide colony countsfor the Exp isolates may have reduced the ability to detect a statistical difference in favor of Exp at passages 3and 11. The CFU results imply that the Exp method may produce isolates with more cells capable of producing colonies.

The neural ganglioside GD2 has been suggested to be a stem cell marker for mesenchymal cells (Xu et al., 2009). This marker may be able to identify stem cell populations without the need for identification of additional markers or characteristics that are currently in use for stem cell detection. GD2 has been shown to be present in freshly isolated bone marrow mesenchymal stem cells as well as these cells later expanded in culture (Martinez et al., 2007). This marker was also found to be present in human umbilical cord derived mesenchymal cells and the GD2 positive cells represented almost all the cells that expressed Oct-4 Nanog, and Sox-2, suggesting that GD2 is useful for identifying primitive cell populations (Xu et al., 2009).

In our experiment the percentage of the cells expressing GD2 was not affected by the isolation method except in passage 11 when Stom isolates were superior to Exp isolates. The interpretation of these results is unclear. It could be that Stom isolates contained cells that on average were more primitive and therefore better able to maintain their stemness to later passage. Interestingly there was a trend for GD2 expression to decrease from passage 3 to 11for the Exo and Dig isolates, but there was no decrease for Stom isolates.

In conclusion we have outlined several measures to assess the proliferative capacity as well as the primitiveness of cells isolated from Wharton's Jelly of the porcine umbilical cord.

We suggest that further work in this area may provide an increased understanding of what is happening to these cells at harvest and over time in culture. This information is important to develop cell isolation procedures that produce the best chance for establishing the WJ cells in vitro for later use in experiments and for biotechnology or cell therapies.

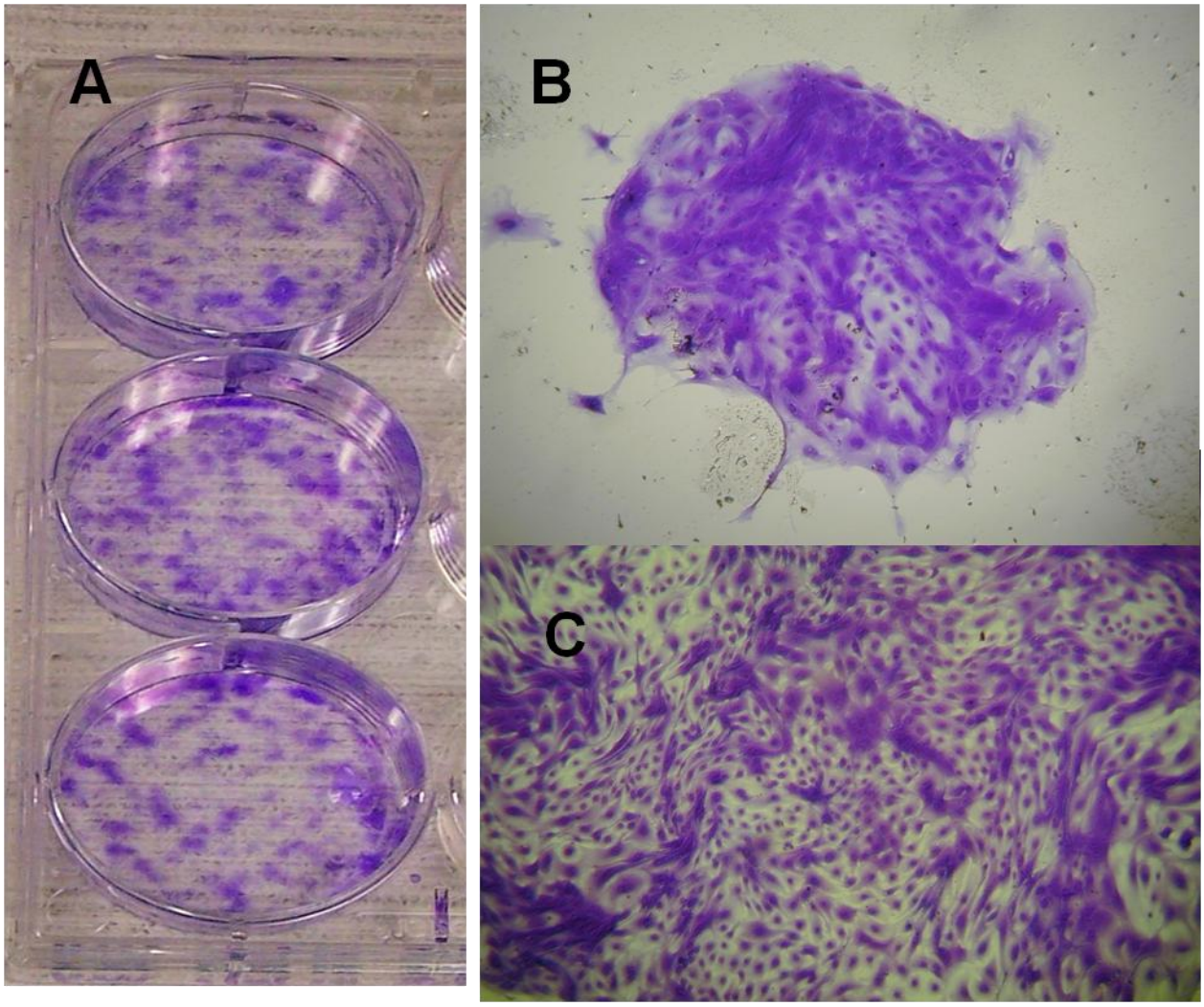

Figure 1. Representative PUC colony forming unit assay.

Colonies in a 6 well plate stained with crystal violet. Cells were plated at 100/cm^2 and grown for 10 days. Large masses indicate colonies **(A). A** typical colony (10x) **(B).** Example of a well with cells that were too confluent to count to identify individual colonies after 10 days (100x) **(C).**

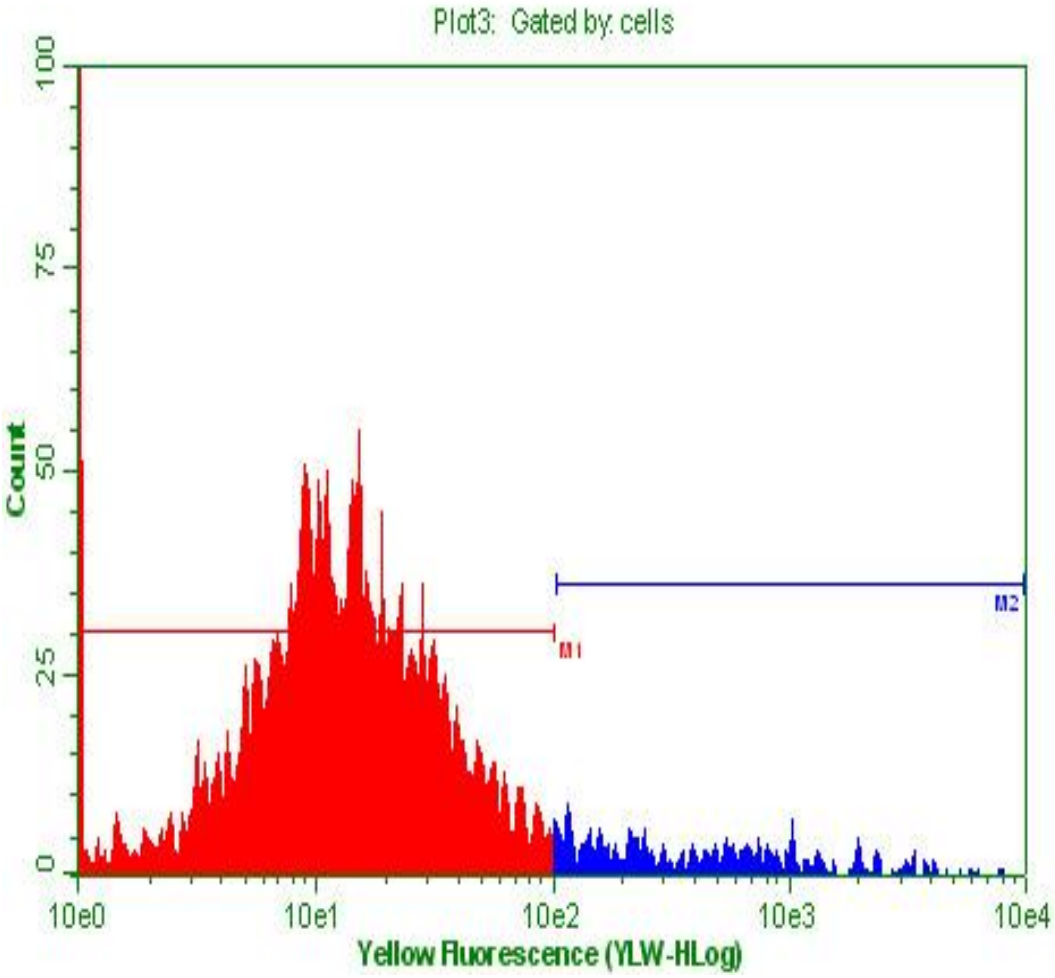

Figure 2. Flow cytometrry illustrating viability staining of PUCs.

Plot illustrating results of a Viacount viability assay for passage 3. Red filled area on the plot and gate M1 indicates viable cells while the blue M2 gate represents nonviable cells.

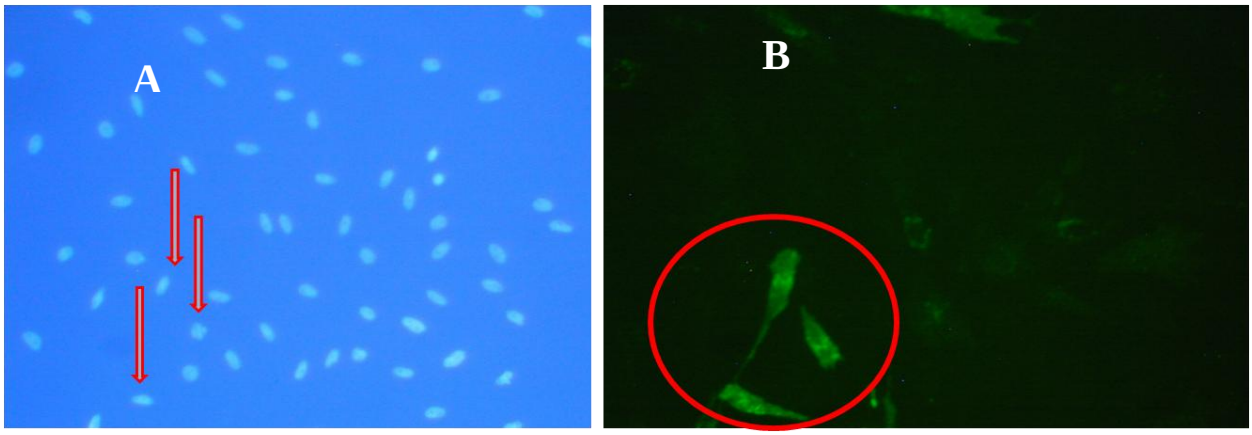

Figure 3. Ganglioside GD2 immunostaining in PUCs.

Passage 8 cells were stained with DAPI to indentify nuclei **(A),** GD2 stained cell surfaces as revealed by green fluorescence (Alexa Fluor 488) **(B).** GD2 positive cells are circled (B) and the cell nuclei are indicated by arrows in A.

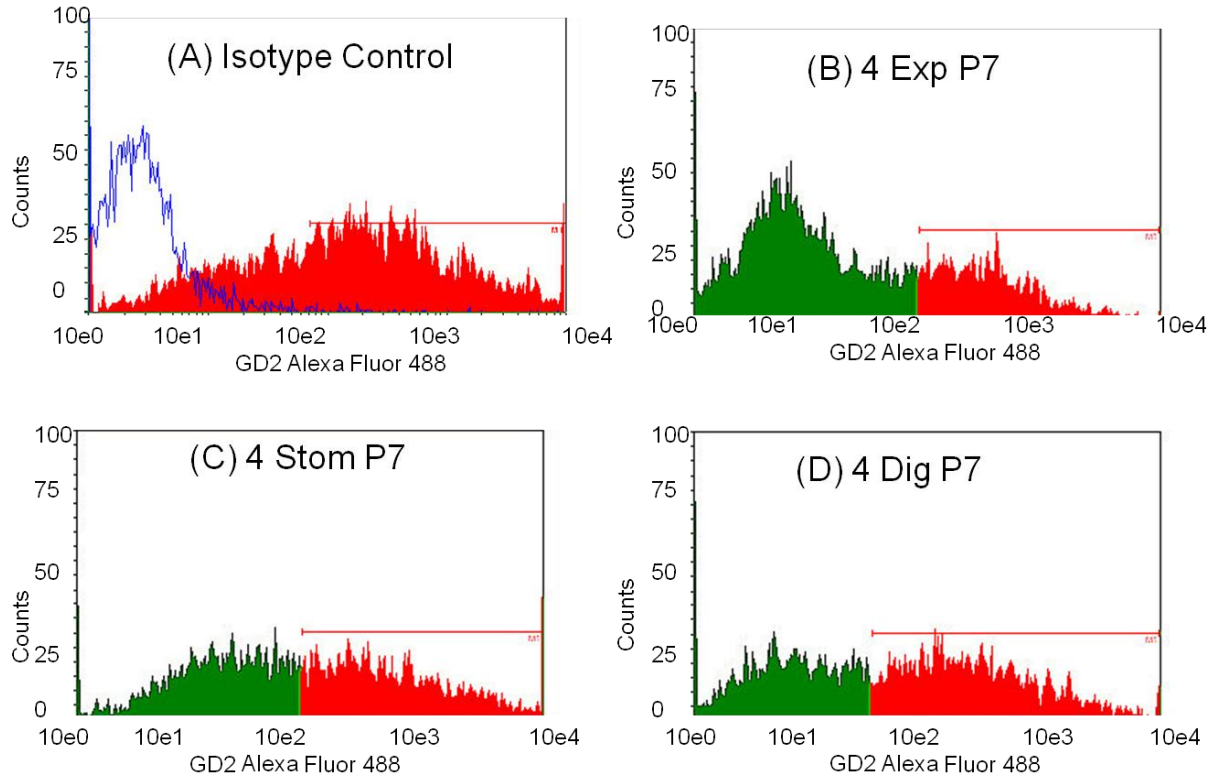

Figure 4. Flow cytometry for GD2 stained PUCs.

Isotype Control **(A),** Exp Passage 7 **(B),** Stom P7 **(C),** Dig P7 **(D),** Representative histograms of cells harvested at early, mid, and late passages, and representing the three treatments explant, enzyme digest, and stomacher aided enzyme digestion. Gated red area indicates GD2 positive cells.

Table 2. 1 Number of cell isolates surviving up to passages 3, 7, and 11.

Treatment	Isolates, n	Established cultures, n	Survived to:		
			P3	P7	P11
Exp	9	9	9	9	9
Stom	9	9	9	8	7
Dig	9	8	8	5	5

Table 2.2 Cumulative population doublings (CumPD) for porcine umbilical cord cells (PUC) isolates to passage 3, 7, and 11.

Passage	**3**			**7**			**11**		
Treatment	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**
n	9	9	8	9	7	5	9	7	5
CumPD	3.57^{a}	1.82^{b}	1.23^{b}	10.37^{a}	3.59^{b}	4.58^{b}	17.76^{a}	5.51^{b}	5.41^{b}
SE	0.55	0.55	0.58	1.28	1.28	1.36	1.84	1.84	1.95

[ab] Means within passage without a common superscript differ (P<0.05).

Table 2.3 Population doubling at passages 3, 7 and 11.

Passage	**3**			**7**			**11**		
Treatment	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**
n	9	9	8	9	7	5	9	7	5
PD	1.57^{a}	0.44^{b}	0.13^{b}	2.35^{a}	0.42^{b}	0.57^{b}	1.57^{a}	0.53^{b}	0.11^{b}
SE	0.35	0.35	0.37	0.29	0.29	0.39	0.33	0.37	0.44

^{ab} Means within a passage without a common superscript differ ($P<0.05$).

Table 2.4 Population doubling after thawing PUCs frozen at passages 3, 7 and 11.

Passage frozen	**3**			**7**			**11**		
Treatment	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**
n	9	9	7	9	8	5	8	4	4
PD	1.82[a]	-0.12[b]	0.23[ab]	0.83[a]	-1.13[a]	1.2[a]	1.38[a]	0.19[a]	3.03[a]
SE	0.54	0.54	0.86	1.07	1.01	1.18	1.11	1.28	1.57

[ab] Means within a passage without a common superscript differ ($P<0.05$).

Table 2.5 Frequency of colony forming units for different cell isolation methods.

Colony Forming Units												
Passage	Exp				Stom				Dig			
	<40	40-99	100-160	>160	<40	40-99	100-160	>160	<40	40-99	100-160	>160
3	5	3	3	0	5	1	2	0	5	1	2	0
7	0	0	5	1	2	3	1	0	1	1	1	0
11	0	4	0	5	2	2	0	0	1	3	0	1

Table 2.6 Colony forming units (CFU) for porcine umbilical cord cells at passages 3, 7 and 11 and isolated by different methods.

Passage	**3**			**7**			**11**		
Treatment	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**
n	9	9	8	9	7	5	9	7	5
CFU	50.3[a]	28.8[a]	32.3[a]	93[a]	62.3[b]	58.4[b]	97.7[a]	30.7[a]	61.4[a]
SE	11.45	11.45	11.91	15.20	15.20	17.82	15.29	17.34	20.52

[ab] Means within a passage without a common superscript differ (P<0.05).

Table 2.7 Percent cells expressing ganglioside GD2 (GD2) at passage 3, 7, and11.

Passage	**3**			**7**			**11**		
Treatment	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**	**Exp**	**Stom**	**Dig**
n	9	9	8	9	7	5	9	7	5
GD2, %	55.1[a]	45.5[a]	52.4[a]	29.6[a]	37.3[a]	44.1[a]	18.2[a]	45.6[b]	34.8[ab]
SE	6.95	6.95	7.88	7.65	7.65	9.67	7.91	10.24	11.31

[ab] Means within a passage without a common superscript differ (P<0.05).

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