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Characterization and Potential Utility of Porcine Trophoblast-Derived Stem-Like Cells

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CHARACTERIZATION AND POTENTIAL UTILITY

OF PORCINE TROPHOBLAST-DERIVED STEM-LIKE CELLS

by

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ABSTRACT

Characterization and Potential Utility of Porcine Trophoblast-Derived Stem-Like Cells

by

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In mammals, the trophoblast lineage of the embryo is specified before implantation. It is restricted to become the fetal portion of the placenta. We have isolated and cultured trophoblast-derived cells from day 10 and day 13 porcine embryos. These cells demonstrate morphological and biological characteristics that make them unique. We have demonstrated that these cells can grow *in vitro* in a defined, serum-replacement medium for over a year without showing any signs of senescence. Trophoblast-derived cells placed into serum-containing medium, however, rapidly senesce and fail to proliferate. Gene expression analysis by RT-PCR and Fluidigm analysis of cells in culture from 0-30 days confirmed expression of genes involved in trophoblast function (*CDX2, TEAD4, CYP17A1, HSD17B1, FGFR2, PLET, HAND1*) as well as some genes known to mediate pluripotency (*POU5F1, KLF4, CMYC*). These experiments revealed changes in gene expression over time and in response to serum-containing medium. We have demonstrated that these trophoblast-derived cells are easily stably transfected with an exogenous transgene (eGFP) by a variety of methods, and show the ability to survive

and to be passaged repeatedly after transfection. Also, immunofluorescence analysis results demonstrated that these cells do not only demonstrate epithelial characteristics by the expression of KRT18, but also they show expression of VIMENTIN which is a protein found in mesenchymal cells. These findings contradict studies done by Ramsoondar in 1993 and Flechon in 1995 which reported the negative expression of VIMENTIN in similar cells. In summary, early embryonic porcine trophoblast-derived cells have demonstrated unique characteristics which have taken us to the conclusion that they could be used as valuable tools for laboratory work. Anticipated applications include the study of trophoblast physiology as well as possible solutions for improving efficiency of transgenesis by somatic cell nuclear transfer and for pluripotency reprogramming of cells.

(64 pages)

PUBLIC ABSTRACT

Characterization and Potential Utility of Porcine Trophoblast-Derived Stem-Like Cells

Edison A. Suasnavas

In mammalian pregnancy, the placenta is a very important organ which helps to establish a healthy pregnancy. Its functions could be described in four points: 1) It allows the fetus to receive gases and nutrients from the mother in a safe way; 2) it lets the fetus get rid of waste through the mother's kidneys; 3) it releases essential pregnancy-related hormones and growth factors that let the uterus hold the fetus; 4) it secretes immune response regulators to give the fetus immune protection against the mother. Abnormalities in the placenta can be the cause of death to the fetus. Mammalian trophoblast cells are responsible of forming the placenta. Therefore, the importance of understanding the function of these cells is critical to obtain a successful pregnancy and high fetus survival. Early embryonic porcine trophoblast-derived cells have shown interesting characteristics in gene expression. When these cells have been cultured in a special formula which does not contain fetal bovine serum, they have been shown to grow continuously without showing signs of death. Also, these cells have been shown to be receptive to foreign DNA which they have been able to incorporate and express inside their nucleus. Currently, cloning techniques contribute to a poor successful fetus survival. A better understanding of in vitro trophoblast models and of the characteristics demonstrated by early embryonic porcine trophoblast-derived cells could lead to a better fetus survival rate in cloned animals. Also, these cells could be utilized as new tools in the laboratory to understand cell and tissue reprogramming utilizing these cells.

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LITERATURE REVIEW

In early eutherian mammalian development, trophoblast cells – precursors to the primitive placenta – stand as essential mediators of embryonic growth and survival. The biological mechanism of trophoblast formation is preceded by other biological processes such as gametogenesis, fertilization, implantation, and signaling pathways which lead to the attachment and signaling of the conceptus to the mother's uterine luminal epithelium when the blastocyst embeds into the uterus. Different species follow different biological mechanisms of trophoblast growth, development, and maintenance. A better understanding of the trophoblast differentiation and function will lead to a better understanding of early embryo development and potential mechanisms behind early embryonic failure. Also, it may help to identify new ways to improve biotechnological techniques such as nuclear transfer and cloning. Finally, trophoblast cells may demonstrate unique characteristics that may result in these cells becoming important tools to be utilized in research.

Gametogenesis is the first biological process leading to the formation of the blastocyst. It controls the formation and the development of generative cells called gametes or germ cells. The process involves the chromosomes and cytoplasm of the gametes which prepares the sex cells for fertilization where the male and female gametes unite. During gametogenesis, the chromosome number is reduced by half and the shape of the cells change. The sperm and the oocyte contain half the number of chromosomes which is considered a haploid number. The number of chromosomes is reduced during meiosis which occurs during gametogenesis. This maturation process is called

spermatogenesis in males and oogenesis in females. Gametogenesis is controlled primarily by the hormone FSH (Gromoll et al., 1996).

The biological process of fertilization can be considered as the second major and key step to achieve pregnancy. It is responsible for the union of two gametes to produce an individual. The gamete sperm is produced by spermatogenesis and the gamete oocyte is produced by oogenesis. A mature oocyte forms after the union of the gametes. The nucleus of the mature oocyte is known as the female pronucleus. Inside the cytoplasm of the oocyte, the nucleus in the head of the sperm enlarges to form the male pronucleus. During this process the tail of the sperm degenerates. The morphologies of the male and female pronuclei are indistinguishable, and during the growth of their respective pronuclei, they replicate their DNA. The final stage of fertilization occurs when the male and female pronuclei contact each other. They lose their nuclear membranes and fuse to form a new cell called the zygote (Austin and Bishop 1957).

Formation of the trophoblast

After fertilization, the cleavage of the zygote is observed. Fertilization activates the zygote and stimulates it to go through mitotic divisions which are called cleavage. Cleavage consists of repeated mitotic divisions of the zygote which increases the number of cells. First, the zygote divides into two cells – each of which are known as blastomeres – which then divide into four blastomeres, then into eight blastomeres, and so on. When the blastomeres divide into sixteen cells, they change their shape and tightly align themselves against each other and form a compact ball of cells known as the morula. The compaction acquired in the morula stage by the blastomeres permits greater cell to cell interaction and is a prerequisite for segregation of the internal cells that form

the embryoblast or inner cell mass (ICM) of the blastocyst. Fluid passes through the zona pellucida into these spaces from the uterine cavity. By four to five days after fertilization, the blastomeres are separated into two parts. The first one is a thin outer cell layer called the trophoblast which will eventually give rise to the placenta and other extra-embryonic membranes. The second part is the inner cell mass (ICM) which gives rise to the fetus. The fluid filled spaces fuse to form a single large blastocyst cavity called the blastocoel. At this stage of development, the conceptus is called a blastocyst. The ICM now projects into the blastocoel cavity and the trophoblast cells form the wall of the blastocyst. After the blastocyst has floated in the uterine secretions, the zona pellucida gradually degenerates and disappears (Kliman 1999). In human development, about 6 days after fertilization, the blastocyst attaches to the endometrial epithelium. As soon as it attaches to the endometrial epithelium, the trophoblast starts to proliferate very rapidly. This differentiation causes a division of two layers. The first layer is called an inner cytotrophoblast, also called a cellular trophoblast. The second layer is called an outer syncytiotrophoblast also called a syncytial trophoblast which consists of a multinucleated protoplasmic mass in which no cell boundaries can be observed. Both intrinsic and extracellular factors modulate the differentiation of the trophoblast in careful time sequences (Aplin 1991). By the end of the first week, the blastocyst is implanted in the compact layer of the endometrium and obtains its nutrients from the maternal tissues. The syncytiotrophoblast produces the substances that erode the maternal tissues. This enables the blastocyst to implant in the endometrium. At about seven days, a flattened layer of cells called the hypoblast appears on the surface of the inner cell mass facing the blastocyst cavity.

During the initial stages of implantation, the interactions between the conceptus and the mother are different in domestic animals that have a non-invasive implantation and in rodents, carnivores, and primates that produce an invasive implantation (Bazer et al., 2008). Carnivores, rodents, and primates demonstrate invasive implantation where the blastocyst invades and implants inside the endometrial stroma and then the uterine luminal epithelium is restored over the site of implantation. During initial contact, the trophectoderm forms a syncytiotrophoblast cell layer which develops a stable attachment to the uterine luminal epithelium followed by the penetration of syncytiotrophoblasts into the uterine wall to establish contact with the maternal vasculature. One of the proteins that have been found to be important in the connection and communication between conceptus and mother is SPP1. The expression of SPP1 by the uterus increased 12-fold during the receptive phase in woman and up to 60-fold during pregnancy in rats (Girotti and Zingg 2003). These observations demonstrate that SPP1 plays an important role in conceptus-uterine interactions. SPP1 is secreted by the extracellular matrix in high quantities, and the uterus regulates it during early pregnancy in humans, rabbits, goats, sheep, and pigs (Johnson et al., 2003). The differences from species to species occur because of the interaction between the trophectoderm, which gives rise to the chorion, and the maternal uterus at the connection between maternal and fetal cells that give rise to the placental structures. In pigs, a close contact between the chorion and the mother's uterine luminal epithelium is maintained throughout pregnancy. This is known as an epitheliochorial placenta. In ruminants, their conceptuses form binucleate trophectoderm cells that migrate, connect, and fuse to the uterine luminal epithelium, and they form plaques of multinucleated syncytia. This is known as a synepitheliochorial placenta.

Placental lactogen, pregnancy-associated glycoproteins, and progesterone are produced by binucleate cells (Wooding et al., 1992). In both epitheliochorial and synepitheliochorial placentation, the conceptus remains inside the uterine lumen throughout gestation. In ruminants, contact between the chorion and maternal caruncles which are located in the endometrial mucosa, leads to the development of opposing highly vascularized cotyledons of the chorion which form placentomes. Placentomes can be thought as a bunch of umbilical cords, and they are really important for the exchange of nutrients and gasses across the placenta (Reynolds et al., 2005). The trophoblast is responsible for the formation of the placenta (Yang et al., 2007). The placenta is the organ found in mammals that connects the uterine wall to the developing fetus, causing their blood systems to come together. There are four basic functions which the placenta fulfills: 1) allows the fetus to receive gases and nutrients from the mother in a safe way, 2) lets the fetus get rid of waste through the mother's kidneys, 3) releases essential pregnancy-related hormones and growth factors that let the uterus hold the fetus, and 4) secretes immune response regulators to give the fetus immune protection against the mother. This occurs so that the fetus does not get rejected by the mother's immune system. Abnormalities in the placenta can be the cause of death to the fetus.

Trophoblast Function

Studies utilizing mammalian embryos have demonstrated that a complex of transcription factors regulates the trophectoderm formation. Although there are similarities among species, there are also some differences in some aspects of the regulation of the trophectoderm formation, trophoblast and placental differentiation (Douglas et al., 2009).

In most mammals the trophoblast is the part of the embryo that contributes to the placenta of the fetus. The trophoblast lineage of the embryo is specified before implantation. It is restricted to become the fetal extra-embryonic membranes, which cooperate closely with the maternal uterine cells and the surrounding blood vessels to form the placenta (Tanaka et al., 1998). The trophoblast has unique properties and features which make it important to being studied from species to species. Depending on the species, trophoblast cells have been characterized as having an extensive, invasive and rapid growth, and capable of cell migration. Some of the functions that the trophoblast shows are the support of the embryonic patterning, fetal growth and nutrition.

Most of the research about trophoblast-derived cells has been performed in mice where trophoblast stem cells have been extracted. This separation of these cells has helped in their characterization. One important observation is that mouse trophoblast stem cells can self-renew without signs of senescence under specific growth factors in vitro. When these growth factors are not present, trophoblast stem cells begin to differentiate into different cell types which are present in the fetal part of the placenta. In the mouse, the first appearance of the trophoblast occurs at the blastocyst stage where an epithelial sheet of cells, the trophoblast, separates from the inner cell mass (Takahashi and Yamanaka, 2006; Rielland et al., 2008). Some of the transcription factors which specify trophoblast lineage are *GATA2*, *PPARG*, *MSX2*, *DLX3*, *HAND1*, *GCM1*, *CDX2*, *ID2*, *ELF5*, *TCFAP2C*, and *TEAD4*. Differentiated trophoblast cells express genes as *HSD17B1*, *CYP17A1*, and *STAR* (Ezashi et al., 2011).

Initially present in the morula stage of the embryo, the transcription factor *CDX2* appears to be an important marker for this separation and for the maintenance of the

trophoblast. When *CDX2* is not present, the blastocyst structure starts to form but it promptly deteriorates. As blastocoel structures and epithelialisation progresses, *CDX2* begins to be more present in the nuclei of outer cells than in the inner cells (Strumpf et al., 2005). Another factor found to be important in trophoblast maintenance and specification has been *TEAD4*. This gene is required to be present before implantation. When it is absent, the trophoblast lineage does not appear (Nishioka et al., 2008). The relationship between *CDX2* and *TEAD4* is not defined. *SOX2* and *FOXD3* have been demonstrated to be crucial in the development of trophoblast and epiblast which is a tissue type derived from the ICM in mammals. When either of the genes is not present, the epiblast is not structured and differentiated trophoblast giant cells start to develop. This event causes a loss of the maintenance pluripotency in the embryo (Avilion et al., 2003; Tompers et al., 2005). *EOMES* is another important transcription factor for the development of the trophoblast. This gene is required later than *CDX2* in the maintenance of the embryo. When *EOMES* is not present, the blastocyst can be structured and maintained but it does not implant (Russ et al., 2000). *ESRRB* is expressed in the extra-embryonic ectoderm until the end of gastrulation in the mouse. When *ESRRB* is not present, embryos reach senescence at E10.5 from an arrest in trophoblast development. Thus *ESRRB* appears to be involved in late trophectoderm or trophoblast maintenance (Luo et al., 1997).

In the mouse, the transcription factor *POU5F1* is also initially present in the morula of the embryo. As separation occurs, it becomes more concentrated in the inner cell mass and later in the epiblast. *CDX2* and *POU5F1* have been showed to reciprocally inhibit each other in embryonic stem cells in culture (Niwa et al., 2005).

An interesting mechanism is found in the mouse which explains the importance of utilizing *FGF* hormones as growth factors. In the mouse blastocyst, *FGF4* is detected in the inner cell mass and in the epiblast. The receptor of *FGF4* is *FGFR2* which is found in the trophoblast. The activation of *FGFR2* by *FGF4* causes a signaling of molecules which activates Erk1/2 by phosphorylation and forms P-Erk1/2. The phosphorylated Erk1/2 has been detected in the blastocyst and in the extra-embryonic ectoderm (EXE) of the trophoblast next to the epiblast. Proliferation in the trophoblast is caused by the secretion of *FGF4* by the inner cell mass, which then acts locally through cognate receptors on the trophoblast cells to stimulate proper growth and function of the trophectoderm layer of the developing embryo (Rielland et al., 2008).

Trophoblast stem cells have been best characterized in the mouse. Culture of mouse blastocyst with early stages trophectoderm in *FGF4* permitted the collection of mouse trophoblast stem cells. When *FGF4* was not present, the trophoblast cells differentiated into other trophoblast sub-types in vitro (Rielland et al., 2008). Evidence has demonstrated that the production of stem cell-like characteristics in trophoblast cells depends from signals generated in the ICM and transmitted to the trophectoderm (Tanaka et al., 1998).

An experiment performed at the University of Missouri demonstrated how induced trophoblast cells are a by-product of the reprogramming of porcine fibroblast cells to induced pluripotent stem cells. In this experiment, there were some characteristics observed from the induced trophoblast cells. They showed patches of cells with epithelial phenotypes which formed raised domes when the cells where cultured. Another characteristic observed when these cells were cultured were the formation of floating

spheres consisting of single epithelial sheet. Also, induced trophoblast cells displayed self-renewing populations. When these cells were passaged, they demonstrated the down regulation of *POU5F1*. The signal of this gene was not silenced completely by the passages. After extended culture and multiple passages, induced trophoblast cells did not demonstrate signs of senescence and they continuously exhibited high telomerase activity, probably signs of cellular stem-like potential (Ezashi et al., 2011).

Trophoblast stem cells have been mostly analyzed in the mouse. Although there are similarities among species, the mouse could not be used as a model to study trophoblast stem cells in other species. The findings done on porcine trophoblast-derived cells have described unique characteristics in this cell type. One of the main characteristics is that trophoblast cell lines grow in culture continuously without signs of senescence (Ramsoondar et al., 1993; Ezashi et al., 2011). This principle opens up the possibility of investigating more these cells as tools for laboratory work and as possible solutions for obtaining better percentages of cloning and for the pluripotency reprogramming.

Not much research has been performed utilizing porcine trophoblast stem cells. The idea of working with these cells in a project is really promising because they can serve as an in-vitro model for trophoblast function, and they can help increase cloning efficiency. These cells are responsible for forming the placenta and the connective tissues between the mother and the conceptus.

Porcine trophoblast-derived cell lines show morphological and biological characteristics that makes them unique. They can be grown in culture without showing signs of cell death. Also, they show different morphological characteristics compared to

fibroblast cells which makes them interesting to analyze by gene expression and transfection methods (Ezashi et al., 2011).

INTRODUCTION

In most mammals the trophoblast is the part of the embryo that contributes to the placenta of the fetus. The trophoblast lineage of the embryo is specified before implantation. It is restricted to become the fetal portion of the placenta which forms the maternal uterine cells and the surrounding blood vessels from the maternal placental components (Tanaka et al., 1998). The trophoblast has unique properties and features which make it important to being studied from species to species. Although there are similarities among species, there are also some differences in some aspects of the regulation of the trophectoderm formation, trophoblast and placental differentiation (Douglas et al., 2009).

The trophoblast is an important extra embryonic tissue which forms during the development of mammals. This part of the embryo is usually characterized by the properties of being invasive, having an extensive and fast growth, and cell migration. Some other features that the trophoblast demonstrates are the support of the embryonic patterning, fetal growth and nutrition (Rielland et al., 2008). The placenta is the organ found in mammals that connects the uterine wall to the developing fetus, causing their blood systems to come together. Although the placenta is a temporary organ, disorders portrayed in it can cause long-term effects in the fetus (Godfrey, 2002).

Porcine trophoblast-derived cell lines demonstrate characteristics which make them interesting targets for further study: They have been cultured in vitro for prolonged periods without showing signs of cell death (Ramsoondar et al., 1993; Ezashi et al., 2011), and their origin in early developmental tissues suggests that they might provide unique insight into mechanisms of early embryo development and survival.

The gene expression of trophoblast specific genes such as *CYP17A1*, *HSD17B1*, and *HAND1* could be utilized to test for differentiation characteristics in these cells. Also, the testing of the pluripotency markers such as *POU5F1* and *SOX2* could be utilized to observe if these cells contain any undifferentiated characteristics. In the mouse, the pluripotency transcription factor *POU5F1* is initially present in the morula of the embryo. As separation occurs, it becomes more concentrated in the inner cell mass and later in the epiblast. *CDX2* and *POU5F1* have demonstrated to reciprocally inhibit each other in embryonic stem cells in culture (Niwa et al., 2005). In porcine, an experiment utilizing immunofluorescence analysis was performed to demonstrate that the gene *KRT8* is present in day 14 trophoblast-derived cells lines. Also in the same experiment, it was demonstrated that the gene *VIMENTIN* is not present in day 14 trophoblast-derived cells (Ramsoondar et al., 1993).

Most of the research about trophoblast-derived cells has been performed in mice where trophoblast stem cells have been extracted (Rielland et al., 2008). Not much research has been done with porcine trophoblast-derived cells. A better understanding of the trophoblast and of the cells which form it will help to identify new ways to improve biotechnological techniques such as nuclear transfer, transfection, cell reprogramming and cloning efficiency. Also, it will help to improve the detection of malfunction in the placenta and fetus survival. These cells could serve as an in-vitro model for trophoblast function for laboratory work since trophoblast-derived cells demonstrate morphological and biological characteristics which make them unique. These characteristics can help these cells become important tools to be utilized in research.

MATERIALS AND METHODS

Embryo production and trophoblast isolation

Day 10 embryos were collected in February 2011. Day 13 embryos were collected in June 2011 and July 2012. All embryos were collected utilizing the same procedure. A 12-gauge 8.9 cm gavage needle was used to inject flush medium inside the porcine uterus. Flush medium is composed of glucose (5.55 mM; Sigma #080M01752V), Na-pyruvate (0.32 mM; Sigma #010M15801V), CaCl2 (0.68 mM; Mallinckrodt Chemicals #20403), MgCl2 (0.5 mM; Mallinckrodt Chemicals #21029), gentamycin (40 μg/ml; Sigma #61098053), and Bovine Serum Albumin (BSA; 3 μg/ml; Hyclone #100805458E) in calcium- and magnesium-free PBS (Hyclone; AXA26046). Once the medium was inserted, gentle manual pressure was used to make it circulate through the uterine horn to the cervix. At the cervix, a 50 ml tube was placed into the uterine lumen to collect flushed embryos. Embryos were washed twice in fresh flush medium. Hypodermic needles were then utilized to manually microdissect the ICM (embryonic disc) away from the surrounding tissue. All the trophectoderm (TE) tissue that was left was utilized to be day 10 embryo samples, whereas for the day 13 embryos, a 1 cm piece of trophectoderm tissue which was about 2-3 cm. distal from the ICM was utilized for our samples. No effort was made to culture ICM tissue; ICMs were frozen to utilize for positive controls for PCR. For TE cell isolation, three to five drops of 0.05% trypsin-EDTA (Sigma) were added to the TE tissue pieces in microcentrifuge tubes and the tubes were placed in the incubator at 39° C for 5 minutes. Then tubes were placed in vortex at medium speed until the tissue started to dissociate. Porcine fibroblast medium (with 15% Fetal Bovine Serum) was added to inhibit trypsin, cells were vortexed again for 30 seconds, and then

tubes were spin at a speed of 500 rpm for 2 minutes. After spinning, fibroblast medium was removed completely and about 1000 cells were placed into each of three wells of a 6-well plate with trophoblast medium (see Table 1 for media formulations) and another 1000 cells into each of 3 wells of a 6 well plate with fibroblast medium. Cell culture plates were cultured in a tissue-culture incubator set at 39° C, 100% humidity, and 6% CO_2 in air. Medium was changed every six to eight days from the day of cultivation. The cultivation of trophoblast cells in both types of medium allowed the observation of morphology changes over time.

Cell growth and morphology in different medium preparations

Trophoblast-derived cells were collected from day 10 and day 13 embryos and cultured in non-serum containing medium called "trophoblast medium" and in serum containing medium called "fibroblast medium" (formulations provided in table format in Table 1). The so-called "trophoblast medium" is a repurposed version of a culture medium originally formulated and described for use with porcine skin-derived cells (Dyce et al., 2004). The complete formulation for this medium is presented in Table 1, but the primary components are Dulbecco's Modification of Eagle's Medium/ Ham's-F12 50/50 Mix (DMEM—F12; Cellgro; 10090055), B-27 serum-free supplement (Life Technologies; 17504044), Gentamycin (Sigma; 61098053), Fibroblast growth factor 2 (FGF2) (Sigma; F0291), Epidermal Growth Factor (Sigma; E9644). The primary components of porcine fibroblast medium are Dulbecco's Modification of Eagle's Medium (DMEM; Cellgro; 10017CV), fetal bovine serum (FBS; Atlanta Biologicals; M1030), Fibroblast growth factor 2 (FGF2; Sigma; F0291), and Gentamycin (Sigma; 61098053). FGF2 is added at 40 ng/ml in trophoblast medium and 2 ng/ml in fibroblast

Table 1. Cell culture medium formulation

Component	Trophoblast Medium	Fibroblast Medium
DMEM base medium	49% (v:v)	85% (v:v)
F12 Nutrient Mixture	49% (v:v)	N/A
Fetal Bovine Serum	N/A	15% (v:v)
B-27	2% (v:v)	N/A
Fibroblast Growth Factor 2	40 ng/mL	2 ng/mL
Epidermal Growth Factor	20 ng/mL	N/A
Gentamicin	40 μg/mL	40 μg/mL

medium. The cultivation of trophoblast cells in both types of medium allowed the observation of morphology changes over time.

Clumps and Passaging

Domes and clumps start to form in porcine TE-derived cell colonies which grow in non-serum medium about 8-12 days after placing them in culture (please reference Figure 1). Also, cell spheres are visible 2-3 days after the first collection tissue is placed in culture in non-serum containing medium. In serum containing medium, clumps and domes did not form. Porcine TE-derived cells could be passaged utilizing three methods: trypsin passage, medium passage, and pick-up clump passage. The less efficient method is the trypsin passage because these cells start to lose regenerative characteristics the more they are treated with trypsin. Porcine TE-derived cells can resist about 2-3 trypsin passages before they start to become old, lose regenerative characteristics and senescence. An intermediate method that works efficiently is the medium passage method. As porcine TE-derived cells become more confluent, they start to release cell

spheres and clumps into their non-serum culture medium. This culture medium can be collected and centrifuged (3 minutes at 400x g), and the pelleted cells can be plated into new non-serum containing medium. Sometimes colonies would grow out from this method. It is thought that as cells get older, they release fewer clumps into the medium, therefore no growth would be observed. The most efficient and less harmful method to passage these cells would be through pick-up clump passage. Clumps could be picked up, placed in new fresh non-serum containing medium, and they would form colonies. Through this method, porcine TE-derived cells could be passaged indefinitely without reaching senescence. The most frequent method used in the lab has been to pick up clumps, which was performed every two weeks or sooner depending on the need of cells.

When porcine TE-derived cells are cultured in serum containing medium, they do not generate clumps, domes or cell spheres, and cannot be successfully passaged by any of the three methods described above.

Reverse transcription and PCR analysis

Trophoblast-derived cells cultured in TE and fetal fibroblast (FF) media were harvested at day 15 and day 30 after first culture from an embryo. The inner cell mass and trophoblast tissue from a day 13 embryo (right after collection) were utilized as day 0 controls. RNA was extracted from cells using Total RNA kit 1 (Omega bio-tek), according to the protocol recommended by the manufacturer. RNA was converted to cDNA utilizing the GoScript Reverse Transcription (RT) System (Promega). Five different batches of cDNA were obtained and tested to replicate results. PCR was performed by following the protocol in Hot Star Taq Plus Master Mix Kit (Qiagen). The annealing temperature utilized was 57° C, the extension time was 30 seconds, and the

Table 2. Primer sequences for RT-PCR experiment

Gene Name	Primer Sequence Forward	Primer Sequence Reverse
CYP17A1	5'-GATTGCCTTCGCCGACCATG-3'	5'-TGTCAGAAGCCAATGACTGAGTG-3'
HSD17B1	5'-TAGGGCTTCCCTTCAACGCTG-3'	5'-TTCTCGGGGAAGGCAGTACG-3'
TEAD4	5'-CATGATCATCACCTGCTCCA-3'	5'-AAGTTCTCCAGCACGCTGTT-3'
PLET	5'-TGTTTCTGTGCTTCGGACTGCTG-3'	5'-TGCCAGAAGCCAATGACTGAGTG-3'
CDX2	5'-GGAGCTGGAGAAGGAGTTTCA-3'	5'-TGCAACTTCTTCTTGTTGATTTTC-3'
FGFR2	5'-ACGGCAGCAAATACGGGCCC-3'	5'-GGCTGGCTGCTGAAGTCCGG-3'
POU5F1	5'-GGGGCTCACTTTGGGGGT-3'	5'-TCTCTTCCGGGCCTGCACGA-3'
SOX2	5'-TAAATACCGGCCCCGGCGGA-3'	5'-GTAGCGGTGCATGGGCTGCA-3'
TERT	5'-ACTGCCTGTTCCCCTGGTGC-3'	5'-AGAGCTTGCGGCGCATGTTC-3'
VIM	5'-GGCCCGTCACCTTCGTGAATACC-3'	5'-TTTCGCTGCACAGAGTACATGCAGTT-3'
KRT8	5'-CATCAGTCTGGTTGGAGGTTACGG-3'	5'-CCAGGCCCTGCATGTTGCCA-3'
EIF4A1	5'-AGGATCATGTCTGCGAGTCAGG-3'	5'-ATAGATGCCACGGAGGAGGGAC-3'

number of cycles was 32. These events were performed in an Eppendorf cycler Model # 5341. Samples were tested three times utilizing different batches of cDNA from each sample to make sure results were consistent. Twelve genes were evaluated by RT-PCR: trophoblast specific genes (*CYP17A1*, *HSD17B1*, *TEAD4*, *PLET*, *CDX2*, and *FGFR2*), pluripotency genes (*POU5F1*, and *SOX2*), telomerase activity gene (*TERT*), cytoskeletal genes (*VIM* and *KRT8*), and a housekeeping gene (*EIF4A1*). Also, water and NO RT cDNA samples were utilized as negative controls, and a reference cDNA sample which is a collection of all porcine tissues cDNA was utilized as positive control (Whitworth et al., 2005). Gel electrophoresis was performed utilizing Tris Acetate EDTA (TAE) (Fisher; #120132) and LE Agarose (Lonza; #193329) according to standard laboratory practice. Sequences for qPCR primers are presented in Table 2.

Fluidigm analysis

Fluidigm's protocol 14 was followed for quantitative PCR gene expression. DNA binding dye Evagreen (Biotium, PN 31000) was utilized for gene expression and targeted 48 genes (see Table 3 for primer sequences) in the trophoblast cell samples on Fluidigm's 48.48 Dynamic Array Integrated Fluidic Circuits (IFC). TaqMan PreAmp Master Mix (Applied Biosystems, PN 439112) was utilized as a Specific Target Amplification (STA) to amplify each sample prior to quantitative PCR. Primer mix was prepared by pooling 1 µl of each primer pair (see Table 3 for primer sequence information) at 20 µM each to form a 200 nM primer mix

For STA thermal cycling, each reaction required 1.25 µl of this primer mix, along with 2.5 µl of the TaqMan PreAmp Master Mix, and 1.25 µl of the cDNA. The activation took place at 95° C for 10 minutes and then the amplification was done for 14 cycles (95° C for 15 seconds then 60° C for 4 minutes). After STA thermal cycling was performed, each reaction was treated with Exonuclease I (New England Biolabs; #M0293S) in order to remove any unincorporated primers. The Exonuclease reaction solution was prepared for each reaction utilizing 1.4 µl water, 0.2 µl Exonuclease I Reaction Buffer, and 0.4 µl Exonuclease I. The total volume of 2 µl was added to every reaction and then placed in a thermal cycle at 37° C for 30 minutes in order to allow the digest to take place. The reactions were then inactivated at 80° C for 15 minutes. Each reaction was then diluted 5-fold by adding 18 µl of water to the 7 µl reaction (volume of STA reaction + Exonuclease I) for a total volume of 25 µl. Reactions were then stored at -20° C.

To run the Fluidigm chip on the Biomark, a sample Pre-Mix solution was prepared utilizing 2.5 µl of the 2x TaqMan Gene Expression Master Mix (Applied

Table 3. Genes and primers used for Fluidigm qPCR analysis. Gene names and forward and reverse primer sequences utilized for quantitative PCR.

Gene Symbol	5' - Forward Primer - 3'	5' - Reverse Primer - 3'
ASCL2	CTGGTGAACTTGGGGTTCCA	GAGCGCAGCGTCTCCA
ASH2L	TTTCCGGCCATCTCACTGTA	TCCTTCGGCGGATACTTGAA
ATM	TTAAGAGCTTGGGCTCTGGAA	TGGTACAGCGAGATCACACA
BCL2L1	AGCGTAGACAAGGAGATGCA	TTCAGGTAAGTGGCCATCCA
BMP15	GGCCATTGGTTAATGGAGCAA	GCTACCCGGTTTGGTCTCA
CASP3	TCAGAGGGGACTGCTGTAGAA	CGTCTCAATCCCACAGTCCAA
CYP17A1	GGACACAGATGTCGTCGTCAA	AAGCGCTCAGGCATGAACA
DMAP1	GACGGAGCCATGTTCTTCCA	ACCTGCACCGTCTTGTTGAA
DNMT1	AAAGGCGCTCATAGGCTTCA	ACGCTGAACAGTGGTGCATA
DNMT3A	ATGACCTCTCCATCGTCAACC	CAGGAGGCGGTAGAACTCAAA
DNMT3B	AGCTGTACCCTGCCATTCC	AAGTACCCTGTTGCGATTCCA
EHMT2	ACTTCAGCCTCTACTACGATTCC	GTTCAGCCAGAGCTTCAACC
EIF4A1	GAGAAGCCCTCTGCCATCC	ATTGGGCTTGAGCGATCACA
ELF5	AGTGGCATCAAAAGCCAAGAC	GCAGGTCTCGTACAAATTCCC
EZH2	CGAAGGATCCAGCCTGTTCA	ACGGGATGACTTGTGTTGGAA
GAPDH	AGTGGACATTGTCGCCATCA	CGTGGGTGGAATCATACTGGAA
GDF9	AACACTGTCCGGCTCTTCAC	TCAACAGCAGTAACACGATCCA
GNAS	AAGGCAGAGGAGAAGAAGCA	GATGGGTCCCCTCTTGGAA
GRB10	AAGCACGCGGATGAATATCCTA	AGTGCTGCGTCCTGTGAA
HAND1	GCGAGAGCAAGCGGAAAA	CCTGTGCGCCCTTTAATCC
HDAC3	ATCGATTGGGCTGCTTCAAC	GAGGGATGTTGAAGCTCTTGAC
HPRT1	AAAGAGATGGGAGGCCATCA	GTAATCCAGCAGGTCAGCAAA
HSD17B1	TCGGGTCGCATATTGGTGAC	AAACTTGCTGGCGCAGTAAAC
HSP90AA1	GACCAGAAACCCCGATGACA	GATCCTCCCAGTCATTGGTCAA
IGF2	CAGCCCACAGCGATTCCAA	GAGGCCAAGGCCAAGAAGAC
IGF2R	TGTGGTGGTGGCAAGAGAATA	CAGCACTGGAGCACTCTCTAA
KLF4	GGGAAGGGAGAAGACACTG	TCTTTGCTTCATGTGGGAGA
KRT8	AAGCGTACCGACATGGAGAA	TCCAGCTCGACCTTGTTCA
LIN28A	TTCGGCTTCCTGTCCATGAC	GCCCTCCATGTGCAGCTTA
MOS	GGCTTCGGCTCGGTATACAA	GTTCTTGGTGCATCTGCTCAC
MYC	CGAACCCTTGGCTCTCCA	GCTGCCTCTTTTCCACAGAAA
NANOG	CTTGGAAACTGCTGGGGAAA	CCATGATTTGCTGCTGGGTA
NECD	ATGTGGTACGTGCTGGTCAA	CACTTCTTGTAACTGCCGATGAC
NNAT	TTTCGAAATCCTCCAGGGACAC	CCAGCTTCTGCAGGGAGTAC
NOBOX	CCACTATCCGGACAGCGAAA	CCGGCGATTCTGGAACCA
PEG10	AGTCCTCGCGTGGTGAGTA	CCCAGGTGTAGCTTCACTCC
POU5F1	AGAAGAGGATCACCCTGGGATA	ATGGTCGTTTGGCTGAACAC
RPN1	AGACAGTGGGATCTCCTCCA	GGTGGAAACATTGCCAATCTCA
SIRT1	TGTCAGAGTTACCACCCACAC	ACTGAAGAAGCTGGTGGTGAA
SOX2	CCTGCAGTACAACTCCATGAC	TGCGAGTAGGACATGCTGTA
SRY	GCTCAAACGATGGACGTGAAA	CGTTCATGGGTCGCTTGAC
TAF11	AGAGAAGAAGCAGAAAGTGGATGAA	GGTTCAGCTGCTCCTCAGAA
TEAD4	TGTTGGAGTTCTCTGCCTTCC	GGCCGATGTGCACAAACAA
TP53	TCGCCAGTGCAAAAGAAGAA	GGAACATCTCGAAGCGTTCA
UBE3A	ACTTTTCGTGACTTGGGAGAC	CTTCCACACTTCCTTCATACTCC
XIAP	GAGTGCTCAGAAAGACAATGCA	CCTCAGCTGTTCTTCAGCACTA
ZAR1	CCCTTATCGCGTGGAGGATA	TCCACGTGGCGAAGTTTTAC
ZP3	CACCGTAATGGTGGAGTGTCA	CCTGATGAGCTTCCCGGTAC

Biosystems, PN 4369016), 0.25 µl of 20x DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-0388), 0.25 µl EvaGreen DNA Binding Dye (Biotium, PN 31000), and 2 µl STA Exo I-treated sample. Each sample mix solution was vortexed for 20 seconds and centrifuged for 30 seconds. The assay (primer) mix solution was then made for every primer set by using 2.5 µl of the 2x Assay Loading Reagent (Fluidigm, PN 85000736), 0.25 µl water, and 2.25 µl of each 20 µM Forward and Reverse Primer Mix. All assay mixes were vortexed for 20 seconds and centrifuged for 30 seconds.

Before running the chip in the thermal cycler, the IFC chip was 'primed' by injecting 300 µl of control line fluid into each accumulator on the chip. When the priming run had finished, we pipetted 5 µl of each assay and 5 µl of each sample into their respective inlets on the chip and returned the chip to the IFC Controller. We then ran the Load Mix (113x) script to load the samples and assays into the chip. Once this had been done, the samples were exposed to a 5-minute initial enzyme activation step at 95° C, followed by 35 denaturation/extension cycles (95° C for 15 seconds followed by 60° C for 60 seconds) and a 3-minute final extension cycle at 60° C. After final amplification cycling was complete, samples were subjected to a melt curve cycle to verify the quality of each amplicon.

Data was analyzed via the Fluidigm Real-Time PCR Analysis Software which utilizes the Ct value determined through thermal cycling to quantitate the ΔCt value by normalizing through a selected housekeeping gene (HSP90AA1). A selected calibrator sample (reference cDNA; Whitworth et al., 2005) was utilized to determine the ΔΔCt value for each sample, which is done by subtracting the ΔCt of each experimental sample

by the ΔCt of the calibrator for each gene. Lastly, we determined the fold change for each sample by using the equation $2^{-\Delta\Delta Ct}$.

For our standard curve, we used a reference cDNA composed of a variety of porcine tissue samples.

Immunofluorescence analysis

Immunofluorescence analysis was carried out utilizing 24-well plates to grow porcine TE derived cells and fibroblast cells, as outlined above. Cells were fixed with 10% neutral-buffered formalin (Fisher; #305-510) for 30 minutes at room temperature, washed twice with PBS for five minutes, and exposed to a permeabilization and blocking (P&B) solution for one hour at room temperature. This solution is composed of 5% BSA (w:v), 5% (v:v) Normal Goat Serum (Thermo Scientific; #31872), and 0.1% (v:v) Triton X-100 (Fisher; #11379) in PBS. (To prepare 10 ml of P&B solution, we utilized 0.5 g of BSA, 500 µl goat serum, 10 µl Triton X-100, and added PBS to 10 ml.) After an hour, P&B solution was removed by aspiration. The cells were then incubated with primary mouse monoclonal antibodies directed against *KRT18* (1:200 dilution, Abcam Inc., ab668, Cambridge, MA), mouse monoclonal directed against *VIMENTIN* (1:200 dilution, Abcam Inc., ab8069, Cambridge, MA), or mouse monoclonal directed against *OCT-3/4* (1:200 dilution, Santa Cruz Biotech.) overnight at 4° C. Primary antibodies were diluted with "dilution solution" composed of 0.05% Triton X-100, 1% goat serum, and PBS. (To prepare 10 ml of dilution solution, we utilized 5 µl Triton X, 100 µl goat serum, and 9.9 ml PBS.) The following day, primary antibodies were aspirated and cells were washed twice with dilution solution for five minutes and rinsed twice with PBS for five minutes. Then they were incubated with the secondary antibody Goat Anti-Mouse

Immunoglobulin G (IgG) (1:200 dilution from Thermo Scientific) for 1 hour at 4° C in the dark. The secondary antibody was diluted with dilution solution in the dark. Nucleus staining was performed utilizing 1.0 μg/ml Hoechst 33342 in dilution solution for 15 minutes at 4° C in the dark. Controls were obtained by omitting the incubation of primary antibody in the cells (i.e. pre-immune antisera only). Slides were imaged using an inverted microscope equipped for epifluorescence imaging. Images were obtained by utilizing NUANCE camera (Caliper Life Science; model: N-MSI-420-FL-Flex; 70284). Images were obtained utilizing the same exposure time of 500 ms for the fluorescence images and for their controls.

Transfection

Transfection was performed utilizing the plasmid DNA which included the enhanced green fluorescent protein (eGFP) reporter gene driven by the chicken beta actin (CAGG) promoter. This plasmid also contained a neomycin resistance cassette as a selectable marker (Whitworth et al., 2009). Three different transfection methods were performed over day 10 porcine TE-derived cells, and porcine fetal fibroblast (FF) cells. Both types of cells were grown in 24 well plates. The three methods utilized were electroporation (Neon from Invitrogen), jetPEI (Polyplus), and Fugene (Promega). The transfection performance of the three methods over both types of cells allowed the observation of the receptivity of porcine TE-derived cells, and of porcine FF cells to a foreign gene.

Plasmid DNA containing the green fluorescent protein marker with the neomycin resistance gene was prepared by standard protocols using laboratory strains of E. coli to propagate the plasmid. A restriction enzyme digest to linearize the plasmid was

performed by combining 10 units of the enzyme AseI (New England Biolabs; NEB #R0526) with 1x NEB Buffer 3 and 1.0 μg plasmid DNA, and placed the reactions in a 37° C water bath for 1 hour. After that time period, the AseI enzyme was deactivated by placing the reactions in a thermal cycler at 65° C for 10 minutes. Once digested, the DNA required purification using QiaQuick PCR Purification Kit (Qiagen). The purified DNA was then analyzed on a nanodrop system to measure the precise concentration.

The electroporation method was performed by having the cells be detached in order to properly deliver the DNA into the cell. Cells were able to detach utilizing .25% trypsin (Hyclone Technologies) according to standard laboratory cell culture practices. After detachment, cells were re-suspended in cell culture medium containing serum for deactivation. Once re-suspended, the cells were centrifuged at 400 g for 4 minutes at room temperature. Cells were washed with PBS by a second centrifugation at 400 g for four minutes at room temperature, and cells were suspended in resuspension Buffer R.

Utilizing the Neon Transfection system, transfection was performed in a sterile fume hood to decrease risk of contamination. The cells suspended in Buffer R were mixed with the proper concentration of DNA (5 μg per shock). Using 100 μl Neon® electroporation tips, the loaded pipette was inserted into the transfection chamber tube, and the electroporation sequence was initiated through the Neon® module. Once completed, the loaded pipette was carefully dislodged and the shocked cells were plated into the appropriate well of the pre-incubated plate. Cells were shocked with 1400 millivolts, for 20 milliseconds, with one pulse (parameters predetermined by prior experimentation). These steps were repeated with each cell type and each tip could not be

utilized more than twice (overuse of each tip will decrease the efficiency of each progressive shock).

Once plated, the cells immediately were placed in culture medium. Then they were placed into the humidified 39C/6% CO_2 incubator for proper growth to occur. Four days later, once cell survival was consistent, G418 antibiotic (300 μg/ml; Toku-E #G020) was added to select for the successfully transfected cells. G418 or geneticin is a common antibiotic that functions to inhibit the elongation step of eukaryotic cells. Our transfected cells contained the neomycin gene which confers resistance to G418.

The jetPEI method involved mixing ingredients together, letting them incubate and adding calculated amounts to the adherent cells utilizing 150 mM NaCl and supplied jetPEI reagent. Cells were plated until they reached 50-70% confluency the day of transfection. The cells are not required to detach in order to properly transfect with this method. The DNA was prepared and digested as mentioned above (AseI enzyme), and using the nanodrop system, concentrations were collected. The jetPEI protocol only calls for 1 μg of DNA per well. For organization methods, two "master mixes" were made of the solutions, one for trophoblast cells and one for fibroblast cells.

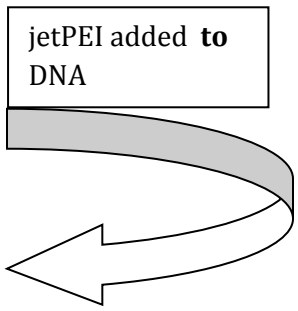
To begin with, the jetPEI and DNA both, separately, needed to be diluted in the NaCl solution:

PEI mix

2 μl jetPEI X 2.2 = 4.4 μl jetPEI
48 μl NaCl X2.2 = 105.6 μl NaCl

DNA mix (DNA was 108.9 ng/μl)

9 μl DNA (1 μg) X 2 = 18 μl DNA
41 μl NaCl X2 = 82 μl NaCl



These two mixes were done for each cell type, for a total of four mixtures. Once complete, the protocol specifically illustrated the jetPEI mix was to be added to the DNA mix, not the other way around, otherwise, transfection efficiency may decrease. After simple vortexing, the combined solution was then incubated for 15-30 minutes at room temperature. One hundred microliters of the solution was then added per 1 ml of media in each well. By gently swirling the plate, a homogenized distribution of the transfection solution was achieved. Four hours later, the medium was changed in order to avoid any adverse cytotoxic effects occasioned by prolonged exposure to the jetPEI reagent.

In the Fugene method, cells were plated similar to the jetPEI, in that they were 50-70% confluent the day of transfection. For the 24 well plate, the FuGene 6 protocol calls for 25 µl total transfection mixture per well: 0.5 µg of DNA (at 117.7 ng/µl = 4.25 µl), 1.5 µl of reagent, with the balance of the volume (19.25 µl) made up with cell culture medium. Tubes were tapped to adequately mix the reagents, and the mixtures were incubated for 5 minutes. Lastly, 25 µl of the mixture was added to each well. Once the incubated solution was added to the cells, it was gently swirled to ensure a homogenized distribution of the transfection mix. For each replicate (day), each cell type was treated twice.

All transfection methods had G418 (300 µg/ml) added to them four days after the procedure. Cells were fixed in 10% neutral buffered formal (as described above) and Hoechst staining (1 µg/ml in PBS) was utilized to stain the cells' nuclei to allow for the comparison of green cells against total cells for all three methods in both cell types. The cells were washed twice with PBS for 5 minutes. Fluorescence and bright-field (BF) images were obtained by utilizing a epifluorescence microscope to detect signal. The

Nuance camera was used to take pictures of green cells, and blue (Hoechst-stained) cells of three replicates at the different time points for both cell types. Percentage data of green cells compared to live cells were collected on day 8 and day 12 after transfection.

RESULTS

Cell Growth and Morphology

At the time of collection, day 10 embryos measured 0.5 cm and day 13 embryos were elongated, filamentous and were estimated to be approximately 1 meter (100 cm) in length. Porcine TE derived cells from day 10 and day 13 embryos (Figure 1A and B), demonstrated different morphological and physiological characteristics when cultured in serum and non-serum containing media. When these cells were cultured in serum containing medium (FF medium), they reached differentiation quicker, their attachment to the underlying substrate is irregular, and the intracellular components of the cells appear rough. In this type of medium, cells do not form domes, floating spheres, or clumps. Also, they demonstrate a great differentiation since they cannot be passaged by any of the three passage methods described above. Cells stopped actively dividing 2-4 weeks after being in culture in serum containing medium.

In non-serum containing medium, cells showed regenerative properties by reforming part of its colony once it became detached and they showed less differentiation (smaller, roundish cells with larger nuclei, as opposed to large, flattened cells with more compact nuclei). They demonstrated a "softer," more regular type of attachment (Figure 1G). After eight to twelve days in culture, these cells generated clumps which could be picked up and transferred to another well to form new porcine TE derived colonies, as long as they are cultured in non-serum containing medium (Figure 1D). Also, small floating spheres and domes started to become visible on top of these cell colonies (Figure 1C). It is believed that clumps and floating spheres are composed of epithelial sheets (Ezashi et al., 2011),

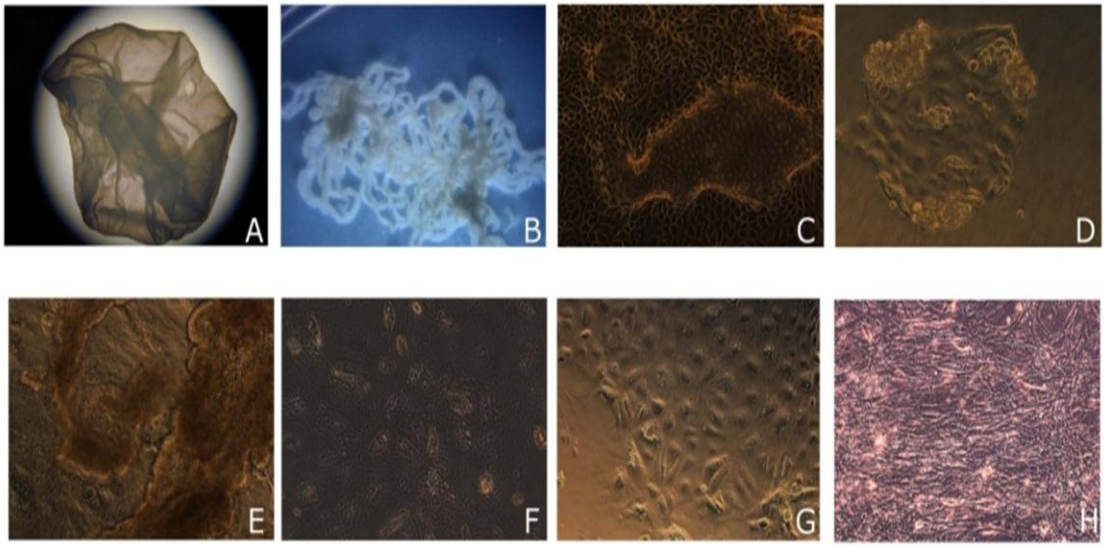


Figure 1 Porcine embryonic trophoblast-derived cells A: Day 10 Porcine embryo; **B**: Day 13 Porcine embryo**; C**: Dome structure formed in a day 10 porcine TE derived cell colony cultured in non-serum containing medium (trophoblast medium); **D**: Clumps attachment and formation of a day 10 porcine TE derived cell colony in non-serum containing medium (trophoblast medium); **E**: Day 10 porcine TE derived cell colony after 30 days of culture in non-serum containing medium (trophoblast medium);**F**: Day 10 porcine TE derived cells colony after 30 days of culture in serum containing medium (fibroblast medium); **G**: Day 13 porcine TE derived cell colony after 15 days of culture in non-serum containing medium (trophoblast medium);**H**: Day 13 porcine TE derived cell colony after 15 days of culture in serum containing medium (fibroblast medium).

which structure is composed of regenerative-like characteristics, since they generate new cell colonies. The cells reached full confluency about 30-40 days after they have been placed in culture. If part of the original, confluent colony became detached from the substrate, new cells started to grow in the bottom of the detached tissue as shown in Figure 1E. The formation of clumps and floating spheres which can be used for pick-up passage, and the regenerative properties that these cells demonstrated, portrays the main characteristic of "no senescence." Therefore, these cells can be passaged indefinitely and they can be grown on the same well and same plate for as long as needed as long as contamination is avoided. Porcine day 10 TE-derived cells have been grown in the laboratory for 20 months.

Reverse Transcription and PCR Analysis

The results from the qualitative reverse transcription-PCR experiments are presented in Figure 2. The RT-PCR results demonstrated some differences in porcine TE derived cells cultured in serum and non-serum containing media over 30 days. CYP17A1 was not expressed in non-serum containing medium at days 15 and 30. It was expressed in all of the other samples. Other trophoblast specific genes as *HSD17B1*, *TEAD4*, *PLET*, *CDX2*, and *FGFR2* were expressed uniformly in all of the samples. *POU5F1* was expressed up to day 15 in the samples, but loses expression at day 30 for serum and non-serum media. *SOX2* did not express in any of the samples, just in the positive control sample. *TERT* demonstrated expression in all of the samples. The *VIM* gene which is expressed in mesenchymal derived cells did not show any expression in TE day 0, but gained expression in days 15 and 30. Also, it was expressed in the ICM day 0 sample.

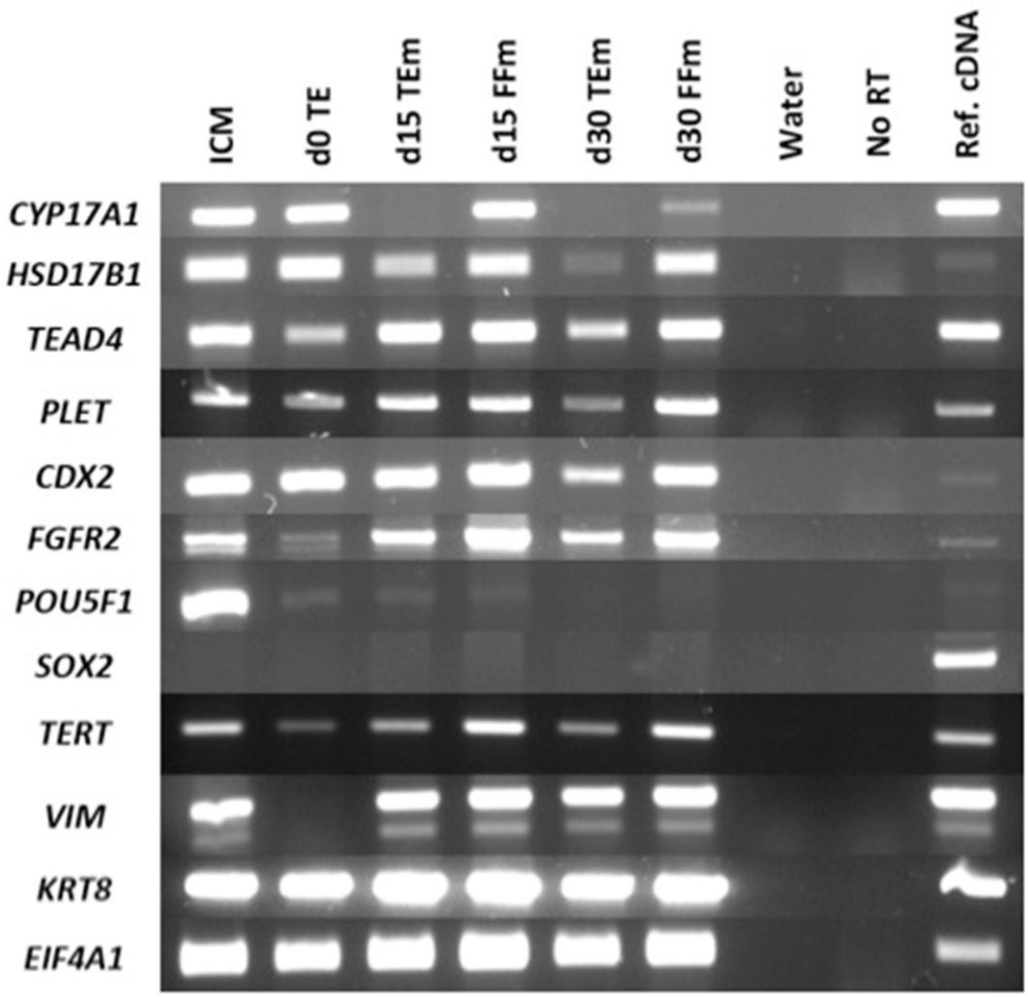


Figure 2. Gene Expression panel for porcine trophoblast-derived cells cultured in non-serum-containing medium and in serum-containing medium. RT-PCR testing of trophoblast specific genes (CYP17A1, HSD17B1, TEAD4, PLET, CDX2, FGFR2); pluripotency gene (POU5F1, and SOX2), telomerase activity gene (TERT); cytoskeletal genes (KRT8 and VIM), and housekeeping gene (EIF4A1) with day 13 trophoblast-derived cell samples grown over serum- and non-serum-containing medium ("FFm" and "TEm", respectively). ICM = cells collected from the inner cell mass of the same embryos used for trophoblast cell isolation. d0, d15, d30 = day 0, day 15 and day 30: day of sample collection, with day 0 being the day of embryo flush and initiation of cell culture. No RT = no reverse transcriptase control. Ref. cDNA = universal reference cDNA sample utilized as a positive control as described in the text.

KRT8 which is a cytoskeletal gene for epithelial cells and EIF4A1 which is a housekeeping gene were expressed in all of the samples.

Fluidigm results

Raw Ct values for the replicate reactions of all the samples tested were subjected to unsupervised hierarchical clustering using the MeV module of the TM4: Microarray Software Suite, which allowed for the mathematical calculation of the similarity of raw Ct values between samples. As presented in Figure 3, those samples (Y-axis) with the most closely related gene expression patterns clustered together. Cells cultured in vitro (regardless of condition) were quite divergent from primary tissue samples (day 0 ICM and TE). The data show that cell culture conditions (FF vs TE) drove the clustering analysis to a greater degree that did the time of in vitro culture.

When the Fluidigm qPCR data was evaluated on a gene-by-gene basis, some interesting patterns were revealed (see data presented in Figure 4). *CMYC* is more highly expressed in TE cells cultured without serum. Regardless of treatment, expression of *CMYC* appears to increase somewhat over time in culture. *CYP17A1* was highly expressed in the primary trophoblast tissue and to a lesser extent in the primary ICM sample. TE cells cultured in serum-free medium lose *CYP17A1* expression after both 15 and 30 days in culture. In cells exposed to serum, a very low level of *CYP17A1* expression was noted at 15 days, whereas expression was undetectable at day 30. *HAND1* is expressed at very high levels in primary ICM tissue and even more so in primary TE tissue. *HAND1* gene expression is markedly reduced in all TE-derived cells cultured in vitro up to 30 days. Cells cultured in serum for 15 days still expressed *HAND1* at

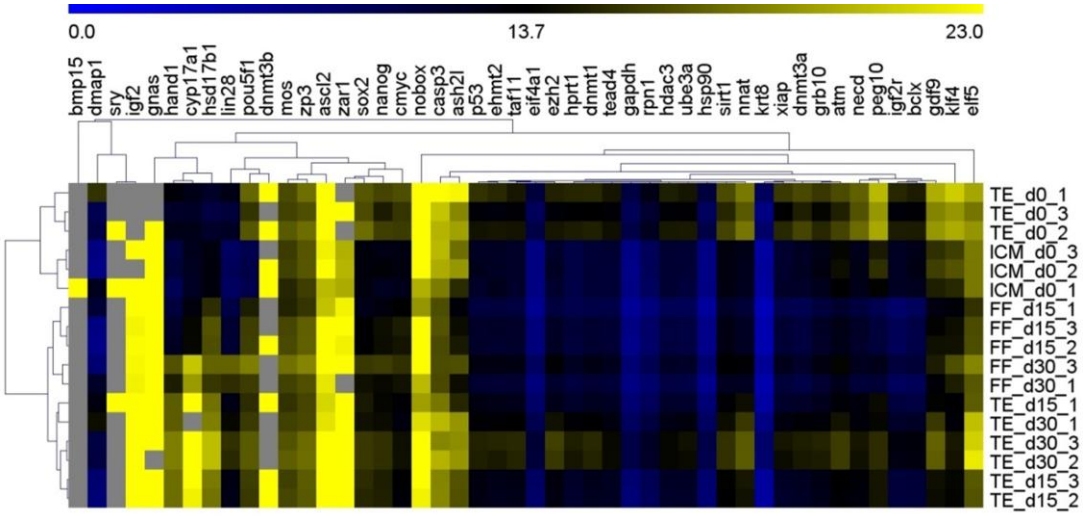


Figure 3. Unsupervised bidirectional hierarchical clustering of Fluidigm quantitative PCR data from trophoblast cells cultured in the presence or absence of fetal bovine serum. Expression levels of 48 genes were tested by qPCR in day 13 porcine trophoblast-derived cells grown in serum or non-serum containing medium ("FF" or "TE", respectively for the day 15 [d15] and day 30 [d30] samples) using the BioMark system from Fluidigm. Data presented here are the raw Ct values for the three replicate qPCR reactions run for each sample types. The figure shows distinct clustering by culture method, suggesting that culture environment drives changes in gene expression to a greater level than does time in culture. TE for the day 0 [d0] samples = primary trophoblast tissue harvested on the day of embryo flushes. ICM = cells from the inner cell mass of embryos harvested for trophoblast cell isolation. 1, 2, 3 = experimental replicates. Sample names are presented on the Y-axis, and gene names are presented above the figure on the X-axis. The color scale bar above the figure shows the range of values presented in the heat-map, with blue representing a very low Ct value (high expression), yellow representing a very high Ct value (no or very low gene expression), and black being intermediate Ct values/expression levels. Grey boxes indicate missing Ct values.

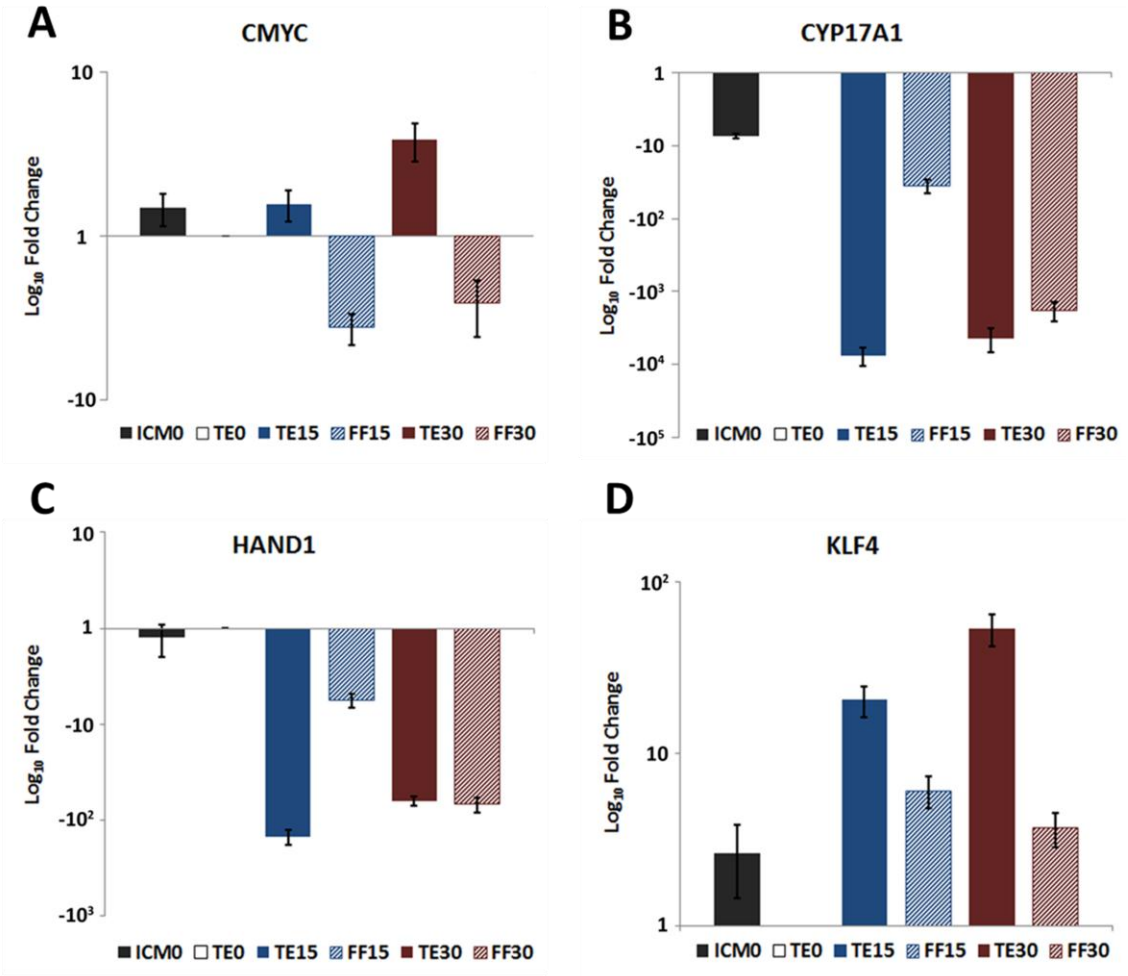


Figure 4. CMYC, CYP17A1, HAND1, KLF4 gene expression in day 13 porcine trophoblast-derived cells. Relative transcript abundance in trophoblast cells cultured with or without fetal bovine serum (FF and TE, above, respectively) was determined by quantitative real time PCR using the BioMark system from Fluidigm. Here CMYC (Panel A), CYP17A1 (Panel B), HAND1 (Panel C), and KLF4 (Panel D) data are reported as Log10 fold-change values in experimental samples, relative to a calibrator sample (day 0 trophoblast tissue [TE0]) and housekeeping gene (HSP90AA1). Experimental samples (X-axis) are as follows: embryonic disc/inner cell mass from day 13 conceptus (ICM0), trophectoderm tissue from day 13 conceptus (TE0), trophectoderm-derived cells cultured in-vitro in serum-free medium for 15 or 30 days (TE15 and TE30, respectively) and trophectoderm-derived cells cultured in vitro in medium containing fetal bovine serum (15% v:v) for 15 or 30 days (FF15 and FF30, respectively). Error bars reflect the standard error of the mean fold-change values calculated for three replicate reactions.

moderate levels, but by day 30, expression was virtually undetectable. Cells cultured without serum showed very little *HAND1* expression at any time during culture in vitro. Expression levels of *KLF4* were relatively low in both ICM and TE primary tissues, but were higher in cells cultured in vitro. *KLF4* expression was higher in cells cultured without serum than either primary tissue or cells cultured without serum, and relative expression levels increased with prolonged serum-free culture in vitro. In contrast, *KLF4* expression did not change appreciably over time in cells exposed to serum.

Immunofluorescence analysis results

Immunofluorescence analysis was performed utilizing cytoskeletal marker *KRT18*, mesenchymally-derived cells marker *VIMENTIN*, and pluripotency marker *POU5F1*. Protein expression utilizing these markers was tested in porcine trophoblast-derived cells (TE) cultured in serum-free conditions and in porcine fetal fibroblast (FF) cells which were utilized as a control.

Figure 5 demonstrates how protein expression is different between both types of cells. *KRT18* is strongly expressed in porcine TE cells where strong red staining is observed around the cells' cytoplasm. Also, it is interesting to observe some cells which demonstrate a smaller cytoplasm, a reason of this effect might be that they are undergoing mitosis. *KRT18* is not expressed in porcine FF cells. As observed in the testing performed, a negative expression is noticed in the red staining protein expression picture and in the control.

VIMENTIN is moderately expressed in porcine TE cells. In contrast with the staining pattern observed with *KRT18*, this protein is not only expressed in the cytoplasm

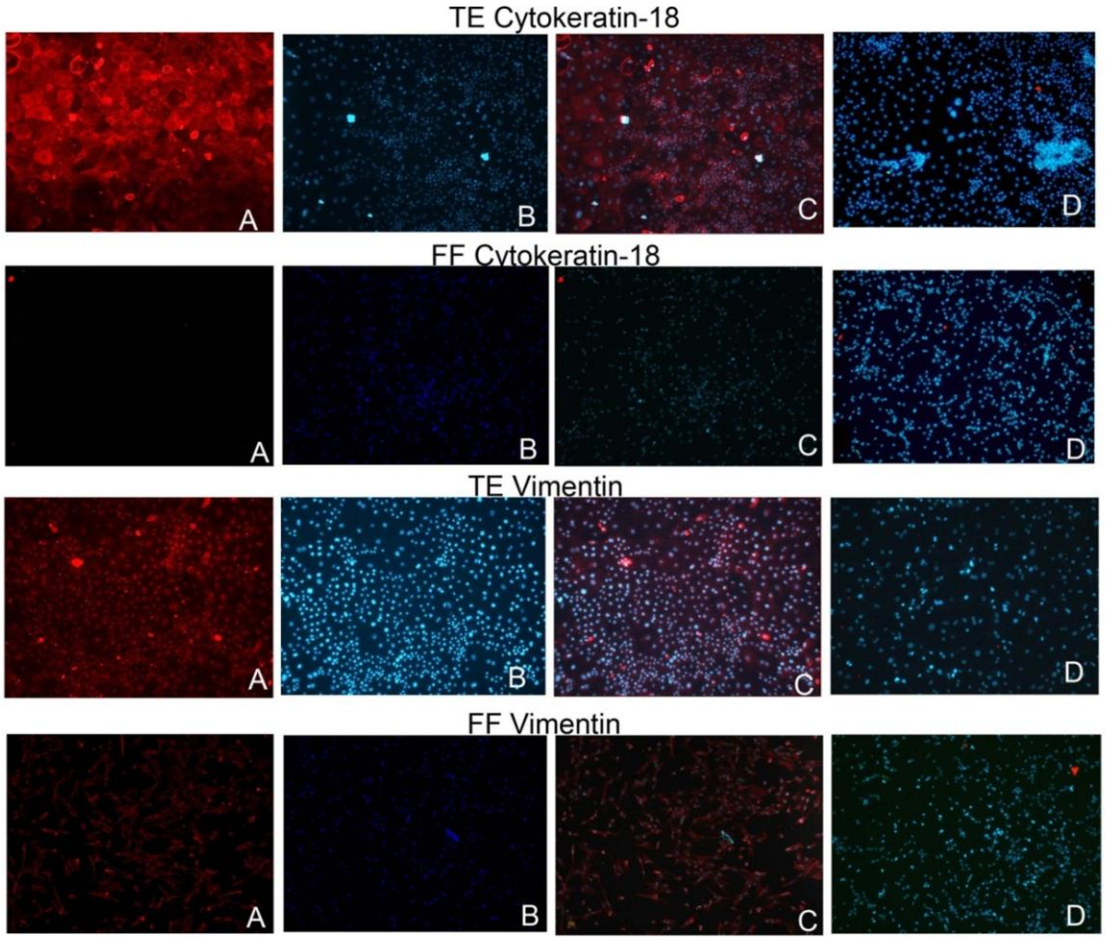


Figure 5. Immunofluorescence analysis of day 10 porcine embryonic trophoblast-derived cells and of porcine fetal fibroblast cells for the KRT18 and VIMENTIN proteins. A: shows the experimental protein staining from each sample; **B**: Hoechst 33342 nuclear staining from the same cells represented in Panels A; **C**: represents merging of panels A and B within a given cell type/protein combination; **D**: represents the merged image (secondary antibody [red] plus Hoechst 33342 [blue]) of control slides (pre-immune serum as primary antibody) to demonstrate the specific nature of the immunodetection (i.e. no or very low background staining [red] in Panels D).

of the cells but also in their nuclei. A differentiation can be seen between small cells which grow in clusters next to each other, and really big cells after the staining is applied. A denser color is noticed in the small cell clusters and a more dim color in the big cells. *VIMENTIN* is highly expressed in porcine FF cells. The target red staining is highly noticed around the cytoplasm of FF cells. Porcine FF cells definitely expressed the protein *VIMENTIN* higher than porcine TE cells as Figure 5 demonstrates.

The signal for the antibody utilized to test *POU5F1* did not appear when tested in TE and in FF cells. Also, the signal did not appear when testing for the controls for both types of cells. All the controls utilized demonstrated a negative signal.

Transfection results

Results were obtained 8 and 12 days after transfection was performed (see Figure 6, panels A and B, respectively). The purpose of picking up these two days was to observe the differences of the three methods of transfection between both cell types four and eight days after G418 was added to the samples (day 8 and day 12 of the experiment, respectively). The differences in transfection efficiency demonstrated that porcine TE cells were different than porcine FF cells not only morphologically but also in their receptivity to a foreign DNA. As shown in Figure 6A, at eight days after transfection was performed (four days after G418 was added), some differences are observed. Porcine fetal fibroblast cells (PFF) demonstrated low GFP receptivity by the Fugene-6 method, and they demonstrate a little higher receptivity by the jetPEI method. The most efficient method to transfect PFF cells was the electroporation method. For porcine trophoblast cells (TE) transfection

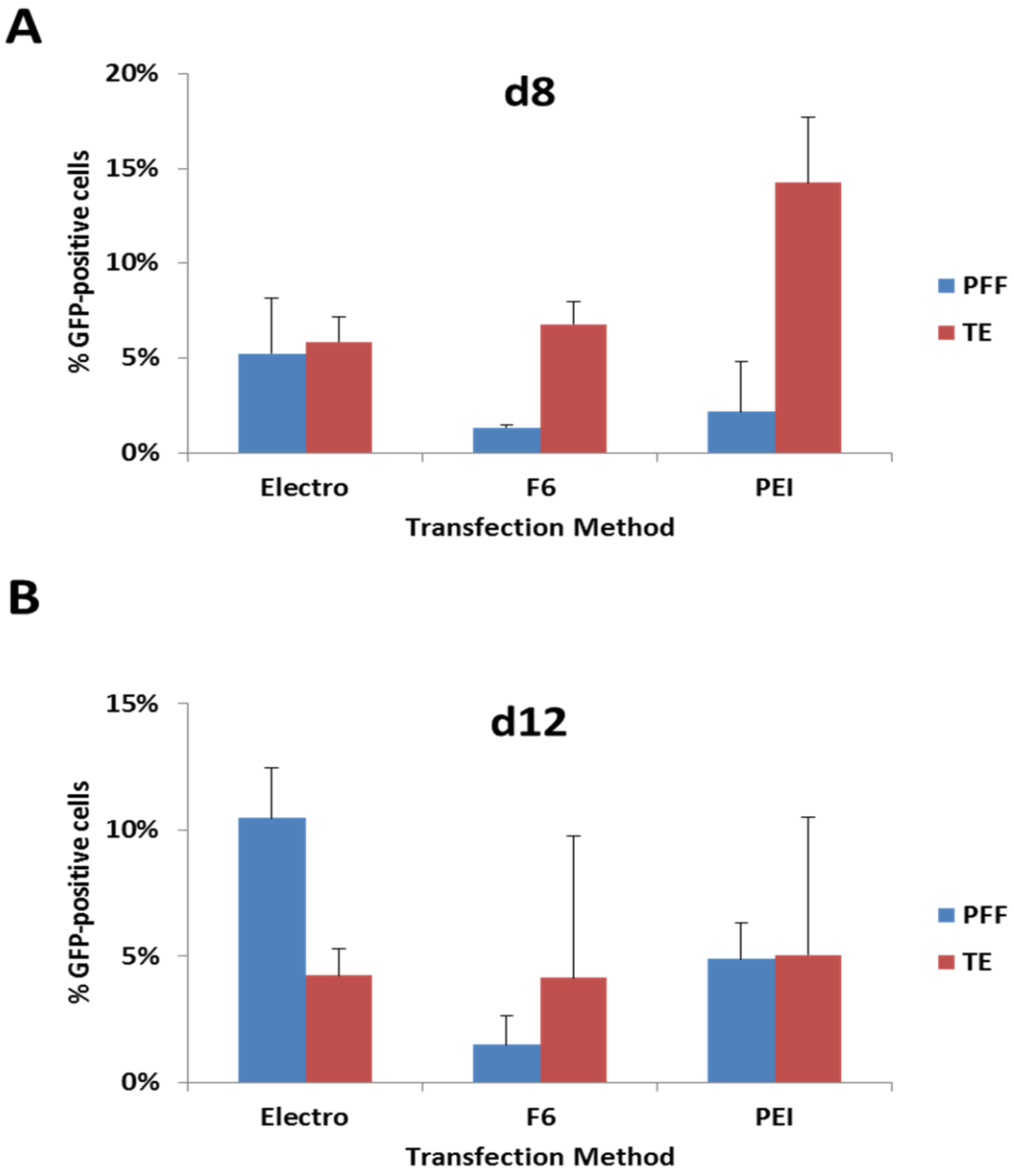


Figure 6. Transfection of porcine trophoblast cells and fibroblast cells using a variety of methods. Porcine fetal fibroblast (PFF) and trophoblast (TE) cells were transfected with a plasmid containing GFP- and neomycin resistance genes using a variety of methods: Electroporation by the Neon ® system (Electro), Fugene 6 (F6), and jetPEI (PEI). On day 4 after transfection, geneticin (G418) was added to the cells, then on day 8 (Panel A) and day 12 (Panel B) after transfection, cells were fixed, stained, and evaluated for retention and expression of the transgene DNA (GFP). Experiments were repeated three times for each time point. Values are presented as the average percentage of the GFP-positive cells, ± the standard error of the mean.

receptivity is backwards from porcine FF cells, having electroporation being the least efficient method to transfect TE cells. The most efficient method for the transfection of TE cells is the jetPEI method which percentage of GFP-positive cells is definitely higher than Fugene-6 and electroporation methods.

Figure 6B demonstrates essentially the same trend as Figure 6A. The most efficient method to transfect porcine fetal fibroblast cells is electroporation, followed by jet Pei and the Fugene 6 being the less efficient method. On the other hand, the most efficient method to transfect porcine trophoblast-derived cells is jetPEI, followed by Fugene 6 and electroporation being the less efficient method. When comparing panels A and B from Figure 6, it is obvious that the percentage of GFP-positive fibroblast cells increases from day 8 to day 12 after transfection. When performing the same comparison in porcine trophoblast derived cells, the percentage of GFP-positive cells decreases from day 8 to day 12 after transfection. Even though a decrease in GFP cells survival was visible in the jetPEI transfection of porcine TE cells from day 8 to day 12, proliferation of GFP cells was observed at around day 15 after transfection (Figure 7D). After 15 days of transfection in porcine TE cells, "green" clumps became visible. These "green" clumps were picked up, placed in new-fresh TE medium, and they formed green colonies. Stable transfected colonies were accomplished since bright field pictures looked identical to GFP pictures, and also GFP cells were able to be passaged indefinitely as long as contamination was avoided (Figure 7C-H).

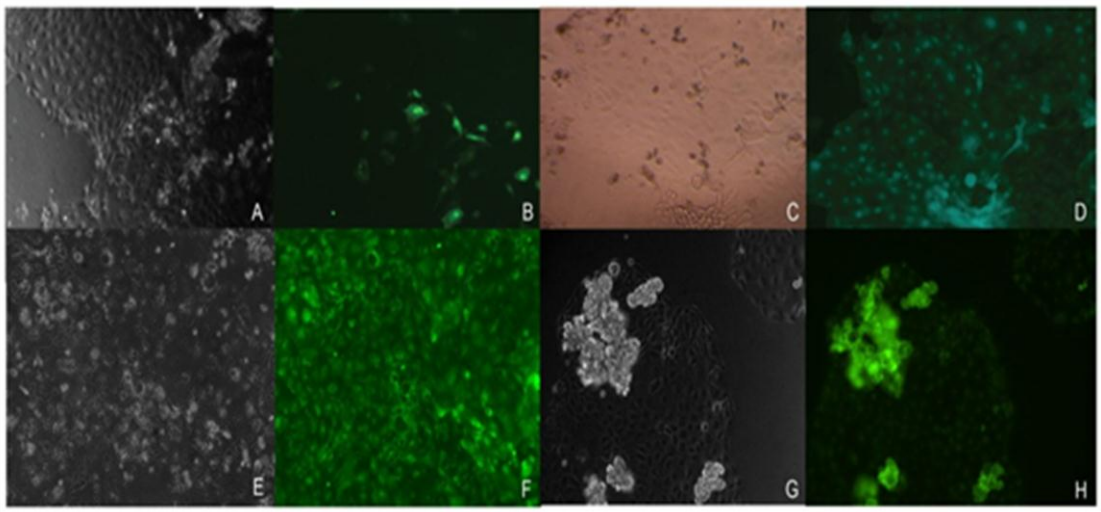


Figure 7. Transfection of day 10 porcine trophoblast-derived cells through jetPEI method. A: Bright field image representation of porcine trophoblast-derived cells 4 days after jet –Pei method transfection; B: Epifluorescence image of porcine trophoblast-derived cells 4 days after jetPEI method transfection; C: Bright field image of porcine trophoblast-derived cells 15 days after jetPEI method transfection; D: Epifluorescence image of porcine trophoblast-derived cells GFP image 15 days after jetPEI method transfection (same field of view as in C); E: Bright field image of porcine trophoblast-derived cells after jetPEI method transfection and six months of continuous G418 selection (300 μg/ml); F: Epifluorescence image of porcine trophoblast-derived GFP-expressing cells six months after jetPEI method transfection (same cells as in E); G: Bright field image of "clumps" generated by jetPEI method transfected porcine trophoblast-derived cells; H: Epifluorescence image of GFP expression in "green clumps" of porcine trophoblast-derived cells transfected by jetPEI method.

DISCUSSION

Porcine trophoblast-derived cells have been shown to possess regenerative characteristics. The formation of these characteristics is dependent on the growth factors which are contained in non-serum containing medium (TE medium). Two of the medium components, FGF2 and B-27, have been demonstrated to be essential to the formation of stem-like characteristics in these cells (data not shown). In the mouse model, FGF4 is utilized as a stimulating growth factor for trophoblast stem cells. The trophoblast and its FGFR2 receptor are a target tissue for the embryonic fibroblast growth factor signal (FGF) which is generated at the ICM (Tanaka et al., 1998). The expressions of FGF4 from the ICM and of FGFR2 from the trophoblast are necessary to maintain the proliferation of the trophoblast (Arman et al., 1998). When these two genes are not present in mice, they demonstrated similar peri-implantation lethal phenotypes (Tanaka et al., 1998). Also in mice, it is believed that the expressions of the gene *NODAL* and FGF signaling are essential for the maintenance of the expression of *CDX2*, *EOMES*, and *ESRRB* which are necessary for the maintenance and development of the trophoblast. In the absence of FGF, differentiation genes like *ASCL2* (*MASH2*), a marker of spongiotrophoblasts, begin to manifest their expression (Guillemot et al., 1994). The growth factor utilized in our medium has been FGF2 which is a hormone utilized in porcine skin stem cell medium (Dyce et al., 2004). Similar characteristics have been observed utilizing FGF2 in porcine cells compared to the FGF4 in mice. B-27 is a Serum Free Supplement developed for the long term-culture of rat hippocampal and cortical neurons. It is believed to induce proliferation in primary cells, stem cells, and glial cells (Life Technologies).

One of the most important characteristics observed utilizing porcine trophoblast medium containing FGF2 and B-27 has been the formation of clumps. This is one of the most remarkable differences observed between porcine and mouse trophoblast-derived cells grown over similar growth components. The generation of clumps in TE medium have allowed for indefinite passage of these cells. Another characteristic that have allowed for indefinite passage has been the formation of cell spheres which become visible 2-3 days after the first culture of trophoblast tissue. This characteristic is observed in porcine and in mouse trophoblast-derived tissue culture. We believe that clumps generate from the detachment of domes which are epithelial phenotype cells which grow under 20% oxygen conditions. Also, it is believed that cells spheres are composed of a single epithelial sheet whose cells were tethered laterally by desmosome-like structures (Ezashi et al., 2011). Clumps and domes became visible 8-12 days after the first culture from trophoblast tissue. Clumps and cell spheres are believed to possess regenerative characteristics which still have to be investigated. The main reason behind this thought is because they generate new porcine trophoblast-derived colonies which characteristics are identical to cell colonies generated after the first culture of the first tissue collection. Another regenerative mechanism that trophoblast-derived cells have demonstrated in non-serum containing medium has been the generation of new tissue after the colony becomes confluent. As Figure 1E demonstrated, 30-40 days after culture, trophoblast-derived cell colonies become confluent. When they run out of space to grow, part of the colony detaches and the cell colony keeps growing under the detached tissue. This demonstrates the ability of these cells to regenerate in the same plate, in the same well as long as the medium is changed every 6-8 days and avoid contamination. When porcine

trophoblast-derived cells have been grown in serum containing medium, they have demonstrated a faster differentiation, no generation of clumps or cell spheres, and no passage has been accomplished even with trypsin. In serum containing medium, porcine trophoblast-derived cells stopped actively dividing in culture 2-4 weeks after the first culture of trophoblast tissue. On the other hand, porcine trophoblast-derived cells have been kept in the laboratory for 20 months after the first tissue collection. There is a difference in morphology and characteristics when cells are cultured in serum containing and in no-serum containing media. Serum affects trophoblast-derived cells in their differentiation causing them to have a rough attachment, not being able to passage them and reaching senescence sooner. When porcine trophoblast-derived cells have been grown in non- serum containing medium, they have a more regular-regenerative attachment, they generate domes, clumps, and cell sphere which allowed for the continuous passaging of these cells. Also, these cells have demonstrated regenerative properties in the same well in the same plate when grown in non-serum medium which makes them avoid senescence. The continuous passage and the regenerative properties when cultured in non-serum containing medium, which has FGF2 and B-27 as part of its components, demonstrate the characteristics of stem-like cells. Therefore, porcine trophoblast cells cultured in non-serum containing medium could be considered porcine trophoblast stem-like cells.

As morphological differences became visible, RT-PCR gene expression was performed in our day 13 porcine trophoblast-derived cells samples to observe gene expression differences when these cells were cultured in serum and non-serum containing media. An experiment performed by Asanoma et al. (2011) in rat blastocyst demonstrated

that when FGF4 was present in growing medium, key transcriptional regulators as *CDX2* required for trophoblast stem cell renewal and pluripotency were present. In the same experiment, when FGF4 was removed from the growing medium, the activation of genes such as *CYP17A1* and *HSD17B1* which are expressed in mature trophoblast cell lineages as trophoblast giant cells were present (Asanoma et al., 2011). A similar path was observed in day 13 porcine trophoblast-derived cells: *CYP17A1* was not expressed in the sample growth in higher amounts of FGF and non-serum conditions. In Fluidigm analysis, *CYP17A1* is expressed mostly in trophoblast day 0 samples and in the cell sample grown over fibroblast (FF) medium for 15 days. Thus, *CYP17A1* is mostly expressed in samples that are going through differentiation. The RT-PCR and Fluidigm gene expression experiments confirms that the expression of *CYP17A1* is mostly expressed in trophoblast differentiated samples or sample grown in serum-containing medium. *HSD17B1* was expressed in all of our samples, with an obvious difference between the samples grown in serum compared to the ones compared in non-serum. The differentiated samples portrayed a stronger expression of the gene compared to the un-differentiated samples where weak signal bands are observed. On days 11 and 12 of pregnancy, porcine growing conceptuses secrete estrogen for maternal recognition of pregnancy, therefore it is reasonable to observe components of the steroid biosynthesis pathway present in these samples (Bazer and Thatcher 1977). Another gene which has been shown to be present in trophoblast-derived cells is *HAND1*. In mice, *HAND1* has been demonstrated to be essential in placental development since it promotes differentiation of trophoblast giant cells. Trophoblast giant cells develop at gestation by the terminal differentiation which are first present in the ectoplacental cone and then in

the spongiotrophoblast layer of the placenta. *HAND1* and *MASH2* genes regulate this process, their expression patterns overlap in the ectoplacental cone and in the spongiotrophoblast. *MASH2* is required for the maintenance of giant cell precursors and its overexpression avoids giant cells differentiation. Also, it is down regulated as trophoblast cell populations differentiate into giant cells. Therefore, the development of the trophoblast lineage is regulated by the coordination of the antagonistic activities of *HAND1*, *MASH2*, and their cofactors (Scott et al., 2000). Fluidigm analysis performed in *HAND1* demonstrated how this gene is mostly present in differentiated samples. Even though a lower expression of the gene was observed in undifferentiated samples, the path was clear that *HAND1* had a bigger fold change of its gene expression in our tested differentiated samples. These results are reasonable when analyzing the function of *HAND 1* of promoting the differentiation of trophoblat giant cells.

A similar pathway to the Asanomas et al (2011) experiment referenced above was observed in our sample when the expression of *CDX2* was analyzed (Asanoma et al., 2011). *CDX2* was expressed strongly in all of the samples. This gene is initially present in the morula stage of the embryo, and it appears to be an important marker for the separation of the trophoblast from the ICM. Therefore, it is reasonable to observe a strong signal in the porcine day 13 samples since this gene is responsible for the maintenance of the trophoblast. When *CDX2* is not present, the blastocyst structure starts to form but it promptly deteriorates. As blastocoel structures and epithelialisation progresses, *CDX2* begins to be more present in the nuclei of outer cells than in the inner cells (Strumpf et al., 2005). In the mouse, the transcription factor *OCT4* is also initially present in the morula of the embryo. As separation occurs, it becomes more concentrated in the inner

cell mass and later in the epiblast. *CDX2* and *OCT4* have been showed to reciprocally inhibit each other in embryonic stem cells in culture (Niwa et al., 2005). This pathway is observed in day 13 porcine trophoblast-derived cell samples, since an expression of *OCT4* is observed up until day 15 in both the serum and non-serum media samples. The reason might be that as *CDX2* gains expression as a trophoblast maintenance and proliferation gene, the pluripotency gene *OCT4* loses expression since these samples might lose pluripotency properties after 30 days of being in culture. Therefore, our porcine samples provide further suggestion that *CDX2* and *OCT4* reciprocally inhibit each other. Another gene found to be important in trophoblast maintenance and specification in the mouse has been *TEAD4*. This gene is required to be present before implantation, when it is absent, the trophoblast lineage does not appear (Nishioka et al., 2008). *TEAD4* is expressed very uniformly in our sample, confirming the need of the gene to be present to aid in the trophoblast maintenance. *PLET* which is a gene which is expressed highly in porcine placenta was uniformly expressed in all of our samples, confirming the trophoblast specific origin from them.

In the mouse, the pluripotency factor *SOX2* has been demonstrated to be essential for both trophoblast and epiblast development. In its absence, the epiblast is lost and the extra-embryonic ectoderm differentiates completely into trophoblast giant cells, thus pluripotent populations are not maintained in the embryo. This event causes a loss of the maintenance of pluripotency in the embryo (Avilion et al., 2003). As observed in the RT-PCR analysis, the *SOX2* mRNA utilized was not expressed in any of our samples except in the control sample. Further analysis needs to be performed to confirm that these results are correct.

The genes *SOX2* and *OCT4* in combination with the genes *KLF4* and *C-MYC* have been utilized to induce pluripotent stem cells from mouse embryonic and adult fibroblast cells (Takahashi and Yamanaka, 2006). *KLF4* has been demonstrated to be expressed in terminally differentiated epithelial cells at the villus borders of the mucosa in the intestinal epithelium. It is a factor that regulates biological processes as proliferation, differentiation, development and apoptosis. *KLF4* appears to play a critical role in regulating differentiation and possibly lineage specification of the intestinal epithelium. This gene is activated in response to stress as when DNA is damaged in the cell or when tissue is injured. *KLF4* and *KLF5* are crucial components for restoring tissue homeostasis; *KLF4* induces growth arrest to allow for DNA repair. *KLF4* plays a critical role in the activation of cell cycle checkpoints which prevent the progression in the cell cycle to maintain DNA integrity. Also, *KLF4* has been found to be involved in goblet cells maturation and in skin differentiation, these roles suggest that *KLF4* functions in the switch from proliferation to differentiation (McConnell et al., 2007). Gene expression in *KLF4* through microarray analysis demonstrated that it activates genes that encode negative regulators of the cells cycle and suppress the expression of genes that promote cell cycle progression. Therefore, *KLF4* could be classified as a growth arrest-associated gene that inhibits cell proliferation (Chen et al., 2003). In the Fluidigm analysis, *KLF4* was shown to be highly present in undifferentiated samples. The longer undifferentiated samples were in culture, the higher the expression of *KLF4*. As it has been mentioned, porcine trophoblast-derived cells have demonstrated regenerative properties which make them renew themselves to avoid senescence and differentiation. One of the characteristics of *KLF4* is that it is a crucial component for restoring tissue homeostasis which is really

applicable to these cells. *CMYC* is another factor which has been mostly present in our undifferentiated sample in Fluidigm analysis. In the mouse, *CMYC* is expressed at elevated levels in stem-cells, this gene regulates the pathway of self-renewal and maintenance of pluripotency. The absence of *CMYC* antagonizes self-renewal and promotes differentiation (Cartwright et al., 2005). Therefore, it makes sense that in Fluidigm analysis in our samples *CMYC* demonstrates a higher fold change in gene expression difference in our undifferentiated samples. The longer undifferentiated samples were in culture, the higher the expression of *CMYC*.

In an experiment performed to observe the gene expression in pregnant and non-pregnant sows, the mRNA coding for the growth factor FGF9 was highly expressed in pregnant sows. It is known that FGF9 binds with high affinity to FGFR2 and FGFR3. In the same experiment, another fibroblastic growth factor, FGF7, was identified as a strong factor which takes part in the FGFR2-mediated signaling (Ostrup et al., 2010). In mouse, FGF4 generates in the ICM and it binds the FGFR2 receptor for the proliferation of trophoblast stem cells. When FGF4 was not present, the trophoblast cells differentiated into other trophoblast sub-types in vitro (Rielland et al., 2008). Further investigation about the FGFR2 receptor has demonstrated that the isoform of this receptor, either the b or c exon, is dependent upon cell lineage. The b exon appears to be expressed in epithelial tissue and the “c” exon is expressed in mesenchymal tissue. Also, additional switching in the expression from FGFR2b to FGFR2c has resulted in the progression of prostate cancer from a non-malignant epithelial tumor to an invasive undifferentiated tumor. FGF2, FGF4, FGF7, and FGF9 have demonstrated high binding activity towards the FGF2 c form receptor (Ornitz et al., 1996). In the RT-PCR gene expression

experiment, all of our samples demonstrated expression of the FGFR2 receptor. These results confirm the conclusion that the expressions of FGF signaling and the receptivity of FGFR2 from the trophoblast are necessary to maintain the proliferation of the trophoblast (Arman et al., 1998). *KRT8* was tested in the RT-PCR experiment and *KRT18* was tested in the immunofluorescence analysis experiment. *KRT8* is a keratin protein that is utilized together with *KRT18* and *KRT19* to differentiate epithelial cells from hematopoietic cells in blood cells (Allard et al., 2004). *KRT18* is one of the most common products of the intermediate gene family next to *KRT8,* and it is expressed mainly in single layers of epithelial tissue of the body. Day 13 trophoblast-derived cells samples expressed strong bands for the test of *KRT8* in all of the samples. In immunofluorescence analysis, day 10 trophoblast-derived cells express the protein staining of *KRT18*. There is a strong staining at the cytoplasm of trophoblast cells. The staining allowed for the observation of smaller aggregated cells and bigger cells. It is believed that the smaller cells are less differentiated than the bigger ones, but further analysis needs to be done to confirm this. Some cells are observed to have a condensed chromatin; the reason might be that they are undergoing mitosis. Also, in the same immunofluorescence analysis, porcine fibroblast cells do not express the protein staining for *KRT18*. These results confirm a difference between porcine trophoblast-derived cells and porcine fibroblast cells. Porcine trophoblast-derived cells are part of epithelial tissues in the body. Another filament intermediate utilized to test in our day 13 and day 10 samples was *VIMENTIN*. *VIMENTIN* is expressed as a marker of mesenchymal cells and of cells undergoing an epithelial to mesenchymal transition. In the current RT-PCR gene expression experiment, *VIMENTIN* was not expressed in the trophoblast day 0 sample, but it seemed to gain

expression as the sample was cultured over the days in serum and non-serum. In the immunofluorescence analysis experiment, day 10 porcine trophoblast-derived cells demonstrated a lower staining for *VIMENTIN* than when they were stained for *KRT18*. Porcine trophoblast-derived cells were stained not only in the cytoplasm, but also in their nuclei when tested for *VIMENTIN*. Also, porcine fibroblast derived cells demonstrated a higher staining than porcine trophoblast-derived cells for *VIMENTIN*. The reason might be that porcine trophoblast-derived cells possess epithelial characteristics but might just have some mesenchymal characteristics in them also. The results also suggest that porcine fibroblast cells are mainly composed of mesenchymal characteristics. These results contradict the results obtained by Ramsoondar et al. (1993) where a positive expression of cytokeratin and a negative expression of *VIMENTIN* in day 14 porcine trophoblast-derived cells was observed through immunocytochemistry analysis. A possible explanation for this contradiction of results in cells derived from very similar sources is that the trophoblast cells currently described were cultured in a different medium, specifically in the absence of serum or other non-specific protein sources (such as feeder fibroblasts, etc.) that might promote differentiation and prevent vimentin gene expression.

The gene *TERT* for telomerase activity was expressed in all of our samples, suggesting an explanation for the regenerative properties demonstrated by day 10 and day 13 porcine trophoblast-derived cells. The housekeeping gene *EIF4A1* which was utilized as a control, confirmed the correct expression of our sample when tested for all the genes.

The transfection experiment described an important characteristic of day 10 porcine-derived trophoblast cells: These cells can be receptive to a foreign DNA. This

opens the possibility of future testing of cell reprogramming in these cells. In this case, a foreign DNA was able to penetrate the cell membrane and stably form part of porcine TE cells nuclei. The jetPEI method was the most efficient in transfecting porcine TE cells. The main difference in transfecting both types of cells is that after 12-13 passages GFP porcine fibroblast cells reach senescence and GFP porcine TE cells transfected by jetPEI method have been passaged for over a year in our laboratory. The clump pick-up passage allowed for the generation of stably transfected GFP colonies. Therefore, stably transfected porcine trophoblast-derived cells could be used as markers in other experiments.

The medium components utilized for the growth and development of porcine trophoblast-derived cells have allowed for continuous passage and renewal. Preliminary data has demonstrated that modifying the components of the serum-free medium causes these cells to lose their capacity for indefinite passage and self-renewal. The removal of the factors EGF and FGF have caused these cells to stop producing regenerative clumps and to eventually differentiate and senesce. Likewise, the B-27 serum replacement product was essential to maintaining the morphological and behavioral characteristics of these cells. Conversely, when serum was taken out of the serum-containing medium and replaced with B-27, the TE cells started to act like they were in an undifferentiated stage. But, they did not produce regenerative clumps and eventually started to differentiate. Also, charcoal filtered serum was utilized in the serum-containing medium to observe if steroid hormones caused differentiation of porcine trophoblast-derived cells. The results demonstrated that this was not the case since cells portrayed similar characteristics as when they were grown on the original serum containing medium. Therefore, the medium

components utilized in this experiment for non-serum containing medium are still ideal for the investigation of the clump formation and renewal of porcine trophoblast-derived cells.

Day 10 and day 13 porcine trophoblast-derived cells have been shown to be a cell line which could be utilized as a laboratory tool for the investigation of trophoblast and placental function, cell reprogramming and nuclear transfer analysis. The generation of clumps in these cells allows for indefinite passage and self-renewal. Also, that the laboratory can have access to an interesting cell line which could be passaged indefinitely and not senesce after 12-15 passages like fetal-derived fibroblast cells do. Another advantage of utilizing these cells is that they have been able to grow over plastic. This is interesting because most of the publications have indicated that to grow porcine trophoblast-derived cells, a feeder layer of mitotically-inactivated fibroblasts is utilized (Ramsoondar et al., 1993; Flechon et al., 1995; Ezashi et al., 2011). The fact that we have been able to grow these cells without a feeder layer and that they demonstrate such regenerative characteristics as cell spheres, domes and clumps makes these cells unique. These cells have been receptive to a foreign DNA through transfection. This is a really important step in utilizing them as laboratory tools since they could be used as markers to observe nuclear transfer efficiency rates. Because of their self-renewal characteristics and their differentiation to become the placenta, it is hypothesized that these cells could be more efficient at cloning than utilizing fibroblast cells. Also, the idea of cell reprogramming could be an option in these cells, since they have demonstrated to be receptive to foreign DNA.

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