Conversion of equine umbilical cord matrix mesenchymal stem cells to the trophectoderm lineage using the Yamanaka reprogramming factors

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Animal and Poultry Sciences

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10 June 2015
Blacksburg, VA

Keywords: Induced, trophoblast, spheres, monolayer

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ABSTRACT

Induced pluripotent stem (iPS) cells that possess embryonic stem (ES) cell-like properties are generated through the use of the Yamanaka transcription factors, *OCT4*, *SOX2*, *KLF4*, and *MYC* (*OSKM*). Advanced transgene delivery methods utilizing non-integrating viruses for transduction of target cells has provided new opportunities for regenerative medicine in humans and other species. We sought to use this technology to generate equine iPS cells to address challenges in equine regenerative medicine. Umbilical cord matrix mesenchymal stromal cells (MSC) were transduced with the non-integrating Sendai virus encoding for the *OSKM* transcription factors. The cells initially were cultured on mouse embryonic feeder cells supplemented with LIF (10 ng/mL) and FGF2 (4 ng/mL). Transduction generated 21 initial colonies. Of these, four survived beyond 20 passages. The transduced equine cells morphologically resembled ES cells and expressed cell surface antigens indicative of ES cells. Molecular evaluation revealed the cells maintained expression of endogenous *OSKM* while the exogenous *OSK* transgenes were extinguished, but *MYC* was maintained. The transduced equine cells did not express the ES marker *NANOG*, but did express the trophectoderm markers *CDX2* and *TFAP2A*. Both OCT4 and CDX2 were colocalized to the nucleus. The transduced equine cells were termed equine induced trophoblast (iTr) cells. Culture of the iTr cell in suspension resulted in formation of blastocyst-like spheres rather than solid cell aggregates.

indicative of ES and iPS cells. The iTr cells were transitioned to a feeder free monolayer culture. Transformation of the iTr cells to the spherical arrangement stimulated expression of genes that mark differentiation of trophoblast cells and up-regulated 250 transcripts over the monolayer arrangement. The iTr monolayer arrangement up-regulated 50 transcripts compared to the spherical arrangement. The iTr spheres respond to BMP4, EGF, and FGF2 by phosphorylating signal transduction proteins. Addition of BMP4, EGF, or FGF2 in combined pairs was able to alter *TFAP2A*, *NEU1*, and *SLC35A1* expression. The generation of iTr cells by transduction of the Yamanaka reprogramming factors is not unique to equine cells. However, this report marks the generation of the first equine trophoblast cell line capable of recapitulating early equine trophoblast development. The new iTr line could prove valuable in gaining greater understanding of equine trophectoderm development.

ACKNOWLEDGEMENTS

I am a first generation college graduate. I was home educated from second grade until the day I began college. I thank my cousin Trista for all the effort she put forth tutoring me in subjects my parents could not. Those tutoring sessions propelled me toward earning a GED and entry into college. A long journey and a lot of work took me from those early days on a hog farm in Indiana to the cusps of earning a PhD.

I believe the mentoring process is very important to success. I want to thank Sally Johnson for the opportunity to advance my skills under her guidance. She has been a patient and helpful mentor that embraced my strengths and cultivated my weaknesses. I thank my committee members Jia-Qiang He, Rob Rhoads, and Eric Wong for their direction and experience in guiding me through my dissertation. Many thanks to Alan Ealy for his suggestions and expertise as the tide of reproductive and placental biology rose around me during this project. I want to take this opportunity to thank my past mentors. From my youth, to undergraduate, to non-academic positions and through this PhD program, I have had too many mentors to name. The unexpected nuances of daily activities with mentors over the years, such as car trips or sampling days, have had lasting impacts on the way I view situations. I make every attempt to learn how each one has taught and guided me so one day when I can successfully mentor students.

Many thanks to… Jennifer Bradley who has been one of the best co-workers one could ask for. Her help in experiments and making everything run smoothly has been invaluable! I have thoroughly enjoyed our conversations and comradery over the last two plus years. My lab and office mates Vicki McCracken, Meghan MacGhee, Sarah McCoski, Robert Jacobs for making the work days so enjoyable. My longtime friend Angela Pavuk for lending an ear and for her encouragement during this process. Ben Riggins, Jason and Tracy Scheffler, Steve Kasten, and Brittni Bailey for their friendship and comradery over the years. My brother James Reinholt, my father Michael Reinholt, and my mother Dana McPheron for continued support and patience as I have been ‘in school’ for the last ten years. Last but not least, I thank Maria Ritenhouse for tolerating me. I met her at the conclusion of my Masters project and, with help from some of the aforementioned friends (Tracy!), she became what I hope is a permanent part of my life two years ago. I do not tell her enough how much her love and support means to me.

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BMP4, bone morphogenic protein 4

CDH1, cadherin-1,

CDX2, caudal type homeo box transcription factor 2

CTGF, connective tissue growth factor

DHRS9, dehydrogenase/reductase (SDR family) member 9

DPPA4, developmental pluripotency-associated 4

EGF, epidermal growth factor

ELF3, E74-like factor 3

EOMES, eomesodermin

ES, embryonic stem

ETS2, v-ets avian erythroblastosis virus E26 oncogene homolog 2

FBS, fetal bovine serum

FGF, fibroblast growth factor

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GATA2, GATA-binding protein 2

GATA3, GATA-binding protein 3

GNE, glucosamine (UDP-N-Acetyl)-2-epimerase/N-acetylmannosamine kinase

HAND1, heart and neural crest derivatives-expressed protein 1

HESX1, homeobox expressed in embryonic stem cells 1

HEXIM2, hexamethylene Bis-Acetamide Inducible 2

ICM, inner cell mass

IGF-I, insulin-like growth factor-I

iPS, Induced pluripotent stem

iTr, induced trophoblast

KLF4, kruppel-like factor 4

LIF, leukemia inhibitory factor

LIN28, lin-28 Homolog A

MEF, mouse embryonic fibroblasts

MSC, mesenchymal stromal cells

MOI, multiplicity of infection

MYC, avian myelocytomatosis viral oncogene homolog

NANOG, nanog Homeobox

NEU1, neuraminidase 1

OCT4, octamer-binding transcription factor 4

REX1, zfp-42 (reduced expression 1)

SALL4, spalt-like transcription factor 4

SOX2, sex determining region Y-box 2

SLC, solute carrier family

SSEA4, stage-specific embryonic antigen 4

TCL1A, T-cell leukemia/lymphoma 1A

TE, trophectoderm

TEAD4, transcriptional enhancer activator domain family member 4

TFAP2A, transcription factor activating protein 2-alpha

TGM3, transglutaminase 3

TRA-1, trafalgar antigen 1

VEGF, vascular endothelial growth factor

CHAPTER I

Literature review

Introduction

Musculoskeletal injuries are a significant concern for the equine industry. Estimates of up to 30% of racing injuries are tendon related alone (Goodship et al. 1994). Moreover, 43% of injured animals will suffer recurrent injuries requiring treatment that often results in prolonged recovery periods (Dyson 2004). As reported by The Jockey Club, catastrophic musculoskeletal injuries occurred at a rate of two deaths per 1,000 starts in Thoroughbred race horses (2015). Poor vascularization and stem cell populations with limited mitotic activity reduce self-repair ability of these tissues (Borjesson and Peroni 2011). Experiments over a decade ago demonstrated the potential for mesenchymal stromal cell (MSC) use in equine regenerative medicine (Smith et al. 2003). Demand for cytotherapy treatments has spawned several commercial options that take advantage of adipose tissue (AT)-derived and, to a lesser extent, bone marrow (BM)-derived MSCs (Marx et al. 2015). Mesenchymal stem cell therapy in horses is widely used, but with variable long-term repair success. Poor success rates are primarily attributed to the small number (0.01- 0.1%) of authentic MSCs harvested and the limited proliferative ability of these cells *in vitro* (Borjesson and Peroni 2011; Vidal et al. 2006). Structural improvements to tendons are reported in horses using equine fetal fibroblasts suggesting more plastic cell populations may improve repair (Watts et al. 2011). Embryonic stem (ES) cells can provide a cell source that can proliferate indefinitely and can readily contribute to all tissues of the body. However, isolation of stable ES cell lines from livestock has been unsuccessful (Koh

and Piedrahita 2014). Efforts were shifted from ES cells to induced pluripotent stem (iPS) cells when it was discovered that somatic cells could be reprogrammed to induce an ES cell-like state by ectopic expression of a defined set of transcription factors (Takahashi and Yamanaka 2006).

Embryonic stem cells

In mammals, the first differentiation event of the zygote partitions cells into either the trophectoderm (TE) or the inner cell mass (ICM). The ICM further differentiates into the epiblast and the hypoblast. The epiblast is pluripotent and constitutes each germ layer (ectoderm, mesoderm, and endoderm) that gives rise to all the tissues of the organism. The TE and hypoblast are extraembryonic tissues that provide nutritional resources, protection, and a fetal-maternal interface critical for pregnancy success (Gardner and Beddington 1988). Embryonic stem cells are derived from the ICM of early blastocyst stage embryos. Epiblast stem (EpiS) cells are isolated from the epiblast of late blastocyst stage embryos near or at the time of implantation. Both are pluripotent and are capability of self-renewal. Because of these properties they represent tools to study disease *in vitro* as well as provide hope to repair damaged tissue *in vivo*.

The first pluripotent embryo cells were isolated by Martin Evans and Matthew Kaufman in July of 1981 (Evans and Kaufman 1981). The term “embryonic stem cell” was introduced by Gail Martin after she successfully isolated the ICM from 76 hour mouse blastocysts (1981). The cells isolated met the criteria for ES cells by indefinite propagation *in vitro* and ability to differentiate into the three germ layers. These first mouse ES (mES) cultures were established on a mitotically inactive mouse embryonic

fibroblast (MEF) feeder layer in the presence of teratocarcinoma-conditioned media. These conditions were based on earlier observations that embryonal carcinoma cells showed greater propagation in the presence of a “contaminating” fibroblast cell type (Martin and Evans 1975). The authors suspected an unknown factor produced by the fibroblast cells, or a combination of factors, supported the continued propagation of the carcinoma cells. It was determined that the MEFs provide growth factors, including leukemia inhibitory factor (LIF), that promote maintenance of pluripotency (Smith et al. 1988). For many years derivation of mES cells was limited to the 129 and C57BL/6 strains. Later studies showed the unknown factor that maintained propagation to be LIF. Activation of the LIF receptor stimulated the signal transducer and activator of transcription 3 (STAT3) (Conover et al. 1993; Nagy et al. 1993). Related cytokines such as ciliary neurotrophic factor or oncostatin M. have a similar effect. Later, the mechanism of action was revealed to be STAT3, which drives demethylation of pluripotency gene promotor regions. Phosphorylation by STAT3 blocks the action of the DNA methlytransferase 1 (DNMT1) and histone deacetylases 2, 3, and 8 on the promotor regions of genes required for pluripotency (Tang and Tian 2013). Inhibition of mitogen-activated protein kinase/ extracellular-signal-regulated kinases1/2 (MAPK/ERK1/2) and glycogen synthase kinase-3 (GSK3) signaling pathways combined with LIF receptor activated STAT3 could generate ES cells from mouse strains previously thought to be non-permissive (Buehr and Smith 2003; Umehara et al. 2007). Fibroblast growth factor 4 activation of MAPK stimulates expression of mesodermal genes (Burdon et al. 1999; Thiery and Sleeman 2006). Inhibition of MAPK prevents differentiation and supports pluripotency. Beta-catenin is a signal transducer for the Wnt

signaling pathway. Phosphorylation of β-catenin by GSK3 makes it a target for degradation. Inhibition of GSK3 leads to the stabilization and nuclear translocation of β-catenin that stimulates Canonical β-catenin signaling that activates genes driving cell proliferation and maintenance of pluripotency (Kaldis and Pagano 2009). A single network directly links STAT3 signaling with MAPK and GSK3 inhibition to regulate expression of pluripotency genes (Ye et al. 2013). Chemical inhibition of GSK and ERK1/2 (2i) promotes maintenance of ground state pluripotency. Ground state is defined as a fully unrestricted cell that harbors the necessary developmental potency to generate all tissue of the organism. Ground state is characterized by two active X-chromosomes (in XX cells) (Nichols et al. 2009). In the absence of the 2i, ES cells from non-permissive mouse strains are unstable and differentiate to a more specialized post-implantation primed EpiS cell state. Primed ES cells hold all properties of ground state ES cells, with the exception that they only have 1 active X-chromosome and cannot form germ line chimeras (Nichols and Smith 2009). Therefore, activation of the STAT3 and Wnt/β-catenin signaling pathways along with inhibition of MAPK signaling are important for the successful isolation and propagation of pluripotent ES cells in mice.

The first derived mouse ES cell lines were characterized based on morphological similarities to teratocarcinoma cells such as high nucleus-to-cytoplasmic ratios (Martin 1981). The first widely accepted molecular markers used to identify ES cells were the cell surface proteins Stage Specific Embryonic Antigen3 (SSEA3)/SSEA4 and Trafalgars antigen-1-60 (TRA-1-60)/TRA-1-81 (Reubinoff et al. 2000; Thomson et al. 1998). These cell surface markers are present in the ICM, but absent from trophoblasts of blastocyst stage embryos and absent from the morula. When differentiation is

initiated, SSEA3/4 and TRA1-61/81 become down-regulated and SSEA1 is up-regulated. Other markers used to identify ES cells are the cell surface proteins GCTM2, GCTM343, CD24, CD9, and the cell membrane protein alkaline phosphatase (Oka et al. 2002; Pease et al. 1990; Pera et al. 1988; Polanco et al. 2013).

The transcription factors *NANOG*, *OCT4*, and *SOX2* are molecular markers of uncommitted ES cells (Table1-1) (Clark et al. 2004; Reubinoff et al. 2000). Expression of *NANOG* is absent until the morula stage, then confined to the ICM in both mice and humans (Chambers et al. 2003). Expression of *NANOG* is increased by Wnt signaling, supporting the use of GSK3 in ES cell culture (Ho et al. 2013). Unfertilized oocytes express *OCT4* and zygotes expresses maternally derived *OCT4* until the ten cell stage and embryonic genome activation. In the mouse, when maternally driven *OCT4* shuts down, *OCT4* is restricted to the ICM. Contrary to the mouse, *OCT4* is expressed in both the ICM and TE of human blastocysts through the implantation period, with higher *OCT4* levels in the ICM (Cauffman et al. 2005). Transcripts for *SOX2* are detected in the morula and localize to the epiblast of late blastocysts. Cultured EpiS cells from *SOX2* knockout mice differentiate into trophoblasts and extraembryonic endoderm. This suggests that SOX2 is critical in maintenance of the epiblast (Avilion et al. 2003). Other genes used in ES cell identification and markers of pluripotency are *DNMT3β*, *LIN28*, *REX1*, and *UTF1*. Methyltransferase 3β (DNMT3β) has been shown to affect the methylation status of *NANOG* and *OCT4*, and is expressed in the ICM, epiblast, and embryonic ectoderm of mice in a pattern similar to that observed for *OCT4* (Watanabe et al. 2002). The transcription factor *UTF1* appears in the primitive ectoderm and extraembryonic ectoderm of late blastocysts. Although not completely clear, evidence

suggest UTF1 may function as a co-activator that enhances transcription of *OCT4, SOX2, NANOG*, *REX1*, *KLF4*, and *MYC* (Okuda et al. 1998). Mice homozygous null for *LIN28* exhibit decreased cell proliferation and increased apoptosis of ES cells (Xu et al. 2009). High levels of *LIN28* expression at low cell densities leads to differentiation toward extraembryonic lineages (Darr and Benvenisty 2009). The role of *REX1* in ES cell pluripotency is to inhibit differentiation and is regulated by OCT4 where OCT4 is reported to bind to the *REX1* promoter. As differentiation progresses, decreased OCT4 expression causes an abrupt down-regulation of REX1 (Ben-Shushan et al. 1998).

Continued analysis of ES cells identified *CDH1*, *DPPA4*, *HESX1*, *SALL4*, and *TCL1A* as genes that can aid identifying pluripotent cells (Table1-1). Moon and colleagues (2011) discovered *DPPA4* and *HESX1* were up-regulated significantly in human ES (hES) cells compared to human embryoid bodies. Ectopic overexpression of *DPPA4* prevents mES cell differentiation (Masaki et al. 2007). Homeobox expressed in ES cells 1 (*HESX1*) is specific to the mouse ICM (Webb et al. 1993). The *CDH1* gene encodes for the E-cadherin protein which is critical for cell-cell adhesion. High expression of *CDH1* is important for maintaining close association of the ICM and cultured ES cells. Dissociated mES and hES cells cultured *in vitro* rapidly differentiate when cell-to-cell contact is lost (Soncin et al. 2011). Proliferation of both mES and hES cells is impaired with a prolonged G1 phase in the absence of *SALL4* and *TCL1A* (Ivanova et al. 2006; Yuri et al. 2009). Neither *CDH1*, *DPPA4*, *HESX1*, *SALL4* nor *TCL1A* are specific to ES cells, but they are up-regulated in pluripotent cells where it is hypothesized they support the highly proliferative properties of ES cells.

Cell potency is the ability of a cell to differentiate into different and more specialized cell types. The degree of potency of ES cells can be measured both *in vitro* and *in vivo*. *In vitro* differentiation potential is assayed through the ability to form embryoid bodies. Embryoid bodies are three-dimensional structures formed by spontaneous aggregation of undifferentiated ES cells in suspension (Itskovitz-Eldor et al. 2000). Pluripotent cells spontaneously differentiate into cells representative of each germ layer. Histochemical analysis can determine if cells from each lineage are present. *In vivo* differentiation potential is determined by the capacity of ES cells to form teratomas. This was first accomplished by injecting ES cells in athymic mice (Evans and Kaufman 1981; Martin 1981). Now, severe combined immunodeficient (SCID) mice are used. *In vivo* differentiation ability is considered a more stringent assay over *in vitro* assays (Ben-David and Benvenisty 2011). As with embryoid bodies, histochemical analysis of the teratomas is performed to determine the differentiation potential of the injected cells.

Human ES cells were isolated by James Thomson and colleagues in 1998 from an *in vitro* produced blastocyst (Thomson et al. 1998). As of April 2015, 307 hES cell lines exist that are eligible for NIH funded research (NIH 2015). Phenotypically, mES and hES cells are indistinguishable in culture, however there are several distinct differences. Unlike their mouse counterparts, hES do not require LIF to maintain pluripotency (Daheron et al. 2004). This is possibly due to low expression levels of LIF receptor and the LIF receptor signal-transducing subunit glycoprotein 130 (gp130), and high expression levels of LIF signaling inhibitors in hES (Ginis, 2004). Human ES cells are thought to compensate for decreased LIF receptor activated signaling through

alternative pathways independent of the STAT3 pathway, potentially through transforming growth factor-β (TGFβ) signaling or through an unidentified pathway (Chen et al. 2011; Dani et al. 1998). Unlike the mouse, canonical Wnt/β-catenin signaling induced hES cell differentiation and reduces the expression of *OCT4*, *SOX2*, and *NANOG* (Sumi et al. 2008). Human ES cells respond to FGF2 stimulated MAPK signaling by promoting self-renewal and expression of *NANOG* (Greber et al. 2010). Fibroblast growth factor 2 preserves the undifferentiated state by promoting cell adhesion and compact hES cell colony formation (Eiselleova et al. 2009). For these reasons the majority of hES cell culture conditions include FGF2. Unlike mES, virtually all hES cell line exhibit partial or complete X-inactivation (Lengner et al. 2010). These properties suggest mES and hES cells use divergent signaling pathways to maintain a pluripotency state.

Ectopic *OCT4* overexpression alone in mES cells stimulates differentiation into mesoderm and endoderm (Niwa et al. 2000). By contrast, ectopic *OCT4* overexpression in hES induces endoderm formation (Rodriguez et al. 2007). Repression of *SOX2* results in differentiation into TE in both mES and hES cells (Fong et al. 2008). In both mES and hES cells, inhibition of *NANOG* initiates differentiation and overexpression maintains pluripotency (Schnerch et al. 2010). Mouse ES cells express *SSEA1*, but not *SSEA4* and hES cells exhibit reciprocal expression of these markers. Mouse ES cells do not express TRA-1-60/81 whereas hES cells do (Ginis et al. 2004). A significant difference between hES cells and mES cells is the ability of hES cells to differentiate into trophoblasts (Odorico et al. 2001). Human ES cells harbor detectable levels of genes associated with the TE lineage that are absent from mES cells (Ginis et al. 2004).

These suggest hES cell more closely resemble mEpiS cells, rather than ground state mES cells (Nichols and Smith 2011).

Embryonic stem cells in domesticated species

Six putative equine embryonic stem (eES) cell lines are reported to date (Guest and Allen 2007; Li et al. 2006; Saito et al. 2002). Saito and colleagues (2002) dissected the ICM from blastocysts recovered at day 6 to day 7 post-ovulation and established primary cultures of putative ES cells on mitotically inactive bovine embryonic fibroblasts. They were able to propagate the cells for over 50 passages, but both cell lines displayed abnormal karyotypes after passage 20 and were only able to differentiate into ectoderm and mesoderm lineages. Li and coworkers (2006) employed immunosurgical procedures to isolate the ICM from day 8 equine blastocysts. This procedure releases the cells of the ICM by lysing the surrounding trophoblast cells with complement once they have been exposed to serum from an unlike species. Three additional eES lines that maintained proliferative ability for more than 26 passages and displayed the ability to differentiate into all three germ layers *in vitro* were established. A follow-up study to Li and coworkers produced a sixth putative eES cell line (Guest and Allen 2007). In all studies, embryoid bodies were formed, but differentiation into the three germ layers was reported in only one study and embryoid bodies were irregularly shaped compared to those from mouse and human.

Bovine ES (bES) cells were some of the earliest attempts at ES cell isolation due to the advanced *in vitro* reproduction techniques developed in cattle compared to other livestock species (Saito et al. 1992; Strelchenko and Keefer 1995). Several reports have

documented bES cell generation with extended passage and the ability to differentiate into each germ layer (Mitalipova et al. 2001). However, several lines displayed chromosomal instability at later passages (Kwon et al. 2009). Molecular markers in bES are more consistent with those in the mouse than human. Pluripotency markers *OCT4*, *SOX2*, *NANOG* are expressed in purported bES cells like in mES and hES, but expression of cell surface markers more closely resembles mouse being TRA-1-60/81 negative and SSEA1 positive (Han et al. 2011).

Early attempts of blastocyst collection from swine involved laparoscopic surgery to flush day 10 embryos from artificially inseminated females (Strojek et al. 1990). Talbot and colleagues (1993) were the first to establish alkaline phosphatase staining as a reliable marker of EpiS cells in any species. Successful chimeric pigs were produced from stem cells derived from primordial germ cells, but the chimeric animals generated were not germline chimeras (Rui et al. 2004). Most recent research into successful establishment of pES cells has focused on culture conditions and propagation protocols (Brevini et al. 2010). Porcine ES cells are more sensitive to non-mechanical passage techniques and buffers compared to other livestock species. After only minutes of exposure to Ca^{2+}/Mg^{2+} free PBS, pES cells rupture and completely disintegrate within an hour.

Induced pluripotent stem cells

In 2006, Takahashi and Yamanaka showed that mouse embryonic fibroblasts could be reprogrammed into pluripotent stem cells (Takahashi and Yamanaka 2006). They demonstrated that exogenous expression of the transcription factors *OCT4*,

SOX2, *KLF4*, and *MYC* (*OSKM*) were necessary and sufficient to reprogram mouse fibroblasts into pluripotent cells. This was despite the fact *NANOG*, which appears to be critical for ES cell maintenance, seemed dispensable for somatic cell reprogramming. The reprogrammed cells were termed induced pluripotent stem (iPS) cells. The iPS cells possessed a great capacity for self-renewal, a critical component to ES cells. The reprogrammed cells also displayed endogenous activation of *OSKM* and *NANOG*. The ability to differentiate into cells representing each embryonic lineage was confirmed by both *in vitro* embryoid body formation and *in vivo* teratoma formation. The iPS cells generate germline chimeras in mice and rats, indicating their functional equivalency to ES cells of the ICM proving they displayed all measurable properties of definitive mES cells. (Okita et al. 2007).

The first generation of iPS cells used retroviral delivery of the exogenous human *OSKM* transgenes into embryonic and adult fibroblasts (Takahashi et al. 2007; Takahashi and Yamanaka 2006). The delivery method employed lentivirus, which integrated genetic material of viral origin into the host cells. The use of the oncogenes *KLF* and *MYC* enhanced tumorigenic risks with the reprogrammed cells. Since the discovery of the four transcription factor reprogramming system, several methods are now used to package and deliver the reprogramming factors to target cells. Transfection of target cells, which can deliver mRNA molecules, recombinant proteins, and transposons coding for the reprogramming factors, can successfully generate human and mouse iPS cells (Kim et al. 2009; Stadtfeld et al. 2008; Warren et al. 2010).

Virally induced iPS cells comprise the majority of the literature. This is primarily because of the high efficiency, in terms of number of iPS cell colonies generated, at which retroviruses and lentiviruses reprogram mammalian cells, (Takahashi and Yamanaka 2006). Retroviruses and lentiviruses are single-stranded RNA viruses that convert their RNA genomes into DNA through reverse transcription. The transcribed viral genome is then integrated into the host genome via integrases. The viral particles are then replicated utilizing the host cell's machinery. Lentivirus can infect non-dividing cells which makes it a more attractive platform for reprogramming somatic cells. However, the integration of viral DNA limits the clinical applicability of these systems since the integrated genetic material can induce tumorigenic side effects and negatively alter the host genome (Hacein-Bey-Abina et al. 2003).

To overcome the integration events of the retro- and lentiviral approaches, investigators sought non-integrating platforms to initiate reprogramming (Tashiro et al. 2009). Adenoviruses are double-stranded DNA viruses that do not need to reverse transcribe their genetic material to take advantage of the host's machinery. Additionally adenoviruses provide efficient transduction of both dividing and non-dividing cells making them a more diverse vector. Other non-integrating reprogramming approaches use Sendai viral vectors (Fusaki et al. 2009). Sendai virus vectors replicate in the form of negative-sense single-stranded RNA in the cytoplasm, compared to the nucleus, of infected cells, which do not integrate into the host genome. Sendai viruses enable efficient production of iPS cells with subsequent elimination of the viral vector over a fixed period of cell divisions.

Non-viral episomal expression of transgenes to induce pluripotency has been achieved through plasmids (Yu et al. 2009) and minicircle vectors (Jia et al. 2010). Minicircle vectors have advantages over traditional plasmids because they do not contain bacterial genomic material. Typically, they are small (≤4kb) which makes them more readily transfected. The main drawback to the episomal vectors to deliver the transgenes is decreased reprogramming efficiency. The episomal vectors are lost over subsequent cell divisions resulting in a reduction in the number of initial iPS cell colonies formed compared to virus-based systems.

Transposable elements (transposons) offer an eloquent delivery method for reprogramming factors. Plasmids carrying the insertion sequence of the transgene, flanking inverted terminal repeats, and transposase enzyme are transfected into the target cell. The desired transgene becomes integrated by a cut-and-paste mechanism, and the plasmid backbones are degraded. The integrated transposon can be removed by supplying the transposase in trans (Woltjen et al. 2009) Two platforms that have been shown to successfully reprogram somatic cells are the Sleeping Beauty (Grabundzija et al. 2013) and piggyBac (Woltjen et al. 2009) systems. The main drawback to transposon based reprogramming is the low number of initial colony number.

Non-viral iPS production in mice and humans currently focuses on using recombinant proteins and mRNA molecules (Plews et al. 2010; Zhou et al. 2009). Protein based systems are not widely used due to the low reprogramming efficiency and the need to administer repeated treatments owing to the short half-life of the

recombinant proteins. Messenger RNA has been employed to efficiently generate iPS cells. Messenger RNAs encoding reprogramming factors are an ideal vehicle for reprogramming since they do not harbor the risk of genomic integration. They can be transfected with high efficiency, and they can be combined in specific ratios of each reprograming factor (Plews et al. 2010). Disadvantages of mRNAs, like the recombinant proteins, is the short half-life of the RNAs and potential silencing by endogenous microRNAs.

Induced pluripotent stem cells in different species

The first generation mouse iPS (miPS) cells were epigenetically and developmentally indistinguishable from mES cells (Jaenisch and Young 2008). The second-generation miPS cells produced germline competent cells that could contribute to the entire organism (Meissner et al. 2007). Functional equivalency to mES cells was demonstrated by germline contribution of the miPS cell to chimeric animals (Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009). In an effort to eliminate tumorigenic potential, *MYC* and *KLF4* were removed from the reprogramming cocktail. Successful reprogramming occurred, but with a reduction in the number of colonies formed (Huangfu et al. 2008). Human fibroblasts demonstrated the same response to the human *OSKM* reprogramming factors as mouse cells. This included indefinite propagation, *in vivo* and *in vitro* differentiation and marker expression of ES cells (Takahashi et al. 2007). One similarity between miPS and human iPS (hiPS) cells is the absolute requirement of *OCT4* for reprogramming (Huangfu et al. 2008). Transcriptome analysis revealed more than 1,200 genes with at least five-fold change in relative expression between miPS and hiPS cells (Schnerch et al. 2010). The majority of these

genes were associated with transcriptional regulation, cell signaling, and cellular differentiation. Culture conditions are consistent with the ES culture system where miPS and hiPS cell cultures typically include LIF and FGF2, respectively.

The first report of equine iPS (eiPS) cells was by Nagy and colleagues (2011). They used a piggyBac transposon-based method to deliver the mouse *OSKM* transgenes into equine fetal fibroblasts. The investigators employed the LIF, FGF2 and 2i (GSK3 and MAPK inhibitors) culture system. The putative eiPS cells were able to differentiate into each of the three germ layers *in vivo*, but displayed chromosomal instability by passage 26 and genomic integration of the transgenes. The authors cited difficulty in obtaining adequate antibodies compatible with the horse for the cell surface markers (TRA-1-60/81 and SSEA1) and the pluripotency markers (*OCT4*, *SOX2*, and *NANOG*). A later study demonstrated derivation of eiPS cells with retroviral transduction of only human *OSK*, removing the oncogene *MYC* from the reprogramming cocktail (Khodadadi et al. 2012). The authors used adult fibroblasts as the starting cell type and added both LIF and FGF2 to the culture medium, but without inhibitors. The putative eiPS cells were maintained over 27 passages and exhibited *in vivo* teratoma differentiation. Breton and colleagues established eiPS cells from adult fibroblasts using retroviral delivery of mouse *OSKM* transgenes in the presence of both LIF and FGF2, but without the 2i (2013). They generated four lines that were propagated for over 30 passages. Two of the lines expressed *OSKM*, *NANOG*, *REX1*, *LIN28, and DNMT3β* and two expressed all of the aforementioned markers of ES cells except *LIN28*. The eiPS cells could differentiate *in vitro* into embryoid bodies and *in vivo* into teratomas that were comprised of all three germ layers. A more recent attempt to establish eiPS cells

used human *OSKM* without supplemented FGF2, but with the 2i (Whitworth et al. 2014). These investigators generated putative eiPS cells that expressed *OCT4*, *NANOG*, and *TERT* and exhibited *in vivo* and *in vitro* differentiation. The authors reported a more ground state pluripotency in the eiPS cell generated in LIF only, due to X chromosome activation, than other eiPS cell reports that were both LIF and FGF dependent. The reprogrammed eiPS cells, however, expressed human *OCT4* through passage 25. Sharma and colleagues established feeder cell independent eiPS cells from adult keratinocytes through retroviral expression of mouse *OSKM* transgenes in the presence of both LIF and FGF2, but without the 2i (2014). They demonstrated, for the first time in eiPS cells, differentiation into functional neurons.

Transduction of bovine fetal fibroblasts with bovine and human *OSKM* transgenes could only be passaged 6 times and showed incomplete reprogramming with supplemented FGF2 in the culture media. When *LIN28* or *NANOG* were included into the reprograming cocktail, the number of ES-like colonies increased and the purported bovine iPS (biPS) cells could be passaged up to 40 times, expressed endogenous *OSKM*, *LIN28*, and *NANOG*, and differentiated *in vitro* into embryoid bodies and *in vivo* into teratomas that were comprised of all three germ layers (Han et al. 2011; Sumer et al. 2011). Huang and colleagues used an episomal viral vector containing bovine *OSKM* transgenes along with the LIF and 2i system in an attempt to generate non-integrated biPS cells (2011). The authors were able to detect endogenous *OSKM* and *NANOG* and the biPS-like cells differentiated into the three germ layers both *in vitro* and *in vivo*. Using bovine testicular cells, Lin and coworkers reported the generation of biPS cells with retroviral transduction of only *OCT4* in the presence of LIF

(2014). The reprogrammed cells expressed endogenous *OCT4*, *SOX2*, and *NANOG* and were able to differentiate into teratomas comprised of the three germ layers. This suggests that the need for *OCT4* is conserved between species. Putative ovine iPS (oiPS) cells generated by Liu and colleagues from ovine embryonic fibroblasts demonstrate silencing of transgene expression by passage 17 and do not require *NANOG* for reprogramming (2012). This was in stark contrast to a 2011 study that required eight reprogramming factors (*OSKM*, *NANOG*, *LIN28*, *SV40 large T antigen*, and *TERT*) to induce pluripotency in adult fibroblasts isolated from the ovine ear (Bao et al. 2011). A key difference between these two studies might be the starting cell type. It is possible the embryonic fibroblasts used by Liu and colleagues are more amenable to the reprogramming process. One report demonstrated chimeric contribution of oiPS cells to born lambs (Sartori et al. 2012), but this was not germline transmissible and integration from the lentiviral delivery system was evident.

Several groups have demonstrated putative porcine iPS (piPS) cells using retroviral (Fujishiro et al. 2013), lentiviral (Ezashi et al. 2009; West et al. 2011), transposon (Kues et al. 2013), and plasmid (Hall et al. 2012) based platforms. The most challenging obstacle in piPS cell derivation has been consistent detection of ES markers. Several reports indicate varied expression of the cell surface proteins SSEA3/4 and TRA-1-60/81, and *LIN28* and *REX1* genes (Ezashi et al. 2009; Fujishiro et al. 2013). This has made it difficult to discern if swine possess different ES cell markers or if pluripotency was not achieved. West and colleagues demonstrated germline transmission of piPS cells when reprogrammed cells were injected into porcine embryos (2011). These investigators used a lentivirus to deliver human *OSKM*, *LIN28*,

and *NANOG* in porcine MSC derived from bone marrow supplemented with FGF2, but not LIF or the 2i. Like other reports in swine, the piPS cells immunostained weakly for TRA-1-81, SSEA4, and had low endogenous *NANOG* expression. The piPS cells did express endogenous *OCT4* and *SOX2* and differentiated into each germ layer *in vivo* and *in vitro*. The piPS cells were injected into embryonic day 4.5 embryos and transferred into asynchronous sows. The offspring displayed incorporation of the piPS cells into tissue from each embryonic and extraembryonic tissue. Evidence of germline transmission was detected in the F2 generation. The efficiency was low at approximately 6%, and the chimeric swine showed genomic integration of the human *OCT4* and *NANOG* genes that were used as markers of piPS cell integration. The authors did not report if the integrated human transgenes were detected at the mRNA level. This was the first report of germline transmission resulting from ES or iPS cells with any non-rodent species.

Alternate cell types generated during reprogramming

Recent reports indicate that the *OSKM* reprogramming factors can induce transient acquisition of pluripotency followed by rapid differentiation, or can initiate transdifferentiation without a pluripotent intermediate. Kim and colleagues abbreviated *OSKM* transgene exposure to mouse fibroblasts under a doxycycline-inducible system to 3-6 days. Mouse fibroblasts were converted to neural progenitor cells, a mesoderm to ectoderm transition. The reprogrammed cells expressed only low levels of *NANOG* and *REX1*, but expressed high levels of *OCT4* (2011). The media contained FGF4 for neural tissue differentiation (Kosaka et al. 2009). Mouse fibroblasts are transdifferentiated to hepatocytes (a mesoderm to endoderm switching) under inducible exposure to only the

OSK transgenes for less than 10 days (Zhu et al. 2014). The authors concluded an intermediate pluripotent state was not achieved considering neither *OCT4* nor *NANOG* expression was detected. Ezashi and colleagues reported a significant fraction of cells that had distinct phenotypic and genotypic characteristics indistinguishable from trophoblasts and termed them induced trophoblast (iTr) cells (2011). The investigators used lentiviral delivery of human *OSKM* transgenes to reprogram umbilical cord and fetal porcine MSCs on MEFs. The iTr cells expressed endogenous *OCT4* and *SOX2*, but *NANOG* expression was not reported therefore it was unclear if the iTr cells first passed through a transient pluripotent phase. The iTr cells did express multiple markers of TE that have been documented in other species and genes that are considered more specific to porcine. The iTr colonies appeared approximately a week prior to the appearance of the piPS colonies. The early appearance was directly attributed to abbreviated exposure since the human *OCT4* and *SOX2* transgenes could be detected through six passages. The culture conditions were supplemented with FGF2, but without the 2i, which is considered standard reprogramming conditions for non-murine iPS cell derivation. Therefore, it remains unclear whether the iTr cells transdifferentiated, or rapidly differentiated towards TE as a default pathway.

Trophectoderm lineage and markers

The extraembryonic lineage gives rise to placental tissue and the extraembryonic membranes (i.e. amnion and chorion) that surround the extraembryonic coelom. Human ES and iPS cells have an intrinsic ability to contribute to the extraembryonic TE lineage (Chen et al. 2013a; Odorico et al. 2001). However, mES and miPS cells can only differentiate into extraembryonic tissue when expression of the transcription factor

caudal-related homeobox protein (*CDX2)* is forced (Blij et al. 2015; Tolkunova et al. 2006).

The *CDX2* transcription factor is one of the earliest expressed trophoblast-specific genes in mammals (Beck et al. 1995; Chen et al. 2013b). Gene knockout experiments demonstrated that *CDX2* null mice embryos fail to implant (Chawengsaksophak et al. 1997). Interestingly, *OCT4* is expressed in trophoblasts of human and domesticated species, but not in mice (Genbacev et al. 2011; Kirchhof et al. 2000; Palmieri et al. 1994). In the mouse, the presence of *CDX2* and absence of *OCT4* strictly demarcates the trophectoderm. The contemporary understanding in non-rodent species is the CDX2 and OCT4 proteins form a repressor complex and the mere stoichiometric balance of each factor dictates the ICM or trophectoderm cell fate (Table1-1) (Chawengsaksophak et al. 2004).

The core transcriptional network that regulates TE specification and function is governed by *CDX2* and is responsible for suppressing *OCT4* and *NANOG* leading to definitive TE (Niwa et al. 2005). In the mouse, the transcription factor *TEAD4* is necessary for *CDX2* expression at the 2-8 cell morula stage (Ralston et al. 2010; Yagi et al. 2007). Trophoblast stem cell self-renewal is stimulated by ETS2, which is enhanced by *CDX2* over expression (Schiffmacher and Keefer 2013; Wen et al. 2007). Studies in the mouse demonstrate that *CDX2* and eomesodermin (*EOMES*) are necessary in parallel for early trophoblast maintenance (Russ et al. 2000). However, bovine trophoblast cell lines do not express *EOMES* (Strelchenko and Keefer 1995). Additionally, *EOMES* expression in bovine, ovine, and equine TE is greatest at days 17,

21, and 22, post-ovulation, respectively, followed by subsequent reduced transcript abundance (de Mestre et al. 2009; Sakurai et al. 2013). Mice null for *EOMES* arrest development at the preimplantation blastocyst stage. The *EOMES* null mouse embryos displayed a block in the continued development of TE, but not in establishing trophoblasts (Russ et al. 2000). These data suggest *EOMES* is not critical for early TE specification, but plays a role in the preimplantation stage.

The GATA2 and GATA3 zinc finger transcription factors play important roles in blastocyst development where they are preferentially expressed in the TE over the ICM (Bai et al. 2013; Iqbal et al. 2014). There is evidence supporting their role in the *CDX2* core network of TE regulating genes. Knockdown of *GATA3* reduced expression of *CDX2* in mice (Home et al. 2009). Overexpression of *GATA2*, not *GATA3*, increased *CDX2* expression in bovine (Bai et al. 2011). Mice homozygous null for *GATA2* or *GATA3* are lethal at embryonic days 10.5 and 11.5, respectively. Mortality was primarily attributed to several hematopoietic abnormalities not directly related to the placental defects (Pandolfi et al. 1995; Tsai et al. 1994). This suggests *GATA2* and *GATA3* are not directly necessary for trophectoderm specification and early development. However, a hypothesized compensating effect by *GATA2* in *GATA3* knockout mice or the reciprocal has not been eliminated (Home et al. 2009).

As development continues, the outermost trophoblasts differentiate and become more specialized. In most mammals, this differentiation event stimulates formation of binucleated or giant cells. Ruminants, equids, and humans develop binucleated trophoblasts which primarily arise from endoreplication or the fusion of two trophoblast

cells. Giant cells typically maintain a single nucleus with amplified DNA content resulting from endoreplication and occur in rodents and rabbits. Binucleated and giant cells exhibit migratory or invasive properties, facilitate fusion of the fetal-maternal interface and produce proteins and hormones necessary for successful pregnancy (Hoffman and Wooding 1993). The formation of binucleated and giant cells is dependent on HAND1, a member of the basic helix-loop-helix transcription factor family, and GCM1 (Anson-Cartwright et al. 2000; Riley et al. 1998). HAND1 and GCM1 are able to arrest cell proliferation and promote differentiation in the presence of ICM derived FGF4 (Hughes et al. 2004). In horses, *HAND1* and *GCM1* are expressed at 21-25 days post-ovulation, a time point when expression levels of *CDX2* are decreasing (de Mestre et al. 2009). There is evidence for direct regulation of *HAND1* by *CDX2* in the bovine CT1 trophoblast cell line (Schiffmacher and Keefer 2013) and of *GCM1* by *ETS2* in mice (Odiatis and Georgiades 2010).

Early equine embryonic development

The morula to blastocyst transition is less pronounced in horses than in cattle or swine as the equine ICM is more dispersed (Betteridge 2007). The conceptus enters the uterus around day 6 post-ovulation. Days 6 through 10 post-ovulation are defined by rapid growth as the spherical conceptus moves freely throughout the uterine lumen (Ginther 1983). The outer layer of cells surrounding the conceptus form trophoblasts which are associated with desmosome and gap junction formation between cells (Klonisch et al. 1997). A characteristic of the mare not found in most mammals is the formation of an acellular capsule around the conceptus. Approximately day 6.5 after ovulation, the trophoblast cells synthesize a glycoprotein membrane deposited between

the developing TE and the zona pellucida (Oriol et al. 1993a). The capsule is comprised of many different proteins, but some that have been specifically identified and shown to be up-regulated during the early stages of biosynthesis are *GNE*, *NEU1*, *SLC17A5*, *SLC35A1*, and *TGM3* (Table1-1) (Iqbal et al. 2014). The capsule protects the conceptus and facilitates movement throughout the uterine lumen necessary to maintain pregnancy (Betteridge 2007).

At 9 days post-ovulation, the blastocyst hatches and shortly thereafter, between days 9 and 14, releases an unknown factor, or factors, responsible for the maternal recognition of pregnancy (Klein and Troedsson 2011). By day 13 post-ovulation, the trophoblasts are cuboidal-columnar shaped and immunostain more intensely for the intermediate filament cytokeratin relative to the embryo proper (Gaivao et al. 2014). In horses, gastrulation begins approximately 12 days post ovulation, somites are first observed at day 16, and establishment of the embryo proper is complete by 18 days post ovulation (Gaivao et al. 2014). Intrauterine mobility of the embryo ceased around day 18 due to the increasing size of the conceptus fixing the embryo in place (Ginther 1983). The cells comprising the TE further differentiate to form two layers of cells termed allantochorion trophoblasts and chorionic girdle cells. Allantochorion trophoblasts make up the inner layer and are considered the trophoblastic stem cells that undergo further differentiation to form specialized binucleated chorionic girdle cells approximately 40 days post ovulation (Noronha and Antczak 2010; Wooding et al. 2001).

Experimental objectives and hypothesis

Objective: Embryonic stem cells have the potential to provide greater efficacy in treating equine sport related injuries than mesenchymal stromal cell populations. However, no stable equine ES cell lines have been successfully isolated to date. The OSKM Yamanaka factors can induce pluripotency in somatic cells reprogramming them to an ES-like state. All attempts to induce pluripotency to date in horses have been clinically irrelevant due to integration of viral genetic material. The objective of this dissertation was to generate protocols for the derivation of integration free induced pluripotent stem cells from equine mesenchymal stromal cells. This includes providing evidence of induction of pluripotency, absence of integrated viral genetic material, and the ability to be differentiated into tenocytes capable of contribution to compromised tissues.

Hypothesis: Lineage restricted umbilical cord matrix mesenchymal stromal cells would more readily reprogram by exposure to the Yamanaka reprogramming factors over less plastic cell populations, such as skin fibroblasts, to a pluripotent state and would provide an ideal starting population for proof of concept studies.

Table 1-1. Putative function of genes related to pluripotency, the inner cell mass, and the trophectoderm, and equine capsule biosynthesis

Pluripotency	Inner cell mass/ Epiblast	trophectoderm	Capsule biosynthesis
CDH1	DNMT3β	CDX2	GNE
DPPA4	NANOG	DHRS9	NEU1
HESX1	REX1	ELF3	SLC17A5
KLF4	OCT4	EOMES	SLC35A1
LIN28	SOX2	ETS2	TGM3
MYC	SSEA1	GATA2	
SALL4	SSEA3	GATA3	
TCL1A	SSEA4	HAND1	
	TRA-1-60	HEXIM2	
	TRA-1-81	OCT4	
	UTF1	TEAD4	
		TFAP2A	

CHAPTER II

Generation of induced trophoblast cells from equine mesenchymal stromal cells with the Yamanaka reprogramming factors

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Running Head: Induced equine trophoblast cells

Author disclosure: Brad M. Reinholt, Jennifer S. Bradley, William C. Golden, Robert D. Jacobs, Alan D. Ealy, and Sally E. Johnson have no conflicts of interest.

Introduction

Conversion of somatic cells to a pluripotent embryonic stem (ES)-like cell is accomplished by ectopic expression of the transcription factors *OCT4*, *SOX2*, *KLF4*, and *MYC* (Takahashi and Yamanaka 2006). The reprogrammed cells, conventionally referred to as induced pluripotent stem (iPS) cells, are capable of differentiation into any of the three germ layers (ectoderm, mesoderm, or endoderm) (Okita et al. 2007) and some extraembryonic tissues (Bai et al. 2012). The ES-like cells provide tremendous opportunities for cytotherapies in both humans and animals that are currently reliant on mesenchymal stromal cells (MSC), a less plastic cell population. Additional concerns exist over potential immunorejection by the recipient of allogeneic MSC therapy. The iPS cells may overcome these barriers by provision of an autologous source of ES-like cells. Human and pig iPS cells generated by reprogramming fibroblasts and myeloid cells have been directionally differentiated into retinal ganglion cells (Zhong et al. 2014; Zhou et al. 2011). This technology is approaching clinical relevance in humans. However, clinically applicable iPS cell generation and directed differentiation systems are not yet available for the horse.

Musculoskeletal injuries are a serious concern for the equine industry. Over 30% of racing injuries affect the tendon specifically (Goodship et al. 1994). Recovery can last one year or more with frequent reoccurrence after return to work (Dyson 2004). Delivery of bone marrow (BM)-derived and adipose tissue (AT)-derived MSCs into tendon, ligament and bone lesions demonstrates mixed success. Injection of BM-MSC into sites of tendon damage offers few advantages in repair rates, but does lessen scar-tissue formation and promote a mature fiber crimp pattern that offers protection from reinjures

(Smith et al. 2013). By contrast, others report no improved structural integrity following injection into collagenase-induced tendon lesions (Nixon et al. 2008). The discrepant results point to variable damage models, a heterogeneous MSC population, inconsistent cell cultivation practices, and the poor engraftment capabilities of the donor MSCs (Guest et al. 2010; Vidal et al. 2006). A more plastic donor cell, such as fetal-derived induced pluripotent cells, may alleviate some of these obstacles to therapeutic success (Watts et al. 2011).

The first generation iPS cells in human and mouse cells used retroviral delivery systems to transduce somatic cells. These systems can integrate viral genetic material into the genome of the host cell. Recent advances have developed integration free iPS cells, thus making clinical studies possible. Several groups report derivation of putative equine iPS cells (Breton et al. 2013; Nagy et al. 2011). Past attempts used a transposon-based vector system to successfully reprogram equine fetal fibroblasts (Nagy et al. 2011). When transgene expression was shutdown, the iPS cells readily underwent differentiation. Brenton and colleagues (2013) established equine iPS cells from fetal and adult fibroblasts that could be propagated long-term and differentiate into the three germ layers. The authors reported incomplete silencing of the transgenes indicating the prolonged pluripotent state is likely driven by the exogenous reprogramming factors. These data suggest that sustained transgene expression is required to maintain the pluripotent state. Investigators have demonstrated supplementation with leukemia inhibitory factor (LIF) can help maintain iPS cell cultures by preventing differentiation (Hirai et al. 2011). In bovine, additional transgenes such as

LIN28 and *NANOG* are necessary for induction of ES cell-like characteristics (Han et al. 2011; Sumer et al. 2011).

Ezashi et al. (2011) reported that porcine iPS cells contained a subpopulation that displayed features similar to trophectoderm (TE) cells. The induced trophoblasts (iTr) exhibited a flat, epithelial-like morphology with a granular cytoplasm and the iTr cells formed dome-shaped structures in culture. When cultured on a non-adherent surface, the iTr cells formed hollow spheres consisting of a single layer of cells with a blastocoele-like cavity. In addition to phenotypic resemblance to porcine trophoblasts, the iTr cells expressed transcription factors associated with TE lineage and differentiation including *CDX2*, *EOMES*, *ETS2, GATA2*, *GATA3*, *HAND1*, *TEAD4*, and *TFAP2C.* This discovery provides evidence that ectopic expression of OSKM in fibroblasts can generate cells with trophoblast-like characteristics.

This report provides evidence for the generation of iTr cells from equine MSC transduced with Sendai-expressed OSKM. Initial observations indicated ES cell-like features. However, thorough analysis revealed the reprogrammed cells possessed similarities to trophoblast cells including the expression of several genes specific to the TE developmental lineage. These reprogrammed cells fail to form embryoid bodies in non-adherent culture surfaces, but readily form spherical and outgrowth structures similar to equine blastocysts. With limited *in vitro* systems to model TE in equine, these cells provide a new tool to elucidate mechanisms and properties unique to early equine TE development during a defined developmental window.

Materials and methods

Estrous synchronization and embryo recovery

Four non-pregnant light-horse mares were used for this study. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. Mares were maintained on a single pasture and remained on pasture throughout the breeding and collection period. Mares were evaluated by transrectal ultrasonography daily to map follicular development and determine stage of estrous cycle. Examinations were conducted once daily until a 35 mm follicle was observed along with appropriate edema. Once a follicle of this size was observed a single dose (1 mL) of deslorelin acetate (SucroMate, BioNiche Animal Health, Louisville, KY) was administered via intramuscular injection. Mares were bred to a single stallion of known fertility (500 x 10^6 motile spermatozoa) 24 hours following deslorelin injection. At the time of insemination, mares were evaluated via transrectal ultrasound to determine ovulation status. Mares that had failed to ovulate were bred again approximately 40 hours post deslorelin injection.

Approximately 7 or 12 days post-ovulation, embryos were collected. Mares were sedated with IV xylazine (0.6 mg/kg). The vulva and surrounding areas were thoroughly cleansed with soap and warm water in order to prevent contamination of the reproductive tract. A sterile Foley catheter (36 French diameter) was inserted transcervically and secured in the uterus by inflation of the cuff. The uterus of the mare was flushed a minimum of three times with 1 L of warmed flush media (BioLife Advantage Complete Flush Medium, AgTech Inc., Manhattan, KS). Prior to the final flush, mares were administered a single intravenous dose (20 IU) of oxytocin to ensure

optimal fluid removal from the uterus. The outflow from the catheter was connected to an in-line embryo filter (AgTech Inc., Manhattan, KS). The filter was washed with collection medium and the flushings were examined using a stereoscopic microscope. All embryos were rinsed, snap frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Cell culture and transduction

Equine umbilical cord matrix mesenchymal stromal cells (MSC) were isolated by collagenase digestion of Wharton's jelly and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin supplemented with 4 ng/mL recombinant human fibroblast growth factor 2 (FGF2). Human foreskin fibroblasts (BJ, ATCC, Manassas, VA) and mouse embryonic fibroblasts (MEF; SNL76/7 expressing human LIF, ATCC) were cultured in growth media (GM) comprised of DMEM supplemented with 10% FBS, and 1% penicillin-streptomycin at 37°C and 5% CO_2. MEFs were mitotically inactivated by incubation with mitomycin C (10 µg/mL) in GM for four hours. Equine MSCs and human fibroblasts were transduced with Sendai virus encoding for human *OCT4*, *SOX2*, *KLF4*, and *MYC* at an MOI of 12, according to the manufacturer's directions (Cytotune, Invitrogen). After 48 hours, the media containing the Sendai viral reprogramming factors was removed and discarded. Seven days after initial exposure to Sendai viral reprogramming factors the MSCs and fibroblasts were enzymatically passaged with TypLE™ (Life Technologies) onto mitotically inactivated MEF cells and cultured in DMEM/F-12+GlutaMAX medium supplemented with 20% knockout serum replacement, 1% penicillin-streptomycin, 1% non-essential amino acids (NEAA), 0.1 mM β-

mercaptoethanol, recombinant human LIF (10 ng/mL, Millipore, Billerica, MA) and FGF2 (4 ng/mL). This media will be referred to as reprogramming media. Colonies were passaged (1:3) by physical dissociation with a tungsten microdissection scalpel (Fine Science Tools, Foster City CA). Human cells were maintained in the reprogramming media for the entirety of the experiment. After 25 passages, equine cells were maintained in a feeder-free system in DMEM supplemented with 10% FBS, 1% NEAA, 0.1 mM β–mercaptoethanol and 1% penicillin-streptomycin on Matrigel™ (BD Biosciences, Franklin, NJ) coated tissue cultureware.

Sphere formation and trophectoderm outgrowths

Equine iTr colonies were dissociated from the MEF feeder layer with L7™ hPSC Passaging Solution (Lonza, Walkersville, MD), or physically scraped from Matrigel coated petri dishes, and passed through a 20 G needle twice to generate a single cell suspension. The cell suspension was passed through a 100 µm Falcon™ cell strainer (Fisher Scientific, Hampton, NH) to remove cell aggregates and seeded in ultra-low attachment tissue culture plates (Corning, Corning, NY) in growth media. After three to seven days, cell spheroid structures had formed and were visualized on an EVOS® cell imaging system (Life Technologies). Spheroids or day seven equine blastocysts were transferred to Matrigel™ coated tissue cultureware and allowed to attach and form adherent outgrowths.

RNA isolation and PCR

Total RNA was isolated from iTr, MSC and day 12 equine embryos using TRIzol reagent (Life Technologies). The RNA was purified with PureLink® RNA mini kit

according to manufacturer's directions (Life Technologies). Isolation of RNA from day 7 equine embryos and iTr spheres was performed with the ARCTURUS® PicoPure® RNA isolation kit (Life Technologies), according to manufacture's recommendations. Purity and quantity of RNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA (50 ng) was treated with 2 units of amplification grade DNAse I and first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit, according to the manufacturer's directions. End-point amplification was performed with GoTaq® Green Master Mix (M712, Promega, Finchburg, WI) with 10 pmols of each equine (Appendix A), human (Appendix B) or Sendai(Appendix C) gene specific forward and reverse primers and cDNA template. End-point cycle parameters were 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Reactions were terminated by a 72°C step for five minutes and stored at 4°C. The PCR amplicons were resolved on 2% agarose gels containing SYBR® Safe DNA Gel Stain (1:10,000) and visualized with UV light on a Gel Doc™ (Bio-Rad, Hercules, CA). Real-time PCR amplification was carried out with Power SYBR® Master Mix with 10 pmols of gene specific forward and reverse primers. Real-time cycle parameters were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds. All PCR reactions were performed on an eppendorf realplex^2 mastercycler epgradient PCR machine. Relative expression level of target genes between treatments was calculated using $2^{-\Delta\Delta Ct}$ method, defined as: $\Delta\Delta Ct = (\Delta Ct^{spheres}) - (\Delta Ct^{monolayer})$.

Immunocytochemistry

Live cell imaging was performed by adding anti-SSEA4 and anti-TRA1-81 (Life Technologies) to culture media at a final concentration of 5 μg/mL and incubating for one hour at 37° C. After washing three times in warmed media, cells were incubated with goat anti-mouse AlexaFluor488 (1:200, Invitrogen) and goat anti-mouse AlexaFluor568 (1:200, Invitrogen) for SSEA4 and TRA1-81 visualization, respectively. Equine cells fixed with 4% (vol/vol) formaldehyde (Polyscience, Inc. Warrington, PA) in PBS for 10 min at room temperature, followed by washing with PBS three times. Non-specific antigen sites were blocked with 5% FBS in PBS containing 0.1% (vol/vol) Triton-X100 for 30 minutes at room temperature. Fixed cells were incubated with anti-CDX2 (1:500, LifeSpan BioScience, Inc., Seattle WA) and anti-OCT4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS containing 0.5% FBS for one hour at room temperature. After washing three times in PBS, fixed cells were incubated with goat anti-mouse AlexaFluor568 (1:200, Invitrogen) and goat anti-mouse AlexaFluor488 (1:200, Invitrogen) for CDX2 and OCT4 visualization, respectively. Nuclei were visualized with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI, 5 μg/mL) for nuclear counterstain. Immune complexes were visualized by epifluorescence with a Nikon Eclipse TS100 (Nikon Imaging Inc. Melville, NY). Representative images were captured with a Nikon eclipse TS100 (Eclipse Ti microscope system) (Nikon Imaging Inc. Melville, NY) connected to a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) and digitized with NIS-Elements AR Ver4.13.00 software (Nikon). Magnifications are indicated in the figure legends.

Statistical analysis

Data were analyzed using ANOVA followed by *t*-test pairwise comparison using JMP Pro 11 software (SAS Institute, Inc., Cary, NC). A difference was considered statistically significant when $P < 0.05$. Data are presented as means ± standard error of the mean (SEM).

Results

Transduction, colony viability, and phenotypic characterization of equine and human cells

Conventional creation of iPS cells is achieved by viral-mediated ectopic expression of *OCT4*, *SOX2*, *KLF4* and *MYC* (OSKM). The presence of viral DNA sequences prevents their cytotherapeutic utility in a clinical setting. To overcome this barrier, equine multipotent MSCs were transduced with non-integrating Sendai virus capable of expressing human *OSKM*. Transduction of human fibroblasts served as a positive control. Seven days post-transduction, the cells were passed on to mitotically inactive MEF feeder cells. After 7 days on the MEF layer visible dome-shaped colonies of cells with a high nuclear:cytoplasmic volume and distinct exterior edges were observed (Fig. 2-1 Phase contrast). The colonies were similar in morphology to human iPS cells and expressed the cell surface markers, SSEA-4 and TRA-1-81 (Fig. 2-1). All visible colonies were picked for serial passage (P) after 14 and 21 days post transduction. Six of 11 initial colonies (identified as individual clones) that formed during the first week of MEF culture remained viable after P5 with one surviving several passages beyond P15. Nine of 10 individual colonies isolated 21 days post-transduction remained viable up to P5. Four of the 10 survived through P10 and only three (2C, 5C,

7C) remained viable beyond P20 and able to be archived (Fig. 2-2). No additional colonies formed during the third week of culture or beyond on MEF cells.

Expression analysis of transgenes and endogenous markers of pluripotency

The four equine clones were analyzed for expression of exogenous reprogramming factors and the endogenous homologs by RT-PCR (Fig. 2-3A). Viral expression of human *OCT4* and *SOX2* was extinguished in all equine ES-like cells (Fig. 2-3A). Exogenous *KLF4* was detected in clones 2C and 5C with endogenous *KLF4* detected in all equine lines. Residual expression of exogenous *MYC* remains in the equine ES-like cells through P50 (data not shown). Endogenous expression of *OCT4*, *KLF4*, and *MYC* were detected in all reprogrammed equine cell lines with only 4B and 7C expression endogenous *SOX2*.

A total of 10 ES-like human lines were isolated and analyzed for expression of ectopic and endogenous expression of the reprogramming factors by RT-PCR (Fig. 2-3B). All exogenous *OCT4*, *SOX2*, and *KLF4* expression was shutdown with the exception of low abundance detection of exogenous *OCT4* in line 4. These data are consistent with the elimination rate of Sendia virus mediated transgenes (Macarthur et al. 2012b). Exogenous *MYC* remained detectable in all reprogrammed human lines through P10. Endogenous *OCT4*, *SOX2*, and *MYC* expression was detected in each reprogrammed human line. Endogenous *KLF4* expression was varied with detection in half of the human lines (4,7,11,20,42) and absence of *KLF4* expression in the remaining half (10,27,29,37,43) (Fig. 2-3B).

The transcription factor *NANOG* is critical for ES cell maintenance. Equine and human reprogrammed ES-like cells were analyzed for species specific endogenous

NANOG expression after transduction (Fig. 2-3C). The equine ES-like cell lines lacked *NANOG* expression. Only two human ES-like cell lines (4 and 43) failed to express *NANOG* at a similar passage number compared to the equine cells.

The genotypes of the putative equine iPS cells were examined by RT-PCR amplification of genes associated with pluripotency. As shown in Figure 2-3D, the starting MSCs were absent for all but two genes in a panel of factors selected based off of their expression pattern in ES cells. The exceptions were *LIN28* and *TLC1A. E*xpression of *CDH1*, *DPPA4*, *HESX1*, *LIN28*, *SALL4,* and *TCL1A* were present in both the putative equine iPS cells and an equine embryo seven days post-ovulation (ED12). The reprogrammed equine cells expressed low amounts of *REX1*.

These results indicate that the MSC do not express a stable ES-like genotype although several ES cell associated genes are detected. Due to the costs associated with iPS maintenance, one equine clone (7C) was selected for continued characterization.

Markers of trophectoderm lineage

Closer examination of the colonies revealed a flat, cuboidal-shaped cell was present around the central cluster of small ES-like cells with phenotypic resemblance to a trophoblast cell. Markers of TE lineage were analyzed by RT-PCR. Figure 2-4A shows the gene expression pattern of a representative equine cell line for the transcription factors *OCT4*, *CDX2*, *TFAP2A*, and *EOMES*. The starting MSC population was positive for only *OCT4*. The reprogrammed cells were positive for *OCT4*, *CDX2*, and *TFAP2A*, but not *EOMES*. Fixed colonies were immunostained for CDX2 and OCT4 to confirm expression at the protein level (Fig 2-4B). Both proteins were evident in the nuclei of

cells. Colocalization of OCT4 and CDX2 was evident in a population of the cells but not all. Subpopulations exhibiting high immunofluorescent intensity of OCT4, CDX2 and both proteins are evident. The morphology and gene expression pattern led to identifying these cells as equine induced trophoblast (iTr) cells. These results indicate that the iTr cells exhibit asynchronous expression of the TE marker proteins which may lead to developmental heterogeneity within the population consisting of trophoblasts that are more advanced in their differentiation along the TE lineage.

Cellular arrangement and sphere formation

Cell culture conditions were adjusted to standard TE cell line culture conditions at approximately P25. Growth factors that promote pluripotency were gradually reduced to zero without a decrease in number of cells that transition through the S-phase of mitosis (Fig. 2-5). Additionally, iTr cells were transitioned to feeder-free culture conditions by plating on Matrigel coated cultureware (Fig. 2-6). The culture of single cell suspensions of iTr cells on non-adherent cultureware promotes the formation of sphere-shaped vesicles. Spheres can be observed by the third day of culture in suspension. Sphere formation media conditions were optimized to generate the most spheres with a 72 hour test in 24 well ultra-low attachment cultureware. Low serum content of 5% resulted in the fewest ($P < 0.01$) spheres formed per well. Knockout serum replacement at a 20% concentration formed fewer spheres ($P < 0.05$) than the 10% serum content (Fig. 2-7). However, media containing 15% serum and 30% knockout serum replacement were not different ($P = 0.073$) (Fig. 2-7). These results show 10% serum content yielded the most spheres and was used in the base sphere formation media.

After seven days of culture in suspension the iTr spheres bear morphological and size similarities to equine embryos with the exception that the iTr spheres lack the prominent ICM structure evident in the equine embryo 7 days post-ovulation (ED12) (Fig. 2-8A, B). Similar to the monolayers, the iTr spheres stain positive for OCT4 protein in some, but not all, cells (Fig. 2-8C, D arrow heads). The iTr spheres attach to extracellular matrix coated cultureware and form outgrowths indistinguishable from an ED7 blastocyst (Fig. 2-8E, F). Expression of TE lineage markers and the ability to form structures reminiscent of native blastocysts provides evidence for creation of a stable equine TE cell line. Sphere organization implies that the iTr cells retain a memory for early tissue morphogenesis.

The impact of cellular arrangement and blastocyst-like configuration on TE gene expression patterns was examined in iTr cells maintained as monolayers or spheres by quantitative RT-PCR. No differences were noted in expression of the definitive TE markers, *CDX2*, *ETS2, TEAD4,* and *TFAP2A* or potentially important equine TE genes *DHRS9,* and *HEXIM2* (Fig. 2-9A). The abundance of *GATA2* transcripts was greater ($P < 0.05$) in monolayers than spheres (Fig. 2-9A).

The equine embryo is unique compared to most domesticated livestock species in that it builds an acellular glycoprotein capsule between the zona pellucida and the TE. Proteins that comprise the capsule are secreted by the early trophoblast cells. Expression of sialic acid and carbohydrate transporters (*SLC35A1*, *SLC17A5*) and enzymes involved in the biosynthesis of sialic acid (*GNE*, *NEU1*) were unaffected by culture system (Fig. 2-9B). By contrast, a large reduction ($P < 0.001$) in expression of the *transglutaminase 3*, (*TGM3*) was apparent in iTr spheres by comparison to their

monolayer counterparts. These results suggest that culture as a sphere may promote developmental progression of the TE. Expression of *EOMES*, *GATA3,* and *HAND1* was examined in ED7 and ED12 embryos, and iTr spheres and monolayers (Fig. 2-9C). Expression of *EOMES* was absent in RNA isolates from ED7 embryos but present at ED12 (Fig. 2-9C). Expression of *EOMES* and *GATA3* was stimulated by culture of iTr cells as free-floating spheres. In a similar manner, *HAND1* mRNA abundance was relatively greater in iTr spheres than monolayers. These results provide evidence that tissue morphogenesis promotes developmental differentiation of iTr.

Discussion

Equine trophoblast cell lines were developed through immortalization of chorionic girdle cells isolated from a 33 day conceptus (Thway et al. 2001). Studies on the differentiation and invasion of equine trophoblasts after day 17 post-ovulation have also been performed (Cabrera-Sharp et al. 2014a; de Mestre et al. 2009). However, tools for investigating early equine TE development are lacking. We have derived stable equine induced trophoblast (iTr) cells that express markers of early TE and readily form blastocyst-like spheres that resemble early equine embryos.

Umbilical cord matrix MSC were chosen for this study because they hold a high degree of plasticity and would theoretically be more readily reprogrammed to a pluripotent state. This was based off of the endogenous *OCT4* and *LIN28* expression in this population of cells. However, *OCT4* is not specific to the ICM in the early conceptus in many mammals. It is also expressed in the early TE of many species including bovine, swine, and humans (Hansis et al. 2000; Kirchhof et al. 2000), but not in mice (Palmieri et al. 1994). The TE lineage is specified by the expression of the transcription

factor *CDX2* which activates down-stream genes that regulate TE differentiation and function (Beck et al. 1995). The CDX2 and OCT4 proteins form a repressor complex and the stoichiometric balance of each factor dictates cell fate to the ICM or the TE of the early conceptus (Chawengsaksophak et al. 2004). The micro-RNA binding protein *LIN28* and the transcription factor *NANOG* can replace *KLF4* and *MYC* in conjunction with *OCT4* and *SOX2* in reprogramming human somatic cells (Yu et al. 2007). However, *LIN28* and *NANOG* are not necessary for reprogramming, but function as reprogramming enhancers improving the number of iPS cell colony formation (Yu et al. 2007). Specifically, *LIN28* stimulates cell proliferation by up-regulating the cell cycle genes *Cyclin A* and *Cyclin B* (Xu et al. 2009). The expression of *OCT4* and *LIN28* in the MSC starting population offers no advantages to iPS formation.

Exogenous *OSK* expression stimulates endogenous *NANOG* expression (MacArthur et al. 2012a), The apparent function of *NANOG* is to counteract differentiation and specifies the pluripotent epiblast (Silva et al. 2009). Expression of *NANOG* was not sustained in any of the equine cell lines described in this report. However, *NANOG* expression was detected in 8 of 10 human ES-like cell lines. The lack of *NANOG* is a likely factor that prevented complete reprogramming or, more probable, resulted in an unstable pluripotent phase. The positive immunostaining for the cell surface markers SSEA4 and TRA-1-81 are typical indicators of ES-like cells. However, SSEA4 and TRA-1-81 also are present on the surface of equine trophoblast cells (Guest and Allen 2007). This indicates that SSEA4 and TRA-1-81 are not unique to an ES-like cell. This supports the observations of Abujarour and colleagues (2013)

that SSEA4 and TRA-1-81 are poor indicators of true iPS cells and do not highly correlate to *NANOG* expression.

Unique to the equine iTr cells is activation of the *CDX2* and *TFAP2A* genes, which were absent from the starting population. A network of repression and activation exists between the *CDX2*, *EOMES, OCT4*, and *NANOG* genes. Enhanced expression of the TE markers *CDX2* and *EOMES* leads to rapid down regulation of *NANOG* within 24 hours (Chen et al. 2009). In contrast, *OCT4* expression levels decline slowly over a 48 hour period. These observations were detected at both the transcript and protein level in the mouse (Chen et al. 2009). The authors reported reciprocal results with *NANOG* over-expression which resulted in diminished *CDX2* and *EOMES* expression. This cross regulation is attributed to reciprocal DNA–protein binding motifs in both the promotor and coding regions of *NANOG*, *OCT4*, *CDX2*, and *EOMES*. Binding sites for NANOG are found in the regulatory regions of both *CDX2* and *EOMES*. Binding sites for OCT4 have also been identified in the CDX2 regulatory regions. Conversely, the CDX2 protein can bind to the *NANOG* promoter approximately 5.5 kb upstream of the transcription start site. Each of these interactions has been verified by CHIP assay (Chen et al. 2009). However, the mechanism underlying the timing and abundance of expression in specific cells of *OCT4*, *CDX2*, *NANOG*, and *EOMES* that drives them toward a specified cell fate remain elusive.

Transcription factor activating (enhancer binding) protein -2 alpha (TFAP2A) is preferentially expressed in the TE of early equine blastocysts (Iqbal et al. 2014; Kuckenberg et al. 2012). Multiple groups have provided evidence that *TFAP2A* and *CDX2* can stimulate reciprocal expression of one another. Evidence exists for

repression of both *OCT4* and *NANOG,* by TFAP2A, but *NANOG* does not regulate *TFAP2A* expression (Berg et al. 2011; Kuckenberg et al. 2012). Activation of *TFAP2A* during the reprogramming event might have an inhibitory effect on *NANOG* expression resulting in a developmental lineage shunted towards TE.

When the iTr cells are dissociated and cultured on non-adherent cultureware they form spherical structures resembling an equine blastocyst lacking an ICM. When the iTr cells are cultured in a monolayer configuration, they alter their gene expression profile. The monolayers increase expression of *GATA2*. The iTr spheres begin expressing *EOMES* and *GATA3* which are absent in the iTr monolayers. The GATA family of transcription factors bind to consensus GATA motifs found upstream of many genes including genes associated with giant cell formation in rodents (Merika and Orkin 1993; Ray et al. 2009). Thus, the increase of *GATA2* in the monolayer was contrary to our expectations. Bai and colleagues (2011) reported *GATA2* binding sites upstream of *CDX2.* Overexpression of *GATA2* stimulated *CDX2* expression in bovine. In the same set of experiments, overexpression of *GATA3* did not increase *CDX2* expression. These data suggest *GATA2* is an important regulator of early trophoblast development by regulating *CDX2*. Day 7 equine blastocysts are devoid of transcripts for the T-box transcription factor *EOMES*. However, by day 12 low abundance *EOMES* transcripts are detected. This expression pattern is mirrored in the iTr monolayer versus the spheres. Studies suggest *EOMES* plays a critical role in trophoblast differentiation, but not in lineage specification. Mice homozygous null for *EOMES* arrest development at the preimplantation blastocyst stage and display a block in the development of TE, but not in establishing the TE lineage (Russ et al. 2000). In ovine and bovine, the greatest

EOMES expression was reported at days 17 and 22 post-ovulation, respectively (Sakurai et al. 2013). Soon after, *EOMES* expression declines. These peaks appear at a time when *CDX2* expression is decreasing in both ovine and bovine. Likewise in the horse, *EOMES* transcript expression is higher at day 21 post-ovulation with reduced abundance before and after (de Mestre et al. 2009). This suggests equids appear to follow a trend of delayed *EOMES* expression relative to lineage markers *CDX2* and *TFAP2A*. Trophoblast giant cells are the first cells of the trophoblast lineage to terminally differentiate. Trophoblast giant cells function, in part, to establish the fetal-maternal interface between the placenta and the endometrium (Hu and Cross 2010). In many species, *HAND1* is directly involved in terminal differentiation of trophoblast cells into giant cells. Suasnavas and colleagues (2015) demonstrated an increase in *HAND1* transcripts in porcine trophoblast cells cultured in conditions that decrease proliferation and induce senescence. Similarly, when equine iTr cells form a spherical structure, they exhibit a subtle increase in *HAND1* transcripts compared to the monolayer conformation. The activation of *EOMES* and *GATA3* along with the increased *HAND1* expression, coupled with the decrease in *GATA2* expression, suggests the spherical cellular arrangement promotes specialized TE development. Comparison of the equine ED7 and ED12 blastocysts to the equine iTr cells suggest the iTr cells recapitulate a defined window in early equine TE development between 7 and 12 days post-ovulation.

The acellular glycocalyx capsule produced by the trophoblast cells provides protection for the mobile equine conceptus. Several gene transcripts are expressed in the day 8 post-ovulation equine conceptus including several sialic acid transporters and transglutaminases (Iqbal et al. 2014; Klein and Troedsson 2011). Among those

evaluated in this study, only *TGM3* was different between the sphere and monolayer conformation with expression down regulated in the spheres. This was contrary to expectations where an increase in genes associated with capsule biosynthesis was expected. The conceptus must be in the uterine environment to form a functional capsule as *in vitro* produced embryos secrete capsular proteins, but fail to form a functional capsule (Tremoleda et al. 2003). Expression of these genes suggests the iTr cells possess the capacity to synthesize the proteins that comprise the capsule. Alternate approaches such as transferring the spheres into a non-pregnant mare or co-culture with endometrial cells may yield a functional capsule.

This report establishes the conversion of equine umbilical cord matrix MSC to a TE lineage using the OSKM reprogramming factors. The iTr cells express a gene profile similar to early trophoblast cells and readily form cell spheres rather than embryoid bodies and have been propagated for over 50 passages. It is unclear whether the iTr cells first passed through a transient pluripotent phase or were directly reprogrammed to the TE lineage. Periodic detection of *NANOG* transcripts during the early passages does suggest that reprogramming was achieved, but not maintained under current culture conditions. The culture conditions during reprogramming were those optimized for human and mouse iPS cell generations. The Sendai viral deliver system eliminates transgene expression within 15 passages. True to the design, exogenous *OSK* were largely extinguished by P15 with the exception of *MYC* which remains through P50. Prior equine iPS cell generation used delivery systems that integrated or had extended transgene expression (Breton et al. 2013; Nagy et al. 2011). Therefore the abbreviated

OSK expression offered by the Sendai system may not be adequate to fully reprogram equine MSCs.

Figure 2-1.

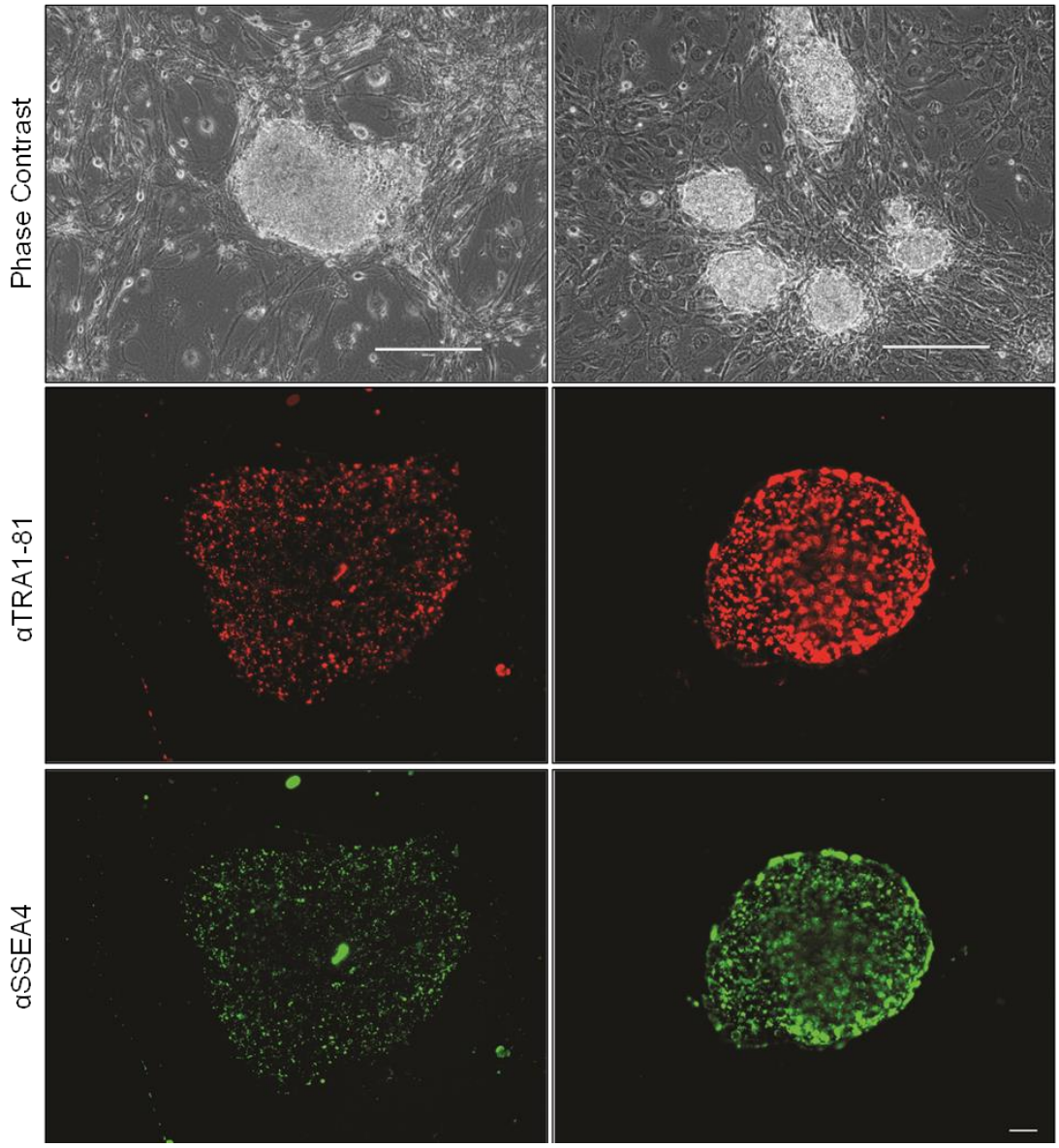

Figure 2-1. Morphological analysis of transduced human and equine cells
Representative phase contrast photomicrographs of initial equine (left column) and human (right column) colonies. Epifluorescent images of the live cell immunostaining for TRA-1-81 and SSEA4 in equine (left column) and human (right column). Scale bars equal 400 µm (top row) or 100 µm (color panels).

Figure 2-2.

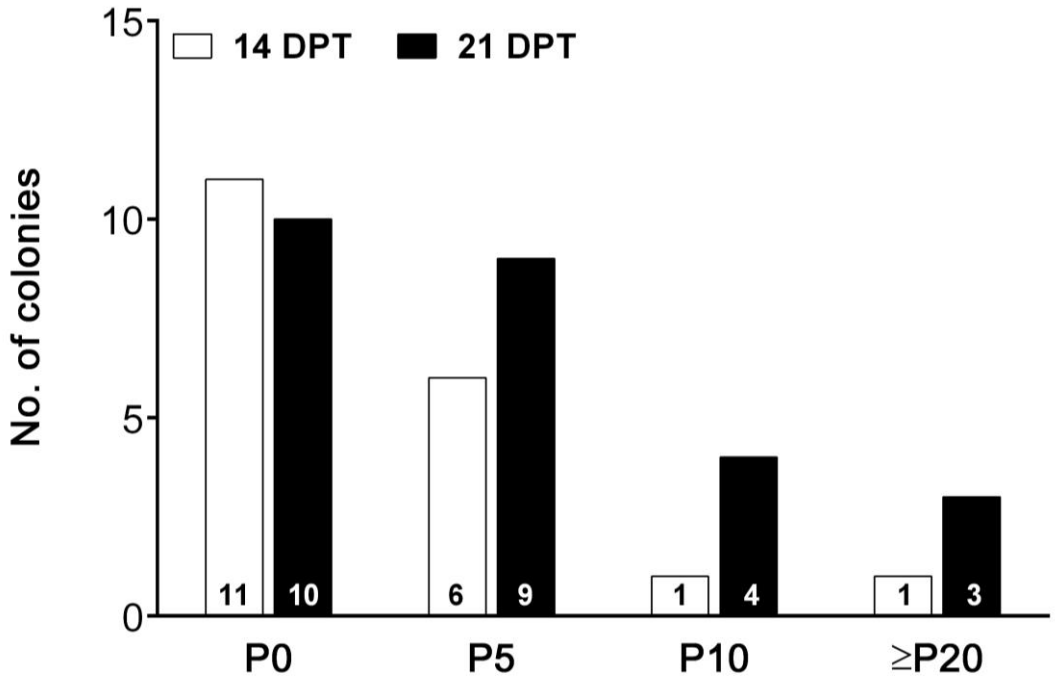

Figure 2-2. Number of reprogrammed equine clones remaining over serial passages. Reprogrammed equine colonies were manually isolated at 14 or 21 days post-transduction (DPT) and passaged (P) approximately every seventh day. The number of surviving colonies identified as individual clones is shown inside the bars.

Figure 2-3.

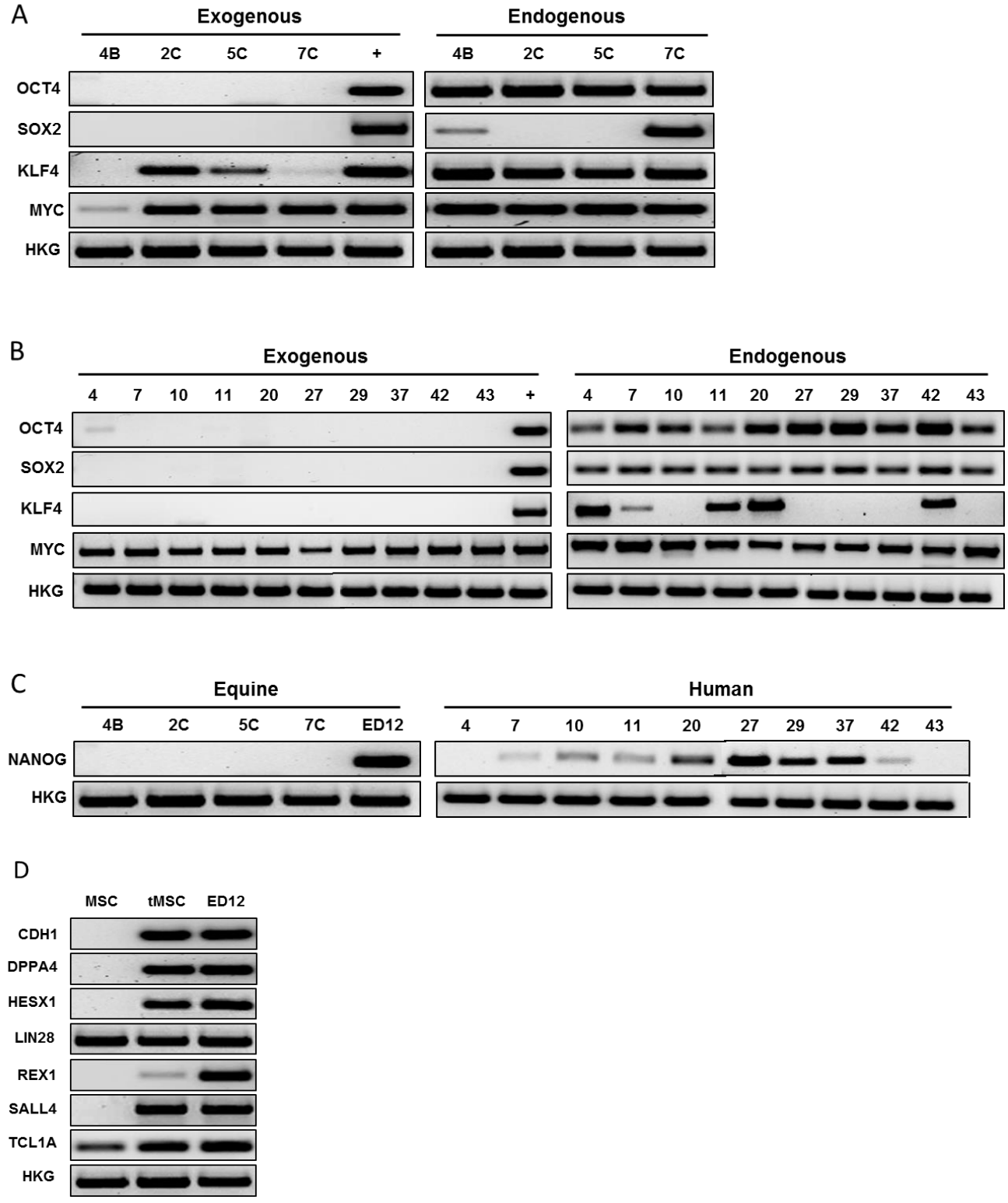
A
Exogenous
Endogenous
4B 2C 5C 7C +
4B 2C 5C 7C
OCT4
SOX2
KLF4
MYC
HKG
B
Exogenous
Endogenous
4 7 10 11 20 27 29 37 42 43 +
4 7 10 11 20 27 29 37 42 43
OCT4
SOX2
KLF4
MYC
HKG
C
Equine
Human
4B 2C 5C 7C ED12
4 7 10 11 20 27 29 37 42 43
NANOG
HKG
D
MSC tMSC ED12
CDH1
DPPA4
HESX1
LIN28
REX1
SALL4
TCL1A
HKG

Figure 2-3. Genotypic evaluation of transduced equine and human cells using semi-quantitative RT-PCR analysis. **A)** All 4 clones (4B, 2C, 5C, and 7C) of transduced equine ES-like cells at passage 10 post-transduction were examined for exogenous Sendai OSKM transgene and endogenous OSKM transcript detection. Cells from early passages served as a positive control for exogenous OSKM. **B)** All 10 clones (4, 7, 10, 11, 20, 27, 29, 37, 42, and 43) of transduced human ES-like cells at passage 10 post-transduction were examined for exogenous Sendai OSKM transgene and endogenous OSKM transcript detection. Cells from early passages served as a positive control for exogenous OSKM. **C)** All equine and human ES-like cells at passage 10 post-transduction were examined for endogenous NANOG expression. **D)** Starting equine mesenchymal stromal cell (MSC) population and transduced equine cells (tMSC) were examined for transcripts associated with pluripotency. Total RNA isolated from an equine embryo 12 days post-ovulation (ED12) was used as a positive control. Housekeeping genes (HKG) were equine GAPDH and human β-ACTIN.

Figure 2-4.

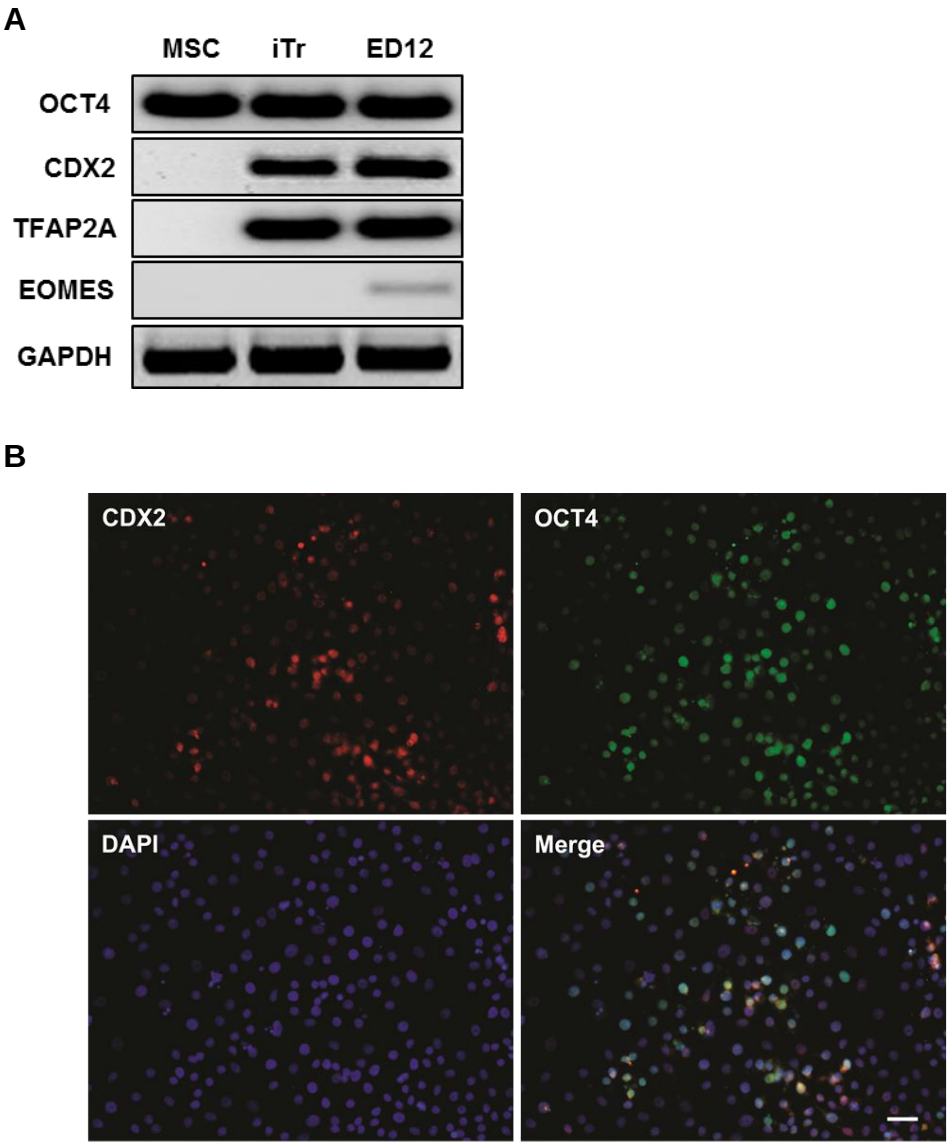

Figure 2-4. Biochemical evaluation of equine induced trophoblast (iTr) cells for markers of trophectoderm. **A)** The starting mesenchymal stromal cell (MSC) population and an iTr cell line were analyzed for *OCT4*, *CDX2*, *TFAP2A*, and *EOMES* by semi-quantitative RT-PCR. Total RNA isolated from an equine embryo 12 days post-ovulation (ED12) was used as a positive control. Equine GAPDH served as the housekeeping gene. **B)** Representative epifluorescence images showing detection of CDX2 (red) and OCT4 (green) immunocomplexes. Colocalization to the nucleus was verified with DAPI (blue) nuclear counterstain in conjunction with OCT4 and CDX2 overlays (Merge). Scale bar equals 50 µm.

Figure 2-5.

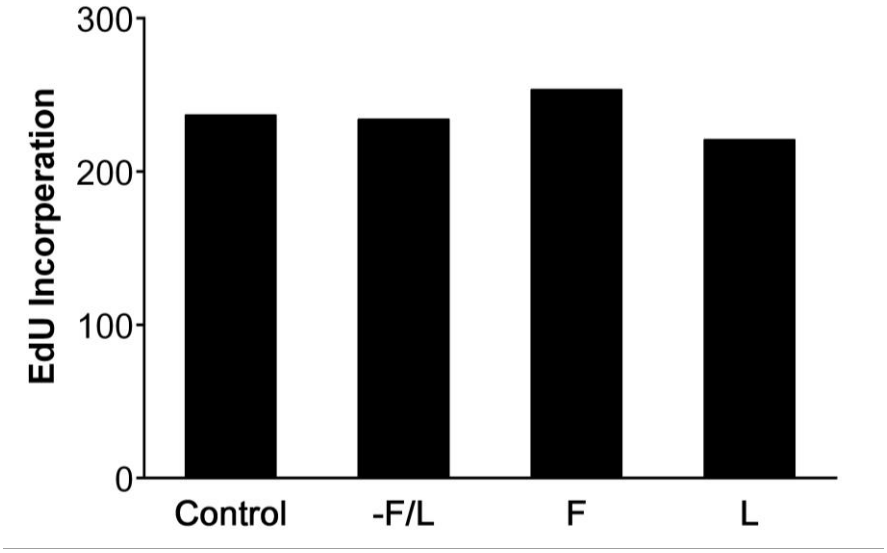

Figure 2-5. Number of traversing S-phase in the presence and absence of growth factors on feeder cells. EdU incorporation was compared for control treatment of standard iPS cell media containing FGF2 and LIF to no growth factor (-F/L), FGF2 only (F) and LIF (L) only. The concentration 4 ng/mL for FGF2 and 10 ng/mL for LIF.

Figure 2-6.

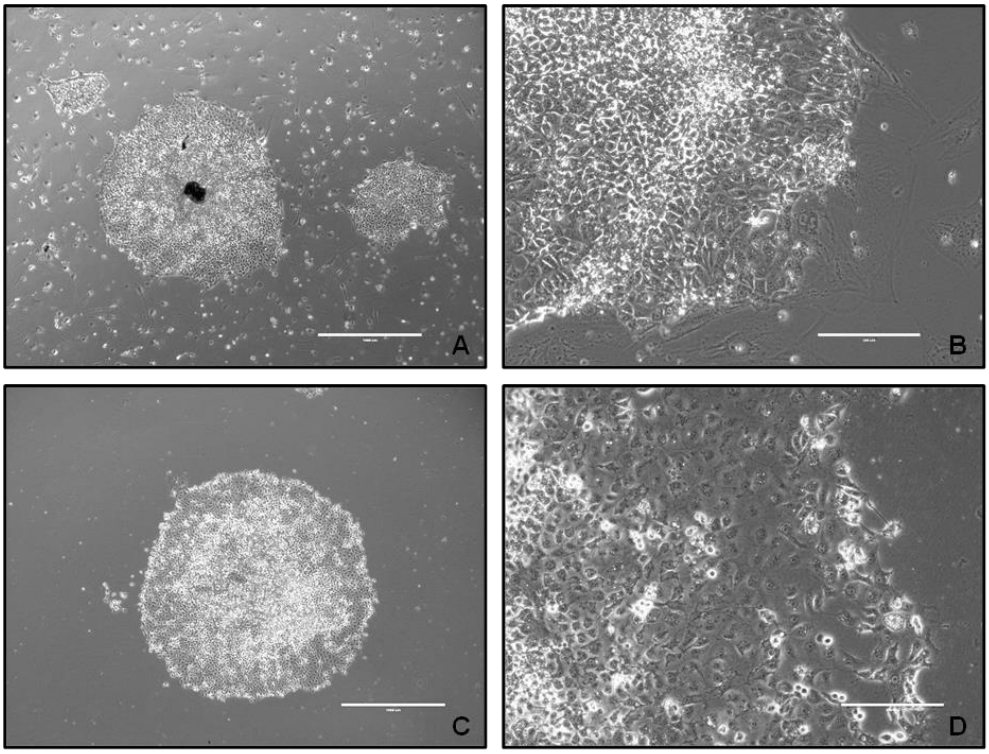

Figure 2-6. Transition of equine induced trophoblast (iTr) cells to feeder-free conditions. **A)** Equine iTr cells on mitotically inactive mouse embryonic feeder cells. **B)** High magnification of **A**. **C)** Equine iTr cells on Matrigel coated cultureware. **D)** High magnification of **C**. Scale bar equals 1000 µm **(A,C)** 200 µm **(B,D)**.

Figure 2-7.

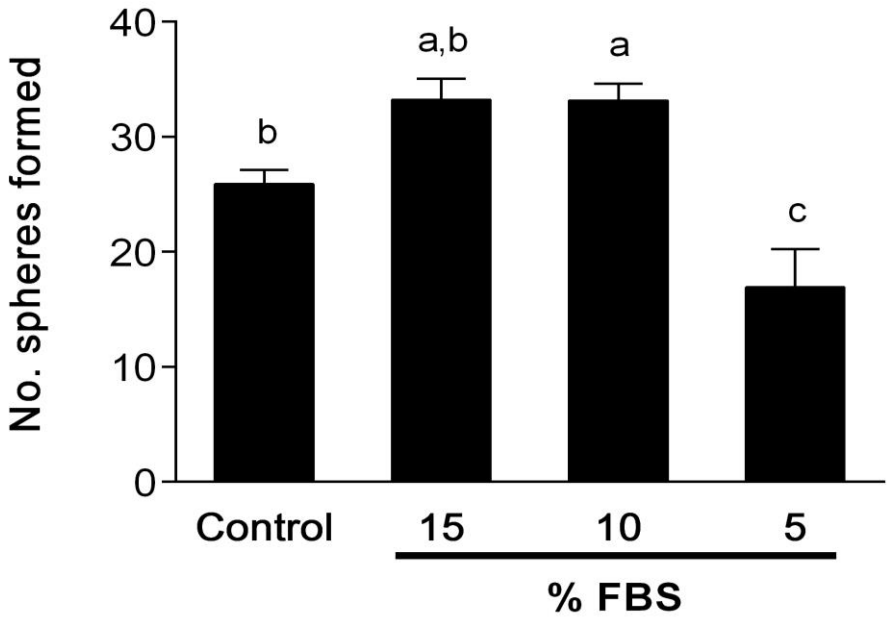

Figure 2-7. Number of spheres formed per well of 24 well plate after 72 hour culture in varying fetal bovine serum (FBS) concentrations. Control treatment contained high serum media containing 20% knockout serum replacement. Results shown are ± SEM of triplicates where levels not connected by same letter are significantly different ($P < 0.05$).

Figure 2-8.

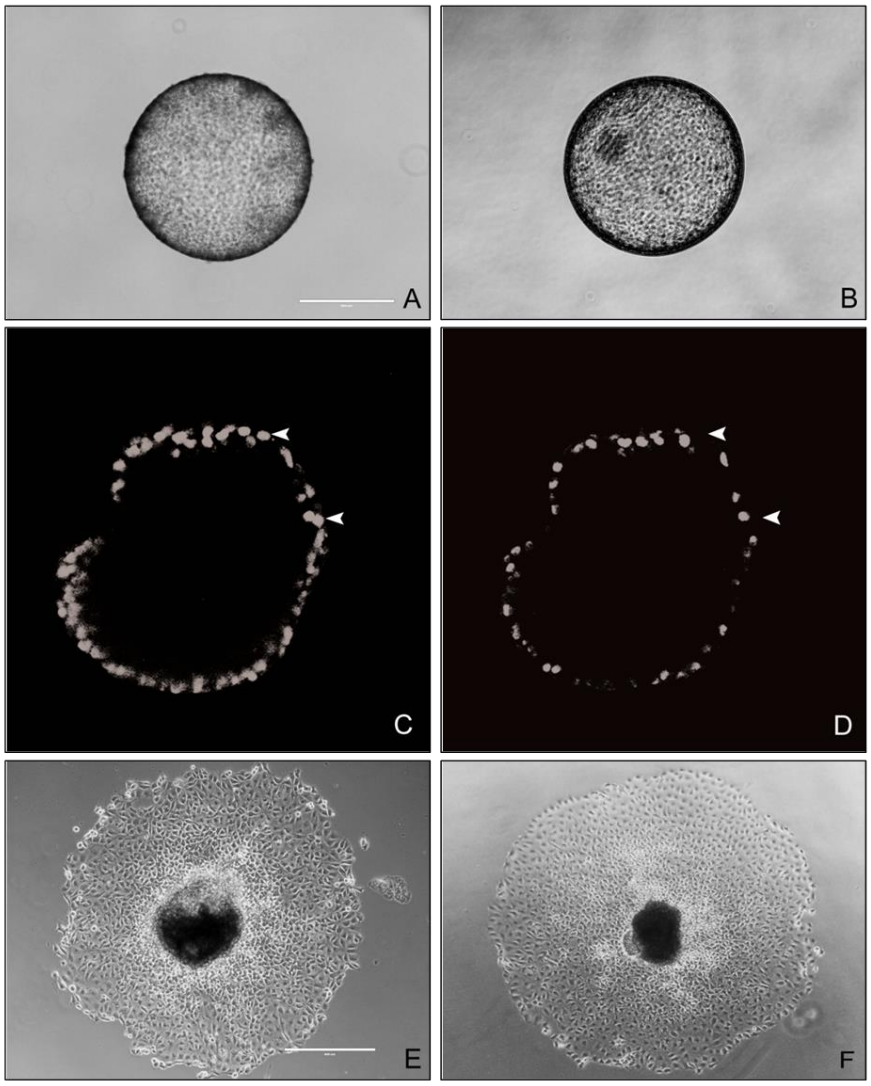

Figure 2-8. Morphological evaluation of a representative equine induced trophoblast (iTr) sphere and an equine embryo 7 days post-ovulation. **A)** Sphere formation after seven days on non-adherent petri dishes. **B)** Equine embryo seven days post-ovulation. Confocal microscopy detection of DAPI nuclear stain **(C)** or OCT4 **(D).** White arrow heads identify cells that do not recognize OCT4 immunocomplexes. Outgrowth formation of iTr spheres **(E)** and an equine embryo **(F)** three days after transfer to Matrigel-coated petri dishes. Scale bars equal 400 µm.

Figure 2-9.

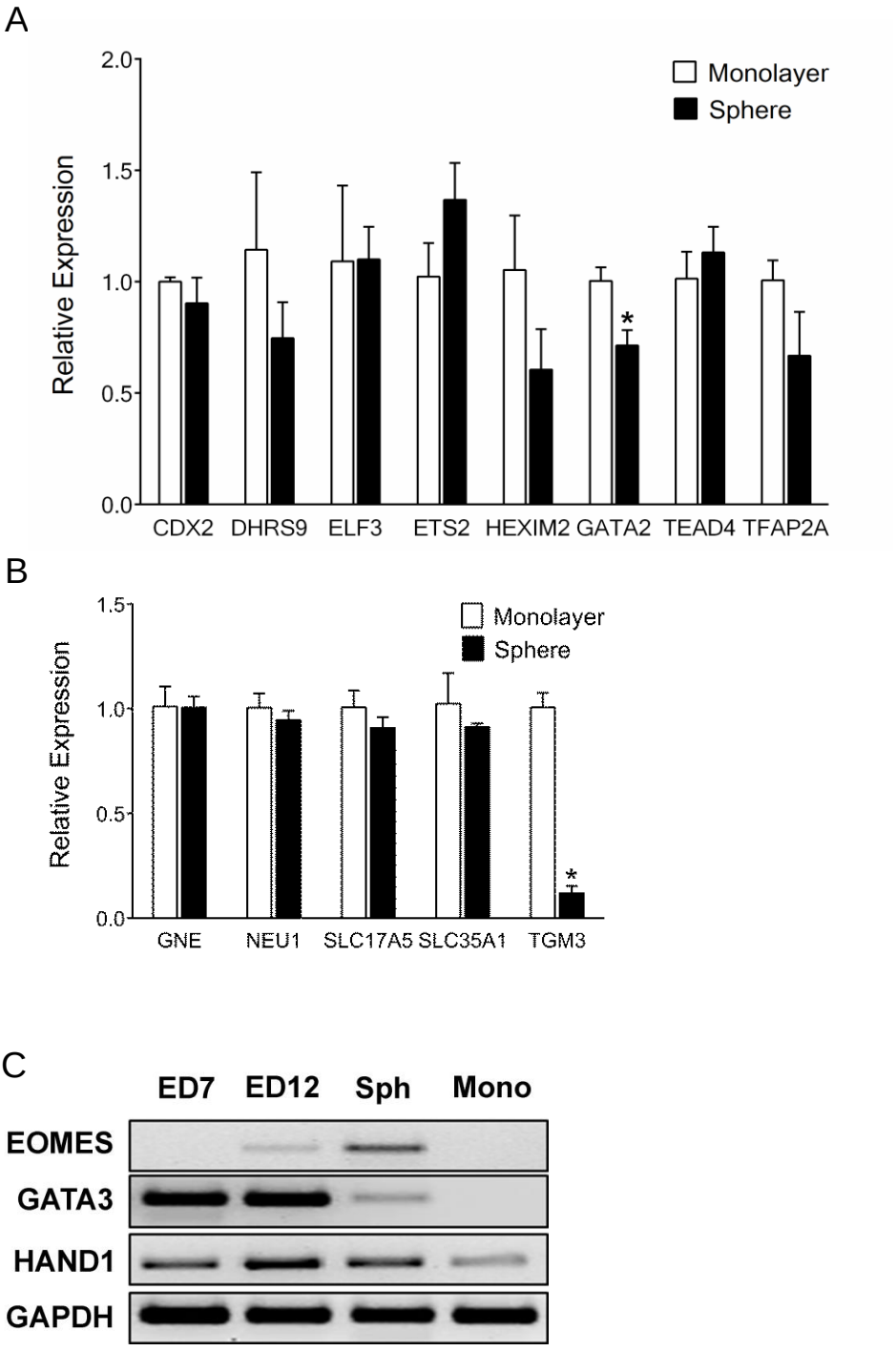
A
Monolayer
Sphere
Relative Expression
2.0
1.5
1.0
0.5
0.0
*
CDX2
DHRS9
ELF3
ETS2
HEXIM2
GATA2
TEAD4
TFAP2A
B
Monolayer
Sphere
Relative Expression
1.5
1.0
0.5
0.0
*
GNE
NEU1
SLC17A5
SLC35A1
TGM3
C
ED7
ED12
Sph
Mono
EOMES
GATA3
HAND1
GAPDH

Figure 2-9. Quantitative gene expression in induced trophoblasts (iTr) monolayers and spheres. Total RNA was isolated and Realtime PCR measurements of trophectoderm lineage **(A)** and capsule biosynthesis **(B)** transcript abundance are shown. Results shown are ± SEM of triplicates. Asterisk denotes differences ($P < 0.05$) in relative mRNA abundance between cellular arrangements. The reference gene was *β-ACTIN* for each experiment. **C)** *EOMES*, *GATA3*, and *HAND1* expression as measured by semi-quantitative RT-PCR in the iTr spheres (Sph) and monolayers (Mono). Total RNA was isolated from equine embryo seven (ED7) or twelve (ED12) days after ovulation was used as a positive controls. Housekeeping gene was *GAPDH* for semi-quantitative analysis.

CHAPTER III

Cell polarity and the combination of EGF, FGF2, and BMP4 differentially drive equine trophectoderm gene expression

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Running Head: Polarity and combination growth factors differentially drive trophectoderm gene expression

Author disclosure: Brad M. Reinholt, Jennifer S. Bradley, Alan D. Ealy, and Sally E. Johnson have no conflicts of interest.

Introduction

Cell arrangement undergoes dynamic changes during embryogenesis. Cleavage of the embryo results in blastomeres that form a spherical structure. Further cellular differentiation results in the formation of a morula followed by the blastocyst with a hallmark blastocoele cavity. Continued morphogenesis is driven by changes in cell adhesion properties creating adherens junctions and actomyosin networks that result in cellular polarity (Rauzi et al. 2010). The outermost cell layer of the blastocyst, or trophoblasts, form the polarized trophectoderm (TE) (Vanderpuye et al. 1988). Tight junctions between trophoblasts create distinct separation between the uterine environment and the blastocoele cavity (Kalt 1971). Further development of the TE is reliant on signals received by both the apical and basal membranes that interact with the uterine or blastocoele environment, respectively.

Vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor 2 (FGF2) are secreted by the endometrium and present in the uterine lumen (Paiva et al. 2011). In humans and rodents, VEGF is secreted from differentiating trophoblasts and instills migratory properties in first trimester trophoblast cells (Achen et al. 1997; Lash et al. 2003). In humans, EGF-dependent expression of *TFAP2A* plays a role in trophoblast invasion, and stimulates differentiation through extracellular-signaling-related kinase1/2 (ERK1/2) activation (Biadasiewicz et al. 2011; Johnstone et al. 2005). Fibroblast growth factor 2 confers a variety of mitogenic and survival properties on numerous cell types, including trophoblasts (Ornitz and Itoh 2001). In cattle, FGF2 can stimulate *interferon-τ (IFNT)* expression which is important for fetal-maternal communication and maintenance of pregnancy (Michael et al. 2006).

Human embryonic stem (ES) and induced pluripotent stem (iPS)cells rely on FGF2 signaling to prevent differentiation and to maintain self-renewal ability (Coutu and Galipeau 2011). Conversely, bone morphogenic protein 4 (BMP4) can stimulate differentiation of human ES and iPS cells to the TE lineage in the absence of FGF2 (Bernardo et al. 2011; Golos et al. 2013). Bone morphogenic protein 4-dependent pathways are important in the regulation of equine trophoblast differentiation *in vivo* and primary trophoblast differentiation *in vitro* via activation of the Sma- and Mad-related protein1/5 (SMAD1/5) pathway (Cabrera-Sharp et al. 2014b). This suggests that growth factor stimulated development might be conserved between species. Additional growth factors expressed specifically in equine endometrium are *FGF9* and *connective tissue growth factor (CTGF)*. These growth factors are differentially expressed in the endometrial tissue from pregnant mares 14 days post-ovulation and may affect the early equine conceptus (Klein et al. 2010).

The spherical equine conceptus enters the uterine lumen on or around day 6 post-ovulation at the late morula or early blastocyst stage (Betteridge et al. 1982). At this time, the TE begins depositing a thin acellular membrane between the trophoblast cells and the zona pellucida called the equine capsule. The expanding conceptus then ruptures and sheds the surrounding zona pellucida leaving only the capsule surrounding the conceptus. Although the function of the capsule is not completely clear, it is thought to aid in the survival of the conceptus as it is propelled throughout the uterine lumen by myometrial contractions. The capsule may also play a role in the unknown equine maternal recognition of pregnancy that occurs between days 12 and 14 post-ovulation (Allen and Stewart 2001; Ginther 1983; Raheem 2015). The conceptus ceases

movement and becomes fixed due to its increased size and increased tone of the endometrium (Ginther 1983). Much of the developmental process that occurs in the conceptus between migration into the uterine lumen and fixation are poorly understood.

An equine induced trophoblast (iTr) cell line was created that mimics equine TE development between day 6 and 12 post-ovulation. Changes in cellular arrangement causes up-regulation of genes associated with TE differentiation. To understand global changes that occur during this developmental window, whole transcriptome analysis was performed on equine iTr cells maintained as monolayers or spheres. This information was used to help identify factors that could be important during the differentiation and survival of the early equine embryo once in the uterine lumen. Additionally, transcript abundance of genes associated with TE lineage and differentiation were measured in response to BMP4, EGF, or FGF2 and combinations thereof. Results of these experiments could improve our understanding of the molecular changes that occur during TE morphogenesis and the uterine-derived growth factors that mediate their expression.

Materials and methods

Cell culture and growth factor assay

Equine induced trophoblast (iTr) cells were cultured in growth medium comprised of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), 1% non-essential amino acids (NEAA), and 0.1 mM β-mercaptoethanol. The monolayer cells were cultured on petri dishes that were coated with Matrigel® (1:10 in serum-free DMEM). Spheres were formed by

physical dissociation of iTr cells with a rubber scraper and passage through a 20 G needle twice to generate a single cell suspension. The cell suspension was passed through a 100 µm Falcon™ cell strainer (Fisher Scientific, Hampton, NH) to remove cell aggregates and seeded in ultra-low attachment tissue culture plates (Corning, Corning, NY) in growth media.

Spheres (n=50; ranging between 300 - 700 um diameter) were cultured in DMEM supplemented with 5% FBS with recombinant human forms of bone morphogenic protein 4 (BMP4), connective tissue growth factor (CTGF) epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), fibroblast growth factor 9 (FGF9), insulin-like growth factor-I (IGF-I) or vascular endothelial growth factor (VEGF) at a final concentration of 10ng/mL. All recombinant proteins were analyzed for homology to the equine forms through the Basic Local Alignment Search Tool provided by the National Center for Biotechnology Information (Appendix D). Lysates were collected in NuPAGE® LDS buffer containing protease and phosphatase inhibitors (Life Technologies) at the beginning (T0) and end of the 20-minute treatment period (T20). For gene expression assay, three independent experiments of 50 iTr spheres in duplicate were treated once for 48 hours in DMEM supplemented with 5% FBS, 1% P/S and 10 ng/mL BMP4, EGF, or FGF2 and each combination.

RNA isolation and qRT-PCR

Total RNA was isolated from monolayer iTr cells using TRIzol reagent (Life Technologies). The RNA was purified with PureLink® RNA mini kit according to manufacturer's directions (Life Technologies). Isolation of RNA from iTr spheres was performed with the ARCTURUS® PicoPure® RNA isolation kit (Life Technologies),

according to manufacturer's recommendations. RNA quantification and integrity was evaluated with a standard-sensitivity RNA chip with the StdSen Analysis kit on the Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). Total RNA (50 ng) was treated with 2 units of amplification grade DNAse I and first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Life Technologies). Real-time PCR amplification was carried out with Power SYBR® Master Mix, 10 pmols of each gene specific forward and reverse primers (See Appendix A for sequences) and cDNA template. Real-time cycle parameters were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds. All PCR reactions were performed on an eppendorf realplex^2 mastercycler epgradient PCR machine. The ΔCt measurments from duplicates were averaged. Relative expression level of target genes between treatments was calculated using $2^{-\Delta\Delta Ct}$ method, defined as: $\Delta\Delta Ct = (\Delta Ct^{transcript\ of\ interest}) - (\Delta Ct^{control})$.

RNA sequencing and analysis

Total RNA was processed for library construction by Cofactor Genomics (St. Louis, MO) according to the following procedure: RNA was amplified using the Ovation® RNA-Seq V2 amplification system (NuGEN, San Carlos, CA). Double-stranded cDNA was sheared to the desired size using the Covaris S2 focused-ultrasonicator (Covaris, Woburn, MA). Indexed adaptors were ligated to sample DNA, and the adaptor-ligated DNA was then size-selected on a 2% SizeSelect™ E-Gel (Invitrogen, Carlsbad, CA) and amplified by PCR. Library quality was assessed by measuring nanomolar concentration and the fragment size in base pairs. Libraries were sequenced on the NextSeq500 following the manufacturer's protocols. Data collection

and analysis was performed by Cofactor Genomics (Saint Louis, MO). Raw sequence data in Fastq format were assessed for quality and ribosomal RNA content (FastQC). NovoAlign was used to align reads to the reference genome. The resulting alignments were combined to create clusters of reads (or patches) which represent non-redundant regions in the reference genome sequence. Cluster boundaries were created using all samples and only uniquely mapping reads were taken into consideration. Transcripts included in Tables 1 and 2 were present in each biological. The manually annotated Uniprot database was used to categorize the genes regarding their putative molecular function in which they are involved.

Western blotting

Equine iTr cells were lysed in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen), sonicated for 10 seconds at 30% amplitude twice and stored frozen at -80°C. Protein lysates were denatured by heating to 95°C for 5 minutes with 2.5% (vol/vol) β-mercaptoethanol to reduce disulfide bonds. Proteins were separated through 10% Tris-Glycine gels (Life Technologies) and transferred to a nitrocellulose membrane using the iBlot system. The blots were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 30 minutes to block non-specific antigen sites. Subsequently, blots were washed three times in TBST for 5 minutes. Primary antibodies used were anti-AKT, anti-ERK1/2, anti-SMAD1/5, anti-α-tubulin (1:1000) or phosphoAKT, phosphoERK1/2, phosphoSMAD (1:2000) and incubated overnight at 4°C with agitation. All antibodies were purchased from Cell Signaling Technologies (Danvers, MA). After three TBST washes for 5 minutes each, the blots were incubated with appropriate peroxidase-labeled secondary antibody for 45 minutes

at room temperature with agitation. Immune complexes were visualized by chemiluminescence (Novex ECL kit; Invitrogen) using a Bio-Rad Universal Imaging system (Hercules, CA). All protein samples were normalized to α-tubulin prior to detection of phosphorylated proteins. Activation of the signaling pathway was determined by relative qualitative phosphorylated protein content to the time 0 sample.

Statistical analysis

Data were analyzed using ANOVA followed by *t*-test pairwise comparison using JMP Pro 11 software (SAS Institute, Inc., Cary, NC). A difference was considered statistically significant when $P < 0.05$. A tendency was considered when $P < 0.1$. Data are presented as means ± standard error of the mean (SEM). The RNA sequencing data were based off of normalized expression. Pairwise *P*-values were determined among the samples using Welch's *t*-test for unequal variance. The resulting comparative expression was visualized in ActiveSite (Cofactor Genomics), and loci of interest were chosen.

Results

Overview of RNA sequencing

Transcriptome analysis was performed to identify gene expression differences between iTr cells cultured as monolayers (n=3) vs. spheres (n=3). A total 24,521 unique transcripts were identified from an average of 47 million reads per sample. Data parsing reveals that 250 genes are up-regulated by two-fold or more in spheres by comparison to monolayers (Table 3-1). Of transcripts up-regulated two-fold or greater in the spheres, 137 were annotated novel proteins. Of the 137 annotated transcripts 51 were

unnamed annotated proteins and 86 were pseudogene annotations. The spherical arrangement stimulated up-regulation of 19 small nuclear RNA (snRNA) transcripts, commonly called U-RNA for their propensity to be rich in uridine. A subset of the up-regulated snRNA were 3 small nucleolar RNA (snoRNA) transcripts. The remaining 16 were variants of the highly conserved U6 snRNA, or U6-derived pseudogenes (Marz et al. 2008). The U6 snRNAs function in splicing out introns of pre-mRNA. The *early growth response gene 1* (*EGR1*) exhibited 9-fold up-regulation in the spheres by comparison to the monolayer. Transcripts of Kruppel-like factor 4 (*KLF4*), *myelocytomatosis viral oncogene* (*MYC*), and *undifferentiated embryonic transcription factor 1* (*UTF1*) were 8, 4.5, and 2-fold more abundant in the spheres. These three genes are associated with iPS cell reprogramming (Zhao et al. 2008) (Table 3-1).

Substantially fewer genes (50) were down-regulated two-fold or greater when comparing spheres to the monolayer arrangement (Table 3-2). Of those down-regulated two-fold or greater in the spheres, nine were unnamed annotated novel proteins. The annotated transcripts revealed five protein-coding, two mitochondrial tRNA, and two non-coding pseudogene annotations. Also, three variants of the microRNA eca-mir-302 were upregulated in the monolayer arrangement. *Nephroblastoma overexpressed (NOV)* which encodes for the CNN3 protein, had the greatest change being down-regulated 29-fold greater in spheres (Table 3-2). *Cysteine-rich angiogenic inducer 61 (CYR61)* encodes for the CCN1 protein and was more than 5-fold down-regulated in the iTr spheres. Both of these genes, along with *CTGF* are members of the CCN intercellular signaling protein family. This protein family is comprised of extracellular matrix associated signaling proteins that are involved in cell adhesion, proliferation and

differentiation (Holbourn et al. 2008). The autocrine growth factor *Amphiregulin (AREG)*, a member of the EGF family that binds to the EGF receptor, was down-regulated more than 5-fold in the spheres compared to the monolayer.

A set of differentially expressed genes was validated using quantitative real-time PCR (qRT-PCR). A similar trend of expression was observed in the five genes selected that were up-regulated in spheres and the four genes selected that were up-regulated in the monolayer (Table 3-3).

Growth factor assay

Equine iTr cells were treated with panel of growth factors that known to regulate trophectoderm development. Growth factors and each combination was administered to a pooled collection of 50 iTr spheres which was determined to be the optimal sphere number for accurate protein analysis (Fig 3-1). Human recombinant VEGF, EGF, IGF-I, FGF 2 and 9, HGF, CTGF, and BMP4 were tested for the presence of a functional cell surface receptor by Western analysis of downstream signaling mediators. Results demonstrate that treatment of iTr spheres with BMP4, EGF, or FGF2 caused phosphorylation of downstream signaling proteins (Fig. 3-1). Treatment with BMP4 activated SMAD1/5 indicating the presence of functional BMP4 receptor dimer, but did not activate the MEK/ERK signaling module. Spheres treated with EGF exhibited activation of both ERK1/2 and Protein Kinase B (AKT) signaling proteins. By contrast, only ERK1/2 was phosphorylated in response to FGF2. Neither ERK1/2 nor AKT was phosphorylated in response to FGF9, HGF, IGF-1 or CTGF treatment.

The effect of BMP4, EGF, and FGF2 treatment on TE development and capsule biosynthesis gene expression was examined. Spheres were incubated for 48 hours with 10 ng/mL of BMP4, EGF, or FGF2, and each combination and total RNA was isolated. Results show that neither BMP4, EGF, nor FGF2 caused a measurable change in *CDX2*, *DHRS9*, *ELF3*, *ETS2*, *GATA2*, HEXIM2, *TEAD4* or *TFAP2A* expression (Fig. 3-2A). There was a tendency for BMP4 to increase *ELF3* (P = 0.073) and *GATA2* (P = 0.086) expression, and EGF tended to increase (P = 0.075) *GATA2* expression. In a similar manner, the growth factors did not alter expression of genes associated with capsule formation (Fig. 3-2B).

Combinations of growth factors can have an additive effect to enhance cell survival and proliferation (Richmon et al. 2005). Equine iTr spheres were treated with EGF+BMP4, EGF+FGF2, or BMP4+FGF2 and gene expression was measured. Quantitative PCR demonstrates that all of the growth factor combinations caused a reduction in *TFAP2A* expression (P < 0.001) compared to the negative growth factor controls (Fig. 3-3A). The combinations of EGF+BMP4 and FGF+BMP4 showed a tendency (P = 0.08) to reduce *NEU1* expression compared to the no growth factor control. A pairwise comparison of treatment with EGF+BMP4 to the no growth factor control and FGF2+BMP4 compared to the no growth factor control did show reduced (P < 0.05) *NEU1* expression. Spheres treated with EGF+BMP4 tended to down-regulate (P = 0.07) *SLC35A1* expression. Similarly, a pairwise comparison between treatment of EGF+BMP4 compared to the no growth factor control did detect reduced (P < 0.05) *SLC35A1* expression in response to EGF+BMP4 (Fig. 3-3B).

Discussion

The transforming growth factor-beta superfamily member BMP4 can drive differentiation of human ES and iPS cells to the TE lineage and promotes continued differentiation of equine trophoblasts (de Mestre et al. 2009; Golos et al. 2013). Treatment of iTr spheres with growth factors was hypothesized to further advance development. Supplementation with BMP4 alone was able to initiate signals through SMAD1/5 but the event did not result in a change in expression of the core TE transcriptional network. The EGF-like growth factors activate the transmembrane receptor tyrosine kinases EGFR/ERBB1 and related ERBB family receptors (ERBB2, ERBB3, and ERBB4) which exhibit signaling redundancy though ERK1/2 phosphorylation (Holbro and Hynes 2004). The iTr spheres up-regulated expression of ERBB2 regulatory proteins, indicating the spheres may be responsive to EGF signaling. Treatment of EGF initiated signals through ERK1/2 and AKT, and FGF2 initiated signals through ERK1/2, but neither EGF nor FGF2 altered expression of genes measured.

Only BMP4, EGF, and FGF2 were further pursued for activation of TE genes due to their documented activation of downstream signaling molecules. Alternative growth factors may require longer exposure time to initiate phosphorylation. For example, Yang and colleagues (2007) demonstrated a 30 minute exposure period to CTGF was necessary to stimulate phosphorylation of ERK1/2 in mouse kidney fibroblasts. The authors used a 100 ng/mL concentration to elicit a response. It is possible that the growth factor concentrations used in this study were insufficient to elicit a response.

Each growth factor combination (EGF+BMP4, FGF2+BMP4, or FGF+EGF) reduced *TFAP2A* expression when compared to the no growth factor control. This demonstrates a combination of growth factors alter *TFAP2A* expression compared to the no growth factor control. Expression of *TFAP2A* increases as human trophoblasts differentiate *in vitro* (Debieve et al. 2011; Richardson et al. 2001). This suggests the combination of each growth factor may inhibit differentiation. However, expression of other transcription factors that identify TE lineage, such as *CDX2*, *ETS2*, and *GATA2*, or differentiation, such as *TEAD4*, remained unchanged.

At day 6.5 post-ovulation the glycoprotein capsule appears between the TE and zona pellucida. It reaches a peak dry weight at the time of fixation around day 17, and is fully degraded by day 22 post-ovulation (Oriol et al. 1993b). This is concurrent with continued development of the TE. A corresponding rise in expression of genes that drive synthesis of capsule synthesis would be expected. These include several solute carrier family (SLC) members and the sialic acid metabolizing enzymes of the neuraminidases (NEU) family. Indeed, expression of *SLC35A1* and *NEU2* are up-regulated in day 14 post-ovulation equine embryos compared to day 8 (Klein and Troedsson 2012; Klein and Troedsson 2011). Iqbal and colleagues (2014) reported higher expression of *NEU1* at day 8 over other neuraminidases at the same time suggesting *NEU1* might be important in day 8 post-ovulation equine TE. The combination of EGF+BMP4 reduced expression of *SCL35A1*, and the combination of EGF+BMP4 and FGF2+BMP4 reduced *NEU1* expression in a pairwise comparison. Treatment of BMP4 alone did not alter expression compared to the no growth factor

control. These results suggest the combination of BMP4, EGF, or FGF2 may impede capsule biosynthesis.

Previous investigations demonstrated that transformation of the iTr cells to the spherical arrangement induces expression of *EOMES* and *HAND1*. Both *EOMES* and *HAND1* are absent from the monolayer and are genes associated with advanced placental cell development. Changes in cell arrangement may promote differentiation (Yamashita and Michiue 2014). To gain insight into other factors that may participate in TE formation and differentiation, a transcriptome analysis was performed. *NRP2, RHOB, NOV,* and *CYR61* were identified as genes that may influence TE development. In the spheres, a more than 4-fold increase in the transmembrane glycoprotein receptor *Neuropilin 2 (NRP2)* was detected. NRP2 is purported to interact with VEGF by acting as a coreceptor that enhances cellular responses induced by VEGF binding (Favier et al. 2006). Transcripts for *the Ras homolog gene family member B (RHOB)* were up-regulated more than 3-fold in the spheres. RHOB promotes AKT signaling and regulates activated EGR receptors by slowing intracellular trafficking of internalized EGF receptor to the lysosome (Gampel et al. 1999; Kazerounian et al. 2013). These data suggest that EGF and EGF-like molecules may, at least in part, be able to stimulate biological responses in the iTr spheres. The NOV and CYR61 proteins belong to the same CNN protein family and were down-regulated 29 and 6-fold, respectively in the spheres compared to the onolayers. Transcripts for *NOV* are expressed in day 8 equine TE and both *NOV* and *CYR61* transcripts are expressed in the day 16 equine conceptus (Iqbal et al. 2014; Klein 2015). The consistently high levels of *NOV* expression from several independent sources likely signify this gene is critical to the developing TE. It is unclear

what the physiological function the high *NOV* levels lead to, but the CNN protein family is implicated in many biological processes including cellular proliferation, differentiation, and adhesion as its true role merits further investigation (Chen and Lau 2009). Several transcripts were also up-regulated in the spherical arrangement that are associated with several common signaling pathways. Coagulation factor II (thrombin) receptor-like 1 (F2RL1), up-regulated almost 3-fold, activates downstream MAPK signaling (Mirza et al. 1996). FBJ murine osteosarcoma viral oncogene homolog (FOS) and ERBB Interacting Protein (ERBB2IB) stimulate SMAD signaling in response to binding of TGF-β to its receptor (Warner et al. 2003; Zhang et al. 1998). Up-regulation of these transcripts indicates that the iTr cells in the spherical arrangement could be more responsive to stimuli than the monolayer.

Transcripts for *fibrinogen, (FGB)* and *fibronectin leucine rich transmembrane protein 3 (FLRT3)* were down-regulated in the spheres compared to the monolayer arrangement. Klein and colleagues (2011) found a similar up-regulation in these two transcripts in the day 12 and 14 compared to day 8 post-ovulation equine conceptuses. In a study by Iqbal and colleagues (2014) the ICM and TE were separated and RNA sequencing analysis was performed. They detected *FGB* and *FLRT3* transcripts in both the ICM and the TE, with higher levels in the ICM. Therefore, it is difficult to conclude whether *FGB* and/or *FLRT3* expression correlate to more developed equine TE or ICM.

In summary, this report identifies genes with altered expression that may be due to cellular arrangement. Equine induced trophoblast cell lines respond to BMP4, EGF, or FGF2 treatment in combination by down-regulating *TFAP2A* expression. These data

suggest that development of equine trophectoderm is complex and influenced by activation of multiple signaling pathways.

Table 3.1. Transcripts showing increased mRNA expression levels in equine induced trophoblast spheres.

Equine gene (*Equus caballus*)	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
Early growth response 1	EGR1	XM_001502603	8.90	Transcriptional regulator, DNA binding
Kruppel-like factor 4	KLF4	XM_005605684	8.02	Transcription factor, DNA binding
FBJ murine osteosarcoma viral oncogene homolog	FOS	XM_001491972	5.27	Signal transduction (SMAD), Protein and DNA binding
v-Myc avian myelocytomatosis viral oncogene homolog	MYC	XM_001497991	4.49	Specific and nonspecific DNA binding
Neuropilin 2	NRP2	XM_005601697	4.38	Receptor activity for semaphorin 3, VEGF, PGF
Solute carrier family 16 (monocarboxylate transporter), member 7	SLC16A7	XM_005611357	4.22	Proton-coupled monocarboxylate transporter, high affinity pyruvate transporter
Choroideremia-like	CHML	NM_001256975	3.78	Substrate-binding subunit of the Rab geranylgeranyltransferase (GGTase) complex, GTPase activator activity
2'-5'-oligoadenylate synthetase 2	OAS2	XM_005612548	3.62	Innate antiviral response, double-stranded RNA binding, ATP binding
Deiodinase, iodothyronine, type III	DIO3	XM_001917216	3.53	Deiodination of T4 (tetraiodothyronine) into T3 and of T3 into T2
Interleukin 6 signal transducer	IL6ST	XM_005604255	3.41	Signal-transducing molecule, IL6, LIF, OSM signaling
Ras homolog family member B	RHOB	XM_005600187	3.28	Signal transduction (AKT), GTP binding, cell adhesion, mediates apoptosis as result of DNA damage
Ligand dependent nuclear receptor corepressor-like	LCORL	XM_001917676	3.15	DNA binding
A kinase (PRKA) anchor protein 12	AKAP12	XM_005608180	3.04	Protein binding, kinase activity
HEN1 methyltransferase homolog 1	HENMT1	XM_005610384	2.98	RNA methyltransferase activity, RNA binding

†Putative molecular function determined UniProt Summary

Table 3.1 continued

Equine gene (*Equus caballus*)	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
Zinc finger protein 407	ZNF407	XM_005613015	2.90	DNA and zinc binding
Coagulation factor II (thrombin) receptor-like 1	F2RL1	XM_005599735	2.87	Signal transduction (MAPK, PLC, Rho), G-protein receptor activity
Tetraspanin 2	TSPAN2	XM_005610541	2.83	Signaling integral component of membrane in oligodendrocytes
NEDD4 binding protein 2	N4BP2	XM_005608811	2.82	Endonuclease activity, protein binding, ATP binding
F-box and leucine-rich repeat protein 17	FBXL17	XM_001918313	2.81	Protein binding, ubiquitin ligase
Nuclear receptor interacting protein 1	NRIP1	XM_005606076	2.80	Modulates transcriptional activation of hormone and steroid receptors such as NR3C1, NR3C2 and ESR1
RAB GTPase activating protein 1-like	RABGAP1L	XM_055609683	2.80	Rab GTPase activator activity,
Tandem C2 domains, nuclear	TC2N	XM_005605404	2.80	-
Runt-related transcription factor 1	RUNX1	XM_005615022	2.72	DNA, protein, and calcium binding, enhances transcription
Growth arrest-specific 2 like 3	GAS2L3	XM_001496518	2.67	Actin and microtubule binding
Nuclear receptor subfamily 1, group D, member 2	NR1D2	XM_001492478	2.60	Transcriptional repressor, steroid hormone receptor activity, governs circadian rhythmicity and is involved in energy homeostasis
Protein tyrosine phosphatase, non-receptor type 4	PTPN4	XM_001492520	2.51	Non-membrane spanning protein tyrosine phosphatase activity, cytoskeletal protein binding
Structural maintenance of chromosomes 5	SMC5	XM_005604966	2.51	Involved in repair of DNA double-strand breaks by homologous recombination, telomere maintenance

†Putative molecular function determined UniProt Summary

Table 3.1 continued

Equine gene (*Equus caballus*)	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
UDP-N-acteylglucosamine pyrophosphorylase 1-like1	UAP1L1	XM_00560606	2.49	Nucleotidyltransferase activity
Ubiquitin specific peptidase 34	USP34	XM_005599957	2.45	Ubiquitin-specific protease activity, signal transduction (Wnt/β-catenin
UHRF1 binding protein 1-like	UHRF1BP1L	XM_001496669	2.42	-
Undifferentiated embryonic cell transcription factor 1	UTF1	XM_005613805	2.41	Transcription coactivator activity
Nuclear receptor coactivator 7	NCOA7	XM_005596975	2.38	Nuclear hormone receptor binding, enhances transcription
Twinfilin actin-binding protein 1	TWF1	XM_005611391	2.37	ATP binding, actin binding
Cyclin-dependent kinase 6	CDK6	XM_001493503	2.31	ATP binding, cyclin-dependent protein serine/threonine kinase activity, cell cycle regulation
Erbb2 interacting protein	ERBB2IP	XM_001915832	2.23	ErbB-2 class receptor binding, component of cytoskeleton, signal transduction
Kinesin family member 18A	KIF18A	XM_001504992	2.23	ATP binding, microtubule motor activity, chromosome congression
Rho GTPase activating protein 5	ARHGAP5	XM_001915062	2.19	GTP binding, SH2 domain binding
STE20-like kinase	SLK	XM_001497698	2.18	Mediates apoptosis, protein serine/threonine kinase activity
AT hook containing transcription factor 1	AHCTF1	XM_001490319	2.17	Sequence-specific DNA binding transcription factor activity, regulates cytokinesis completion
RB1-inducible coiled-coil 1	RB1CC1	XM_005613172	2.15	Signal transduction (TGFβ), protein kinase binding, regulator of myogenesis
Establishment of sister chromatid cohesion N-acetyltransferase 1	ESCO1	XR_290751	2.14	Regulate DNA replication, transferase activity, transferring acyl groups

†Putative molecular function determined UniProt summary

Table 3.1 continued

Equine gene (*Equus caballus*)	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
Programmed cell death 4	PDCD4	XM_001496736	2.14	RNA binding, inhibits translation and signal transduction (MAPK)
PDS5, regulator of cohesion maintenance, homolog A	PDS5A	XM_005608813	2.14	DNA repair, chromatin stabilization
Alstrom syndrome 1	ALMS1	XM_05599904	2.12	Intracellular transport, microtubule function, maintenance of primary cilia
Dynein, light chain, Tctex-type 3	DYNLT3	XM_001489506	2.07	Motor activity, completion of mitosis
TATA box binding protein (TBP)-associated factor, RNA polymerase I, D	TAF1D	XM_001488169	2.03	TATA binding protein associated factor

†Putative molecular function determined UniProt summary

Table 3.2. Transcripts showing decreased mRNA expression levels in equine induced trophoblast spheres.

Equine gene *(Equus caballus)*	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
Nephroblastoma overexpressed	NOV	XM_001496635	29.16	Insulin-like growth factor binding, proliferation
Actin smooth muscle, aorta, α2	ACTA2	XM_005602377	15.79	Constituent of thin filaments, ATP binding
Y box binding protein 3	YBX3	XM_005611039	10.98	Nucleic acid binding and translation repression
Serpin peptidase inhibitor heat shock protein 1	SERPINH1	XM_005612003 4	8.01	Biosynthetic pathway of collagen, serine-type endopeptidase inhibitor activity
Transmembrane protein 88	TMEM88	XM_001504791	7.01	Signal transduction inhibition (Wnt/β-catenin), downstream of GATA factors to specify lineage commitment of cardiomyocyte development
Crystalline α B	CRYAB	XM_001501779	6.48	Cytoskeletal protein and microtubule binding, chaperone activity
Cysteine-rich angiogenic inducer 61	CYR61	XM_001495078	5.78	Promotes cell proliferation, chemotaxis, angiogenesis, and adhesion
Amphiregulin	AREG	XM_001489473	5.44	Cytokine and growth factor activity, EGF receptor ligand
Actin α 1	ACTA1	XM_001497713	4.73	Structural constituent of cytoskeleton, myosin, and ATP binding
Myosin regulatory light chain	MYL9	XM_001502012	4.72	Smooth muscle and non-muscle cell contractile activity, calcium binding
Visinin-like 1	VSNL1	XM_001503420	4.47	Calcium ion binding, rhodopsin phosphorylation inhibition
Caveolin 1	CAV1	NM_001114143	4.45	G-protein receptor binding, mitogen activity
Regulator of G-protein signaling 5	RGS5	XM_005609852	4.21	GTPase activator activity, G-protein signaling inhibition
Lectin, galactoside-binding, soluble, 1	LGALS1	XM_001501032	4.02	Galactoside, glycoprotein binding, signal transducer activity

†Putative molecular function determined UniProt summary

Table 3.2 continued

Equine gene *(Equus caballus)*	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
Fibrinogen β chain	FGB	XM_001500955	4.00	Fibrinogen polymerization, platelet aggregation
Fibronectin leucine rich transmembrane protein 3	FLRT3	XM_005604471	3.68	Receptor signaling protein activity, constituent to extra cellular matricies
Plastin 1	PLS1	XM_001493516	3.48	Structural constituent of cytoskeleton, actin-bundling protein in the absence of calcium
Haloacid dehalogenase-like hydrolase domain containing 3	HDHD3	XM_005605713	3.41	Hydrolase activity, protein binding
SPRY domain containing 4	SPRYD4	XM_001504845	3.21	-
Follistatin-like 1	FSTL1	XM_001500510	3.16	Calcium ion binding, growth factor binding
Protein phosphatase, Mg2+/Mn2+ dependent 1L	PPM1L	XM_005602093	3.1	Catalytic activity, protein serine/threonine phosphatase activity, signaling suppressor (MAPK)
Forkhead box H1	FOXH1	XM_001917145	3.07	Transcriptional activator, signal transducer, nucleic acid binding
Neural cell adhesion molecule 1	NCAM1	XM_005611569	3.07	Neuron-neuron adhesion
Regulator of calcineurin 2	RCAN2	XM_005603972	3.03	Nucleotide binding, calcineurin A, binding
Vimentin	VIM	NM_001243145	2.96	Structural constituent of cytoskeleton in mesenchymal stem cells
Suppressor of Ty 4 homolog 1	SUPT4H1	XM_001503700	2.94	mRNA processing, sequence-specific DNA binding transcription factor activity
Tropomyosin 1 α	TPM1	XM_005603003	2.88	Structural constituent of cytoskeleton, contraction of striated muscle
Trafficking protein particle complex 4	TRAPPC4	XM_001503069	2.85	Protein binding
Carbonic anhydrase 2	CA2	XM_001488490	2.84	Carbonate dehydratase activity, osteoclast differentiation

†Putative molecular function determined UniProt summary

Table 3.2 continued

Equine gene *(Equus caballus)*	Gene symbol	GeneBank accession no.	FC	Putative molecular function†
Harvey rat sarcoma viral oncogene homolog like suppressor 5	HRAS	XM_005598258	2.84	GTPase activity, protein C-terminus binding
Keratin 7	KRT7	XM_001504375	2.44	Structural molecule activity, protein binding, stimulated DNA
Solute carrier family 36 (proton/amino acid symporter), member 2	SLC36A2	XM_005599277	2.37	pH-dependent electrogenic neuronal glycine and proline transporter
Brain abundant signal protein 1	BASP1	XM_005604337	2.24	Transcription corepressor activity, DNA, and protein binding
Cyclin-dependent kinase inhibitor 1A	CDKN1A	XM_001499918	2.17	Cyclin-dependent protein serine/threonine kinase inhibitor activity
ATP-binding cassette C4	ABCC4	XM_005601297	2.14	Nucleotide and ATP binding, 15-hydroxyprostaglandin dehydrogenase (NAD+) and nucleoside-triphosphatase activity

6 Putative molecular function determined UniProt summary

7

Table 3.3. Comparison of RNA-seq and qRT-PCR results for selected genes.

Monolayer/Sphere			Sphere/Monolayer		
Gene	**FC RNA-seq**	**FC qRT-PCR**	**Gene**	**FC RNA-seq**	**FC qRT-PCR**
AREG	5.44	2.53	EGR1	8.90	6.94
FGB	4.00	1.76	GAS2L3	2.67	2.04
LGALS1	4.02	2.85	N4BP2	2.82	2.23
SLC35A2	2.37	1.95	NRP2	4.38	3.62
			TFA1D	2.03	1.68

Figure 3-1.

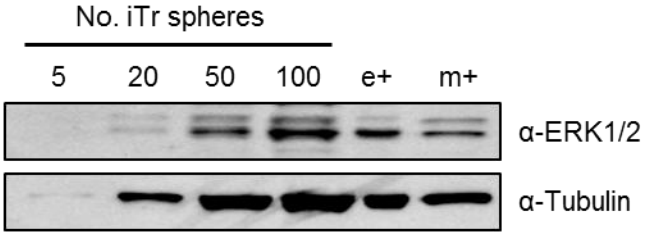

Figure 3-1. Sphere titration assay for protein analysis of equine induced trophoblast (iTr) spheres. Protein lysates from 5, 20, 50, and 100 equine iTr spheres analyzed by Western blot for total extracellular-signal-regulated kinase1/2 (ERK1/2) protein and alpha tubulin protein content. Equine (e+) and mouse (m+) skeletal muscle protein served as positive controls

Figure 3-1.

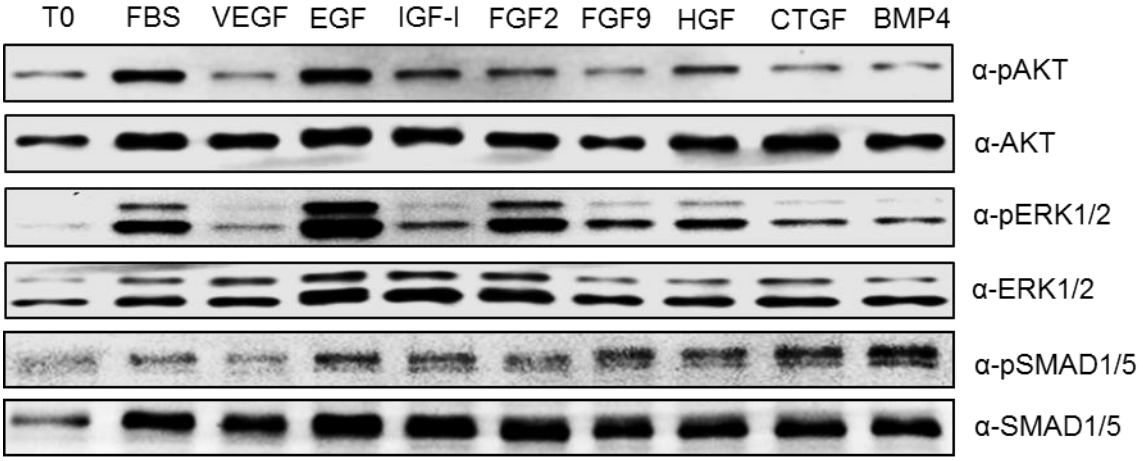

Figure 3-1. Western blot analysis of growth factor mediated signal transduction in induced trophoblast (iTr) cell is shown. Equine iTr spheres were treated with 10 ng/mL VEGF, EGF, IGF-I, FGF2, FGF9, HGF, CTGF, or BMP4. Samples were taken before (T0) and after 20 minutes of treatment. Protein lysates were analyzed by western for the phosphorylated (p) and total intracellular signaling intermediates protein kinase B (α-AKT, Ser473), extracellular-signal-regulated kinases1/2 (α-ERK1/2, Thr202/Tyr204), and SMAD1/5 (α-SMAD1/5, Ser463/465). 10% fetal bovine serum (FBS) served as a positive control for intracellular signal induction.

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Figure 3-2.

A

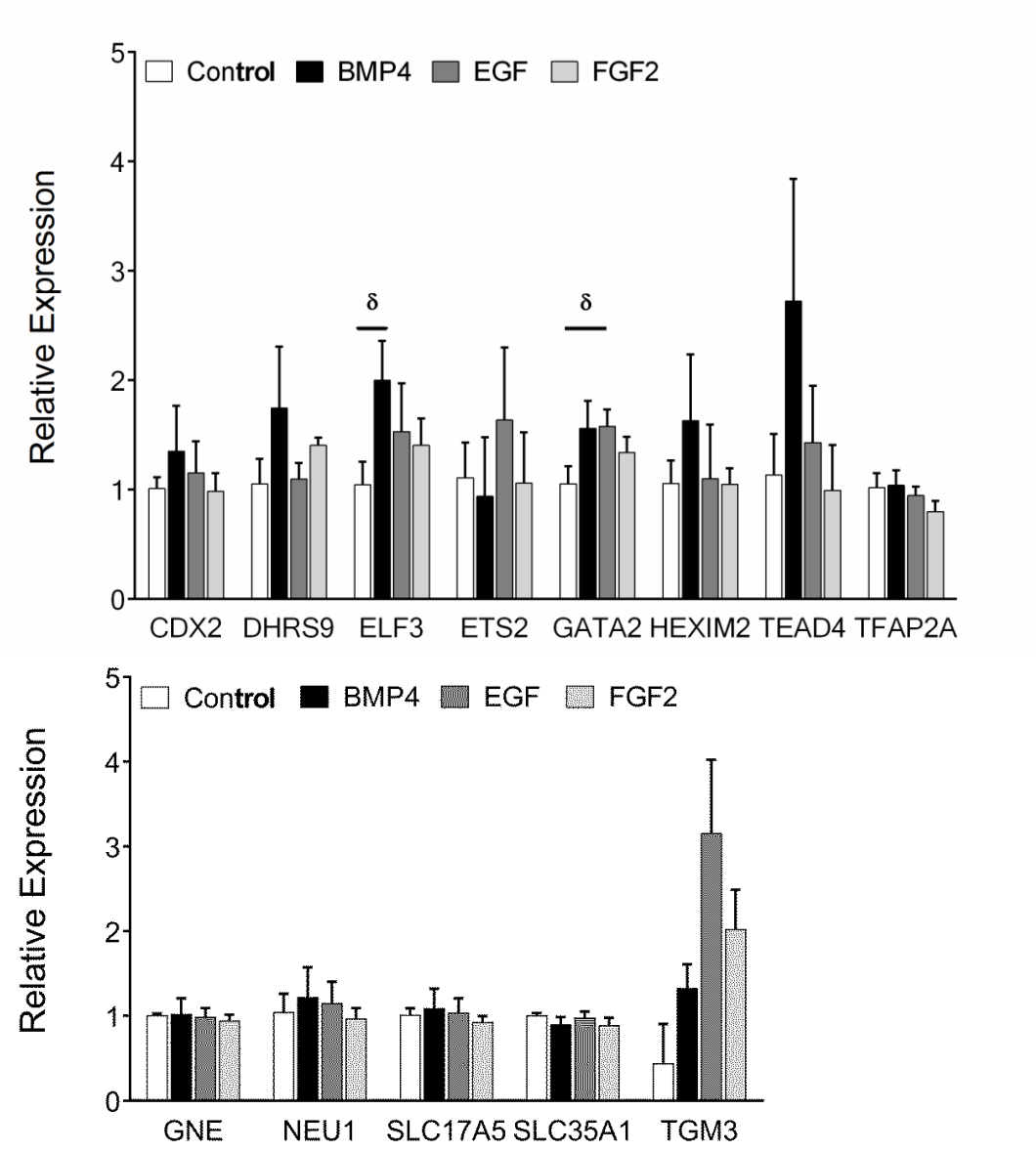

Figure 3-2. Quantitative expression of induced trophoblast (iTr) spheres in response to single growth factor treatment. Total RNA was isolated from iTr spheres treated with BMP4, EGF, or FGF2 for 48 hours Realtime PCR measurement of trophectoderm lineage (A) and capsule biosynthesis (B) transcript abundance is shown. Results shown are ± SEM of triplicates. Delta denotes tendencies (δ, $P < 0.1$) in relative mRNA abundance between the treatment and the negative control using a pairwise comparison. The reference gene was *β-ACTIN* for each experiment.

Figure 3-3.

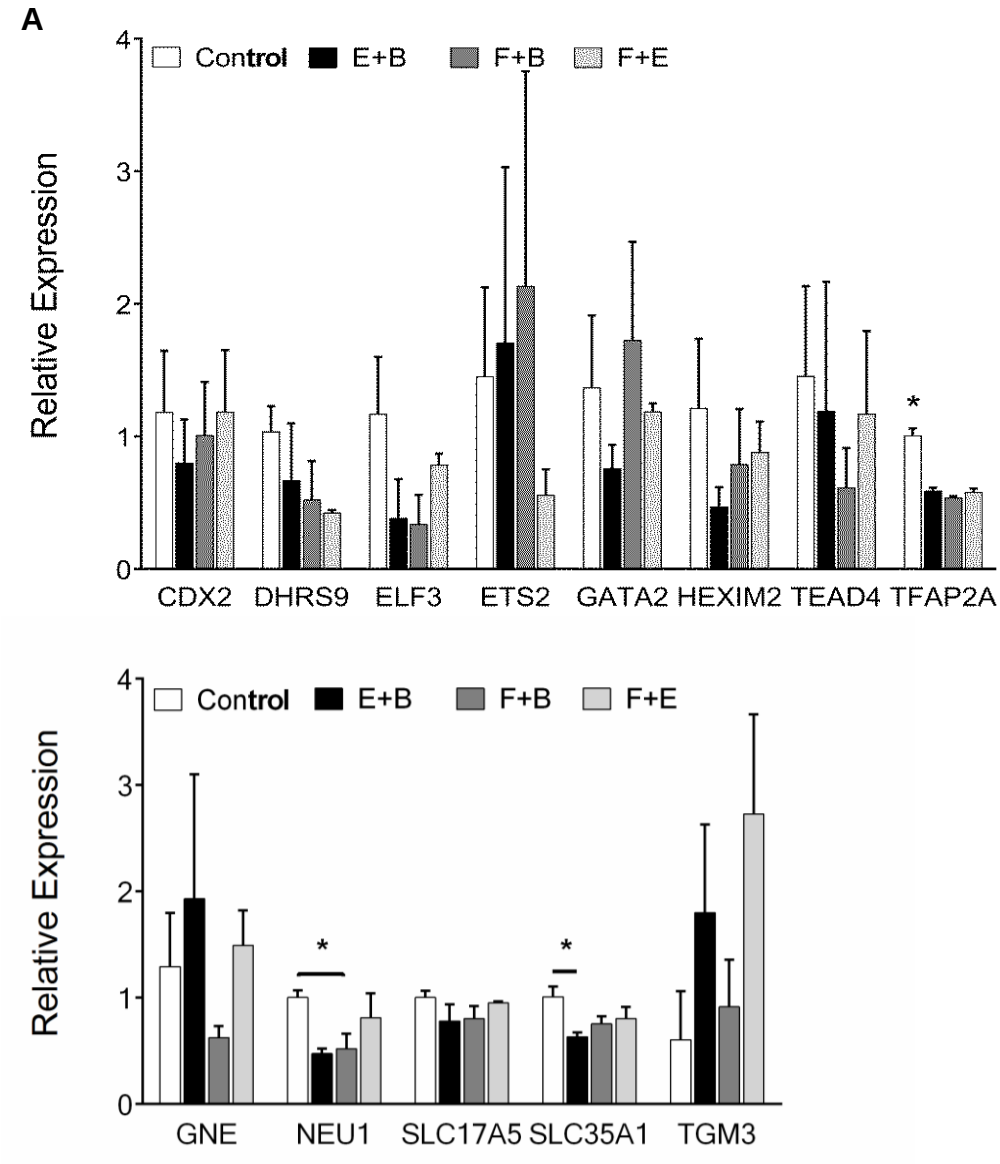

Figure 3-3. Quantitative expression of induced trophoblast (iTr) spheres in response to growth factor combination treatment. Total RNA was isolated from iTr spheres treated with EGF+BMP4 (E+B), FGF2+BMP4 (F+B), or FGF2+EGF (F+E) for 48 hours. Realtime PCR measurement of trophectoderm lineage (A) and capsule biosynthesis (B) transcript abundance is shown. Results shown are ± SEM of triplicates. Asterisk denotes differences (*, $P < 0.05$) in relative mRNA abundance between treatment and the negative control. Delta denotes differences (δ, $P < 0.05$) in relative mRNA abundance between the treatment and the negative control using a pairwise comparison. The reference gene was *β-ACTIN* for each experiment

CHAPTER IV

Implications and future directions

This report establishes new trophoblast cell lines through methods known to induce pluripotency in rodent and human somatic cells. Early gestational trophoblast cell lines exist for humans (Whitley 2006), mice (Sharma 1998), cattle (Talbot et al. 2004), and swine (Ramsoondar et al. 1993), but comparative equine cell lines have not been established. The equine induced trophoblasts (iTr) may be able to recapitulate a defined developmental window of trophectoderm development between days 8 and 12 post-ovulation. An investigation into these equine trophoblast cell lines was extended and whole transcriptome analysis and growth factor response was assayed. A representative cell line cultured in a monolayer or in suspended spherical blastocysts-like structures was analyzed for total mRNA transcripts, and gene expression response to growth factors known to elicit morphological and biochemical changes in trophoblast cells. These cell lines present a powerful new tool to enhance our understanding of early equine trophectoderm development during defined early developmental window.

Survival of the early embryo and maintenance of pregnancy is dependent on cross-talk between the conceptus and the uterine environment. This is chiefly regulated by physical and biochemical signals of the outer trophoblast cells, which communicate with the maternal tissues declaring the presence of the conceptus. Without this recognition of pregnancy signal, uterine derived prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) causes lysis of the corpus luteum (CL) resulting in a decline of progesterone levels and continuation of the estrous cycle. In the mare, this lysis event occurs around day 14-16 post-

ovulation. In the presence of the healthy embryo, $PGF_{2\alpha}$ release is inhibited by a conceptus, or more specifically trophoblast, derived factor that prevents lysis of the CL. This antiluteolytic event interrupts the normal estrous cycle by extending it. The mare then produces growth factors that promote growth of the conceptus and provides an accommodating environment for the developing embryo. The precise factor or factors produced by the trophoblast that prevent lysis of the CL in the mare are unknown, but this event must occur by day 14 post-ovulation. In theory, the newly developed equine trophoblast cell lines harbor this factor, or collection of factors. Understanding the biochemical factor, or factors, produced by the trophectoderm (TE) that prolongs the lifespan of the CL could increase reproductive efficiency by stimulating production or supplementing these compounds in mares that have breeding difficulties.

The equine embryo is completely surrounded by a TE derived acellular glycoprotein capsule at the time between days 7 and 22 post-ovulation, thus making it present at the purported time of fetal-maternal signaling of pregnancy. Removal of the capsule from equine embryos at day 7 post-ovulation and subsequent transfer into reproductively sound recipient mares does not establish a successful pregnancy (Stout et al. 2005). The uterine environment is necessary for capsule formation as equine embryos cultured *in vitro* secrete capsular proteins, but fail to form a functional capsule (Tremoleda et al. 2003). This implicates the capsule as being important in this signaling process. Attempts to stimulate expression of genes that drive the biosynthesis with growth factors *in vitro* in the iTr spheres were largely unsuccessful (see Chapter 3). A more robust response in gene expression may be achieved by treating the iTr monolayer with growth factors and should be analyzed. As with equine embryos, the

equine iTr cell lines may require the uterine environment to assemble this capsule. Transfer of the equine iTr cells into cyclic mares would potentially prove, or disprove, their ability to recapitulate the *in vivo* occurrence. It is understood that the mobility of the equine conceptus extends luteal life span alone. Therefore, the mere presence of the equine trophoblasts in the mare's uterus may extend estrus. Persistent detection of human *MYC* in the iTr cells may make *in vivo* experiments difficult. Co-culture systems with endometrial cell explants have been successful in horses (Vernon et al. 1981). An *in vitro* approach may be more logistically and economically viable until more information is gained on the iTr cell lines. Culture of the iTr cells in the presence of endometrial cells may advance the developmental process of the iTr cells.

The iTr cells described in this report were generated by transduction with transcription factors that reprogram mouse and human somatic cells into iPS cells. Derivation of iPS cells from livestock has been fraught with difficulties and produced various outcomes. The method used to induce the trophoblast cell line employed standard reprogramming factors (*OSKM*) by means of a non-integrating Sendai delivery method (see Chapter 2). The non-integrating system resulted in loss of expression of 3 of 4 exogenous factors more rapidly than most existing reports that used integrating delivery methods. It is irrefutable that the equine MSC were reprogrammed. If the equine MSC gained pluripotency, which is presumed but not empirically proven, the state was not preserved. Two possible reasons for this are the diminishing expression of the exogenous transgenes, and/or the culture conditions in which the cells were maintained. If the duration of transgene expression reduced the pluripotent phase, a delivery system that can easily renew expression or presence of the transgene product

is needed. The culture conditions were known to induce human somatic cells to a stable pluripotent state. It is likely that refinement of the culture conditions is necessary to generate iPS cells in livestock. This could be in the form of additional small molecules, such as the 2i system, or in adjustments in energy substrates and atmospheric conditions of the culture system such as reduced the oxygen level from the standard 20% to a lower concentration to resemble the more hypoxic environment of the uterus. Aerobic to anaerobic transition of energy metabolism has been implicated in proficient reprogramming (Folmes et al. 2011). If a pluripotent state was not achieved, additional, or alternate, transcription factors may be necessary. Alternative reprogramming factors have been explored, but aspects of whether species-specific reprogramming factors are relevant for proper reprograming is not well understood in livestock (Kumar et al. 2015; Maekawa et al. 2011; Sumer et al. 2011).

The RNA sequencing data represent a catalog of all genes that are present in the reprogrammed cells. Published RNA sequencing data exists for the equine ICM and TE (Iqbal et al. 2014). A closer analysis of these data may provide clues to the molecular profile necessary to derive the true equine ES-like cells found in the ICM. Data that may be valuable to include would be RNA sequencing of eiPS cell lines, although not requisite if the RNA sequencing data from the ICM are available. There are likely a set of genes present in the equine ICM that has not been identified in rodents and humans that may be critical for eiPS cell generation and stabilization. This information could be used to tailor a species-specific reprogramming cocktail for horses. Additionally, consideration should be given to garner more information from the starting MSC population. This population holds a high degree of stemness and harbors several genes

implicated in, but not specific to, both trophoblast stem and ES cells. Reprogramming events alter the epigenetic profile of the target cells by changing the methylation status of the genome and activating otherwise silenced genes that are specific to ES or trophoblasts (Pawlak and Jaenisch 2011). Transduction with the OSKM transgenes activated additional genes associated with trophoblast stem and ES cell. Identifying differing methylated regions could give more understanding of the events that underlay the cell fate conversion from a MSC to a trophoblast by revealing the regulatory regions that control expression of critical genes.

In conclusion, the research herein presents the second known report of generation of induced trophoblast cells using standard reprogramming protocols. The other report is documented in swine. Although the goal of stable equine iPS cell generation was not achieved, novel cell lines were generated that have the potential to address a number of unknown processes of early equine trophectoderm development that have been, until now, unanswerable.

APPENDICIES

Appendix A

List of equine primers

Gene	Amplification product (bp)	Primer sequence	Gene function
β-ACTIN	63	F: GGGACCTGACGGACTACCT R: CCGTGGTGGTGAAGCTCTAG	Reference gene
CDH1	155	F: CATGCTGTGTCTTCGAATGG R: ACCTGCATCACAGAGGTTCC	Pluripotency
CDX2	105	F: GCTTCACACTGTGCTTCTGG R: TTGATCAGACCCCAGAGTCC	TE lineage
DHRS9	105	F: GCTTCACACTGTGCTTCTGG R: TTGATCAGACCCCAGAGTCC	TE lineage
DPPA4	178	F: AGGCCTATGGTGTCAAGTGG R: TTGTCCTCCAGGTACAAGGG	Pluripotency
ETS2	226	F: GTGCCTTCCTTCGAGAGCTT R: TGATGAACGACTGGCAGGAC	TE lineage
ELF3	127	F: ACAGCAAGCTCTTCTCCAGC R: GACACTTCTCCAGGCAGACC	TE lineage
EOMES	311	F: CTAAAAGAAGGTGCCAAAGC R: CTTAAGACCCAGCCCTTCTC	TE lineage
GATA2	100	F: GCATGAAGATGGAAAGTGGC R: ACATAGGAGGGGTAGGTGGG	TE lineage
GATA3	224	F: GCATCCAGACCAGAAACCGA R: ATGGTGAGGTCCGAAGGAGA	TE lineage
GAPDH	222	F: GAGACTCCGCCAACATC R: CTGACAATCTTCAGGGAATTGTC	Reference gene
GNE	124	F: GGTGGTACTTGGCTCTCACC R: CATAGCTGCCTCATCTTCCC	Capsule biosynthesis
HAND1	193	F: AGAAACAGGCAAGAAGGCGG R: TGCCCACGAGGTTCATGTTG	TE lineage
HESX1	195	F: GACACCTACGGCTCTTCAGG R: TGGTCTTCGGCCTCTATACC	Pluripotency
HEXIM2	175	F: GAAGGTGGCTACTCCGAAGC R: AGGATCTTCCTCCTCCAAGC	TE lineage
KLF4	222	F: TGGGCAAGTTTGTGTTGAAG R: TGACAGTCCCTGTTGCTCAG	Pluripotency
LIN28	167	F: CATGGGCTCTGTGTCAAACC R: CGGTCATGGACAGGAAGC	Pluripotency
MYC	240	F: GACGGTAGCTCGCCCAAG R: ACCCCGATTCTGACCTTTTG	Pluripotency

F = Forward, TE = Trophectoderm, R = Reverse; each sequence is listed 5'-3'

Appendix A continued

Gene	Amplification product (bp)	Primer sequence	Gene function
NEU1	144	F: ACCTTTCCTTGGATATCGGC R: CACTGAGGAGGCAGAAGACC	Capsule biosynthesis
NANOG	268	F: GTCTCTCCTCTGCCTTCCTCCATGG R: CCTGTTTGTAGCTAAGGTTCAGGATG	Pluripotency
OCT4	363	F: GCTGCAGAAGTGGGTGGAGGAAGC R: GCCTGGGGTACCAAAATGGGGCCC	Pluripotency
REX1	297	F: GACGGGAAAGGCCTGGATAGAAG R: GGCGGTAAGAAGCTGTTGAGAAAGG	Pluripotency
SALL4	240	F: AGAACTTCTCGTCTGCCAGC R: TTTCCTTGGGAAACATCTCG	Pluripotency
SLC35A1	94	F: TGAAGTTAAGTGTGCCGTCG R: GTCACCTGGTACACTGCTGC	Capsule biosynthesis
SLC17A5	125	F: GCAGATTTTGGAGTTGGAGC R: AAGCTTGCTTCTCTCAAGCG	Capsule biosynthesis
SOX2	282	F: AACGGCAGCTACAGCATGA R: TGGAGTGGGAGGAAGAGGTA	Pluripotency
TCL1A	105	F: ATCAGAGATGGACAGCAGCC R: ATCAAAGGCAGCAGGTAAGG	Pluripotency
TEAD4	193	F: GCTACATCAAGCTCCGGACA R: TTACTGTGGAAGGCTGTGGC	TE lineage
TFAP2A	110	F: AATGCTTTGGAAACTGACGG R: ATTGACCTACAGTGCCCAGC	TE lineage
TGM3	128	F: GGTGTCTGTGAACATGACGG R: ATGAGATCTTCACGGGATGC	Capsule biosynthesis

F = Forward, TE = Trophectoderm, R = Reverse; each sequence is listed 5'-3'

Appendix B

List of human primers

Gene	Amplification product (bp)	Primer sequence	Gene function
β-ACTIN	152	F: TGAAGTGTGACGTGGACATC R: GGAGGAGCAATGATCTTGAT	Reference gene
KLF4	397	F: ACGATCGTGGCCCCGGAAAAGGACC R:TGATTGTAGTGCTTTCTGGCTGGGCTC	Pluripotency
MYC	328	F: GCGTCCTGGGAAGGGAGATCCGGAGC R: TTGAGGGGCATCGTCGCGGGAGGCTG	Pluripotency
NANOG	154	F: TGAACCTCAGCTACAAACAG R: TGGTGGTAGGAAGAGTAAAG	Pluripotency
OCT4	144	F: GACAGGGGGAGGGGAGGAGCTAGG R: CTTCCCTCCAACCAGTTGCCCCAAAC	Pluripotency
SOX2	151	F:GGGAAATGGGAGGGGTGCAAAAGAGG R: TTGCGTGAGTGTGGATGGGATTGGTG	Pluripotency

F = Forward, R = Reverse; each sequence is listed 5'-3'

Appendix C

List of Sendai primers

Gene	Amplification product (bp)	Primer sequence	Gene function
KLF4	410	F: TTCCTGCATGCCAGAGGAGCCC R: AATGTATCGAAGGTGCTCAA*	Transgene
MYC	532	F: TAACTGACTAGCAGGCTTGTCG* R: TCCACATACAGTCCTGGATGATGATG	Transgene
OCT4	483	F: CCGAAAGAGAAAGCGAACCAG R: AATGTATCGAAGGTGCTCAA*	Transgene
SOX2	451	F: ATGCACCGCTACGACGTGAGCGC R: AATGTATCGAAGGTGCTCAA*	Transgene
SEV	181	F: GGATCACTAGGTGATATCGAGC* R: ACCAGACAAGAGTTTAAGAGATATGTATC*	Sendai genome

F = Forward, R = Reverse; each sequence is listed 5'-3'

*Primer contains Sendai virus genome sequences. Pairing of these primers with transgene specific primers allows detection of transgenes carried by the Sendai virus. Note that the same reverse primer is used for detecting *KLF4*, *OCT4*, and *SOX2* transgenes.

Appendix D

Protein	Species	Accession No.	AA* No.	Identity†	Positives‡	Expect₽	Notes
BMP4	Human	AAP86646.1	143	83%	83%	4e-47	Equine homolog lacks 15 AA N-term segment
	Horse	CAD29178.1	128				
CTGF	Human	CAG46534.1	349	95%	97%	0.0	Equine homolog lacks 71 AA N-term segment
	Horse	XP_001503366.2	278				
EGF	Human	CCQ43157.1	77	63%	81%	2e-21	Equine homolog has additional 36 AA N-term segment
	Horse	AAB32226.1	89				
FGF2	Human	NP_001997.5	288	98%	99%	5e-93	Only last 128 AA of C-term successfully matched
	Horse	XP_005607981.1	351				
FGF9	Human	NP_002001.1	208	100	100	1e-158	-
	Horse	XP_005601151.1	208				
HGF	Human	P14210.2	728	94%	97%	0.0	-
	Horse	XP_001487899.3	730				
IGF-I	Human	1BQT_A	70	100%	100%	6e-52	Equine homolog identified was precursor with 52 AA N-term segment
	Horse	AAA68952.1	122				
VEGF	Human	NP_001273973.1	163	93%	95%	7e-114	-
	Horse	BAB20890.1	190				

* AA = amino acid

† Extent to which amino acid have the same residues at each positions in alignment

‡ Amino acids that have a biochemical equivalent amino acid in each position of an alignment

₽ Expect lower than 1e-6 was considered biologically meaningful relationship between the homologs

Appendix E

Cell Sphere Formation

This protocol is for forming spheres by picking iTR cells from 2-100cm plates of MEF co-cultures or 1-T75 flask and plating into a 24 well non-adherent plate

1. Wash cells (either on MEF or in monolayer) 2X with DPBS
2. Add enough iTR media* back to sufficiently cover cells
3. If on mouse embryonic feeders (MEFs):
    a. Use microdissection needle to pick cell colonies, avoiding as many MEFs as possible, and collect in 50mL conical tube
    b. Proceed to 5
4. If on a cell matrix coated petri dish in monolayer, scrape with a cell scraper (Corning, #3010), and collect cells in 50ml conical tube
5. Pass the cells 2X through a 20 gauge needle to dissociate cell colonies not dispersed during scraping
6. Pass the cells through a 100µm cell strainer (BD Falcon, #352360) to remove large debris
    a. Optional: Spin for 5 minutes at 0.4 rcf to pellet cells to condense volume if needed
7. Bring to 24ml and add 1mL of cell suspension to each well of a 24 well Ultra-Low Attachment Surface plate (Corning, #3473)

*iTR media

10% FBS

1% Pen/step

1% Non-essential amino acids

100µM β-mercaptoethanol

Appendix F

Mitomycin C Inactivation of mouse embryonic fibroblasts (MEFs)

CARCINOGEN ALL WORK MUST BE DONE IN BIOSAFETY CABINET

1. Reagents
    a. Mitomycin C (Sigma, M4287, 2mg)
        i. Inactivation media is at a 10µg/mL concentration
    b. 196mL growth media*
    c. 4mL sterile PBS or sterile H_2O
2. Method 1
3. Dissolve mitomycin in PBS or H_2O
4. Make 8 aliquots of 500µL and freeze at -20°C until use (up to 12 months)
    a. This gives a concentration of 500µg/mL
5. Add 1 aliquot tube per 25mL growth media to make Inactivation media
    a. This gives a concentration of 10µg/mL
6. Method 2
7. Dissolve mitomycin C in 200mL growth media to get a 10µg/mL concentration of inactivation media
    a. Approximately 4mL dissolves the powder in the mitomycin C container received from Sigma, once dissolved, transfer back to the 200mL of growth media
8. Aliquot 25mL into 50mL conical tubes and freeze at -20°C until use (up to 6 months)
9. Inactivation
10. Grow MEFs to between 60% and 80% confluency
11. Was 2X with DPBS
12. Add inactivation media and incubate at 37°C for 3 hours
13. Remove inactivation media and store at 4° up to 2 weeks
    a. Inactivation media can be used up to 3 times before it must be discarded
14. Wash with DPBS 2X disposing of waste in a container labeled biohazard
15. Wash with DPBS 1X disposing of waste with conventional aspirator

16. If using immediately, add growth media and plate cells to be co-cultured within 3 days. If intended for future uses proceed to 14.
17. Freeze back
    a. After final wash, add an appropriate volume of 0.125% trypsin-EDTA to cover cells and incubate for 5-10 minutes at 37°C
    b. Add 4X trypsin-EDTA volume of growth media to inactivate trypsin, collect in conical tube, and count the cells
        i. e.g. if you add 3mL trypsin, add 12mL growth media
    c. Spin the cells at 0.4 rcf for 5 minutes
    d. Resuspend in freeze media[#] at a concentration of $1.5X10^6$cell/mL and freeze back in 1mL aliquots
    e. Plate 1 tube of $1.5X10^6$cell/mL into a 100cm plate when needed for an 80% confluent feeder layer
    f. Leave overnight to attach
    g. Plate cells to be co-cultured within 3 days

*growth media for MEFs:

    h. 10% FBS
    i. 1% Pen/strep
    j. In high glucose DMEM

[#]freeze media

    k. 10%DMSO
    l. 90% growth media

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