ABSTRACT

Title of Document: THE IMPACT OF CULTURE MEDIA ON THE IN VITRO PRODUCTION OF CAT BLASTOCYSTS AND EXPLANT QUALITY

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Continued improvements in embryo culture media composition allow for the growth of high quality blastocysts, which can be used to derive embryonic stem cells (ESCs). ESCs are capable of becoming any cell type in the body making them a valuable research tool for therapeutic and regenerative research, while furthering our understanding of embryonic development and cell differentiation. The domestic cat is an important model species for both human medicine and wild felids. Cat embryo culture produces blastocysts at a rate far below that of the mouse and initial attempts at deriving cat ESCs have resulted in embryonic stem-like cells, which cannot be maintained indefinitely. In this study we assessed and compared the quality of cat blastocysts produced *in vitro* using two commercial human blastocyst growth media, and the maintenance of pluripotency markers OCT-4 and NANOG in inner cell mass explants from *in vitro* produced blastocysts over 14 days.

THE IMPACT OF CULTURE MEDIA ON THE IN VITRO PRODUCTION OF CAT BLASTOCYSTS AND EXPLANT QUALITY

By

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AKT - also known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase

ATP – adenosine triphosphate

bFGF – basic Fibroblast growth factor

BMP4 – bone morphogenetic protein 4

BSA – bovine serum albumin

C-MYC - myelocytomatosis virus oncogene cellular homolog

cDNA – complementary deoxyribonucleic acid

CFF – cat fetal fibroblasts

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

E2 – Estradiol-2

EpiCS – epiblast derived stem cell

ERK 1/2 – Extracellular signal-regulated kinases

ES-like cells - cells that act like embryonic stem cells that have not yet been validated

ESC – embryonic stem cell

FBS – fetal bovine serum

FGF2 – fibroblast growth factor 2

FGFR – FGF receptor

FITC – a fluorescent probe attached to a secondary antibody

FRS2 - fibroblast growth factor receptor substrate 2

FSH – follicle stimulating hormone

Gab1 – Growth factor receptor-bound protein-associated-binding protein 1

Gata6 – GATA transcription factor, binds DNA sequence “GATA”, endodermal marker

GMEM – Glasgow Minimal Eagles Medium

GP130 - glycoprotein 130

GRB2 – growth factor receptor-bound protein 2

GSK3 – glycogen synthase kinase 3

hESC – human embryonic stem cell

ICM – inner cell mass

iPSC – induced pluripotent stem cells

KSR - Knockout serum replacer

LH – luteinizing hormone

LIF – leukemia inhibitory factor

LIFR – LIF receptor

MAPK – mitogen-activated protein kinases

MEK – mitogen-activated protein kinase kinase (also known as MAP2K)

mESC – murine embryonic stem cell

MET – maternal-to-embryonic transition

MII – metaphase II, in reference to oocyte stage

MMC – mitomycin C

mRNA – messenger RNA

NANOG - a divergent NK2 homeodomain transcription factor responsible for maintaining the self-renewal properties of ESCs

NODAL – a member of the TGF beta superfamily

NOGGIN – TGF-B inhibitor, inhibits BMP4

OCT-4 - Octamer-binding transcription factor 4, a POU domain transcription factor, also known as POU5F1 for POU domain, class 5, transcription factor 1

PBS – phosphate buffered saline

PGCs - primordial germ cells

PI-3k – Phosphoinositide 3-kinase

PLCγ - phospholipase C γ

qRT-PCR – quantitative real time polymerase chain reaction

Raf – proto-oncogene serine/threonine-protein kinase, a member of the MAPK/ERK pathway

Ras – Rat sarcoma protein family, a member of the MAPK/ERK pathway

RNA – ribonucleic acid

RPL-17 – ribosomal protein L17, a house-keeping gene

RTK – receptor tyrosine kinases

SCNT – somatic cell nuclear transfer

SMAD – a portmanteau of the proteins "mothers against decapentaplegic (MAD)," a *Drosophila* protein, and "SMA" (from gene *sma* for small body size), a *Caenorhabditis elegans* protein; an intracellular protein that transduces extracellular signals from the TGF-β ligand

SOX2 – pluripotency transcription factor, Sex Determining Region-Y-box-2

SSEA-1, -3, -4 - stage specific embryonic antigen, a glycosphingolipid

STAT – signal transducer and activator of transcription 3, a transcription factor

TE – trophectoderm

TGF-β – transforming growth factor beta

TX – Triton X 100

WNT – a portmanteau of “Int” (integration 1, a mouse gene) and “Wg” (wingless, a *Drosophila* gene); a group of secreted lipid-modified signaling proteins, this pathway has a role in embryonic development, cell differentiation and cell polarity

Chapter 1: Literature Review

1.1 Introduction

The culture of embryos *in vitro* has allowed for advancements in assisted reproductive technologies such as embryo transfer, embryo sexing, genetic diagnosis, and has provided a better understanding of early embryonic stages. The composition of the media used in embryo culture has evolved over the years from simple salt solutions and complex media more suited to tissue culture to media specifically designed for optimal development (Herrick et al., 2007). Yet despite this progress there is still plenty of room for advancement. Continued improvements in media composition allow for the growth of high quality blastocysts, which can be used in advanced technologies such as embryonic stem cell (ESC) derivation.

Derived from various stages of preimplantation embryos, ESCs can remain in a pluripotent state and are capable of becoming any cell type in the body. This plasticity makes ESCs a valuable research tool for both therapeutic and regenerative research, while simultaneously furthering our understanding of embryonic development and cell differentiation. While ESCs hold much promise for research via their ability to be genetically manipulated and differentiated in culture, it has been thirty years since the first naïve mouse ESCs (mESCs) were derived, and advancements in other species has been limited and slow. Naïve or ground-state ESCs are characterized by the ability to become any type of cell present in the three germ layers (including germ cells), maintain pluripotency

markers, self-renewal capabilities, and have a normal karyotype. To be a validated naïve ESCs, the cells must be able produce a chimeric embryo and to contribute to the germ line of the resulting chimeric animal. Primed ESCs are similar to naïve but have slightly different morphology, cytokine dependencies, and do not contribute to the germ-line (Boyer et al., 2005).

Validated ESCs from the domestic cat would be advantageous as cats are a unique model species for both human medicine and exotic and rare wild felids. Derivation of domestic cat ESCs have shown promising results, however these initial attempts have resulted in embryonic stem-like (ES-like) cells that cannot be maintained indefinitely in culture without differentiating or losing the ability to self-replicate. Domestic cat embryos have been cultured *in vitro* for years, yet the rates of blastocyst formation are far below those of the mouse, demonstrating room for improvement in culture techniques. Techniques developed for the domestic cat can be easily translated to large and small wild felids for conservation biology purposes.

The main objectives of this study were to:

1. Determine whether protein source affected embryo quality. Our hypothesis was that Medium A with a defined protein source would be superior to Medium B with a semi-defined protein source for growing domestic cat blastocysts. More specifically, Medium A contained serum protein substitute while Medium B contained both fetal bovine serum (FBS) and bovine serum albumin (BSA), which can have differing, and sometimes contradictory, effects if they are added at *in vitro* maturation,

fertilization or later staged culture (Dobrinsky et. al., 1996; Karja et al., 2002; Wood et. al., 1995).

2. Derive naïve ESC using 3 different culture conditions. Our hypothesis was that cat inner cell mass (ICM) explants would show preference for a cocktail of small inhibitory molecules rather than previously used cytokine cocktails for cat ES-like cells or the currently used cytokine cocktail for mESC culture. By necessity, cytokines used in cat studies are of human or mouse origin, and we believe that the small molecules will be able to overcome the species-specificity that has been shown with cytokine receptors (Wilcox et al., 2009).

It is hoped that the findings of this research will further elucidate the effects of protein source on embryo culture and provide insights into pluripotency pathways in the domestic cat, helping to remove some of the difficulty inherent with deriving ESCs in novel species.

1.2 The domestic cat

There are 36 known species of felids in the world today, of which 16 are listed as at least vulnerable, if not endangered, on the IUCN Red List. Twenty-nine species are considered decreasing in population size ("IUCN Red List of Threatened Species," 2012). The domestic cat has been used as a model species for wild cats for artificial reproductive technologies for many years (Graves-Herring et al., 2011; Wildt et al., 2010). Cats are smaller and easier to obtain than their exotic cousins, allowing for more research to be conducted more readily. The domestic cat reproduces well in a laboratory setting; it reaches maturity quickly and lives longer than mice, providing an advantageous model for human biomedical research.

There is an ever-growing pool of information about culturing embryos in the domestic cat. Embryonic development in the cat is very similar to other eutherian mammals. *In vitro*, an oocyte can be matured in the presence of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2). Matured oocytes can be fertilized and cultured until they reach the blastocyst stage. After fertilization, the domestic cat embryo undergoes an initial wave of embryonic transcription (Waurich et al., 2010). At 1-day post insemination, the embryo divides into 2 cells, and at 2 days post insemination, the embryo consists of 4 cells (Waurich et al., 2010). At the 5 to 8 cell stage, a second wave of embryonic gene transcription occurs also referred to as maternal-to-embryonic transition (MET) (Filliers et al., 2011; Hoffert et. al., 1997, Waurich et al., 2010). The embryo reaches 8 to 16 cells at day 3, and morula at day 4 to 5 (Waurich et

al., 2010). As the morula develops into the blastocyst stage, the cells differentiate into two populations, the cells of the trophectoderm (TE) and those of the ICM. The embryo enters the blastocyst stage 6 days post insemination, expands by day 7 and hatches out of the zona pellucida by 7.5 days (Filliers et al., 2011). *In vivo,* domestic cat embryos enter the uterine horn between day 6 to 7, during the transition from morula to early blastocyst, and attach to the uterus through an endotheliochorial placenta 12 to 13 days post insemination (Boomsma et. al., 1991). The ICM will undergo a second differentiation into primitive endoderm and epiblast. The epiblast will become the embryo proper while primitive endoderm contributes to the extraembryonic membranes (Bianchi et. al., 1993; Luckett, 1978).

Studying *in vitro* embryo production media in various species has revealed that the requirements of preimplantation embryos are species and stage specific. Multiple protocols have been developed to culture cat embryos *in vitro* with varying success in blastocyst formation (Waurich et. al. 2012). One interesting study sought to make a feline optimized culture medium by identifying precise molar concentrations of ions, carbohydrates, amino acids, vitamins and serum necessary for early embryonic growth (Herrick et al., 2007). Previous studies have examined the importance of protein source when culturing embryos of cat and pig *in vitro* (Dobrinsky et al., 1996; Karja et al., 2002; Wood et al., 1995). Complex biological macromolecules are used in many culture systems across species as a source of protein and nutrients. However, FBS has been found to cause poorer oocyte maturation than BSA in cat, bovine and rat, yet FBS allowed

more oocytes to be fertilized (Wood et al., 1995). Interestingly, more embryos reach the hatching blastocyst stage and have higher cell number with FBS rather than BSA for fertilization and culture (Dobrinsky et al., 1996; Han & Niwa, 2003; Herrick et al., 2007; Karja et al., 2002).

Further complicating these studies is the effect of FBS on oocyte and embryo tolerance to cryopreservation. Several studies in the bovine have reported increased success in blastocyst survival after cryopreservation when cultured in a serum free system (Abes et. al., 2002; George et al., 2008; Gomez et al., 2008; Mucci et al., 2006; Ohboshi et al., 1997; Rizos et al., 2003; Shamsudden et. al., 1994). Studies in the cat has shown that addition of FBS to culture media increased embryo sensitivity and resulted in decreased post-thaw viability of expanding blastocysts after vitrification, compared to serum free culture conditions (Murakami et al., 2011; Swanson et. al., 1999). Addition of serum can cause an accumulation of lipid droplets, which contribute to this decrease in freezing success. As a defining characteristic of cat oocytes is the dark cytoplasm due to a high concentration of lipid droplets, a serum free culture method that would not add to the already high concentration of lipids in cat oocytes would be preferable.

1.3 Embryonic Stem Cells

Background

In mammals, stem cells can be divided into two categories: adult and embryonic. ESCs have the potential capacity to grow *in vitro* indefinitely without differentiating. ESCs were derived first from the ICM of mouse blastocysts (Evans & Kaufman, 1981; Martin, 1981). Currently mESCs can be derived from an 8-cell embryo (Tesar, 2005), morula (Tesar, 2005), and primitive ectoderm of implantation-delayed blastocysts (Prelle, et al. 2002). ESCs can be differentiated *in vitro* into the three germ layers (endoderm, mesoderm, and ectoderm), including further induction into more differentiated cell types by applying the proper culture conditions. ESCs are also capable of differentiating into primordial germ cells (PGCs), another pluripotent cell type that is isolated from the genital ridge of post-implantation embryos (S. Liu et al., 2004). Due to the ability to differentiate into so many cell types, stem cells are an invaluable research model for the study of development (O'Shea, 1999) and disease (Comizzoli et al., 2010; Travis et al., 2009; Wildt et al., 2010).

Validated ESCs are those which can contribute to the germ line after being injected into a blastocyst stage embryo, producing chimeric offspring that have the ESC descendants represented throughout the organism, including germ cells (Kehler et al., 2005). Currently, fully validated ESCs have been derived in the mouse (Evans & Kaufman, 1981; Martin, 1981), and rat (P. Li et al., 2008). Human ESCs (hESCs) cannot be tested for germline capabilities due to ethical considerations, but are considered semi-validated (Thomson, 1998).

There are two types of ESC: naïve ESC (e.g., typical ICM derived mESC), and epiblast derived stem cells (EpiSC, e.g., hESC and mouse EpiSC derived from the epiblast). Naïve mESC lines are dependent on leukemia inhibitory factor (LIF)/Signal transducer and activator of transcription 3 (STAT3) signaling for maintenance of pluripotency (Hall et al., 2009) and bone morphogenetic protein 4 (BMP4) for self-renewal and resistance to differentiation (Ying et al., 2003). When fibroblast growth factor (FGF2) and ACTIVIN/ Transforming growth factor beta (TGF-β) signal transduction pathways are activated, mESCs have a tendency to differentiate (Li et al., 2009). Naïve ESC can be genetically manipulated via the addition or deletion of genes before being injected into a blastocyst, giving rise to a modified whole animal chimera (Evans, 2005; Wobus & Boheler, 2005). This ability allows the mouse to be a wonderfully useful mammalian model for biomedical research and may lead to other, physiologically more relevant models such as domestic cats upon production of validated ESC in these other species.

Human and primate ESCs are examples of primed ESCs. Their colonies look different from mESC colonies in that they have a more flattened colony morphology and have dependence on FGF2 and TGF-β / ACTIVIN/NODAL signaling rather than on LIF/STAT3 for maintenance of their pluripotency (Dvorak et al., 2005; Xu et al., 2005). When exposed to BMP4, hESC differentiate quickly (Das et al., 2007; Xu et al., 2002). The term "ES-like" is used for cell lines that have not yet had proven germline transmission. While these ES-like cells can be used to create cloned animals via somatic cell nuclear

transfer (Keefer et al., 1994), they typically cannot be maintained in culture and may have abnormal morphology or expression patterns of pluripotency markers. ES-like cells have been developed for the rabbit (Fang et al., 2006; Intawicha et al., 2009), baboon (Simerly et al., 2009), rhesus macaque (Mitalipov et al., 2006), marmoset (Thomson et al., 1996), cynomolgus monkey (Suemori et al., 2001), chicken (Pain et al., 1996), mink (Sukoyan et al., 1993), hamster (Doetschman et al., 1988), pig (Brevini et al., 2007; Wheeler, 1994), bovine (Mitalipova et al., 2001; Saito et al., 2003; Stice et al., 1996; Wang et al., 2005), water buffalo (Huang et al., 2010), ovine (Dattena et al., 2006; Notarianni et al., 1991), canine (Hatoya et al., 2006; Hayes et al., 2008; Wilcox et al., 2009), and goat (Behboodi et al., 2011), along with initial progress in other species. Putative success has also been achieved in the domestic cat (Gómez et al., 2010; Yu et al., 2008). ES-like cells cannot yet produce chimeric offspring nor can they be maintained undifferentiated indefinitely in culture.

Derivation and culture of ESCs

Derivation of ESCs from preimplantation embryos can be accomplished by placing a hatched or zona-free blastocyst-stage embryo on a layer of feeder cells and culturing the ICM region *in vitro*. Multiple techniques involving culture of both intact and dissected blastocysts have been used successfully in mice (Evans & Kaufman, 1981; Kobolak et al., 2010). The ICM may be enzymatically separated from the TE of the blastocyst, as is done during typical hESC culture (Pera et al., 2000). Immunosurgery involves labeling the TE and then lysing the

cells to remove them from the ICM (Solter & Knowles, 1975). Microdissection can also be used to mechanically separate the ICM from the TE and cultured separately. Although some TE may remain attached, this method has been used successfully in hESC and mESC derivation (Illmensee & Hoppe, 1981; Skottman & Hovatta, 2006; Ström et al., 2007).

In most procedure, the ICM explants are grown on a layer of mitotically-blocked feeder cells (Evans & Kaufman, 1981; Gómez et al., 2010; Y. Liu et al., 2011). For domestic cat ES-like cell culture, cat dermal fibroblasts provided better cell support for the initial attachment of plated ICMs, generated higher numbers of ES-like cells and maintained more prolonged self-renewal in an undifferentiated state than using mouse feeder cells (Gómez et al., 2010). This demonstrated that cell-surface and soluble factors that support self-renewal and prevent differentiation provided by feeder layer cells may be species specific. The optimal conditions for deriving novel ES cell lines may differ from those required for maintenance. In the pig, homogenous feeder cells were better for starting initial cultures but mouse cells proved better for maintenance (Strojeck et al., 1990). For dog ES-like cells, homologous and heterologous feeders equally supported initial cultures, but mouse feeder cells were superior for continued growth (Wilcox et al., 2009). Having a dense feeder layer is important and should not be overlooked.

Stem cell progress in the domestic cat

Several attempts have been made to develop domestic cat ES-like cells

(Gómez et al., 2010; Serrano et al., 2005; Yu et al., 2008). *In vivo* produced cat blastocysts generated cat ES-like cells in culture for up to 8 passages (Yu et al., 2008). Knockout serum replacer (KSR) medium allowed ICM cells to attach better and form domed colonies, while FBS medium formed flat colonies and had higher cell proliferation (Yu et al., 2008). When ICM cells from *in vitro* blastocysts are explanted for ESC culture, the colonies produced had a tightly packed, dome shaped morphology, alkaline phosphatase activity, and readily expressed the markers genes OCT-4, SSEA-1, NANOG, SOX2 and C-MYC, similarly to what is seen in whole blastocysts, although expression levels of colonies were at a lower level than blastocysts (Gómez et al., 2010). ES-like cells have also been shown to be positive for SSEA-1, SSEA-3, and SSEA-4 (Yu et al., 2008). Several studies have verified that cat ES-like cells seem to survive undifferentiated and proliferate longer when grown on a homologous feeder layer of blocked cat fibroblast cells (Gómez et al., 2010; Serrano et al., 2005; Yu et al., 2008). Reports on the best way to passage domestic cat ES-like cells are conflicting, both passaging via enzymatic and mechanical dissociation can stimulate cells to differentiate (Gómez et al., 2010; Yu et al., 2008). These studies have demonstrated short-term culture can be obtained, however neither has been able to maintain ESC characteristics greater than 102 days (Gómez et al., 2010; Yu et al., 2008).

The quality of ESCs produced is reliant on the quality of the initial starting material. It has been shown in other species that *in vivo* produced embryos have more developmental competence than *in vitro* produced embryos (Bavister,

2004). Typically, *in vivo* produced embryos have higher expression levels of the common pluripotency markers, however *in vitro* embryos are much easier to obtain for many species. It is important for the continued advancement of ESC culture that high quality blastocysts be produced *in vitro*. A domestic cat ESC line will be a valuable non-murine model for studying comparative diseases and developmental disorders in the human. As the domestic cat is already used as a model of assisted reproductive technologies for wild felids (Brown 2011), the domestic cat can be used as a foundation for future ESC research in endangered felids. Establishing efficient ESC derivation techniques in the domestic cat will drastically reduce the number of embryos needed from rare feline species. When future attempts are made at producing rare felid ESCs, the proper ESC culture conditions can be more quickly obtained by starting with the cytokine conditions that best maintain domestic cat ESCs.

Optimization of ESC Culture Systems

Cytokines are small cellular molecules that, when added to the culture medium, can activate or inhibit receptors. For the culture of ESCs, cytokines are added to the medium to stimulate appropriate pathways within the cells, promoting a continuation of the stem-state, or differentiation if desired. Mouse and human ESCs are cultured using different cytokines, possibly reflecting the difference between naïve and EpiSC stem cell types. Traditionally, mESCs are cultured using LIF (leukemia inhibitory factor), sometimes in combination with BMP4 (bone morphogenetic protein-4), depending on the presence of serum in

growth medium used (Ying et al., 2003). LIF activates STAT3 (signal transducer and activator of transcription-3) which directs ESC self-renewal (Matsuda et al., 1999; Niwa et al., 1998). In vertebrate embryos, BMPs are well known anti-neural factors (Wilson & Hemmati-Brivanlou, 1995; Wilson & Edlund, 2001) and have been shown to antagonize neural differentiation of ESCs (Tropepe et al., 2001; Ying et al., 2003). When given alone, BMP promotes differentiation of ESC into non-neural fates, but in combination with LIF self-renewal is enhanced, resulting in highly pure populations of undifferentiated ESCs after 2 to 3 passages (Ying et al., 2003).

When attempting to derive ESCs from a novel species, cytokines like LIF may not yet be available for the novel species; it is critical to use a type of LIF that is closely related to and will cross react with the species of interest. One study found that canine and mouse LIF receptors (LIFR) are responsive to human LIF, but human LIFR is not responsive to mouse LIF. As human and canine are 75% homologous to murine, they used human LIF in this study (Wilcox et al., 2009). The optimal cytokines would be from the species of interest, however isolating or producing recombinant proteins is time consuming and costly. An alternative to species-specific cytokines exists. Small molecules have been developed that have parallel functions compared to known cytokines, without the species-specific requirement of the cytokine. These small molecules usually exhibit a longer life in culture medium than the counterpart cytokine. The addition of small molecules allows scientists to perfect the growth medium and

better maintain undifferentiated proliferation of pluripotent ESCs for indefinite periods of time.

Small molecules should be more useful across species for maintenance of pluripotency, rather than using the traditional semi-defined media supplemented with cytokines, such as LIF. A novel protocol for isolation of mouse and rat ESC involves cocktails of two or three small inhibitory molecules, called the "2i" or "3i" method (Li et al., 2009). These inhibitory small molecules block specific cellular pathways, allowing for continued self-renewal or maintenance of pluripotency factors. Small inhibitory molecules have been shown to be useful in non-mouse species to maintain pluripotency. There are several combinations of inhibitors that one might choose; we chose one version of "3i," which consists of CHIR99021, PD0325901, and PD173074. This 3i cocktail targets the FGF, MEK, and GSK3 pathways. This particular cocktail has enabled efficient derivation and subsequent propagation of germline competent ESCs from several mouse strains, as well as maintaining OCT-4 and NANOG expression longer than in traditional mouse culture (Buehr et al., 2008; Ying et al., 2008). CHIR99021 selectively inhibits both GSK3α and GSK3β (Buehr et al., 2008; Murray et al., 2004). The molecule inhibitor PD0325901 inhibits MEK (via ERK), eliminating the MAPK signal that induces differentiation (Lee et al., 2012). When CHIR99021 and PD0325901 were used on reprogrammed porcine embryonic fibroblast cells, the cells demonstrated a similar colony morphology to naïve mESCs, were dependent on LIF to remain undifferentiated, expressed pluripotency markers, exhibited high telomerase activity, a short cell cycle

interval, a normal karyotype, and were able to generate teratomas (Telugu et al., 2010). This example supports our hypothesis that a "3i" method will support maintenance of pluripotency in domestic cat ES-like cells and initial ICM explants.

1.4 Markers of Pluripotency

Major Transcription Factors

OCT-4, NANOG, and SOX2 work cooperatively to regulate expression of genes in pluripotent cells. Due to their essential role in early embryonic development, NANOG, OCT-4 and SOX2 are the most commonly used markers of pluripotency in ESCs (Boyer et al., 2005). A null mutation for any of these genes is lethal to an embryo; the embryo cannot maintain pluripotent cells and cannot differentiate appropriately to undergo normal gastrulation (Masui et al., 2007). Homeodomain transcription factors, such as NANOG and OCT-4, are characterized as being evolutionarily conserved in sequence, but have species-specific difference in expression patterns

While these genes have distinct roles, the pathways they affect are interconnected and disruption of one factor may result in developmental consequences for the embryo (Boyer et al., 2005). The three factors influence each other's transcription, leading to a rapid loss of pluripotency if one factor is altered. SOX2 and OCT-4 can form a dimer before or after the proteins bind DNA; both proteins are often necessary for efficient binding of the promoter and expression of a gene (Boyer et al., 2005). In fact, more than 90% of the promoter regions of genes that are bound by both OCT-4 and SOX2 are also bound by NANOG in close proximity (Boyer et al., 2005). These three proteins together occupy a minimum of 353 genes, demonstrating their broad control of transcription in hESC (Boyer et al., 2005). As is seen with SOX2, over-expression of OCT-4 and NANOG can inhibit their own promoters (Kuroda et al.,

2005; Rodda et al., 2005). Rescue of expression can occur in some instances: for example over-expression of *OCT-4* can rescue the pluripotency of *SOX2*-null mESCs (Boer et al., 2007).

OCT-4

OCT-4 is a POU domain transcription factor, also known as POU5F1 for POU domain, class 5, transcription factor 1. The POU family of proteins is characterized as being highly conserved across species. POU is an acronym for Pituitary-specific Pit-1, Octamer transcription factor protein and neural Unc-86 transcription factor. It is expressed in unfertilized oocytes, early embryos, ICM cells, epiblast, pregastrulation embryos, and in primordial germ cells of mice (Babaie et al., 2007). It is also associated with lineage differentiation during blastocyst formation (Babaie et al., 2007). Maternally derived *OCT-4* mRNA can be detected in the mouse ovum through the 4-cell stage and the bovine ovum through the 8-cell stage (Kurosaka, et al. 2004). In the mouse, zygotic expression of OCT-4 begins at the 4-cell stage and later becomes restricted to the ICM cells of the blastocyst (Boiani & Schöler, 2005; Yeom et al., 1996).

In human and mouse ESCs, OCT-4 expression becomes reduced when these cells differentiate and lose their state of pluripotency (Ginis et al., 2004). A knockdown of OCT-4 in mESCs resulted in elevated expression of TE genes (*cdx2*), and endodermal markers (*gata6*) (Hay et al., 2004). In mESCs, differentiation into endoderm and mesoderm, or into TE, can be induced by increasing or decreasing *OCT-4* gene expression beyond 50% of its physiological

range (Niwa et al., 2000). Similarly, up-regulation of OCT-4 in mESCs can induce extra-embryonic endoderm and mesoderm lineages, which is functionally similar to a lack of LIF (Niwa et al., 2000). Exclusive OCT-4 expression is not sufficient to maintain undifferentiated mESCs; a cooperative LIF signal is required to maintain pluripotency (Niwa et al., 2000).

NANOG

NANOG is a divergent NK2 homeodomain transcription factor responsible for maintaining the self-renewal properties of ESCs (Mitsui et al., 2003). NANOG has a role in many important cellular mechanisms, including the regulation of transcriptional repression in the epiblast and the transcription of histone methyltransferases and telomeric proteins (Villasante et al., 2011). In the mouse, *Nanog* mRNA has been detected as early as the morula stage, and can be found later in the ICM of blastocyst stage embryos (Avilion et al., 2003), as well as in the proximal mouse epiblast at E6, and in the nascent gonad at E11.5 to E12.5 (Hart et al., 1994).

In mESCs, differentiation causes a reduction in *Nanog* expression (Hart et al., 2005). *Nanog* over-expression in mESCs promotes growth independent of LIF supplementation (Chambers et al., 2003), as well as a resistance to induced differentiation (Loh et al., 2006). The over-expression of *Nanog* in hESCs enables continued propagation of pluripotent cells over several passages (Chambers et al., 2003).

SOX2

The transcription factor SOX2 (SRY-related HMG box 2) is essential for pluripotent cell development. In the mouse, *SOX2* is expressed in the 4-cell embryo, and remains in both the ICM and TE cells in blastocysts (Masui et al., 2007), later becoming restricted to the epiblast (Avilion et al., 2003). Trophoblast stem cells cannot be obtained from *Sox2*-null mouse embryos, signifying that SOX2 is needed for the development of trophoblast (Masui et al., 2007). Interestingly, *Sox2*-null embryos cannot express *Cdx2* (Masui et al., 2007). Expression of *Sox2* can be rescued by forced expression of *OCT-4* (Masui et al., 2007). Due to the dimeric nature of SOX2 and OCT-4, the expression patterns of the two proteins are very similar. *Sox2* was present in higher levels at the morula stage than the blastocyst stage in domestic cat *in vitro* embryos, as well as higher levels of *Sox2 in vitro* than *in vivo* (Filliers et al., 2011). In mouse embryos, higher levels of *Sox2* were found in blastocysts compared to morulae (Avilion et al., 2003).

Relevant Pathways

The activation and inhibition of cellular signaling pathways is the key to maintaining pluripotency in ESCs. While many core pathways are conserved, species-specific differences necessitate further analysis in each new ESC derivation. The culture techniques used for mouse and human ESCs provide a technical starting point for ESC culture of new species. The relevant pathways in these two models are similar and, in both the mouse and human, there is cross talk and redundancy between the pathways. These pathways include phosphorylation

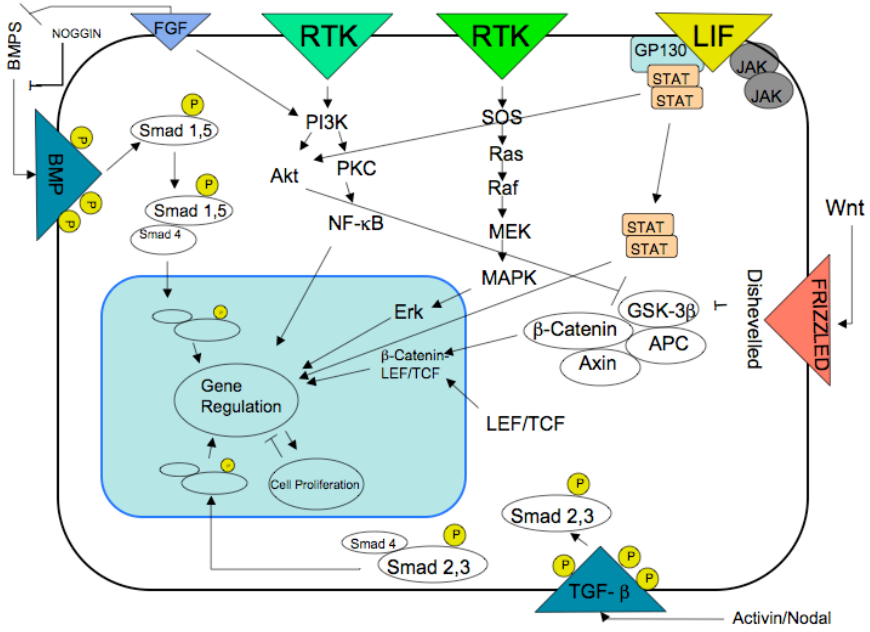
cascades in the presence of ligand-bound receptors, the signal from which is transferred to the nucleus (Figure 1). Here, transcription of key genes can be enhanced or impaired. These genes control various cellular functions including differentiation and self-renewal. Small molecules, or cytokines, can be used to further activate or inhibit these pathways to promote the growth of desired cell types.

LIF/Jak-STAT Pathway

Leukemia inhibitory factor is a member of the LIF-oncostatin M-I1-6 superfamily of cytokines. Supplementation with LIF has eliminated the need for the mitotically inactivated fibroblast co-culture system when culturing mESCs (Evans & Kaufman, 1981; Smith et al., 1988). LIF participates in a heterodimeric cell surface receptor complex consisting of LIFR (the LIF receptor subunit, Gearing et al., 1992) and glycoprotein 130 (GP130, Davis et al., 1993). In the absence of maternal LIF, blastocysts enter a stage similar to delayed implantation (Stewart, 1994). Embryos that lack either LIFR or GP130 are able to develop beyond gastrulation (Dani et al., 1998); since these embryos make it to gastrulation, this suggests that there might be an alternative mechanism to maintain pluripotency *in vivo* than what has been seen for *in vitro* ESC cultures.

When LIF binds to the LIFR, it induces the formation of a LIFR-GP130 heterodimer. This mechanism activates the Jak pathway, causing the tyrosine phosphorylation of GP130 and providing a binding site for proteins on the activated receptor complex (Matsuda et al., 1994). The phosphorylation of

Figure 1: A summary of pathways relevant to embryonic stem cells: RTK, PI3K, BMP, TGF-β, and canonical Wnt Pathways based on Gilbert 2010.

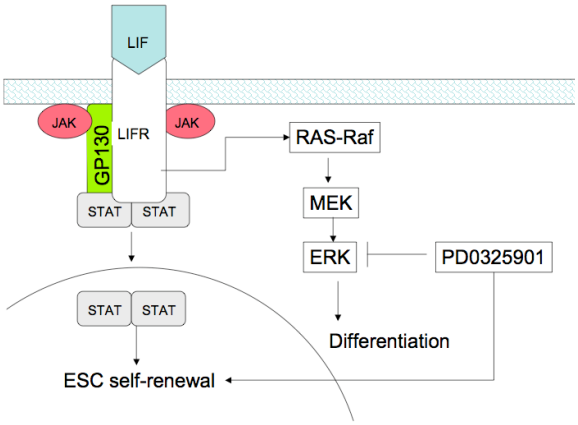


GP130 leads to the activation of STAT 1 and 3, as well as ERK 1 and 2 and PI-3K, as seen in Figure 2 (Cavaleri & Scholer, 2003). Stimulation of Ras/Raf/MEK/ERK leads to differentiation in mESCs (Burdon et al., 1999). LIF maintains pluripotency in mESCs by restricting the GSK3 pathway, and allowing β-catenin to translocate to the nucleus and lead to transcription (Buehr et al., 2008). Conversely, it has been shown that the activation of MEKs or ERKs, can overcome differentiation in mESCs and promote self-renewal (Burdon et al., 1999). A dominant form of STAT3 can be used in place of LIF for mESC pluripotency maintenance (Matsuda et al., 1994). While hESCs similarly express LIF and GP130 receptors, using dominant active STAT3 is insufficient to maintain the undifferentiated state (Humphrey et al., 2004). In hESCs, the use of added LIF is insufficient in maintaining pluripotency, and BMPs induce rapid differentiation.

TGF-β Pathway

The Transforming Growth Factor-β (TGF-β) pathway has two branches: the TGF-β/Activin/Nodal and the BMP subfamily. Both branches of the pathway are activated by a ligand inducing two Type-I receptors and two Type-II receptors, thus forming a complex of four receptors and one ligand. In the first branch, BMP ligands bind the extracellular ligand-binding domain of two Type-I BMP receptors. This complex then binds the Type-II receptor, resulting in a tetramer. On the other branch of the pathway, a TGF-β or Activin ligand will bind to the two Type-II receptors, which then joins with two Type-I receptors (Shi & Massagué, 2003). Phosphorylated receptor-mediated SMADs (SMAD 1 and 5)

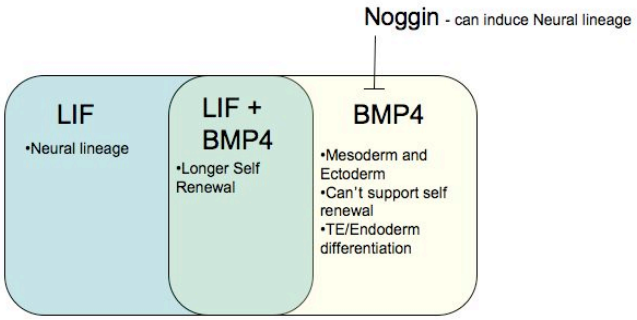
Figure 2: The interactions of LIF and the MEK/ERK and JAK-STAT pathways: When LIF is present in embryonic stem cells, the cells are able to self-renew, while alternative pathways keep differentiation, which is promoted by activation of the MEK/ERK pathway, reduced. The addition of PD0325901 exhibits a similar reaction in this cell type, inhibiting differentiation and promoting self-renewal.



form a complex with SMAD 4, which can then translocate to the nucleus and bind to promoters of target genes, affecting transcription (Varga & Wrana, 2005). SMAD 6 and 7 can inhibit SMAD 1 and 5 and both branches of the TGF-β signaling pathway, respectively. Activation of TGF-β/Activin/Nodal branch through SMAD 2 and 3 is associated with pluripotency.

The other branch of the TGF-β family is the BMP subfamily. BMP4 induces differentiation into mesoderm and ectoderm when not in the presence of LIF in mESCs (Ying et al., 2003, Figure 3). When LIF is present without the influence of BMP4 or FBS, cells undergo neural differentiation. Thus, BMP4 can be said to block neural differentiation. It also is responsible for inhibiting the MAPK pathway (Qi et al., 2004), blocking differentiation. When BMP and LIF are present together, mESCs experience extended self-renewal (Ying et al., 2003). BMP signaling itself is unable to support self-renewal and is associated with trophoblast or extraembryonic endoderm cell differentiation in hESCs. In hESCs, BMPs promote differentiation. NOGGIN is responsible for preventing endoderm differentiation and has been used to induce differentiation into neural lineage by inhibiting BMP4 (Pera et al., 2004). Basic FGF and BMP4 lead to TE differentiation in hESCs grown without a feeder layer (Xu et al., 2002). However, it has been found that a balance of FGF signaling, TGF-β/Activin and BMP inhibition can maintain hESCs in an undifferentiated state (Gilbert, 2010).

Figure 3: The Role of LIF and BMP4 in mESCs: LIF and BMP4 have differing effects on ESCs, however, when the two are combined, an alternative phenotype is seen: longer self-renewal in ESCs. Noggin can inhibit BMP4 and induce neural differentiation, similarly to the addition of LIF without serum.

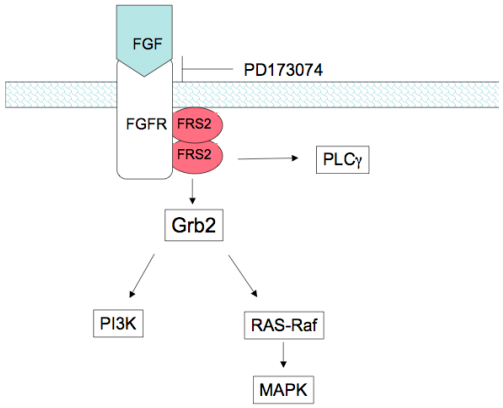


FGF

Fibroblast growth factors (FGFs) affect several areas of embryonic development such as cell proliferation, differentiation, and migration. FGFs bind and activate several types of receptor tyrosine kinases (RTKs), designated as FGF Receptors 1-4 (FGFR1-4). During development, FGFs play a diverse and critical role in organogenesis, demonstrated by the lethal null mutations of several FGF molecules (Eswarakumar et al., 2005). FGF-stimulation can lead to an activation of Ras/MAPK pathway and PI3K signaling (Eswarakumar et al., 2005). Interestingly, an FGF2 (also known as basic FGF or bFGF) enhancer region exists downstream from the coding region activated by OCT-4 and SOX2 (Eswarakumar et al., 2005). Once present, FGF2 can increase the continued proliferation of undifferentiated hESCs (Amit et al., 2000) as well as activate PI-3K to promote self-renewal without serum or feeder cells (Wang et al., 2005). AKT, (which is activated downstream by PI3K) also promotes cell proliferation, survival, growth, and motility.

When the FGF ligand binds the extracellular portion of the fibroblast growth factor receptor, two fibroblast growth factor receptor substrate 2 (FRS2) molecules bound to the domain inside the cellular matrix become phosphorylated, activating phospholipase Cγ (PLCγ) and growth factor receptor-bound protein 2 (Grb2, Figure 4). Grb2 activates both the Ras/Raf (leading to activation of the MAPK pathway and differentiation of mESC), and Gab1 (leading to PI3K activation). When FGF binds its receptor and activates canonical MAPK/ERK pathway in mESC, it represses Nanog. Therefore, it is necessary to inhibit the

Figure 4: FGF Pathway Interactions: When FGF ligand is present, the MAPK and PI3K pathways are activated. This stimulates differentiation in naïve ESCs, but promotes the stem state in EpiSCs. The addition of PD173074 to culture medium inhibits the FGF receptor, deactivating the pathways that are usually stimulated by FGF ligand, promoting the stem state in naïve ESCs.



MAPK/ERK pathway in order to maintain pluripotency. The small molecule PD173074 inhibits fibroblast growth factor receptor 3 (FGFR3). This receptor molecule is ATP-competitive and cell-permeable, and FGFR3 loss in null mice results in bone overgrowth (Eswarakumar et al., 2005). With PD173074 bound, differentiation is blocked. PI3K

Phosphoinositide 3-kinase (PI-3K) is a group of enzymes that are responsible for phosphorylating phospholipids at the plasma membrane (Figure 5). When activated, the PI3K pathway leads to the activation of AKT, which is subsequently translocated to the plasma membrane. Active AKT results in cell proliferation and survival via phosphorylation of many molecules. When AKT is inhibited, blastocyst hatching can be significantly delayed (Riley et al., 2005). PI-3K appears to be very important for the maintenance of undifferentiated mESCs and hESCs based on dominant-negative mutant experiments and inhibitor treatments (Murakami et al., 2004; Paling et al., 2004). PI-3K inhibits the RAS/MAPK pathway, possibly promoting self-renewal in mESCs and hESCs (Li et al., 2007). NANOG transcription and protein levels decrease when PI3K was inhibited (Storm et al., 2007).

WNT

The canonical WNT pathway inhibits GSK-3 mediated B-catenin destruction and leads to the stabilization, nuclear localization and transactivation of B-catenin (Figure 6). WNT ligands have been shown to inhibit differentiation and promote proliferation. When there is no WNT present, B-catenin is degraded,

Figure 5: Relevant Interactions of the PI-3K Pathway: RAS/MAPK and AKT activation via PI3K lead to increased self-renewal in ESCs. mTOR and AKT activation via PI3K lead to increased proliferation of ESCs.

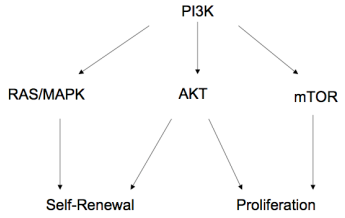
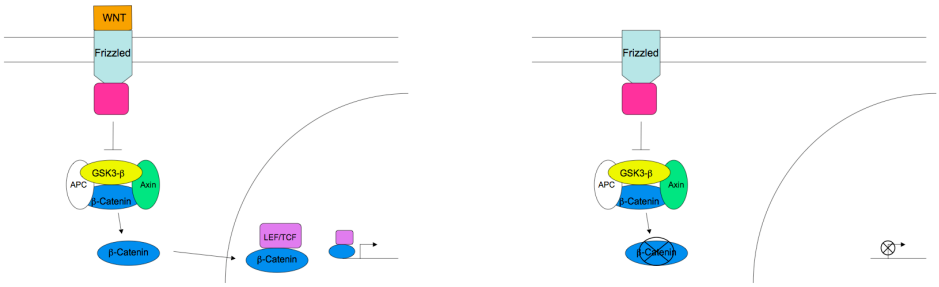
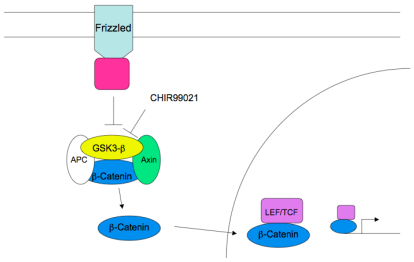


Figure 6: Wnt Pathway: When Wnt ligand is present, β-catenin can translocate to the nucleus and lead to transcription. When Wnt is not present to inhibit GSK3-β, β-catenin is degraded. Addition of CHIR99021 inhibits GSK3-β, allowing β-catenin to accumulate and be translocated to the nucleus to assist in transcription. Either the addition of CHIR99021 or the presence of Wnt ligand promotes pluripotency in naïve ESCs.





resulting in low cytoplasmic presence. In the presence of WNT, free B-catenin will accumulate in the cytoplasm and translocate to the nucleus where it binds downstream target gene promoters and results in differentiation into the neural lineage in hESC (Otero et al., 2004). In mESCs, WNT ligand acts via the canonical pathway. WNTs and LIF work synergistically to regulate STAT3 activity; WNT leads to an increase in STAT3 mRNA and LIF promotes phosphorylation of STAT3 proteins, activating the pathway (Hao et al., 2006). B-catenin interacts with OCT-4 to up-regulate *Nanog*, it also interacts with NANOG and OCT-4 to assist in LIF dependent self-renewal in mESCs (Hao et al., 2006). NANOG reacts with SMAD1 to interfere with BMP and limit mesoderm differentiation, while also acting downstream of the LIF-Jak/STAT pathway (Gilbert, 2010).

The small molecule CHIR99021 selectively inhibits GSK3β, and thus differentiation. This is similar to the LIF activity in which the presence of LIF deactivates GSK3β via the PI3K/AKT pathway, β-catenin can accumulate and move into the nucleus where it binds DNA with Oct3/4 protein to up-regulate *Nanog* and other WNT target genes in mESCs (Gilbert, 2010).

1.5 Comparative Mammalian Blastocyst Development

A necessary pool of maternal RNA is present in the mammalian oocyte to help the oocyte complete maturation and begin its growth after fertilization. The maternal pool of RNA sustains the new embryo until the maternal- embryonic transition, at which time the maternally provided RNA declines and the embryo's genome begins to transcribe its own RNA. The transcription factors controlling blastocyst development in the mouse are well studied.

Maternal pools of *cdx2* help polarize blastomeres before the formation of the blastocyst (Jedrusik et al., 2010). In the mouse, embryonic transcription of *cdx2* appears at 8-cell stage. At the late morula stage, the cells of the embryo begin to sequester into two populations, the TE and ICM. *Cdx2* is gradually restricted to, and up-regulated in, the population of cells that will become TE (Strumpf et al., 2005). *OCT-4* and *Nanog* are repressed by *cdx2* to the population of cells that will become the ICM, allowing TE to differentiate (Berg et al., 2011; Masui et al., 2007). While *cdx2* is necessary for the maintenance of self-renewal in TE cells, it is not what causes the differentiation of the TE itself (Masui et al., 2007). Embryonic transcription of *Sox2* becomes active at the morula stage, and reaches maximum levels at blastocyst stage (Avilion et al., 2003; Li et al., 2005).

Maximum *Nanog* levels occur between the late morula and mid blastocyst stage, and then undergoes down regulation just before implantation when *Nanog* becomes restricted to the epiblast in the mouse (Chambers et al., 2003). The blastocyst will then implant into the endometrium and *OCT-4* down-regulation happens quickly along with a rapid expansion of the TE (Berg et al., 2011). In the

ICM, cells express *Nanog* or *Gata6* in a salt and pepper pattern by the late blastocyst stage, which allows the primitive endoderm to form exclusively from the *Gata6* expressing cells (Chazaud et al. 2006). *Nanog* is necessary to maintain the epiblast in embryo, and it represses Gata6, preventing the differentiation of the entire ICM into primitive endoderm (Filliers et al., 2011).

Bovine and human embryos share many similarities throughout early development. At day 7 of bovine gestation, the cells of the TE continue to express *OCT-4*, as do the cells within the ICM (Berg et al., 2011). *Nanog* and *gata6* are expressed in ICM cells in salt and pepper pattern, much like the mouse (Filliers et al., 2011). In the bovine, however, the blastocyst undergoes TE expansion and gastrulation before attachment, two weeks after the blastocyst is initially formed. *OCT-4* is expressed in both the TE and ICM cells in the expanded blastocyst (Berg et al., 2011). In the human, *OCT-4* mRNA and protein were detected in both ICM and TE, with levels in the ICM 31 fold higher than in the TE (Cauffman et al., 2006). Embryonic expression of NANOG was observed with the onset of gene activation and morula formation (Kimber et al., 2008). During the expanded blastocyst stage of embryo development, nuclear expression of *Nanog* becomes restricted to a subpopulation of the ICM.

The domestic cat has more similarities to bovine and human embryo developmental models than the mouse. During *in vivo* development, domestic cat embryos enter the uterine horn between day 6 to 7, during the transition from morula to early blastocyst, and attach to the uterus through an endotheliochorial placenta 12 to 13 days post fertilization (Boomsma et al., 1991). Similarly to

bovine, blastocyst implantation in the domestic cat occurs later than in the mouse and as such, it is reasonable that the domestic cat blastocyst will show similar expression patterns of *OCT-4* as the bovine blastocyst, with *OCT-4* being expressed in both the ICM and TE.

There have been a few preliminary studies involving mRNA expression patterns of early domestic cat embryos. Initial studies show a loss of mRNA during maturation of *in vitro* oocytes, presumably while maternal pools are used up (Waurich et al., 2010). *OCT-4* mRNA has been shown to be detectable from germinal vesicle stage oocytes through hatching blastocysts (Filliers et al., 2011). *OCT-4* does not increase in 8 to 16-cell embryos, but increases seven fold at the morula stage (Filliers et al., 2011), and further still during the blastocyst stage (Waurich et al., 2010). OCT-4 protein is localized to the nuclei of ICM and TE cells (Gómez et al., 2010). *OCT-4* begins to decrease at the hatching blastocyst stage (Filliers et al., 2011). *Cdx2*, the TE transcript, is not found at the oocyte stage, but is most abundant in hatching blastocysts (Filliers et al., 2011).

At the 5 to 8-cell embryo stage *Sox2* begins to be detectable, and its expression continues to increase through to the compact morula stage (Filliers et al., 2011). *Sox2* expression was found to be sevenfold higher at the morula stage than at the earlier cleavage stages, but expression dropped off during early blastocyst stage, and increasing again at hatching (Filliers et al., 2011). *Gata6* expression is similar to *Sox2*, at the compact morula stage it is 28-fold higher than earlier stages and 3-fold higher than the hatching blastocyst stage (Filliers et al., 2011).

Surprisingly, *Nanog* is detectable and abundant at all early stages of development in the cat, from the germinal vesicle through the 5 to 8 cell stage, after which it is down-regulated resulting in levels 23-fold lower in hatching blastocysts than MII oocytes (Filliers et al., 2011). This is contrary to results found in the mouse and bovine (Khan et al., 2012), but has some similarity to findings in the human. A maternal pool of *NANOG* was detected in human pronucleate embryos, although not in early cleavage stages. Embryonic transcription of *NANOG* began at the 8-cell stage in humans (Kimber et al., 2008), similar to that observed in ruminants (He et al., 2006) and mice (Chambers et al., 2003). A confounding factor that could affect this study is the presence of multiple *NANOG* pseudogenes and newly identified isoforms, all of which may not have the same biological activity (Booth & Holland, 2004; Singh et. al., 2012).

Chapter 2: Source of protein supplementation during *in vitro* culture does not affect the quality of resulting blastocysts in the domestic cat

This chapter is based on an accepted manuscript by Nestle, E., Graves-Herring, J., Keefer, C., and Comizzoli, P. to be published in *Reproduction in Domestic Animals*

Contents

The objective of this study was to assess and compare the quality of cat blastocysts produced in vitro using commercial blastocyst growth media supplemented with different sources of proteins (serum protein substitute from in vitro maturation through embryo development vs. 4 mg/ml of bovine serum albumin for maturation and 5% fetal bovine serum for fertilization and embryo development). Impact was specifically examined on the proportion of blastocyst formation, total number of blastomeres, proportion of ICM and expression of pluripotency marker proteins NANOG and OCT-4. Blastocyst formation per total cleaved embryos was similar (P>0.05) regardless of the protein supplementation. There were no differences (P>0.05) between culture conditions regarding average number of cells and proportion of ICM in each embryo. Presence of OCT-4 protein was detected in nuclei of both TE and ICM region, with a stronger signal in the latter regardless of the culture medium. NANOG protein also was present in the ICM regardless of the in vitro culture condition. We, therefore, demonstrated that serum protein substitute was as good as semi-defined protein

sources for the production of good quality blastocysts and embryonic stem cells. In addition, a single defined medium could be successfully used for cat oocyte maturation, in vitro fertilization, and embryo development.

Introduction

The domestic cat (*Felis catus*) is an excellent biomedical model as well as a good model for rare felid species. Maintenance of genetic diversity in cat populations can be optimized by the use of assisted reproductive techniques including the *in vitro* production and transfer of embryos at the blastocyst stage. Studies in the domestic cat also allow the exploration of new technologies taking advantage of embryonic stem cells derived from blastocysts (Gómez et al., 2010). Both stem cell derivation and embryo transfer necessitate quality blastocysts.

Blastocyst quality can be assessed using several methods. Counting the total number of blastomeres (the higher the better) as well as the ratio between ICM and TE after 7 days of *in vitro* culture are simple and commonly used methods of determining the quality of a blastocyst (Gómez et al., 2010). In addition, levels of NANOG and OCT-4 transcription factors in the ICM of a blastocyst can be used to determine blastocyst quality. Studies in the mouse, rat and human have shown that the ICM of a blastocyst can be used to generate embryonic stem cells, the pluripotency of these stem cells being reflected by levels of NANOG and OCT-4 proteins – two critical transcription factors for the maintenance in an undifferentiated stage (Behboodi et al., 2011). Studies have shown that embryos produced *in vivo* have higher expression of these transcription factors and also produce high quality embryonic stem cells

(Behboodi et al. 2011, Roth et al. 1994, du Puy et al. 2011, Yu et al. 2008). Therefore, *in vitro* produced embryos that have high levels of NANOG and OCT-4 are closer to the *in vivo* phenotype and thus are considered of higher quality. However, when culturing embryos *in vitro*, the composition of the culture medium can significantly affect the quality of produced embryos mainly in terms of morphology, metabolism and gene expression. The quality of the blastocyst subsequently influences the success of the implantation, fetal growth, and general health of produced offspring (Dobrinsky et al., 1996).

Studying *in vitro* production media in various species has revealed that the requirements of preimplantation embryos are species and stage specific. Multiple protocols have been developed to generate cat embryos *in vitro* with varying success in blastocyst formation (Waurich et al. 2012). One interesting study sought to make a feline optimized culture medium by identifying precise molar concentrations of ions, carbohydrates, amino acids, vitamins and serum necessary for early embryonic growth (Herrick et al., 2007). A commercially available defined medium would actually be preferable to better investigate and understand the embryo formation in order to standardize embryo culture between laboratories.

Previous studies have examined the importance of protein source when culturing embryos of cat and pig *in vitro* (Dobrinsky et al., 1996; Karja et al., 2002; Wood et al., 1995) . Complex biological macromolecules are used in many culture systems across species as a source of protein. However, FBS has been found to cause poorer oocyte maturation than BSA in cat, bovine and rat, yet FBS

allowed more oocytes to be fertilized (Wood et al., 1995). Interestingly, more embryos reach the hatching blastocyst stage and have higher cell number with FBS rather than BSA for fertilization and culture (Dobrinsky et al., 1996; Han & Niwa, 2003; Herrick et al., 2007; Karja et al., 2002). Contrarily, FBS can diminish post-thaw survival following cryopreservation (Murakami et al., 2011; Swanson et. al., 1999).

The objective of this study was to measure the impact of protein supplementations (defined vs. semi-defined) on the success of blastocyst formation. The hypothesis was that a defined medium (serum protein substitute) could provide blastocysts with high cell number more consistently than a culture system that is semi-defined and needs additional protein supplementation (i.e., FBS and/or BSA).

Materials and Methods

Oocyte maturation and embryo production

Oocyte selection and main steps of the *in vitro* culture were performed using standard protocols developed in our laboratory (Waurich et al., 2012; Wood et al., 1995). Briefly, ovaries were obtained from local veterinary clinics and stored in PBS (Gibco) at 4°C for up to 24 hours. Immature cat oocytes (n = 2126 total; 29 replicates) were collected from adult ovaries and matured in 50 μl droplets of Medium A (Sage) or B (Irvine) supplemented with 0.01% (v/v) FSH, 0.01% LH , 0.16% E2 (Hormones: National Hormone and Pituitary Program (NHPP)) and 4% BSA (Sigma-Aldrich) added to Medium B only. The media used in this study had

essentially the same salt composition and components with the exception of protein source. In Medium A, the serum protein substitute contained 88% normal human serum albumin in the presence of 12% α and β globulins. After 26 to 28 hours of maturation, oocytes were fertilized with 5.0 x 10^5 motile sperm ml^{-1} of frozen-thawed epididymal spermatozoa from different males. Oocytes and sperm cells were co-incubated for 16 to 18 hours. A control for parthenogenesis (no sperm cell insemination) was included in each replicate. Presumptive zygotes were then cultured *in vitro* and media was changed at day 4. For *in vitro* fertilization and embryo culture, Medium B was supplemented with 5% FBS (Irvine Scientific) whereas Medium A contained the same protein substitute. After 7 days of embryo development *in vitro*, proportions of different embryo stages were recorded according to previous criteria (Wood et al., 1995).

Immunocytochemistry

Immunostaining was done at 38°C unless otherwise noted. Day 7 blastocysts were fixed in 5% paraformaldehyde (FisherBioTech) in PBS for 30 min. Following fixation, embryos were washed in saturation medium (20% FBS, + 0.5% Triton-X100 (TX100, Sigma-Aldrich) in PBS) for 5 min, then blocked in saturation medium for 30 min before being incubated with a primary antibody (anti-OCT-4 (Millipore, No. MAB4401), 1:200; or anti-NANOG (AbCam #ab80892), 1:200) at 4°C overnight. The following morning embryos were washed in medium (2% FBS + 0.5% TX100 in PBS) for 45 min before being incubated with the secondary antibody (FITC (Sigma-Aldrich, No. F2266) 1:100 in washing medium) for 1 hour. A 30 min rinse in washing medium followed.

Chromatin was stained using 10μg/ml Hoechst 33342 (Sigma-Aldrich) and 5μg/ml propidium iodide (Sigma-Aldrich) for 10 min. Stained embryos were mounted in Vectashield® (Vector Laboratories) on a glass slide, sealed with nail polish, and stored at 4°C.

qRT-PCR

A pool of 5 blastocysts at day 7 post insemination were suspended in lysis buffer (Buffer RLT, RNeasy Protect Micro Procedure Kit, Qiagen) and were snap frozen at -80°C for less than 6 months until processing. Total mRNA was isolated using an RNeasy Protect Micro Procedure Kit (Qiagen) according to the manufacturer's directions. Concentration of mRNA was determined using Nanodrop, and cDNA was produced using a Quantitect RT Kit (Qiagen) according to manufacturer's directions. Quantitative real time PCR (qRT-PCR) was then performed on a BioRad thermocycler using iQ-SYBRGreen Supermix (BioRad) following manufacturer's instructions and using the primers provided in Table 1.

Experimental design and statistical analysis

Domestic cat ovaries were recovered from local veterinary clinics during routine spay procedures. On a given day, recovered oocytes were evenly distributed between the two culture media. Resulting blastocysts (OCT-4, 4 replicates: Medium A, n =12, Medium B, n=18; NANOG, 5 replicates: Medium A, n=27, Medium B, n=11) were fixed and immunostained for the pluripotency proteins OCT-4 and NANOG (and determination of number of cells in the ICM). Blastocysts from the remaining trials were preserved in lysis buffer for future

Table 1: Primers for qRT-PCR: The gene name, forward and reverse primers, amplicon size and annealing temperature are provided. Citations are provided for the original design of these primers. Primers were obtained from Integrated DNA Technologies. The sequence of gene products was confirmed after purification.

Gene	Sequence	Size (bp)	Citation	Annealing Temp
NANOG	F: CCTGAACATACAGCCTGAAGTC	113	Gomez 2010	58.86
	R: AAGGCAGAGATAGAGGACCCTA			59.77
SOX2	F: CATCACCCGCAGCAAATGA	111	Gomez 2010	59.72
	R: AAGAAGTCCAGGATCTCTCATAAA			60.36
OCT-4	F: TGCAGCTCAGTTTCAAGAACA	112	Gomez 2010	59.77
	R: ACAAGTGTCTCTGCTTTGCATA			57.72
RPL-17	F: CTCTGGTCATTGAGCACATCC	109	Penning 2007	60.27
	R: TCAATGTGGCAGGGAGAGC			62.38

gene expression analysis. The remaining non-blastocyst embryos were stained for chromatin using 10μg/ml Hoechst 33342 and 5μg/ml propidium iodide for 10 min to evaluate embryo stage. The rate of cleavage was recorded as well as the percentage of blastocysts produced from total oocytes fertilized. Cell numbers were analyzed using an unpaired T-test. Proportions of cleaved cells reaching 8 cells, 8 to 16 cells, morula, and blastocyst stages were transformed (arcsin) and analyzed using an unpaired two-tailed T-test (Graphpad Prism 5, GraphPad Software, Inc). The ΔCt method and ANOVA was used to analyze the relative difference between qRT-PCR values of samples and the house-keeping gene RPL-17.

Results

Embryonic cleavage

The percentages of cleaved embryos obtained with the two media were not different ($P > 0.05$; Table 2). Parthenogenetic activation was not observed in either culture medium. The proportions of each embryo stage including blastocyst formation were also similar ($P > 0.05$) between the two media (Table 2). A small proportion of blastocysts (~5%) were hatched after 7 days of culture regardless of the medium. There was no significant difference in the number of hatched blastocysts produced in either media (Fisher's Exact Test).

There were no differences ($P > 0.05$) between media regarding average number of cells in blastocysts (Medium A: 158.1 ± 11.1; Medium B: 174.6 ±

Table 2: Percentages of cleaved embryos and proportions of each developmental stage obtained after *in vitro* culture in the two different media: Values are expressed as mean ± SE. Numbers within columns with the same letter were not significantly different (P>0.05, T-test).

Medium	No. of Oocytes	% Cleaved	Proportion of embryo stages (per total cleaved)			
			<8-cells	8 to 16 cells	Morula	Blastocysts
A	1062	74.1 ± 2.0[a]	24.6 ± 2.3 [a]	24.9 ± 2.7 [a]	20.9 ± 2.6 [a]	29.6 ± 3.8 [a]
B	1061	82.5 ± 2.6[a]	29.5 ± 3.4[a]	30.8 ± 3.1[a]	17.4 ± 3.1 [a]	22.4 ± 3.2 [a]

10.2) and proportion of ICM in each embryo (Medium A: 23.7% ± 2.0; Medium B: 24.7% ± 1.5) as represented in Figure 7. There was no difference in the variance of any cleavage state (F-test).

Immunostaining for pluripotency marker proteins

Immunostaining revealed the presence of OCT-4 protein in nuclei of both TE and ICM region, with a stronger signal in the latter (Figure 8B) regardless of the culture medium. NANOG protein also was detected in the ICM (Figure 8E) of embryos from both media.

Analysis of pluripotency marker genes via qRT-PCR

OCT-4 and *SOX2* mRNA was observed in the nuclei of cells of the ICM and TE in embryos produced using both media. There was no significant difference between the two media for any gene ($P > 0.05$). SOX2 had the smallest relative difference between the two media, while OCT-4 had the largest. No trend could be detected between the two media. Results of individual trials are provided in Figure 9 to demonstrate the range of values obtained per embryo pool. There was no significant difference between the average of the three trials for either media for any gene ($P > 0.05$, ANOVA, Figure 10).

Discussion

The quality of an *in vitro* produced blastocyst can be defined by the total number of cells and the presence of pluripotency proteins NANOG, SOX2 and OCT-4. In this study, we found no difference between media and protein sources for the number of cells in a given blastocyst. There was no observed difference in the

Figure 7: Individual blastocyst analysis: Proportion of total cells that occurs in the ICM per blastocyst per medium is shown in A. Cell number is compared per blastocyst per medium in B.

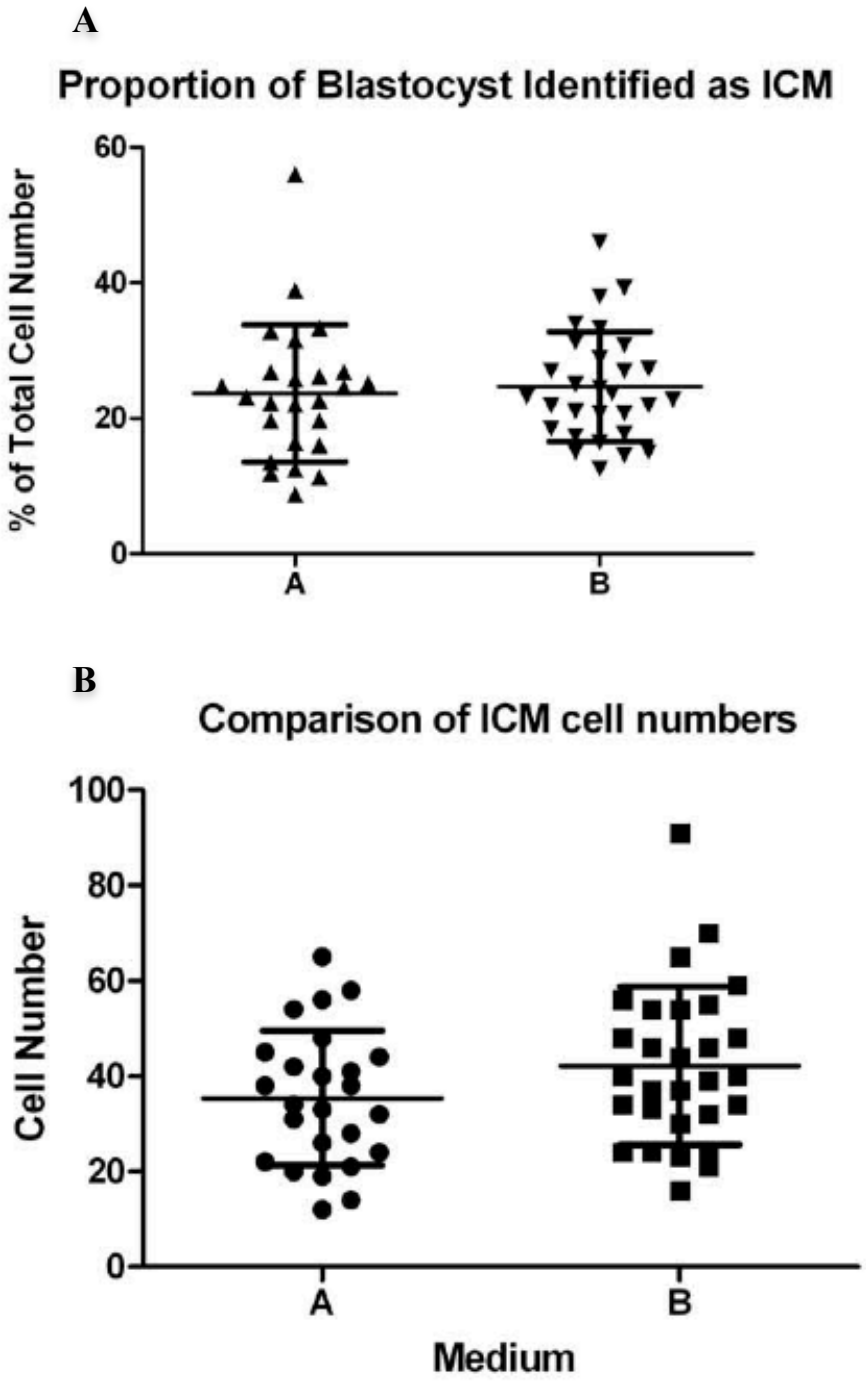


Figure 8: Immunostaining of OCT-4 (B) and NANOG (E): Propidium Iodide staining to show blastomere nuclei (A, D). OCT-4 protein (B). Images C and F have no primary antibody added (negative controls). Bar = 100μm

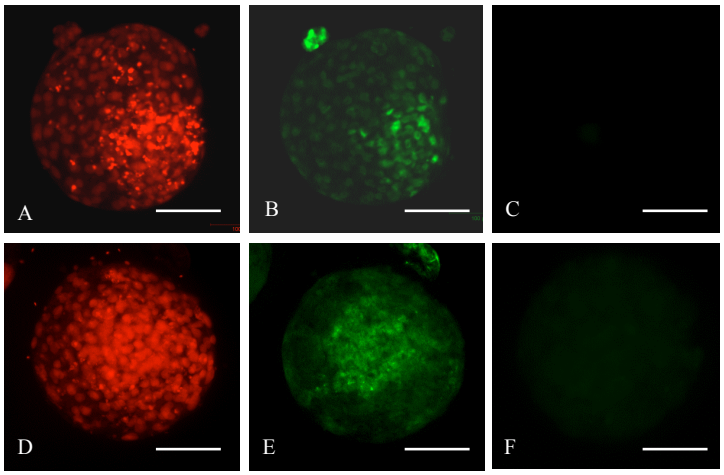


Figure 9: Expression values for Individual Pooled Samples of NANOG, OCT-4, and SOX2 in blastocysts following *in vitro* culture: ΔCt values of NANOG, OCT-4 and SOX2 mRNA in individual pools of 5 domestic cat embryos each. A1-A3 denotes experimental replicates for embryos cultured in Medium A, whereas, B1-B3 represent those cultured in Medium B. Cat fetal fibroblasts (CFF) were run as a negative control. Expression was normalized to the house-keeping gene, RPL-17.

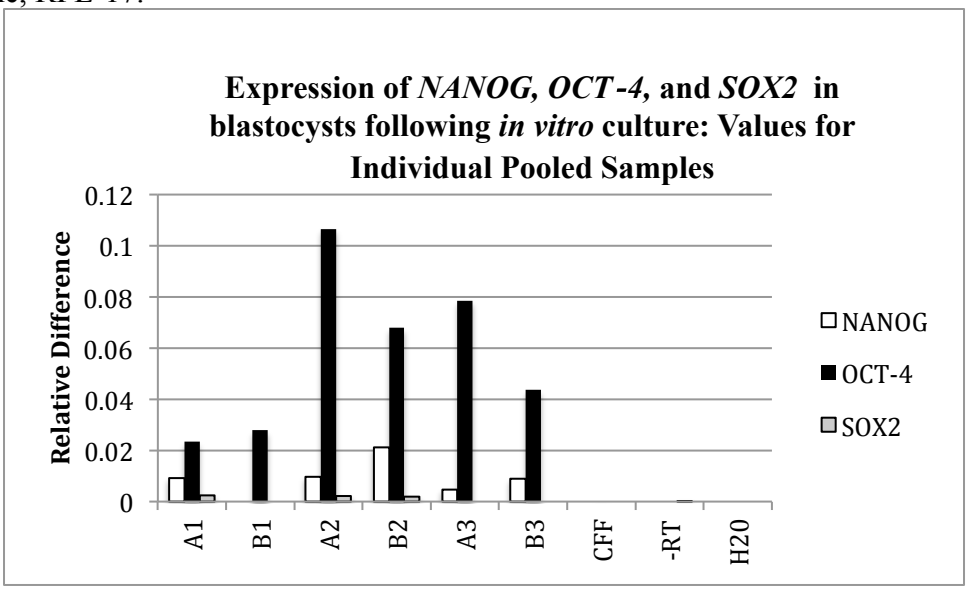
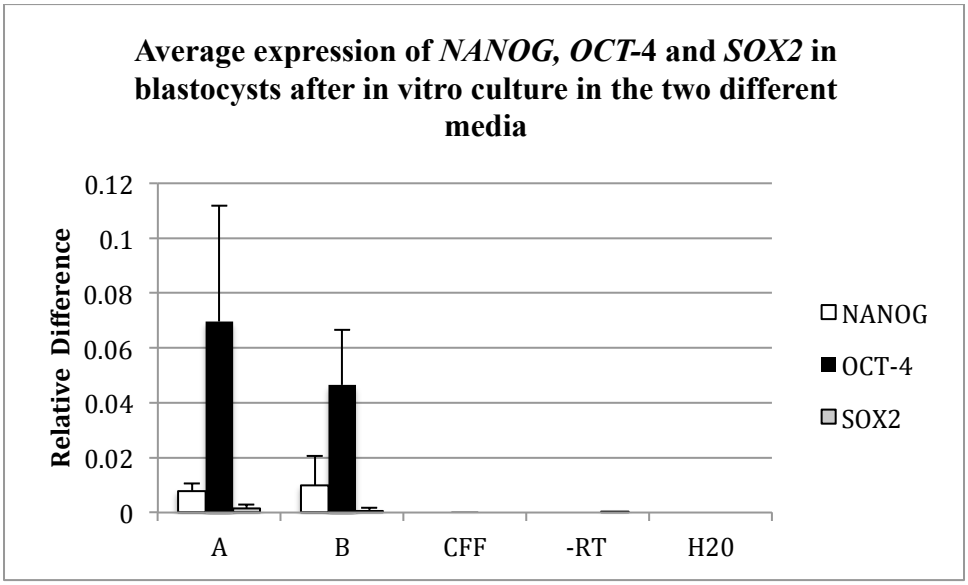


Figure 10: Average expression of *NANOG, OCT*-4 and *SOX2* in blastocysts after in vitro culture in the two different media (number of pools=3): A represents the average of three trials of medium A, and B represents the average of three trials of medium B. Expression was normalized to the house-keeping gene, RPL-17. Standard deviation error bars are shown.



proportion of cells in the ICM between media and protein sources either. Concordantly, expression of the pluripotency proteins NANOG and OCT-4 was similar between the two culture conditions. We also found that the proportion of cleaved embryos for either medium was not significantly different. The cleavage proportions for each embryo stage (less than 8-cells, 8 to 16 cells, morula, and blastocyst) were similar between the two media. Both the rates of cleavage and proportion of embryos to reach the blastocyst stage were comparable to similar studies in the cat (Gómez et al., 2010). Depending on the expertise and selectivity of the scientist, *in vitro* fertilization success rates can be as high as 70 to 80% with nearly 80% of embryos reaching morula stage by 96 hours post insemination (Roth et al., 1994). A typical day 7 cat blastocyst should have at least 100 to 150 cells (Pope et al., 2006; Spindler & Wildt, 2002).

Both media produced good quality blastocysts of high cell number that expressed NANOG and OCT-4 proteins. Our results confirmed that in a blastocyst, OCT-4 protein was present in the nuclei of both the TE and ICM; however, it was more prominently in the ICM. NANOG protein was found to be present in the ICM only. *NANOG* and *OCT-4* mRNA were found to be present at comparable levels in blastocysts grown in both media. There was no significant difference between the relative expression of the genes between the two media. Thus, cat blastocysts expressed pluripotency proteins in a pattern similar to the bovine model (Berg et al., 2011), porcine model (Kirchhof et al., 2000) as well as to previous results reported in the domestic cat (Gómez et al., 2010).

Protein source in culture media has been shown to have a critical effect on the maturation of oocytes and success of blastocyst formation. Previous studies have shown that BSA is best used for oocyte maturation, followed by FBS for fertilization and culture to the blastocyst stage, resulting in more hatched blastocysts with higher cell number comparatively (Dobrinsky et al., 1996; Han & Niwa, 2003; Herrick et al., 2007; Karja et al., 2002; Wood et al., 1995). The media used in this study had the same components and salt composition, with the exception of protein source. In Medium A, the serum protein substitute contains 88% normal human serum albumin with 12% α and β globulins. The high content of polyhydroxy domains in the α and β globulins produces a weak gel-like environment that can enhance embryonic development both in human and mouse embryos (Weathersbee et al. 1995). The serum albumin protein sequences for human and cat are more similar than for cat and bovine, (H:C 82%; B:C 78%); however, there was no beneficial effect of using human serum over bovine. To the author's knowledge, this is the first time human serum (present in the serum protein substitute) has been used successfully for cat embryo culture. Interestingly, the medium containing serum protein substitute performed equally well as the media that used BSA for maturation and FBS for fertilization and culture. Using the same protein source from *in vitro* maturation through embryo culture did not influence the quality of the resulting blastocysts.

This study demonstrates that high quality blastocysts can be culture *in vitro* for the domestic cat utilizing two commercially available media. Producing high quality blastocysts consistently and abundantly *in vitro* will provide superior

starting material for performing embryo transfer, derivation of embryonic stem cells, and for additional research. Further advancement in the understanding of the nutritional requirements of preimplantation embryos will enable improved media to be developed and generation of blastocysts that may more closely resemble *in vivo* derived embryos.

Chapter 3: Comparison of cytokine supplementation on ICM explant growth

Contents

The objective of this study was to assess and compare the maintenance of pluripotency markers OCT-4 and NANOG in inner cell mass (ICM) explants from *in vitro* produced blastocysts over a period of 14 days. ICM explants were cultured using a combination of 3 inhibitors (3i: CHIR99021, PD173074, PD0325901), mouse LIF and BMP4, or mouse LIF and human recombinant bFGF. Explants were imaged with bright field (day 7 and day 14), immunostained for OCT-4 and NANOG (day 14), and samples were analyzed for gene expression using qRT-PCR (day 14). There was no statistical difference between the three conditions for the expression of *OCT-4* or *SOX2*, for the number of persisting explants on day 14, or between the numbers of large or small colonies between the three conditions.

Introduction

Embryonic stem cells (ESCs) hold much promise for therapeutic research. While stem cells have been a topic of research for over three decades, naïve ESCs have been produced and validated only in the mouse and rat. Authenticated naïve ESCs are defined by several characteristics: unlimited self-renewal, rounded colony morphology, expression of specific molecular markers, maintenance of normal karyotype, and the ability to differentiate into any cell type in the body. Furthermore, progress in other species has been limited and slow; each new

species requires specific research to define the best culture conditions to derive and maintain embryonic stem-like cells, eventually leading to truly naïve ESCs. Naïve ESCs can be incorporated into a blastocyst producing chimeric offspring, useful for biomedical and genetic studies. Naïve ESCs can be differentiated into gametes, demonstrating a potential avenue for preserving genetic material for rare genomes such as endangered felids (Kerkis et al., 2007).

A common starting point for deriving ESC lines from novel species is based on knowledge gained from mESCs. A typical culture cocktail for the mouse contains LIF and BMP4 (Ying et al., 2003). LIF acts to promote self-renewal by activating the STAT3 pathway and BMP4 induces expression in the SMAD pathway leading to inhibition of differentiation (Ying et al., 2003). Alternative cytokines are added to the media as successes and failures are observed in nonmurine species. Each change in cytokines reveals potential active and inactive pathways of pluripotency in these novel species. The first attempt at deriving stem cells for the domestic cat used LIF in the culture medium (Yu et al., 2008), soon after, a combination of LIF and human basic fibroblast growth factor (b-FGF) (Gómez et al., 2010) was used, producing ES-like cells that could survive longer in culture than those produced by Yu et. al. 2008. bFGF serves to maintain self-renewal as well as promoting ERK and PI-3K pathways (Honda et al., 2009) and is typical of what is described for EpiSC.

At the time of writing, no validated embryonic stem cell exists for the domestic cat, although several attempts have been made. Yu et al (2008) used *in vivo* produced cat blastocysts to generate cat ES-like cells in culture for up to 8

passages, determining that medium containing KSR allowed ICM cells to attach better and form domed colonies, while medium containing FBS formed flat colonies and with higher cell proliferation. Gómez et al., (2010) generated and maintained ES-like cells derived from *in vitro* produced domestic cat blastocysts for up to 12 passages (102 days). Gómez et al., (2010) found that their ICM explanted colonies produced had a tightly packed, dome shaped morphology, alkaline phosphatase activity, and readily expressed OCT-4, SSEA-1, NANOG, SOX2 and C-MYC, although expression levels of colonies were at a lower level than blastocysts. Several studies have verified that cat ES-like cells seem to survive undifferentiated and proliferate longer when grown on a homologous layer of blocked cat fibroblast cells (Gómez et al., 2010; Serrano et al., 2005; Yu et al., 2008). While typically *in vivo* produced embryos have more developmental competence than *in vitro* produced embryos (Bavister, 2004), *in vitro* embryos are much easier to obtain for many species, including the domestic cat. In earlier studies (Chapter 2), we showed that good quality cat embryos could be produced *in vitro* using a defined protein supplement.

In this study, we compared the use of LIF + BMP4 (mouse condition), LIF + bFGF (cat condition), and a combination of inhibitory molecules that has shown success in induced pluripotent and embryonic stem cells (3i condition) (Buehr et al., 2008; Gómez et al., 2010; Telugu et al., 2010; Ying et al., 2003). We examined the effect of these different cytokine conditions on the growth of ICM explants from *in vitro* produced domestic cat blastocysts to identify an approach

that provides superior conditions for deriving cat ES-like cells and maintaining pluripotency in future studies.

We predicted that the cocktail of inhibitory small molecules would provide a superior environment for maintenance of "stemness" in cat ICM explant cells due to the small molecules having fewer species-specific characteristics. As such, they should overcome any species-specific cytokine receptor differences in pluripotency promoting pathways, and stimulate specific pluripotency pathways and the inhibition of differentiation in domestic cat explant cells. Due to differences between mouse and cat pluripotency promoting pathways, we predicted that the current domestic cat ES-like cocktail condition would be superior to the current cytokine cocktail used in rodent ESC culture. These predictions proved false as demonstrated by the failure of ICM explants to thrive under 3i conditions.

Materials and Methods

Generating and blocking cat fetal fibroblast cells

Primary cat fetal fibroblasts (CFF) were generated from 3 fetuses obtained as waste tissue from one gravid female cat approximately 35 days of gestation after hysterectomy for non-research related reasons. The uterine horns were extracted, placed in a Petri dish and rinsed with PBS. Fetuses were excised from the embryonic sacs, rinsed thoroughly and fetal visceral tissue was removed. Remaining tissue was cut into small pieces and incubated with Trypsin in 5% CO2 at 37°C for 20 minutes with shaking. The cells were centrifuged for 5minutes at 1000RPM. Supernatant was aspirated and cells were resuspended in

Glasgow Minimal Eagles Medium (GMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 2.4 mM L-glutamine (Gibco), 2.4 mM sodium pyruvate (Gibco), 2.2 mL Dulbecco's Modified Eagles Medium (DMEM, Gibco), 10 U/mL penicillin, 10 g/mL streptomycin in 75cm^2 flasks for 7 to 10 days of culture at 38°C in 5%CO_2. After 7 to 10 days, monolayers were disaggregated with 2.5mg/mL of trypsin, resuspended in DMEM (Gibco) supplemented 5% DMSO (FisherBioTech). Cells were cooled at 1.0°C .min to -80°C (Mr. Frosty; Nalgene) and stored in liquid nitrogen.

As needed, CFFs were thawed and blocked following the protocol provided in Gomez et al. 2010. Briefly, cells were thawed from liquid nitrogen, washed, and expanded for at least 3 days in culture. Medium was removed from the culture dishes and 40μg/mL of mitomycin-C (MMC, Sigma-Aldrich) was added for 4 to 5 hours at 37°C at 5% CO_2. MMC was removed and trypsin was added for 2 to 3 minutes at 37°C at 5% CO_2. Cells were rinsed with PBS at least 3 times before being plated on a gelatin coated 4-well plates. Cells were maintained in GMEM and could be used after 2 hours and within 3 days of being blocked.

Aliquots were thawed as needed in 37°C dry baths and swirled gently until thawed. To count total cells, 10μl of cells and 90μl of Trypan Blue were mixed and cells were counted using a hemocytometer. A final concentration of 1x10^5 cells/well (24 well) was added to each gelatin coated well. Plated cultures were incubated at 38.5°C, 5% CO_2, and used within 48 hours of preparation. Feeder

medium was replaced with medium based on Gomez et al. (2010) on the day of plating ICMs.

Explantation of inner cell masses and passaging of cells after explantation

Embryos were produced using the protocol laid out in Chapter 2. Day 6 blastocysts were preincubated in the media cocktail for 24 hours at 38.5°C and 5% CO_2. Mechanical dissection was performed using two 30 gauge needles. The zona pellucida was removed and the embryo was laid out in a single layer. The ICM was removed from the TE and plated separately with as little TE as possible. Cells were cultured at 38.5°C in 5% mixed gas. Brightfield images were taken at D0. Media was changed every 1 to 2 days. After 7 days of culture, brightfield images were taken and culture continued. At 14 days of culture, brightfield images were taken before large colonies were split into 2 to 3 pieces. The first piece was fixed for immunostaining for OCT-4 and NANOG, the second piece was stored in lysis buffer for qRT-PCR analysis and if a third piece could be obtained, it was explanted onto fresh blocked CFFs and culture continued. Small colonies (those that were less than 300 μm) were trypsinized with the CFFs in each well (producing a whole well lysate) and stored in lysis buffer for qRT-PCR analysis.

Preparing cytokine cocktails

Explants were placed into wells containing 1mL of base embryonic stem-like cell growth medium (DMEM/F12 (Invitrogen) supplemented with 15% Knockout

Serum replacement (Invitrogen), 5% FBS (Irvine Scientific), 1mM L-glutamine (Gibco), 0.1mM B-mercaptoethanol, 1.25% non-essential amino acids, 50 Units/mL penicillin and 50ug/mL streptomycin (Gómez et al., 2010). The mouse condition was prepared by the addition of 1000 Units/mL of mouse LIF (Esgro, Chemicon / Millipore) and 8ng/mL mouse BMP4 (R&D Systems). The cat condition was prepared by the addition of 1000 Units/mL of mouse LIF and 5ng/mL human recombinant bFGF (Peprotech). The 3i medium was prepared by the addition of 3μM CHIR99021 (Stemgent), 1μM PD0325901 (Stemgent), and 0.1 μM PD173074 (Stemgent) (Table 3).

Immunocytochemistry

Immunostaining was done at 38°C unless otherwise noted. Day 14 ICM explants were fixed in 5% paraformaldehyde in PBS for 30 min. Following fixation, explants were washed in saturation medium (20% FBS, + 0.5%Triton X100 in PBS) for 5 min, then blocked in saturation medium for 30 min before being incubated with a primary antibody (anti-OCT-4, 1:200, Millipore, No. MAB4401; or anti-NANOG, 1:200, Abcam, No. ab80892) at 4°C overnight. The following morning explants were washed in medium (2% FBS + 0.5% TX100 in PBS) for 45 min before being incubated with the secondary antibody (FITC, 1:100, Sigma, No. F2266; in washing medium) for 1 hour. A 30 min rinse in washing medium followed. Chromatin was stained using 10μg/mL Hoechst 33342 and 5μg/mL propidium iodide for 10 min. Stained explants were mounted on a glass slide, sealed with nail polish, and stored at 4°C.

Table 3: Three cytokine treatments: Small molecules (3i), the currently used cytokine combination for domestic cat ES-like cells, and a currently used cytokine combination used for mouse and rat ESC. The small molecule and rodent-like culture conditions are hypothesized to produce naïve ES-like cells from the cat, as has been shown in the mouse. Condition B, the cat ES-like culture condition, should produce EpiSC, due to the inclusion of FGF.

A: Naïve - Small Molecule		**Inhibits**
PD173074	0.1μM	FGF
PD0325901	1μM	ERK
CHIR99021	3 μM	GSKB3
KSR/Feeder Layer		

B: Control – Cytokines for cat ES-like culture (Gómez et al., 2010)		
LIF	1000 U/mL	Activates Jak/Stat, MAPK
bFGF	5ng/mL	Inhibits BMP induced differentiation
KSR/Feeder Layer		

C: Naïve – Cytokines for rodent ESC culture (Ying et al., 2003)		
LIF	1000 U/mL	Activates Jak/Stat, MAPK
BMP4	8ng/mL	Activates MAPK
KSR/Feeder Layer		

qRT-PCR

Large colonies were divided into 2 to 3 pieces, one of which was stored in lysis buffer for genetic analysis at day 14 post explantation. Small colonies and CFF control wells were trypsinized as whole wells and stored in lysis buffer at day 14 post-explantation as follows. Trypsin was added to wells for 2 to 3minutes at 37°C in 5% CO2. Cells were centrifuged for 3 min at 1000RPM, the supernatant was removed and pellet resuspended with PBS. Cells were centrifuged again and supernatant removed. Cells were then resuspended in lysis buffer (Buffer RLT, RNeasy Protect Mini Procedure Kit, Qiagen, No.74124) and were snap frozen at -80°C and stored for less than 6 months until processing. Total mRNA was isolated using an RNeasy Protect Micro Procedure Kit according to the manufacturer's directions. Concentration of mRNA was determined using Nanodrop, and cDNA was produced using a Quantitect RT Kit according to manufacturer's directions. Equal amounts of cDNA were analyzed by quantitative PCR performed on a Biorad thermocycler using iQ-SYBRGreen Supermix following manufacturer's instructions and using the primers provided in Table 4.

Experimental design and Statistical analysis

Domestic cat embryos were cultured using Quinn's Advantage Protein Plus medium (Sage) for 7 days, as described in Chapter 2. Five oocytes were not fertilized during each trial to observe for parthenogenetic activation. At day 6, blastocysts and morula were evenly distributed and preincubated in each of the three conditions for 24 hours. On day 7, the embryos were transported in a

Table 4: Primers for qRT-PCR: The gene name, forward and reverse sequence, amplicon size and annealing temperature are provided. Primers were obtained from Integrated DNA Technologies and designed based on the cat genome . The sequence of gene products was confirmed after purification.

Gene	Sequences (5'-3')	Size (bp)	Annealing Temp (°C)
OCT-4	F: TGACTATTCGCAACGAGAGG	121	59.02
	R: GAAGTGAGGGCTTCCATAGC		58.89
NANOG	F: GAAGAAGGACGATCCAGCTC	127	58.97
	R: TTCTTGCATCTGCTGGAGAC		59.12
SOX2	F: ATGCACAACTCGGAGATCAG	130	58.83
	R: TATAATCCGGGTGCTCCTTC		59.00
RPL-17	F: AATGCGGCGTAGAACTTACA	125	58.47
	R: GTGCAACCTCCTCTTCTGGT		59.30

portable incubator at 38ºC from the Smithsonian National Zoological Park to the University of Maryland, College Park, where the ICM was subsequently mechanically dissected away from the TE and tacked down onto a homologous feeder layer. Feeder cells were thawed and blocked at least 24 hours in advance of use, and plated into 4 well dishes. At least three explants were placed onto the cat fetal fibroblast feeder layer in the wells of a 4 well dish, under 1mL of the appropriate cytokine conditions. Five additional wells of CFF monolayer were prepared, one well each of the base medium containing each cytokine condition, the base medium with no cytokines, and a basic cell growth medium (DMEM plus 10% FBS, 1% Pen/strep, 1% L-glutamine) as negative controls. Media was changed every 1 to 2 days for 14 days. On day 7, brightfield images were taken and colony number was assessed. On day 14, brightfield images were taken, colony number assessed, and any large colonies were split into 2 to 3 pieces. Large colonies are defined as greater than 300μm, (small colonies were generally ~100μm in diameter and assumed to be non-growing). One piece of the large colony was stored in lysis buffer for gene analysis for OCT-4, SOX2, NANOG, and RPL-17 via qRT-PCR, one piece was immunostained for OCT-4 and NANOG proteins, and the final piece (if available) was replated on a fresh feeder layer to observe further growth. The remaining non-blastocyst embryos were stained with propidium iodide or Hoechst 33342 to evaluate embryo stage. The rate of cleavage was recorded as well as the percentage of blastocysts produced from total oocytes fertilized. Proportions of cleaved cells reaching 8 cells, 8 to 16

cells, morula, blastocyst, and hatching blastocyst were recorded to ensure that all trials were similar.

The number of colonies produced between the three conditions was analyzed using an unpaired t-test. Any differences in morphology were noted throughout growth. The RNA expression level of pluripotency markers (*NANOG, OCT-4,* and *SOX2*) and one reference gene were measured in triplicate using qRT-PCR and results were normalized to the reference gene. qRT-PCR results were analyzed using the Livak (ΔΔCt) Method, with partly enriched cat spermatagonium stem cells (SSC) used as the comparator, and ΔΔCt values compared using ANOVA.

Results

Fertilization and cleavage rates of blastocysts

Parthenogenetic activation was not observed during the duration of this experiment. Blastocyst rates were comparable to study 1 and the literature (Gómez et al., 2010; Yu et al., 2008). Nearly half of fertilized oocytes became blastocysts by 7 days post fertilization and almost 20% of these began hatching by the time of explantation (Table 5). This increase in the number of fertilized oocytes reaching the blastocyst stage was likely due to an increased selection of higher-grade oocytes.

Explant Growth

For each growth trial, at least three blastocysts were mechanically dissected and explanted onto a homologous feeder layer. Often, TE cells could

Table 5: Percentages of cleaved embryos and proportions of each developmental stage obtained in Study 2.

Number of Oocytes	Proportion Cleaved	Proportion of Embryo Stages (per total cleaved)				Proportion Hatching Blastocyst per total Cleaved
		<8-cells	8 to 16 cells	Morula	Blastocyst	
439	69.66	26.78	18.62	11.07	43.53	19.57

not be completely separated from the ICM region. Early blastocysts or those with ICM regions that could not be visualized were dissected into a monolayer and plated as such. No condition had a significant difference in the number of explants that survived to D7 post explantation ($P>0.05$, Table 6). There was no significant difference between the numbers of explants that persisted to D14 between the treatments ($P>0.05$, Table 6). The cat cytokine condition had 52% of explants persisting at D14, while the mouse had 51% and 3i had 46.5%. There was no significant difference between treatments in the number of small or large colonies ($P>0.05$, Table 7). Of the total number of ICM regions explanted, 7 (15.5%) of the mouse colonies grew to a large size (at least 300μm in diameter), while the cat had 3 (7%) and the 3i condition had 1 (2%). Proportion of ICM explants considered to be large was not significantly different between groups owing to the small sample sizes. Explant morphology was documented by brightfield imaging conducted at D7 and D14 post explantation for every persisting explant (Figure 11).

Immunostaining and qRT-PCR

Most of the large colonies had a TE like appearance with occasional smaller tightly packed cells. The TE like cells stained positively for OCT-4 (Figure 12A-C). Immunostaining was only performed on portions of large colonies (Table 7). NANOG staining was either negative or had high levels of background.

Table 6: Description of explants: The number of explanted colonies on day 0, day 7, and day 14 were determined under brightfield using a Leica DML inverted microscope (n=8 trials).

Cytokine Condition	Number Explanted	Number Attached at D7	Number Persisting at D14	Percent Persisting at D14
Mouse	45	29	23	51.1%[a]
Cat	42	25	22	52.3%[a]
3i	43	34	20	46.5%[a]

Table 7: Comparison of colony size: The percentage of colonies smaller or larger than 300μm in size was determined using a Leica DML inverted microscope (n=8 trials).

Cytokine Condition	Number of Small Colonies	Number of Large Colonies	Percent of Large Colonies
Mouse	16	7	30.4%[a]
Cat	19	3	13.6%[a]
3i	19	1	5.0%[a]

Figure 11: Brightfield imagery: Original ICM explants (day 0) are small, less than 100µm (A). A small colony at day 14 of culture is similar in size to an original explant (B), whereas a large colony at day 14 of culture is greater than 300µm in diameter (C). Bar = 100µm

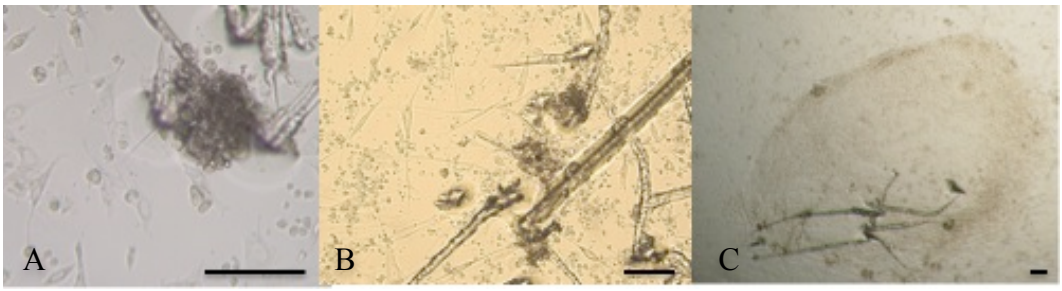
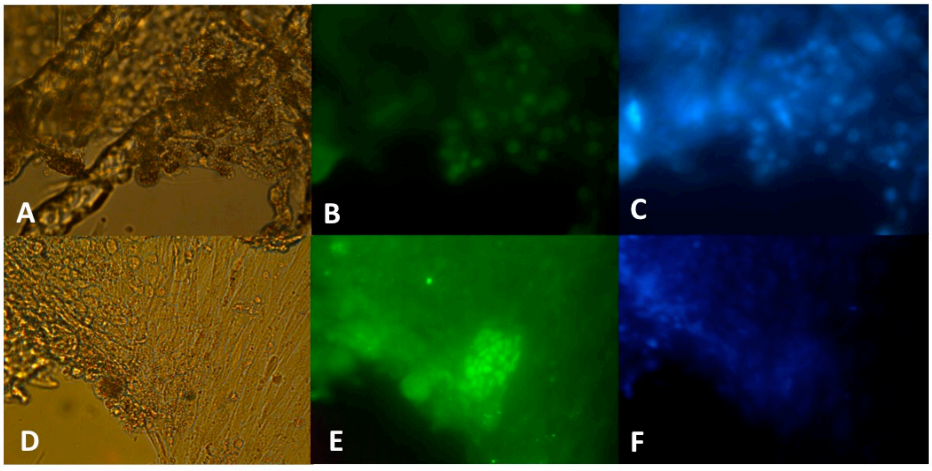


Figure 12: Immunostaining for OCT-4 protein: Brightfield images are shown in A and D. OCT-4 staining is shown in B and E. Hoechst 33342 staining for nuclear material is shown in C and F. Only colonies over 300µm were considered large. Many of these colonies had a TE-like appearance (A-C). Small colony shown in E and D had ESC-like morphology (small, round, tight edged colonies, intense nuclear OCT-4 staining).



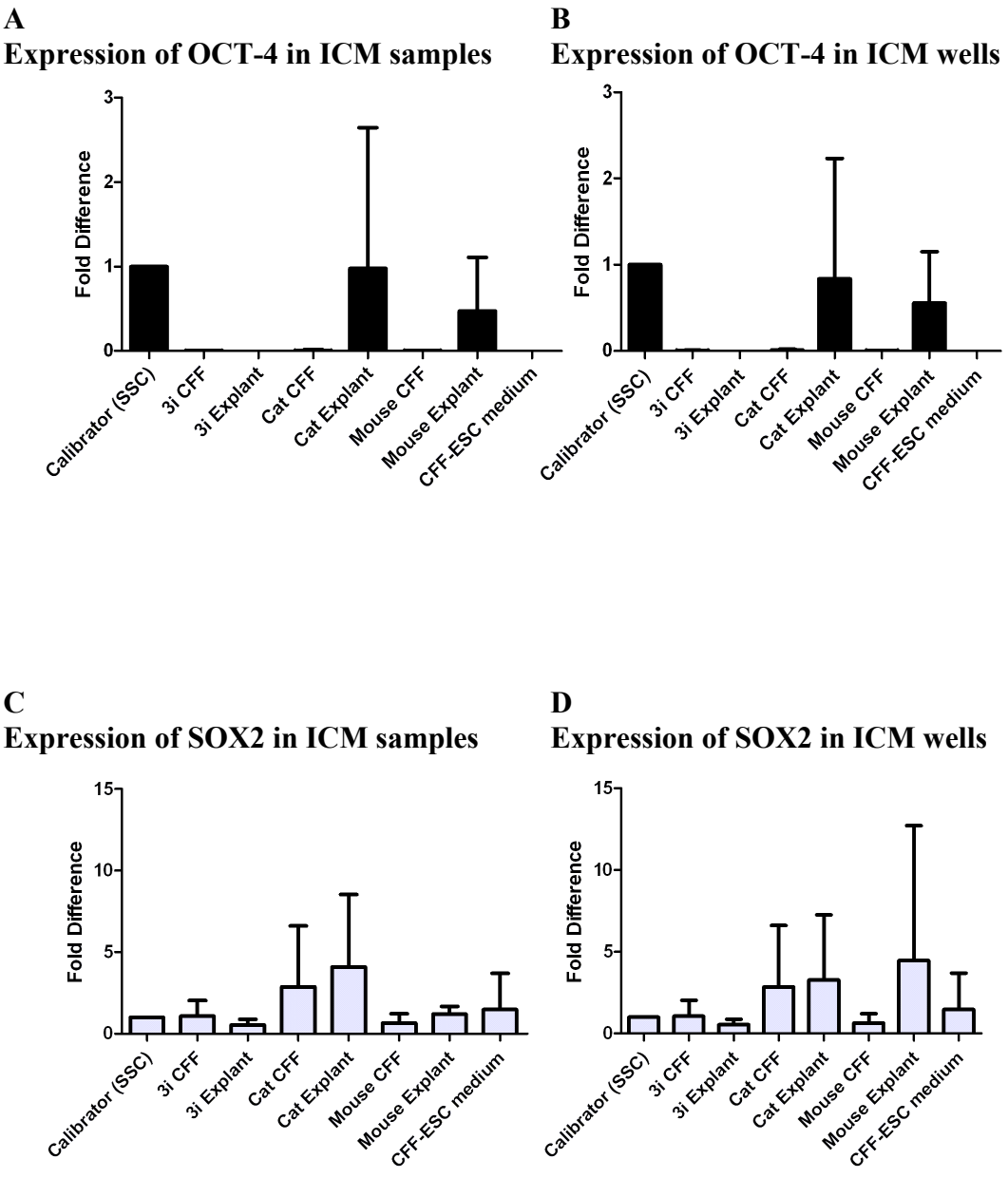
Results for analysis of mRNA for OCT-4 and SOX2 are shown in Figure 13. Genes were normalized to the house-keeping gene RPL-17 and the Livak (ΔΔ Ct) method was used to analyze the fold difference of genes using domestic cat enriched spermatogonial stem cells (SSC) as the comparator. Results obtained from the three trials had large standard deviations. OCT-4 was present in the cat condition (LIF + bFGF) only, both in the ESC lysate and the whole-well lysate (which includes feeder cells and possible small explant colonies). Surprisingly, the whole well lysate had a high level of *OCT-4*. This could be due to the presence of several small explants or remnants of large explants after their removal. *SOX2* was found in all conditions, although no significant difference was seen in the fold difference between cytokine conditions when normalized to RPL-17 and SSC cells ($P>0.05$). *NANOG* was not analyzed due to contamination issues that affected only runs for that gene.

Discussion

There was no significant difference between embryo growth trials. The blastocysts produced using this culture techniques have been previously checked for the production of pluripotency markers using immunocytochemistry and qRT-PCR (Nestle et al., 2012). The rates of blastocyst formation obtained were comparable to the literature and better than during the media trial (Chapter 2; Gómez et al., 2010; Yu et. al., 2008). Hatching and late staged blastocysts were preferentially used for isolation of ICM explants. The increased rate of blastocyst formation and hatching was due the more intense selection of high-grade oocytes.

For the cytokine treatments, our hypothesis was that the 3i condition would perform best, followed by the cat and finally the mouse condition would perform

Figure 13: Results of qRT-PCR in ICM explants: *OCT-4* expression is shown in A and B. *SOX2* is shown in C and D. Each cytokine condition had a sample of cat fetal fibroblast (CFF) cells to confirm that the cytokine condition did not alter gene expression in the feeder cells. The explant columns for cat and mouse in A and C represent the results of analysis of the portions of large colonies removed from the remaining CFFs and potential small colonies, conversely, B and D represent results from an entire well lysate containing all cell types combined. The one large colonies from the 3i condition was not analyzed. Bars represent standard deviation (n=3 trials), no significant differences between values were determined by ANOVA.



the worst. Surprisingly, the cat cytokine condition had 52% of colonies persisting at D14, while the mouse had 51% and 3i had 46.5%, with no significant difference between treatments in the number of small verses large colonies (Table 7). The mouse condition had 15.5% of colonies grow to a large size (at least 300μm in diameter), while the cat had 7% and the 3i condition had 2%. No condition was significantly better based on colony counts, due to the limited numbers available.

Immunostaining was only performed on portions of large colonies. OCT-4 was found to be present in both the tightly packed ES-like explant cells as well as the cells that had TE morphology (Figure 9). The tightly packed colonies were difficult to see with bright field; unlike mESC colonies, which can be easily visualized with brightfield imaging. Due to the removal of colony portions from large colonies for immunostaining, only a limited number of samples were available for qRT-PCR. In the future, larger more homogenous colonies will be needed to generate enough cells for simultaneous confirmation of protein and mRNA expression.

OCT-4 protein and mRNA were present in explants from the cat and mouse conditions. When larger explants were removed and analyzed separately from the rest of the culture well, we saw expression of *OCT-4* mRNA in the whole culture well sample; however, we did not see *OCT-4* mRNA present in the CFF samples. This suggests that some smaller explant colonies or remnants of the larger explants remained in the well and were the source of the *OCT-4*. *SOX2*

mRNA was present in all conditions and the feeder cell control wells. A more complete picture could be obtained by immunostaining for SOX2 protein as well.

We were unable to determine whether or not *NANOG* was present in the explants due to contamination issues that affected only runs for that gene. It is unlikely that substantial *NANOG* was expressed in the explants due to the mostly TE like morphology, and poor results following immunostaining. NANOG is essential for the maintenance of pluripotency and should be evident, if the cells were indeed ES-like cells.

While we predicted that the 3i condition would perform the best, our results show that it did not provide the best conditions for growth. The 3i condition is specifically designed to restrict pathways that lead to differentiation or a lack of self-renewal, similar to the naïve condition, however if the explants were more similar to EpiSC, then the 3i cocktail would not allow their growth. For example, the addition of PD0325901 to inhibit MEK (via ERK) should block any EpiSC from developing because of their dependence on FGF/ERK signaling (Buehr et al., 2008). In the future, addition of LIF to the 3i condition may produce different results. Adding LIF to a 3i cocktail has been shown to have some success for porcine (Telugu et. al, 2010). It has been reported that CHIR99021 may delay chromosome alignment and induce instability in chromosomes in cultured HeLa cells (Tighe et. al., 2007). If the compound has the same effect on cat ESCs, other factors may need to be tested to disrupt the GSK-3β pathway, such as Wnt3a (P. Li et al., 2008; Willert et al., 2003). Wnt3a

supplementation has shown success in maintaining induced pluripotent stem cells and may still hold promise for the domestic cat.

All three conditions produced at least one large colony that persisted to day 14. All large colonies had either a TE like appearance or a combination of TE and an ESC-like colony appearance. All colonies stained similarly for OCT-4 protein; the protein was localized to the nuclei of cells and was more intense in the ES-like small colonies, rather than the TE like colonies.

While significance was not obtained for any of our parameters, the mouse condition (BMP4 and LIF) produced colonies that were large in size, while simultaneously having high levels of mRNA for OCT-4 and SOX2 proteins. Also a small region containing tightly packed cells, which stained intensely for nuclear-localized OCT-4 was observed. Surprisingly, the trend in regards to number of colonies established was the opposite of that predicted with mouse and cat conditions being greater than 3i, however, due to low efficiencies of colony formation, these differences were not significant. For future experiments the 3i conditions should be altered with the goal of improving explant survival and growth. This project has helped to elucidate some of the pathways that may be active in maintaining pluripotency in the cat as well as showing that these conditions have potential for future embryonic stem cell growth.

Chapter 4: Discussion

Embryonic stem cells are a dynamic population that can be manipulated and directed to become any cell type found in the body. Providing conditions, which would allow these cells to maintain pluripotency, has proven to be difficult in species other than the mouse, rat and human. The competency of the starting material determines the initial success of the derived embryonic stem cells, as well as the supporting media they are cultured in. Producing high quality blastocysts that express the pluripotency markers is essential to the derivation and maintenance of these novel cell lines. In these studies, we have demonstrated that a defined medium can support development of good quality blastocysts, which express NANOG, OCT-4 and SOX2. Furthermore, we assessed the ability of resulting ICM explants to maintain expression of these pluripotency makers using three different cocktails of cytokines or small molecules.

Domestic cat embryos have been cultured for decades and yet the rates of blastocyst formation are still below that of the mouse. Culture media have ranged from basic salt solutions to completely defined growth media. Through the two portions of this study, we have demonstrated the importance of culture media on the growth of the resulting embryos and ICM explants. One of the necessary macromolecule ingredients for embryo culture media is protein. Embryos are constantly growing and dividing and need to have access to proteins and amino acids in order to continue their growth. It has long been known that BSA and FBS are needed in sequence for domestic cat embryonic culture. Generally in cat embryo culture, BSA is added for maturation and FBS for fertilization and

culture. However, serum is an unknown biological fluid that may have components that are not yet fully understood. Serum contains three types of proteins: globulins, albumin and fibrinogen. Purified BSA contains mainly albumin, while FBS contains mainly fibrinogen, and human serum protein substitute (used in Medium A) contains mainly globulins. In Study 1, we determined that human serum protein substitute (containing α and β globulins) could provide an adequate protein source for domestic cat embryos, resulting in embryo development similar to that obtained using FBS and BSA. Moreover, human serum protein substitute's globulin could be used as a protein source for all stages and steps of culture. No change in protein source was necessary for the growth of embryos between maturation and fertilization. Maturation rates were not different between the serum protein substitute containing medium and the BSA containing medium, unlike in a previous study where FBS was used in place of BSA for maturation, resulting in limited expansion of cumulous and decreased fertility (Wood et al., 1995). The constituents of serum beg deeper investigation, as many of its components are still undefined.

The components in serum may also have an effect on the culture of embryonic stem cells. Serum can differ between lots, in some cases containing factors that may promote differentiation. Knockout Serum Replacer (KSR) is used in place of serum as a defined nutrient source that contains serum proteins and associated lipids that have proven helpful in the culture of hESCs and mESCs (Garcia-Gonzalo et. al., 2008; Hao et al., 2006). In Study 2, we explanted ICM regions of blastocysts grown in Medium A (containing serum protein substitute)

into three different culture conditions supplemented with serum replacer. We found no significant difference between the growth of explants in these trials, however more trials are necessary to truly elucidate any subtle differences.

The 3i condition was hypothesized to be the superior medium that would stimulate the growth of ES-like explants of the naïve subtype due to the addition of chemical inhibitors, rather than relying on biological molecules that may be species specific. However, in this study the 3i condition trended to be the worst condition for growth. Because the 3i condition is meant to simulate a mouse like condition, the addition of LIF may help aid in the stimulation of pluripotency pathways in future experiments. An evaluation of the cat LIF protein and receptor (LIFR) could reveal which species of LIF (human or mouse) would be most active for the cat LIFR, as was done for the dog (Wilcox et al., 2009).

The cat condition containing LIF and bFGF was hypothesized to be the best at deriving ES-like cells for the domestic cat based on the current literature, however, it performed similarly to the mouse condition. Human ESCs are dependent on FGF; it can increase proliferation in these cells when grown on a feeder layer and it promotes the ERK and PI-3K pathways (Amit et al., 2000; Dvorak et al., 2005; Honda et al., 2009; Wang et al., 2005; Xu et al., 2005). Mouse ESCs are not dependent on FGF, therefore, we can make the assumption that if this condition had fared best, the cells we obtained would have been more like the EpiSC state, and not the naive state.

The mouse condition permitted establishment of large colonies (30% of colonies). This condition utilizes BMP4 and LIF in combination to inhibit the

MAPK pathway and extend self-renewal (Qi et al., 2004; Xu et al., 2002; Ying et al., 2003). This condition produced the best looking colony, which had a tight edge, intense OCT-4 immunostaining, and tightly packed cells. If explants prefer this condition while exhibiting pluripotency proteins and mRNA, it is a promising sign that they are of the naïve type, like mESCs. OCT-4 mRNA and protein were present; however the results for NANOG are inconclusive. Further examination of this condition is necessary, and mandates better methods to visualize NANOG.

Due to the lack of significant results, none of these three conditions can be said to provide ideal conditions for domestic cat naïve ESC-like explants. Naïve or ground-state ESCs are characterized by the ability to become any type of cell present in the three germ layers (including germ cells), maintain pluripotency markers, self-renewal capabilities, and have a normal karyotype. We have shown that these explants expressed proteins for OCT-4 and SOX2 in both the mouse and cat conditions: however the predominant morphology appeared to be that of TE. Future work is needed to better define the ideal culture conditions of domestic cat naïve ES-like explants.

To better our explant results, several recommendations can be made for future experimentation. The mechanical isolation technique led some blastocysts to collapse and for the ICM to become indistinguishable from TE cells. This resulted in a portion of TE being explanted along with the ICM region. Significant improvements in ESC derivation might be obtained if the TE can be completely removed and only the ICM region explanted because TE has been shown to be detrimental to the explant if it remains in culture. Porcine TE can

secrete retinoic acid which causes differentiation of the ICM into epiblast and primitive endoderm (Parrow et al., 1998; Trout et al., 1991; Yelich et al., 1997). Therefore, in the future, an alternative method that completely removes the TE, such as immunosurgery of TE with lysis of the labeled cells, should be attempted. Although both techniques have been successful for explants in the mouse and other species, removal of the TE may prove more beneficial in the cat.

Another essential component of success is having the appropriate markers to determine the quality of the ICM and explants, as well as of the starting blastocyst. OCT-4, an essential pluripotency factor found in embryos and ESCs, is an excellent marker for both mRNA and protein. NANOG however, has proven to be very difficult to study; immunostaining is rarely shown in the literature. We had moderate success with the ICC of NANOG in embryos, and poor results due to high background in explants. Quantifying mRNA was troublesome due to contamination and inadequate primers. The trouble with visualizing NANOG protein could be due to the loss of expression of NANOG with continued embryo culture. Similarly, having a complete picture of SOX2 would enable us to make better conclusions about the state of our explants. A decent anti-SOX2 antibody would be useful to produce a more complete picture. Unfortunately when working in new species the process of confirming new antibodies can be slow and tedious. Other pluripotency markers such as REX1 or KLF4 could be used to increase our panel of genes to assess.

The type of feeder layer can also play a large role in the success of cell growth. While it has been shown in one study in the cat that domestic cat dermal

fibroblasts were superior to mouse feeder cells (Gómez et al., 2010), other species have different results. In the pig, homogenous feeder cells were better for starting initial cultures but mouse proved better for maintenance (Strojeck et al., 1990). For dog ES-like cells, homologous and heterologous feeders equally supported initial cultures, but mouse feeder cells were superior for continued growth (Wilcox et al., 2009). There are cell-surface and soluble factors provided by the feeder layer that allow the embryonic stem cells to prosper. The optimal conditions for deriving novel ESC lines may differ from those required for maintenance, and there may be more beneficial types of homologous feeder layers. We used a cat fetal fibroblast layer that perhaps was inferior to the cat dermal fibroblast line obtained by other labs. Furthermore, cytokines can have dramatic effects on feeder cell morphology – artificial matrixes may eventually prove superior to current co-cultures.

Alternative avenues must also be investigated, such as pursuing induced pluripotent stem cells (iPSCs). These cells are generated by reprogramming somatic cells, forcing them to express the pluripotency transcription factors, thus converting the cell to an ES-like state. The resulting cells can be differentiated in the three germ layers as well. One of the benefits of iPSCs is the ability to generate these cells from any cell type, i.e. an embryo is not necessary. Another technique that is of value is the production of embryos from somatic cell nuclear transfer (SCNT). SCNT embryos are produced by removing the nuclei of a donor cell and placing it into an enucleated egg. These techniques could provide a way to generate stem cell lines from rare and endangered species, for which embryos

are scarce and valuable. ESC from these species could be generated for medical and research purposes, and, more specifically, converting these stem cells into gametes as an alternative method for preserving rare genomes (Byrne et al., 2007).

In summary, we have shown that commercially available media for *in vitro* culture can be used to produce domestic cat embryos of good quality. Human serum protein substitute can be used during maturation as well as fertilization and culture with equivalently good results as BSA and FBS. These embryos express NANOG, OCT-4 and SOX2. The ICM regions of these embryos can be mechanically isolated and cultured for two weeks on a CEF feeder layer and still maintain the expression of OCT-4 and SOX2 under three separate growth conditions. Refining the cytokine concentrations and cocktails could help to determine the relevant pathways of pluripotency in the cat, and these data could be extrapolated to more exotic felines, increasing our ability to work with the embryos and potential ESCs of rare cats. More work is needed to determine the optimal culture condition for naïve cat ESCs and ES-like cells.

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