

ABSTRACT

Dissertation Title: DERIVATION OF PLURIPOTENT STEM CELLS
FROM BLASTOCYSTS AND SOMATIC CELLS
IN THE DOMESTIC CAT (*FELIS CATUS*)

Ran Zhou, Doctor of Philosophy, 2018

Dissertation directed by: Professor Carol L. Keefer,
Department of Animal and Avian Sciences

Pluripotent stem cells in the domestic cat model represent a huge potential for disease modeling, drug screening and regenerative medical treatments for feline species as well as for humans. However, current knowledge on deriving and maintaining stem cells has been obtained primarily from studies in mouse, rat, and human. Difficulties in attaining similar results in cats indicate the necessity to better understand pluripotency in this species. The hypothesis was that inadequate cytokine supplementation results in pluripotency loss along with declining transcription factors

expression. The main goal of this project was to assess the effects of selected growth factors and inhibitors, in maintaining pluripotency in embryonic cells, and to attain pluripotency from fibroblasts by controlling expression of exogenous transcription factors. In the first study, conventional cytokine cocktails, leukemia inhibitory factor coupling with glycogen synthase inhibitor 3, and mitogen-activated protein kinase inhibitor (LIF and 2i) could partially maintain pluripotency regulatory circuitry in the cat. In this condition, embryonic cells reached a state that was not fully defined (neither naive nor primed). Overall, cell characterizations revealed a trend of pluripotency loss over time. In the second study, pluripotency was attained by forced expression of inducible exogenous transcription factors (NANOG, POU5F1, CMYC, and SOX2) and cultured in medium supplemented with the same cytokine combination identified in the first study. Notably, unlike previous reports in the cat, colonies with partial pluripotent features could be maintained after the transgenes were silenced. In addition to the protein and transcript markers for pluripotency, lineage marker dynamics were examined in pluripotent cells and embryoid bodies. The outcome suggested the cells generated with LIF and 2i had developed beyond the undifferentiated stage of ICM in expanded blastocyst. Collective results not only challenged the efficacy of the cytokines combinations LIF and 2i in maintaining feline pluripotency, but also suggest direction of research towards the species-specific signaling requirement in embryonic progression and stem cell derivation.

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SOMATIC CELLS IN THE DOMESTIC CAT (*FELIS CATUS*)

by

Ran Zhou

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in complete fulfillment
of the requirements for the degree of
Doctor of Philosophy
2018

Advisory Committee,

Professor Carol L. Keefer, Chair

Professor Dorothy Beckett

Dr. Pierre Comizzoli

Professor Lisa Taneyhill

Professor Bhanu Telugu

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Dedication

Today is the day, writing these words of thanks is the opening of my dissertation. It has been an intense and exciting period of learning and experimenting, not only in the biological field, but also on a personal level. The time spent in the doctorate program has a great impact on me. I would like to reflect on who have encouraged, helped and inspired me so much throughout the period.

This work would not have been possible without the financial support of Jo Gayle Howard Revocable Trust.

I would first like to thank all the members of the advisory committee for their generous support. You were always willing to offer me advice in every aspect for an emerging researcher. I would particularly like to single out my advisors Dr. Carol Keefer and Dr. Pierre Comizzoli, I want to thank you for your valuable guidance, excellent cooperation and for all of the opportunities I was given to conduct my research and further my dissertation at University of Maryland, College Park as well as in National Zoological Park, Washington D.C. I would like to thank my former and current colleagues, Dr. Lindsey Vansandt, Dr. Halli Weiner, Dr. Pei-Chih Lee, Dr. Lara Mouttham and Dr. Tricia Rowlinson. Without you, I would have been drained by all the samples collections. With you, I was still exhausted yet feel a bit comfort through the shared tiresome. In addition, I would like to thank my interns Ms. Morgan Wooten, Yael Ed, working with you is a delight.

Special thanks to all the staffs in the animal clinics, Animal Birth Control LLC., Spay Now Laurel and Animal Rescue Inc., which have been providing me samples. You are animal loving people. I have felt the passion and been encouraged by that passion for research in the domestic cat.

Nobody has been more important in the pursuit of this project than my parents. My deepest gratitude is distributed for their sympathetic ear. You are always on my side. Finally, there are my friends, in the department and out in the world. We have been supporting each other by deliberating over our issues, thinking and findings, the conversations were so much beyond our papers.

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Abbreviation List

BMP	Bone morphogenetic protein
CFF	Cat fetal fibroblast
CLC	Cardiotrophin-like cytokine
c-MYC	Myelocytomatosis oncogene
CT1	Cardiotrophin-1
EpiSC	Epiblast stem cells
ERK	Extracellular signal-regulated kinases
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
GF	Growth factor
GFP	Green fluorescence protein
GSK3	Glycogen synthase kinase 3
ICM	Inner cell mass
IL6	Interleukin 6
iPSC	Induced pluripotent stem cell
JAK	Janus kinase
KLF4	Kruppel-like factor 4
KOSR	Knockout serum replacement
LIF	Leukemia inhibitory factor
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase

NANOG	Nanog homeobox
OCT4	Octamer-binding transcription factor 4
OSM	Oncostatin M
PC	Positive control
POU5F1	POU domain, class 5, transcription factor 1
p-SMAD	Phosphorylated mothers against decapentaplegic homolog
p-STAT3	Phosphorylated signal transducer and activator of transcription 3
REX1	Reduced expression protein-1 (Zinc finger protein 42)
RT-PCR	Reverse transcription polymerase chain reaction
rtTA	Reverse tetracycline-controlled transactivator
SCNT	Somatic cell nuclear transfer
SMAD	Mothers against decapentaplegic homolog
SOX2	Sex determining region Y box 2
SSEA	Stage-specific embryonic antigen
STAT3	Signal transducer and activator of transcription 3
T	T-box transcription factor T (Brachyury)
TCF3	Transcription factor 3
tet	Trans activator induced expression by tetracycline
TF	Transcription factor
TGF	Transforming growth factor
WNT	Wingless-type mouse mammary tumor virus integration site family member

Chapter 1 Introduction

1.1 Biology significance of pluripotent stem cells in the domestic cat (*Felis Catus*) model

The value of generating pluripotent cells in domestic cats is comparable to that of such versatile cells in mouse and human, for the reasons below,

1) The feline family included many endangered species (<http://www.iucnredlist.org>). The domestic cat has been used as a model for developing assisted reproductive technologies for preserving endangered wild cats (Gomez *et al.*, 2000; Fernandez *et al.*, 2015). Because domestic cats have genetic and anatomic similarity to the other members in Felidae and have a highly conserved genome as compared to primate family (Johanson *et al.*, 2006), they can also serve as a model for studying stem cells. By establishing pluripotent cells *in vitro*, the clinical use of stem cells for endangered species in regenerative medical treatment and drug screening becomes possible. In all, the lessons we will learn from establishing pluripotent cells in domestic cats is of great advantage for expanding such study to other cats.

2) Humans and cats share more than 250 diseases, which afflict in similar ways (Hedges *et al.*, 2006 and Meredith *et al.* 2006). Therefore, even though mice have less evolutionary distance, the cat is a better model in stem cell engineering for some diseases (e.g., FIV and HIV), which share more similarity to the human disease (Lee *et al.*, 2017). In addition, biomedical applications could be tested extensively (e.g.,

transgenic cell or organs) prior to consideration for translation to human medicine.

3) Domestic cats are obligate carnivores and considered the representative for Felidae. This family, which last shared a common ancestor with humans around 92 million years ago, are evolutionary further from human than rodents (Hedges *et al.*, 2006 and Meredith *et al.* 2006). Studies for acquisition of pluripotent stem cells in these animals not only broaden the view of regulating pluripotency, but also deepen the understanding of the how these mechanisms adapt to different evolution paths.

The success of generating pluripotent stem cells for the feline family relies on the knowledge acquired from studies in rodents and humans, as described more fully below.

Pluripotent stem cells are cells that have two features, 1) potential to produce all cell types (>200) in a body and 2) unlimited self-renewal (Bradley A. *et al.* 1984; Takahashi and Yamanaka, 2006). Based on the origin, there are two major types of pluripotent cells *in vitro*, embryonic stem cell (ESC) from embryos and induced pluripotent stem cell (iPSC) from somatic cells (Nakagawa *et al.*, 2008). The first pluripotent stem cells were identified in mouse testicular teratocarcinoma. It was recognized that carcinoma cells could give rise to cells in various types after transplantation into endodermal, mesodermal and ectodermal lineages (Stevens 1960 and 1962). Because these cells shared features with inner cell mass (ICM), which could be used to generate teratomas, ESCs were then directly obtained from mouse pre-implantation embryos (Evans and Kaufman, 1981; Martin, 1981). By now, mouse ESCs are still the best-understood type of pluripotent cells. With the knowledge gained

from ESCs, another equivalent type of pluripotent stem cell in this species was generated in 2006 (Takahashi and Yamanaka, 2006). These cells were reprogrammed somatic cells that have been reversed to the undifferentiated state (Takahashi and Yamanaka, 2006), and they have the same potential to contribute to all cell types as ESCs.

ESCs are derived from an embryo and are not genetically matched to an individual, so they are unlikely to be used in customized medical treatment. They are studied to advance the knowledge of how stem cells sustain, differentiate, and react to defined environments. iPSCs, on the other hand, are reprogrammed from somatic cells, and possess great therapeutic values (Nakagama *et al.*, 2008; Takahashi *et al.*, 2007). iPSCs are genetically matched to their donor, thus they inherit the same genome and partial DNA methylation pattern, which make them useful for drug screening in treating genetic disease (Beevers *et al.*, 2013). This feature minimizes tissue rejection response of the donor hence leads to successful cell and tissue transplantation (Takebi *et al.*, 2013). Gene-edited iPSCs can give rise to mutant or genetic modified offspring, which help scientists understand how different phenotypes emerge from the similar genotypes with minor variation (Lin *et al.*, 2018). These findings have expanded the view of *in vitro* cell modeling of disease.

In addition to what has been learned from mouse ESCs and iPSCs, the robust translation of mechanism in maintaining pluripotency and directing differentiation between these two cell types has encouraged a series of similar studies in other species (Buehr *et al.*, 2008; Carey *et al.*, 2009; Debowski *et al.*, 2015; Gomez *et al.*, 2010).

Looking into mammalian embryogenesis, the early epiblast, the origin of ESC, is a conserved stage during development *in vivo* (Forgács and Newman, 2005). Extensive studies in mouse ESC and iPSC have proven that those pluripotent cells produced *in vitro* are competent when inserted into a recipient embryo to produce a chimera. They contributed to form organs from all three germ layers, and the offspring of chimeric mice inherited genetic markers from those ESC and iPSC cell lines (Cai *et al.*, 2015; Nakagawa *et al.*, 2008; Takahashi *et al.*, 2007). With the understanding that the core networks regulating pluripotency in ESC and iPSC are shared, such machinery is thought to be present in other mammals also. Yet, the same level of success cannot be achieved in most other species tested (Madeja *et al.*, 2015; Okita *et al.*, 2016; Verma *et al.*, 2012).

Rat and human studies demonstrated that additional inhibitors and manipulation are required to generate ESC (Buehr *et al.*, 2008; Ware *et al.*, 2014), while rabbit and bovine studies have not produced the same type of pluripotent cells (Madeja *et al.*, 2015 and Osteil *et al.*, 2016). Neither ESC nor iPSC has been established in domestic cats, although several studies report partial success, in which those pluripotent-like cells either differentiated in a few passages or relied on exogenous gene expressions (Gomez *et al.*, 2010; Yu *et al.*, 2008; Verma *et al.*, 2012). Further efforts to understand how pluripotency is regulated regarding the pluripotency-related transcription factors (TFs) and the species disparities in this regulation are needed, before successful derivation of ESC and iPSC can be achieved in felids and other non-rodent species.

1.2 Literature review

1.2.1 Pluripotency regulatory circuitry

Pluripotency in embryonic and induced stem cells,

In the mouse model, ESCs are derived from ICM in expanded blastocysts on Day 3.5 (Czechanski *et al.*, 2014). In cat, ESC-like cells were generated from ICM in blastocysts with the similar morphology, on a later time – Day 7-9 (Fig. 1.1). The ICMs in different species share the critical TFs, Nanog homeobox (NANOG), POU domain, class 5, transcription factor 1 (POU5F1 aka. octamer-binding transcription factor 4, OCT4), and Sex determining region Y box 2 (SOX2). These TFs are responsible for maintaining pluripotency in ICM and epiblast (Niakan KK *et al.* 2013), as well as iPSCs (Takahashi and Yamanaka, 2006). There are specific signaling pathways that regulate these TFs (Fig. 1.2). Each factor has their own functions in gene regulation and they coordinate with each other in a balanced pluripotent state (Rodda *et al.*, 2005).

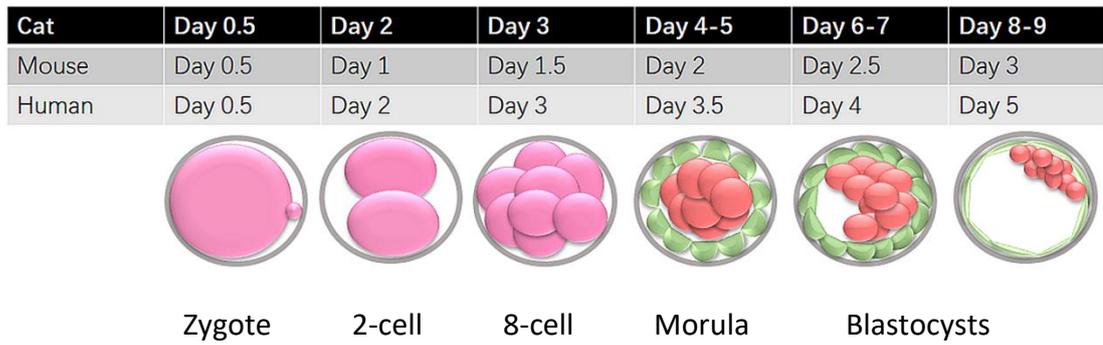
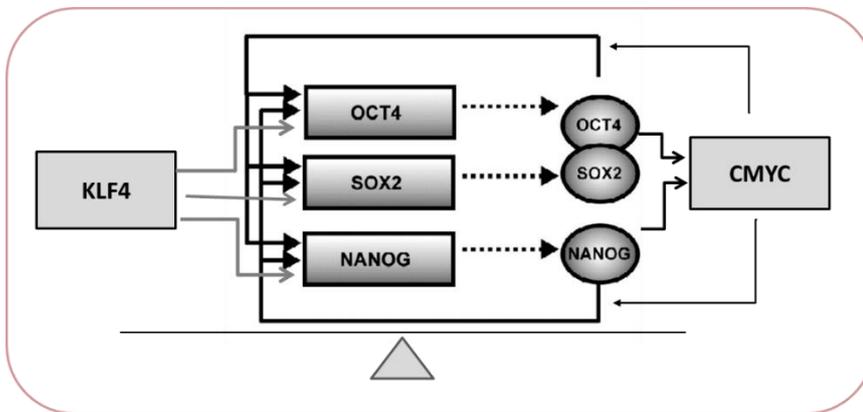


Figure 1.1 Timeline of key stages of early embryogenesis in cat, mouse and human. Pink cells represent totipotent blastomeres from zygote to 8-cell stage, red (ICM) and green (trophectoderm) cells represent different lineage in morula and blastocysts.

A.



B.

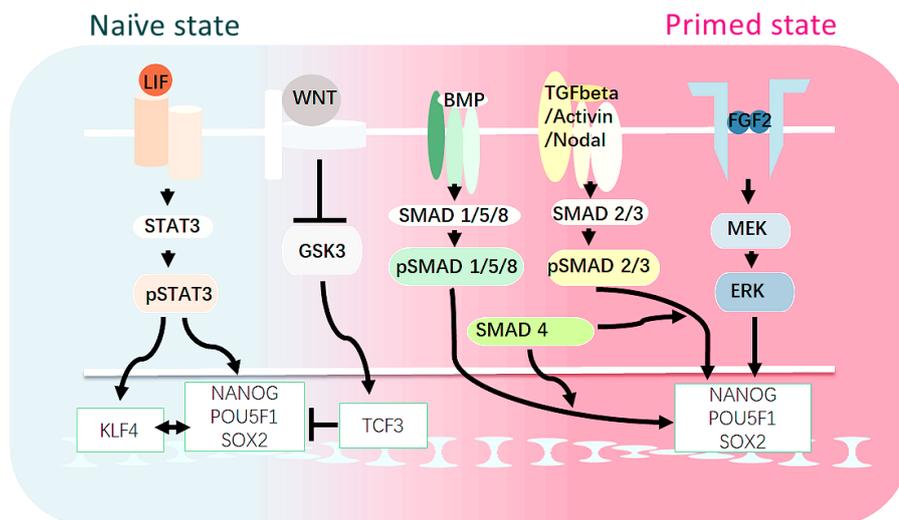


Figure 1.2 A) Pluripotency regulation circuitry between TFs; B) Activation of four major pathways and inhibition of WNT pathway maintain ESC and iPSC pluripotency. In Naïve state, LIF binds to its receptor and signals through phosphorylation of STAT. In primed state, FGF2 binds to its receptor and activates MEK/ERK.

NANOG is the ground state marker and regulator of pluripotent stem cells (Cai *et al.*, 2015; Yates *et al.*, 2005). In ICM or pluripotent stem cells in vitro, over 50% of promoters that are actively regulated are bound by NANOG. Ninety percent of promoters bound by POU5F1 and SOX2 also have NANOG bound. Likewise, 50% of POU5F1 target genes also showed co-occupation by SOX2. These indicate genes are co-regulated by all three TFs. Moreover POU5F1, SOX2 and NANOG bind to their own promoters in a self-regulatory positive/negative feedback loop, and POU5F1-SOX2 hetero-dimer and NANOG also bind to each other's promoters (Rodda *et al.* 2005). NANOG, OCT4/POU5F1 and SOX2 co-bind to each other's enhances to maintain ESC properties, and they also repress each other on the promoter regions when excess expression is not necessary (Cai *et al.*, 2015) (Fig. 1.2A). These three core transcription factors target over 300 hallmark genes of ESC, which are either repressed or activated (Nakagawa *et al.*, 2008; Wang *et al.*, 2012). This basic understanding may apply to various species including felids.

Four pathways [Leukemia inhibitory factor (LIF)/ Signal transducer and activator of transcription 3 (STAT3), Bone morphogenetic protein (BMP), Activin/Nodal, Mitogen-activated protein kinase (MEK)/ Extracellular signal-regulated kinases (ERK)] have been reported and validated in mouse, rat, rabbit, and human, as being involved in maintaining ground state pluripotency (Duggal *et al.*, 2015; Gao *et al.*, 2015; Hayashi *et al.*, 2011; Hou *et al.*, 2013) (Fig 2). In addition to these four pathways, the Wntless-type mouse mammary tumor virus integration site family member (WNT) pathway also contributes to supporting pluripotency in stem cells (Madeja *et al.*, 2015;

Munoz-Descalzo *et al.*, 2015; Pieters and Van Roy, 2014). These pathways activate the core transcriptional regulatory circuitry of NANOG, POU5F1 and SOX2 for maintaining pluripotency and self-renewal in different states, whereas FGF2 pathway takes over the control during development (Evans and Kaufman, 1981; Nicholas and Smith 2009; Thomson *et al.*, 1998).

However, differences were observed between mouse and human ESC, which resulted in the distinction of naive and primed states of ESCs. Naïve ESCs like mouse ESC should have unlimited self-renewal and clonal capacity (i.e., can be propagated as single cells). They also should exhibit pre-implantation epiblast identity and potency, which is identified by the presence of two active X chromosomes in female cells (Bradley A. *et al.* 1984), along with the ability to incorporate into the epiblast and re-enter development to produce various somatic and germ cells when forming chimera embryos. The human ESC is derived from *in vitro* cultured embryos donated to assisted reproduction clinics (Thomson *et al.*, 1998). Initial attempts followed the protocols in mouse. Yet early human ESCs are more like post-implantation epiblast and do not survive passage as single cells (no clonal expansion). Thus, the human ESCs are similar to cells at a more developed stage compared to mouse ESC and exhibit what is known as primed pluripotency (Duggal *et al.*, 2015; Thomson *et al.*, 1998). The major difference is shown in Table 1 (Nicholas and Smith 2009; Rossello *et al.*, 2016).

Table 1.1 Property of Naive and primed state of ESCs

Characteristics	Naive	Primed
Origin	Early epiblast	Egg cylinder/embryonic disc/late epiblast
In vitro phenotype	Rodent ESC	Rodent epiblast stem cells (EpiSCs) and primate ESCs
Pluripotency markers	OCT4, NANOG, SOX2, KLF, KLF4	OCT4, NANG, SOX2
Naive markers	REX1, NR0B1, FGF4	None
Specification markers	None	FGF5, T
Response to LIF/STAT3	Self-renewal	None
Response to MEK/ERK	Differentiation	Self-renewal
Clonogenicity	High	Low
Teratoma formation	Yes	Yes
Chimera blastocyst formation	Yes	No
XX status	Both active	Only one active

Besides the TFs shown in Table 1.1, another TF is important for stabilizing the cell state. Myelocytomatosis oncogene (*MYC*) gene (known for cell proliferation control) is conditionally needed to maintain naive ESC (Scognamiglio *et al.*, 2016). Primed (epiblast) stem cells (EpiSCs) can convert to naive state when expression of Kruppel-like factor 4 (*KLF4*) or *MYC* is forced in EpiSCs with LIF supplementation. LIF supplemented with bovine fetal serum (FBS) plus MEK inhibitor and glycogen synthase kinase beta 3 (*GSKβ3*) inhibitor can be sufficient to induce such conversion (Rossello *et al.*, 2016). Combined knowledge from mouse, rat, and human ESCs studies may provide guidance when developing the protocol for cat ESC.

1.2.2 Approaches to achieve pluripotency balance

Induction of pluripotency

Pluripotency in ESC is built on the natural presence of the *SOX2*, *POU5F1* and *NANOG* (Evans and Kaufman, 1981). For embryo development and cell differentiation, varying levels of these TFs are needed (Rodda *et al.*, 2005). In somatic cells, all the three genes are down-regulated (Le *et al.*, 2014). Thus, the strategy for inducing pluripotency in somatic cells is to reverse differentiation by over-expressing TFs responsible for maintaining pluripotency (Tanabe *et al.*, 2014), and selection of cells reprogrammed into a pluripotent state (Takahashi and Yamanaka, 2006). A summary of recent strategies for transduction of transcription factors used for iPSC derivation is provided in Table 1.2.

Table 1.2 Recent strategies reported in producing iPSC in different species

Basic system	Integration of exogenous genes	Cell/Species	Efficiency	Reference
Retro-virus vector	Yes	Fetal and adult Fibroblast/mouse, rat, rabbit, human	0.1%	Takahashi and Yamanaka, 2006; Brambrink <i>et al.</i> , 2008; Kang <i>et al.</i> , 2009; Hayashi <i>et al.</i> , 2011; Hussein <i>et al.</i> , 2014
Polycistronic vector with several-in-one	Yes	Fetal and adult fibroblast/mouse	0.001%	Carey <i>et al.</i> , 2009
Adeno virus - mediated vector	No	Fetal and adult fibroblast/mouse, human	0.001%	Harui <i>et al.</i> , 1999; Okita <i>et al.</i> , 2008; Stadtfeld <i>et al.</i> , 2008
PiggyBac vector	No	Dividing cells/mouse, human	0.01%	Woltjen <i>et al.</i> , 2009; Debowski <i>et al.</i> , 2015
Sendai virus vector	No	Dividing cells/mouse, rat, human	1%	Skiadopoulos <i>et al.</i> , 2002; Fusaki <i>et al.</i> , 2009; Soares <i>et al.</i> , 2016
Episomal vector	No	Dividing cells/monkey, human	0.2%	Okita <i>et al.</i> , 2011; Su <i>et al.</i> , 2013
Mini-DNA circle	No	Fibroblast/mouse	0.005%	Jia <i>et al.</i> , 2010
Synthesize mRNA	No	Fibroblast/human	4.4%	Warren <i>et al.</i> , 2010
Protein delivery	No	Fibroblast/mouse	0.001%	Kim <i>et al.</i> , 2009
Small chemical molecules	No	Fibroblast/mouse	0.2%	Hou <i>et al.</i> , 2013
Somatic cell nuclear transfer (SCNT)	No	Cells/mouse, cat, dog, rabbit, rat, sheep, monkey	30%	Le <i>et al.</i> , 2014

Every method is based on the foundation of forcing expression of these four TFs- POU5F1/OCT4, SOX2, KLF4 and c-MYC. Regardless the vector/methodology used, a complete reprogramming requires at least 16 days of forced expression of the pluripotent transcription factors and a defined culturing environment for maintenance. In the study using the *piggyBac* system (Tonge *et al.*, 2014), a new type of pluripotent cell - F(uzzy)-class cells (clones having a fuzzy border), expressed endogenous NANOG and POU5F1/OCT4, but not most of the other ESC-associated genes, and they were dependent on the continuous expression of high levels of exogenous reprogramming factor. Only after the silencing of ectopic TFs, were the cells able to obtain the endogenous pluripotent balance and acquire the ability to contribute to chimeras (Wang *et al.*, 2012; Zunder *et al.*, 2015). This finding proved the importance of both establishing the endogenous pluripotency circuit while silencing the exogenous transgenes to complete reprogramming.

1.2.3 Status of generating pluripotent cells in the domestic cat

Like in other species, it should be possible to produce ESC or iPSC in domestic cats. However, attempts of maintaining/inducing stem cell have not been satisfactory (Gomez *et al.*, 2010; Yu *et al.*, 2008; Verma *et al.*, 2012). Yu *et al.* (Yu *et a.*, 2008) used *in vivo*-derived blastocysts to ensure the quality of embryos. ICM was isolated and cultured on cat embryonic fibroblast (CEF) in medium containing 1000U/ml recombinant mouse LIF. Gomez *et al.* (Gomez *et al.*, 2010) used *in vitro*-cultured blastocysts to serve the same purpose but cultured the ICM with 5 ng/ml human

recombinant basic fibroblast growth factor (FGF2). Regardless of supplementation with different growth factors, the putative colonies would differentiate spontaneously. The colonies in late-passage (9-12 passages) lost stem-like features and failed to proliferate. While there seems to be no difficulty in isolating good quality ICM from cat blastocysts produced either *in vivo* or *in vitro*, there is an issue of establishing outgrowth cultures and subsequent propagation. The protocols proven efficient in mouse and human ESC, therefore, do not guarantee the success for cat ESC. The reason might be a varying reliance on each pathway in different species when propagating pluripotent cells from early-stage embryos. In mouse ESC, the LIF/STAT3 signaling pathway plays a major role in maintaining pluripotency while MEK/ERK is the critical pathway for human ESC (Angeles *et al.*, 2015; Nicholas and Smith 2009). A recent study found that human ESC couldn't contribute to chimera formation, unlike mouse ESC (Duggal *et al.*, 2015). Characterization of human ESC indicated that it is equivalent to late epiblast cells (post implantation stage). Human ESC can be transformed to a state like mouse ESC by activating LIF/STAT3 pathway, but human ESC will differentiate if support from MEK/ERK pathway is removed (Nicholas and Smith 2009; Rossello *et al.*, 2016). In cats, neither supplementation of LIF nor Fibroblast growth factor 2 (FGF2 aka bFGF) support the maintenance of pluripotency in cells derived from inner cell mass (ICM) (Gomez *et al.*, 2010; Yu *et al.*, 2008). Furthermore, while forced expression of transcription factors in initiated reprogramming in fibroblasts from a snow leopard; the characterization of the cells showed incomplete reprogramming that lacked activation of endogenous genes

(Verma *et al.*, 2012). In this study, human genes (*OCT4*, *SOX2*, *KLF4*, *cMYC* and *NANOG*) were integrated using Moloney-based retroviral vector into snow leopard genome. Colonies were obtained and split after reprogramming, however, *KLF4* and *cMYC* remained active throughout 36 passages, and thus it was an incomplete reprogramming. These results indicated that using methods for mouse and human cells was not sufficient to reprogram cat somatic cells into pluripotent cells. In all, these studies raise some questions concerning pluripotency in cats. Which pathways are necessary to propagate embryonic pluripotent cells? Which genes are required in reprogramming somatic cells? The answers are necessary for achieving our goal of maintenance of pluripotent cells in domestic cats.

1.3 Project overview

While transgene-free reprogrammed iPSC would be the ultimate objective, allowing for broader utilization of iPSC in applications directed towards regenerative medicine and species preservation, the overall goal in this study was to produce ESC or iPSC in domestic cats. The hypothesis was that inadequate cytokine supplementation results in pluripotency loss. To determine whether cytokines can stimulate pathways that regulate pluripotency in cells derived from cat ICM, the levels of known TFs and their upstream pathways were investigated under varying cytokine supplementation. In the approach of transforming somatic cells to iPSC, human transcription factors *POU5F1*, *SOX2*, *KLF4*, *cMYC* and *NANOG* were used to force reprogramming and the resulting cells assessed for pluripotency characteristics after

culture with the cytokines growth factors identified in the ICM studies. Cells that were generated from these two experiments, could be widely expanded and possessed some of the characteristics of pluripotent stem cells.

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Chapter 2 Combinations of growth factors regulating LIF/STAT3, WNT and FGF2 pathways pluripotency-related proteins in cat embryonic cells

Portions of this chapter have been submitted to Stem Cells and Development.

2.1 Abstract

Propagation of pluripotent cells from early-stage embryos in mouse and human highly depend on LIF/STAT3 and FGF2/MEK/ERK signaling pathways. However, mechanisms for maintaining pluripotency in embryonic stem cells (ESC) using various combinations of growth factors (targeting LIF or FGF2 pathways) and inhibitors (targeting WNT/GSK3 or FGF2 pathways) still have to be deciphered in other models, including the domestic cat. Our objective was to understand how cytokines influence pluripotency in the cat inner cell mass (ICM) outgrowths. Cat ICM were isolated from in vitro produced embryos and outgrowths were cultured for up to 6 days with single or combined cytokines. Cell proliferation was enhanced with almost all single growth factors and cytokine combinations. Based on gene expression and presence of NANOG, POU5F1 and SOX2 as cell state markers, single growth factors could not maintain similar levels in outgrowth as in the original ICM, which is different from the mouse and human. In our conditions, cytokine combinations involving LIF, GSK3 inhibitor and MEK inhibitor resulted in the most robust expression levels. However, further characterization of embryonic cells derived from ICM indicated that the pluripotent state was not fully preserved. Specifically, low levels of *LIF-receptor* and *STAT3* transcripts in ICM demonstrated that the insufficient response to LIF was due

to signaling variation in regulatory mechanisms of early embryonic cells. The absence of *BMP2-receptor* and *SMAD4* expression also suggested that cat specific signaling pathway was needed to ensure pluripotency balance. Collective results reveal more options to improve the production of ESC in that fundamental animal model.

2.2 Introduction

Pluripotent stem cells have inherent capacity to differentiate into any cell type and to self-renew unlimitedly (Bradley *et al.*, 1984; Takahashi and Yamanaka, 2006). The embryonic stem cell (ESC) is a major pluripotent cell type that can be produced and maintained in vitro (Martin, 1981; Sato *et al.*, 2004) including for biomedical research, drug testing, genetic and congenital disease modeling, and regenerative medicine. Most ESC lines and studies have focused, so far, on common laboratory rodents (Martin, 1981; Czechanski *et al.*, 2014). However, our research team has demonstrated the value of a larger-sized model, the domestic cat, for understanding a wide array of reproductive mechanisms analogous to the human (Comizzoli *et al.*, 2018). Moreover, the cat and human share more than 250 diseases (e.g., Feline Immunodeficiency Virus and human Acquired Immune Deficient Syndrome) (Lee *et al.*, 2017). Other groups also have explored the development of domestic cat assisted reproduction technology that could have application to other felid species (Gomez *et al.*, 2000; Fernandez-Gonzalez *et al.*, 2015), many of which are classified as vulnerable to extinction (The International Union for the Conservation of Nature Red List <http://www.iucnredlist.org>). The availability of cat ESC lines will greatly expand

such potential, possible uses include drug safety screening on pluripotent cells and their derivatives after differentiation, and also in vitro tissue generation for cell-based therapies (Stem Cell Basics VII. stemcells.nih.gov).

In earlier investigations, methods developed to establish ESCs in mouse and human did not lead to stabilized, pluripotent stem cells in the cat (Gomez *et al.*, 2010; Yu *et al.*, 2008). This was likely due to insufficient prevention for differentiation in the culture system. From studies in the mouse model, we know how to maintain ESCs in vitro and how these cells differentiate and contribute to organ formation in chimeric embryos (Tesar, 2005). In addition, the core network of pluripotency in mouse (Tesar, 2005; Tesar *et al.*, 2007), rat (Li *et al.*, 2008) and human (Tesar *et al.*, 2007; Wang *et al.*, 2012) ESCs is a regulatory circuitry involving Nanog homeobox (NANOG), POU domain, class 5, transcription factor 1 (POU5F1 aka OCT4) and Sex determining region Y box 2 (SOX2). Furthermore, four conserved pathways (Leukemia inhibitory factor [LIF]/ signal transducer and activator of transcription 3 [STAT3]), bone morphogenetic protein (BMP), activin/nodal, mitogen-activated protein kinase [MEK]/ extracellular signal-regulated kinases [ERK]) are involved in maintaining ground state pluripotency by regulating the NANOG, POU5F1 and SOX2 expressions (Chen *et al.*, 2015; Duggal *et al.*, 2015; Eiseleova *et al.*, 2009). We suspect that those same general mechanisms apply to the maintenance of pluripotency in the domestic cat. However, there are specific differences in embryonic peri-implantation development process in felids (Knospe, 2002). For instance, blastocyst formation occurs about 8 days after conception, and it

will develop further to neurulation stage before implantation. This is quite different from what has been observed in mouse and human (Hogan *et al.*, 1994). Therefore, we hypothesize that there also are species-specific variations for signaling pathway synergy in different mammalian models. To better elucidate the underlying mechanism in pluripotency, evaluation of cytokine effects targeting different pathways in cat embryos can be very informative. The objective of our study was to understand how the growth factors LIF and FGF2, GSK3 inhibitor and MEK inhibitor (known to be efficient for mouse and human ESC maintenance (Sato *et al.*, 2004; Pieters *et al.*, 2014)) affect the pluripotency circuitry in cat embryonic cells. This could lead to the development of proper culture systems and applications for cat ESC.

2.3 Materials and Methods

In vitro maturation and fertilization of oocytes followed by embryo culture

Approval from an Institutional Animal Care and Use Committee was not required as domestic cat ovaries and testes were obtained from local veterinarians following routine owner requested ovariohysterectomies or orchiectomies. These are exempt studies under Title 9, Code of Federal Regulations, Subchapter A- Animal Welfare, Part 1 to 3. Mature cat ovaries and testes were collected from local veterinary clinics, transported and processed within 6 hours of excision (Godard *et al.*, 2009). Grade 1 immature oocytes were recovered from ovarian tissue and matured for 24 hours (Godard *et al.*, 2009) (38.5°C, 6% CO₂) in 50 µl drops of

human Quinn's Advantage Blastocyst Medium (Origi) supplemented with bovine FSH and LH (Sigma) at 1 μ g/ml. Spermatozoa were collected from excised cat epididymis in Ham's F10 medium (Irvine Scientific) (Hribal *et al.*, 2012). After centrifugation (350 g x 8 minutes), spermatozoa were allowed a swim-up for 30 minutes (38.5°C, 6% CO₂.) and oocytes inseminated by co-incubation with motile spermatozoa (1 million/ml) for 24 hours. Resulting embryos with at least two blastomeres (48 hours post insemination) were cultured under the same conditions in Quinn's Advantage Blastocyst Medium until 9 days after fertilization (Nestle *et al.*, 2012). Under our experimental conditions, percentage of cleaved embryos after in vitro maturation/fertilization was $46 \pm 24.5\%$ (mean \pm SD), and the proportion of expanded blastocysts relative to total number of 2-cell stage embryos was $15 \pm 13.1\%$. Expanded blastocysts were selected for RNA extraction (n = 10), immunofluorescence (n = 5) and outgrowth culturing (n = 3-5) per treatment group. A total of 212 expanded blastocysts from 150 in vitro cultures were used during the entire study.

Cat blastocyst outgrowth culture

The zona pellucida of each blastocyst was removed in 1% Protease (Sigma) (Moor and Cragle, 1971) whole embryo was cultured on mouse mitotically inactivated embryonic fibroblasts (mEF) grown on 0.1% gelatin-coated (Sigma) in 4 or 8-well chamber glass slides (Millipore) for 6 days. Blastocysts outgrown for further propagation were cultured in 96-well tissue culture plates (Olympus) until ICM expansion (considered for this investigation to be a two-fold increase in ICM

(Czechanski *et al.*, 2014; Gomez *et al.*, 2010). The medium was composed of 43% Knock-out Dulbecco's Modified Eagle's Medium, 43% Neural Basal Medium, 5% Knock-out Serum Replacement, 2% B27 Supplements and 1% N2 Supplements (Life Technologies). In Study I, nine single growth factor treatments human LIF (Kingfisher Biotech (at 1, 10 or 100 ng/ml; feline LIF (Kingfisher Biotech) at 1, 10 or 100 ng/ml; FGF2 (Peprotech) at 5, 10 or 20 ng/ml), were assigned to each group (n = 4-5 blastocysts/group) from the blastocysts seeding day (Day 0). The concentrations were based on previous studies for different species, FGF2-human (Eiselleova *et al.*, 2009); LIF-mouse (Czechanski *et al.*, 2014). All blastocyst outgrowths were fixed and stained (as described below). The influence of cytokines supplementation in sustaining pluripotent protein markers were classified as follows, 1) 'excellent' (maintaining protein concentration similar as in ICM on Day 0 and higher than control on Day 6); 2) 'good' (maintaining protein concentration lower than Day 0, but higher than control on Day 6); or 3) 'poor' (maintaining protein concentration lower than control on Day 0 and Day 6). In Study II, cat blastocyst outgrowths were cultured in groups (3-4 blastocysts/group) with growth factors in combinations comprised of cytokines having 'good' or 'excellent' effects on at least two protein markers in Study I, GSK3 inhibitor (Sato *et al.*, 2004) and MEK inhibitor (Chen *et al.*, 2015) were added in the treatment combination to enhance proliferation and prevent differentiation (Wang *et al.*, 2012). The combinations tested were numbered in Table 2.1.

Assessment of protein expression in cat blastocyst outgrowths by immunofluorescence

Day 7 to 9 blastocysts and blastocyst outgrowths cultured on glass slides for 6 days were fixed (Nestle *et al.*, 2012) by incubating in 4% paraformaldehyde (PFA) in PBS at room temperature (10 minutes) and then rinsing twice with DPBS (Sigma). Fixed cells were permeabilized by incubating with 0.2% Triton X-100 and 0.1% Tween 20 in PBS at room temperature (10 minutes), then rinsing twice in TBS with 0.05% Tween 20. Outgrowths were rinsed in PBS once followed by blocking with Maxblock Blocking Medium (Active Motif) at 4°C overnight. Cells were incubated with the primary antibodies (anti-human NANOG [500-P236; Peprotech), anti-human POU5F1 [MAB4401; Millipore) and anti-human SOX2 [SC1002; Millipore) at 1.5 µg/ml in Maxbind Staining Medium (Active Motif) overnight (4°C) with gentle rocking. Cells then were washed three times (5 minutes each) with Maxwash Washing Medium (Active Motif). Cells were then incubated with the secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG [H+L), Alexa Fluor 647 goat anti-mouse IgG1, Alexa Fluor 594 goat anti-mouse IgG2a (Thermo Fisher Scientific) at 1 µg/ml in Maxbind Staining Medium (room temperature, 1 hour, in the dark). Then cells were washed four times (5 minutes each) with Maxwash Washing Medium for imaging. Hoechst 33342(Invitrogen) was added during the third wash at 1 µg/ml to stain nuclei. Imaging was done using a Leica DMI 6000 inverted fluorescent microscope. Fluorescence intensities were analyzed using ImageJ for semi-

quantification of proteins (NANOG, POU5F1 and SOX2). Intensities of each stained proteins were normalized to the average intensity in ICMs in Day 7 to 9 blastocysts (control Day 0). Negative controls were blastocysts stained without secondary antibodies.

Table 2.1 Growth factor and inhibitor combinations for study II

		Treatment numbering																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Feline LIF (ng/ml)	1				+			+										
	10					+			+	+	+	+	+	+			+	+
	20														+	+		
	100						+											
Humna LIF (ng/ml)	1	+																
	10		+					+	+	+	+						+	+
	100			+														
FGF2 (ng/ml)	5									+								
	10	+	+	+	+	+	+				+							
GSK3 i (uM)	3											+		+	+	+	+	+
MEK i (uM)	1												+	+		+		+

Cat embryonic cell propagation

Based on the protein intensities measured in Study II, one cytokine combination was selected for further embryonic cell propagation for assessing cytokine effect in synergy for long-term culture (Study III). The percentage of blastocysts (n=41) adhering to mEF feeders and expanding to at least twice original size was $30 \pm 15\%$. Expanded ICMs (n = 13) on tissue culture plates were isolated using two 27.5-gauge needles. These were seeded on mitotically-inactivated mEFs in medium supplemented with 10 ng/ml fLIF, 10 ng/ml hLIF, 3 μ M GSK3 inhibitor plus 1 μ M MEK and 10 μ M ROCK inhibitors (Stemgent). After first single cell passage of the initial outgrowths, the percentage of colony formation (colony formed per ICM) was $10 \pm 9\%$. Cells were disassociated as single cells using Accutase (Innovative Cell Technologies) and seeded onto mitotically-inactivated mEFs. Cells were maintained in a humidified incubator (Thermo Fisher Scientific) (37°C, 5% CO₂ in air) for 4 to 6 days or until colonies occupied 30% to 40% of the well surface area. Colonies were then disassociated as single cells by Accutase and split (ratio of 1,3 to 1,4). Cells were expanded in this manner until passage 10.

Evaluation of propagated embryonic cells

For transcript expression, ICMs were isolated from blastocysts using 30.5g needles to separate cells that were subsequently lysed using extraction buffer (Applied Biosystems). RNAs were isolated using a PicoCure RNA isolation kit (Applied Biosystems). After being collected using cell scrapers (Thermo Fisher Scientific), embryonic cells were centrifuged (200 g, 5 minutes) to produce a pellet

that was subsequently lysed using RLT buffer (Qiagen), RNA then was isolated using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen).

Complimentary DNA was generated by reverse transcription of 100 ng of RNA using the Super Script III First Strand Synthesis System (Thermo Fisher Scientific).

Complimentary DNA samples were amplified using GoTaq Green Master Mix (Promega) in an Eppendorf thermocycler.

Genes selected for amplification and primer sequences are shown in Appendix Table 1. The amplification was performed for 5 minutes at 95°C followed by 32 cycles for 30 seconds at 95°C, 30 seconds at 58°C, 1 minute at 72°C, and 1 cycle for 5 minutes at 72°C for extension. Presence or absence of amplified genes was evaluated by imaging after gel electrophoresis. Fluorescence intensity of PCR products were analyzed using ImageJ for semi-quantification. Then 20 ng of each PCR product was amplified using a Big Dye Terminator V3.1 cycle sequencing kit (following manufacturer's instruction; Applied Biosystem™) and were sequenced using a 96-capillary 3730xl DNA Analyzer (Applied Biosystem™). PubMed Gene and Genome were the reference databases of sequences (Pontius *et al.*, 2007; Irizarry *et al.*, 2012).

For genotyping, cells were expanded in the same medium without mEF feeders for three passages. Genomic DNA was isolated using a Purelink Genomic DNA Mini Kit (Thermo Fisher Scientific) from these embryonic cells. Genomic DNA samples (10 ng/10 µl reaction system) were amplified using AmpliTaq Gold 360 Master Mix (Applies Biosystems) in an Eppendorf thermocycler.

Karyotyping cat embryonic cells

Chromosome spreads (n = 21, 23, 28 for each cell line, respectively) from cat embryonic cells were prepared as previously described (Priscila *et al.*, 2009).

Chromosomes were stained using Hoechst 33342 (Invitrogen) at 1 µg/ml with subsequent imaging using a Leica DMI 6000 inverted fluorescent microscope (Latte *et al.*, 1975). Cat genome contains 38 chromosomes (Ford *et al.*, 1980).

Statistical analysis

Values presented for staining intensity are means ± SD. Nine single factor treatments and 17 combination factor treatments were tested. Each treatment group includes 3 to 4 ICM outgrowths. Transcriptional expressions are normalized to *RPL17* expression. Data were analyzed using a generalized linear mixed model (McDonald *et al.*, 2014) in SAS University Edition with statistical significance set at $P < 0.05$. To select the best growth factor and inhibitor cocktail, average Euclidean distance between each treatment group and the ICM control group was calculated in Perl 5.8.8. A three-dimensional scatter plot diagram for each ICM outgrowth was generated via an IBM SPSS program.

2.4 Results

Presence of pluripotency markers and signaling components in the ICM

Right after the removal of zona, blastocysts collapsed, and re-expanded on day 1. Half of the blastocysts attached to the feeder cells by day 2. About 80% blastocysts built attachment with feeders by day 4; the blastocyst outgrowths unattached by day 4

remained intact and detached to the end of culture. Those attached on mEF feeders expanded further with a dark inner cell mass sitting on top (Fig. 2.1). Without growth factor supplementation, the cell number increased up to day 4 of culturing without cytokines and declined from day 5. The cell number change indicated cell proliferation up to day 4 and cell differentiation or degeneration from day 4 (Fig. 2.2A). NANOG staining co-localized with the nucleus staining. NANOG intensity declined during the culturing. From day 4, NANOG intensities in the ICM outgrowths were significantly lower than that in blastocyst control, which correlated with cell number change in ICM. The loss of NANOG was either due to differentiation or cell degeneration. On day 6, a vague and duller green fluorescence was shown in the cytoplasm and cellular membrane, suggested that this is artifact, not true NANOG staining (Fig. 2.2B, Appendix Fig. 5). POU5F1 was stained in the nucleus like NANOG. The level of POU5F1 remained similar to blastocyst control until Day 4. The large variance of staining intensity showed that some of the cells had very low POU5F1 on Day 4, and POU5F1 levels decreased significantly like NANOG from Day 5. The artifact fluorescence was similar with what has been observed in NANOG (Fig. 2.2C, Appendix Fig. 5). SOX2 was stained in the nucleus as other two transcription factors. It decreased significantly from day 3 of culturing. Artifact observed on day 6 in the whole blastocyst outgrowths was consistent with the other channels. SOX2 declined faster than NANOG and POU5F1 (Fig. 2.2D).

The pluripotent-related proteins NANOG, POU5F1 and SOX2 were detected in blastomeric nuclei of expanded cat blastocysts (Fig. 2.3A). POU5F1 was more

restricted to the ICM compared to *NANOG* and *SOX2* that were prevalent in trophoctoderm nuclei as well.

Transcripts of pluripotent-related genes (*NANOG*, *POU5F1*, *SOX2*, *KLF4* and *CMYC*) were also expressed in the ICM (Fig. 2.3B). Signaling components in the FGF2 pathway (*FGF2-receptor*, *MEK* and *ERK* transcripts) were highly expressed compared to low levels of *LIF-receptor* and *STAT3* transcripts in the LIF pathway of the ICM (Appendix Fig. 1). *Activin A-receptor* was modestly expressed in the ICM only, whereas other signaling components from the BMP and TGF/activin pathways (specifically *BMP2-receptor* and *SMAD4* transcripts) were nearly absent (Fig. 2.3B, Appendix Fig. 1). Presence of *BMP2-receptor* and *SMAD4* transcripts in fetal ovary validated the primers used for detecting these cat mRNAs. All PCR products were of the expected length (Appendix Table 1), upper band for *NANOG* was a larger transcript variant and upper band for *ERK* was a 5'-primer product with larger size. Bands at the bottom the lanes for *CMYC* and *BMP2-receptor* were unspecific, and not fragments of any anticipated transcript (Fig. 2.3B).

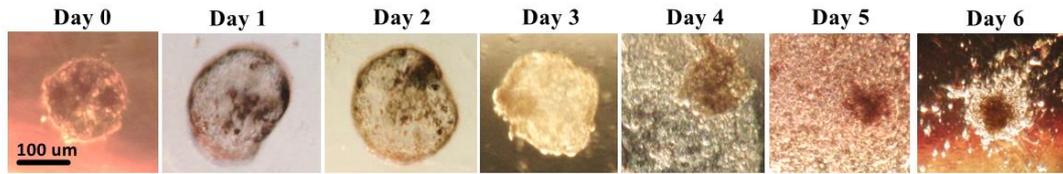


Figure 2.1. Morphology change of blastocyst outgrowths in 6 days of culture without cytokine supplementations: blastocyst outgrowths expanded from day 1, attached to MEF feeders from day 2, and degenerated from day 5.

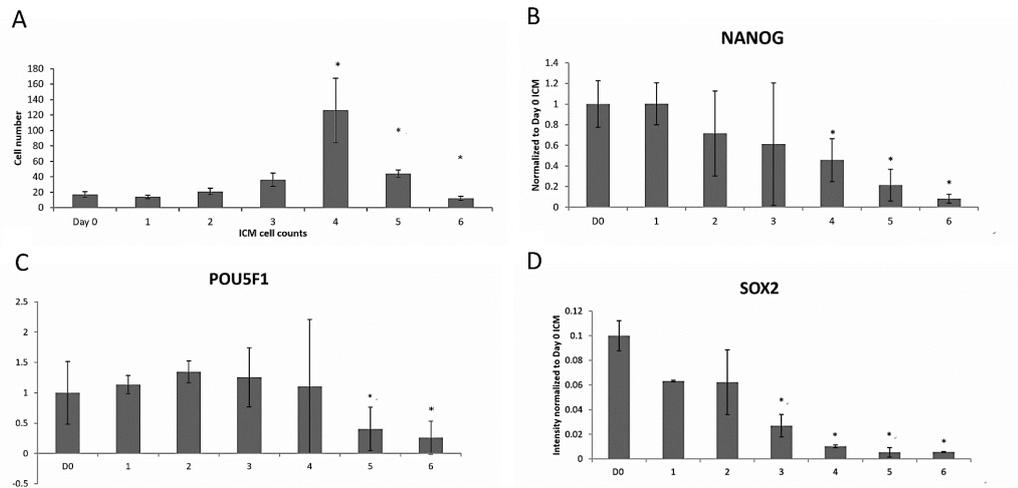


Figure 2.2 Blastocysts were cultured without cytokine supplementations for 6 days. A) Cell numbers in ICM outgrowths. Relative fluorescence intensity in cat ICM outgrowths of, B) NANOG; C) POU5F1 and D) SOX2. Fluorescence intensity values were relative to the average of control ICM. Values were expressed as mean \pm standard deviation (N=4-5 replicates). * $P < 0.05$, compare to Day 0.

Assessment of single growth factor supplementation on ICM outgrowths

Because we detected signaling components in LIF/STAT3 and FGF2 pathways, ICMs were cultured for 6 days in medium supplemented with FGF2, human LIF or feline LIF at various concentrations. Cell numbers in ICM outgrowths were increased ($P < 0.05$) in most treatment groups after 6 days compared to the ICM control (Day 0) or control without growth factor (Day 6) (Fig. 2.4A). An exception that was not significant ($P > 0.05$) was treatment with 5 ng/ml FGF2.

With the exception of supplementing with 10 ng/ml hLIF, other post-treatment results did not meet our ‘excellent’ classification, as defined in the methods, in sustaining NANOG. However, most treatments met our ‘good’ criteria at enhancing this protein (Fig. 2.4B). Three treatments (5 ng/ml FGF2, 1 or 10 ng/ml fLIF) were ‘excellent’ at maintaining POU5F1 in ICMs while several supplementations (5 or 10 ng/ml FGF2, 10 ng/ml hLIF, 1 or 10 ng/ml fLIF) were ‘good’ at preserving POU5F1 (Fig. 2.4 C). Treatments with 5 ng/ml FGF2 or 100 ng/ml fLIF were ‘excellent’ at maintaining SOX2 in ICM outgrowths. Lastly, all three growth factors were capable of stimulating ‘good’ to ‘excellent’ results for SOX2 for at least one of the concentrations tested (Fig. 2.4D). However, not a single factor maintained all three markers ($P > 0.05$) at levels comparable to ICM controls.

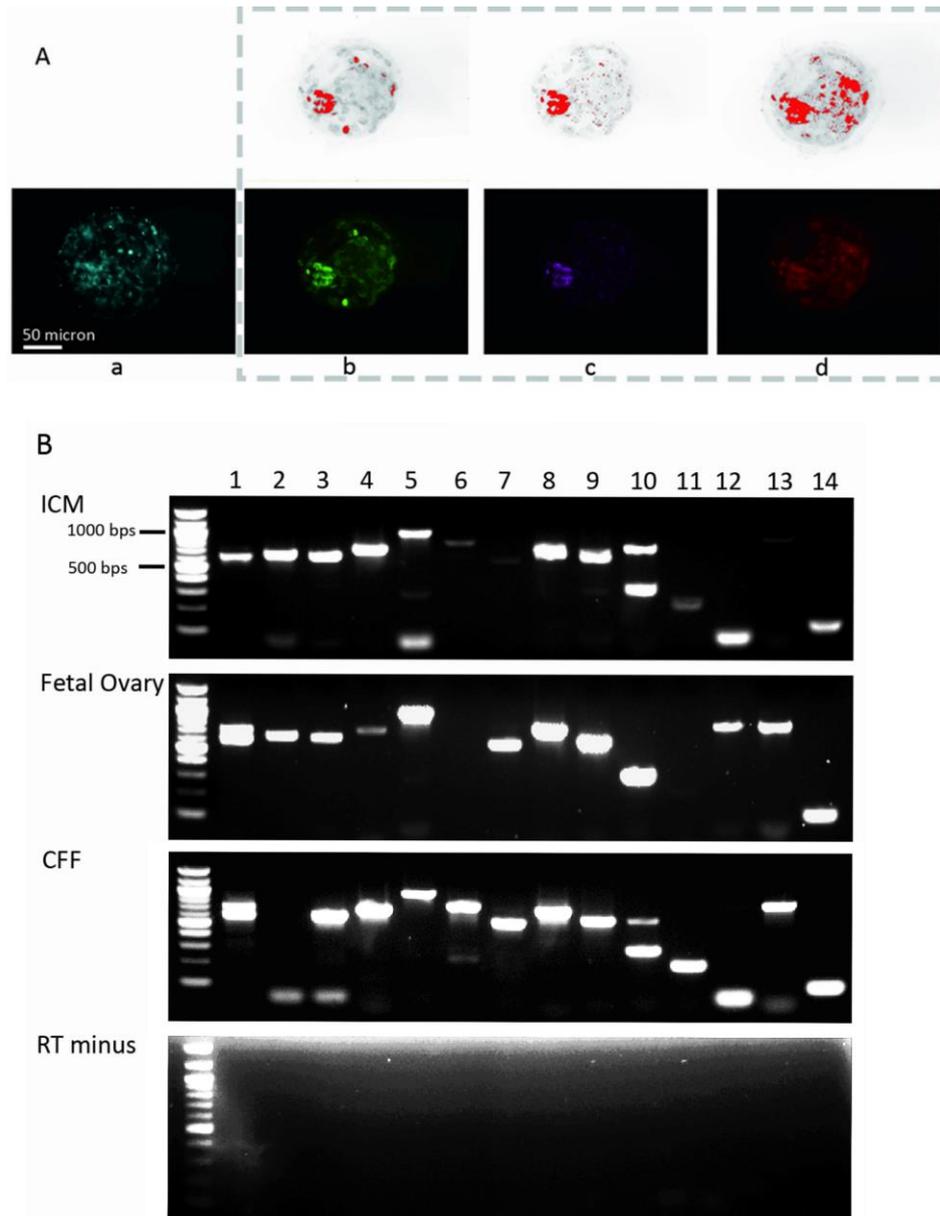


Figure 2.3. Presence of pluripotency proteins and expression of genes related to pluripotency and signaling in the inner cell mass (ICM) of cat blastocysts. A) Whole blastocyst immuno-staining of major pluripotency markers, chromatin (a), NANOG (b), POU5F1 (c), SOX2 (d). Quantified areas in ImageJ are displayed above fluorescence images. B) RT-PCR detection of pluripotent and signaling component mRNAs in ICMs, fetal ovary and cat fetal fibroblasts (CFFs), lanes were numbered as follows, DNA ladder, *NANOG* (1), *POU5F1* (2), *SOX2* (3), *KLF4* (4), *CMYC* (5), *LIF-receptor* (6), *STAT3* (7), *FGF2-receptor* (8), *MEK* (9), *ERK* (10), *Activin A-receptor* (11), *BMP2-receptor* (12) and *SMAD4* (13). *RPL17* (14) was used as a reference gene.

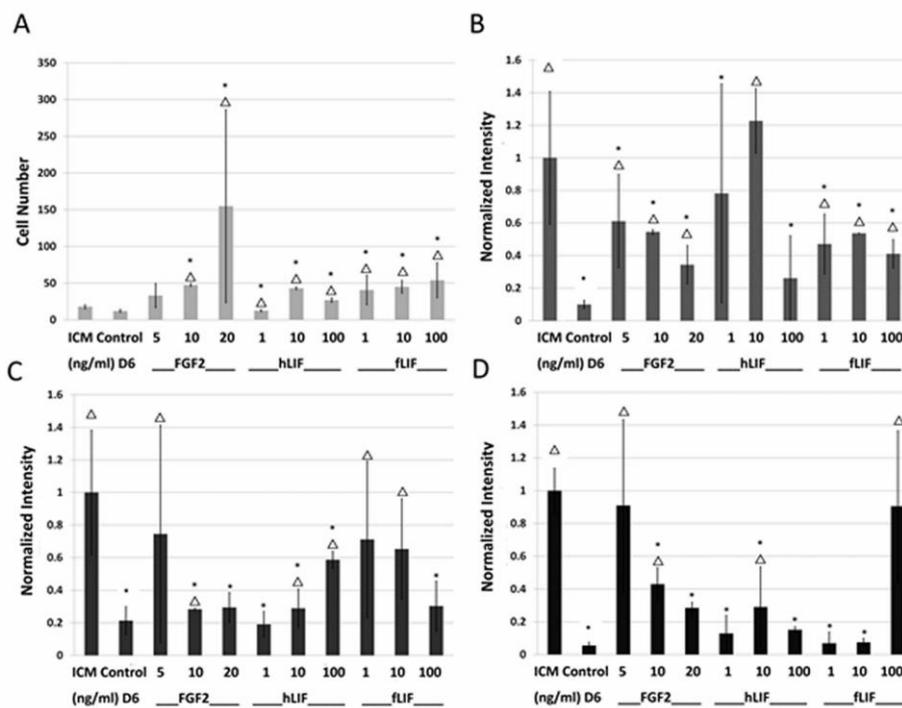
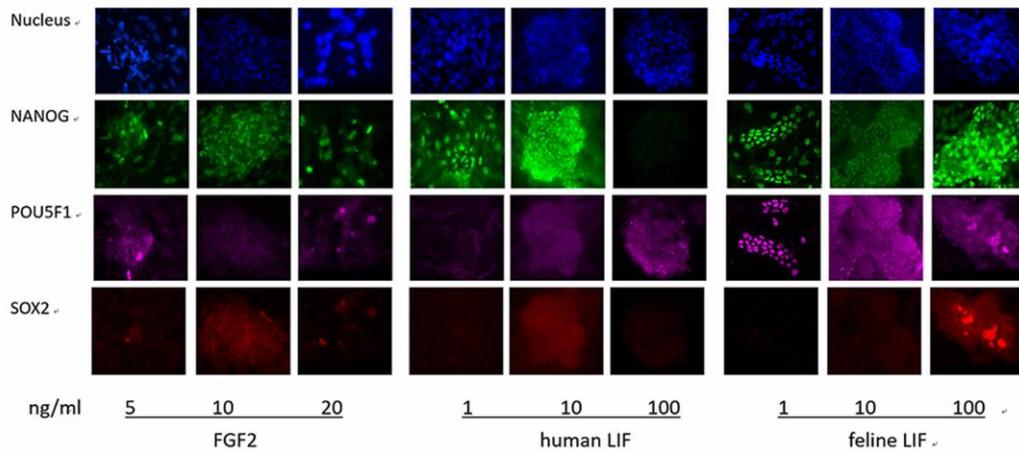


Figure 2.4. Characterization of inner cell mass (ICM) outgrowth cultured in vitro for 6 days with single growth factors (FGF2, hLIF, fLIF) at different concentrations. Positive controls were ICM in expanded blastocysts on Day 0 of culture (ICM). Other control groups were ICM outgrowths cultured for 6 days without growth factor supplementation (Control D6). A) Cell numbers in ICM outgrowths. Relative fluorescence intensity in cat ICM outgrowths of, B) NANOG; C) POU5F1; and D) SOX2. Fluorescence intensity values were relative to the control ICM. Values were expressed as mean \pm standard deviation (N = 4-5 replicates); * above the bars indicates a difference ($P < 0.05$) from ICM; Δ above the bar indicates a difference ($P < 0.05$) from Control D6.

Assessment of combinations of growth factors and inhibitors on ICM outgrowths

We formulated combinations of growth factor treatments based on testing of single cytokines (above) that resulted in ‘good’ or ‘excellent’ results as defined in the Material and Methods. Thus, we evaluated two to four factor-containing cocktails comprised of fLIF at 1, 10, 20 or 100 ng/ml with hLIF at 1, 10 or 100 ng/ml and/or FGF2 at 5 and 10 ng/ml (Table 2.1). All combinations, except Treatment 2, increased ($P < 0.05$) cell number in ICM outgrowths (Fig. 2.5A) compared to control Day 6. ‘Excellent’ treatments for NANOG were combinations no. 11, 12, and 17 (Fig. 2.5B). For POU5F1, ‘excellent’ treatments were combinations no. 4, 5, 9 and 17 (Fig. 2.5C). For SOX2, ‘excellent’ treatments were combinations 9, 14 and 17 (Fig. 2.5D). NANOG, POU5F1 and SOX2 intensities were increased ($P < 0.05$) compared to Control D6 in most of the treatments, although no combination sustained ($P < 0.05$) all three protein expressions compared to ICM (Fig. 2.5).

To better illustrate the capacity of each treatment to collectively sustain production of all three protein markers, relative intensities of each in individual ICM outgrowths were plotted three-dimensionally (Fig. 2.6). One treatment in particular was highly similar to the ICM control, 10 ng/ml feline LIF plus 10 ng/ml human LIF plus 3 μ M GSK3i plus 1 μ M MEKi (Treatment 17) giving the shortest Euclidean mean. This combination then was selected for further propagation of embryonic cells derived from ICM outgrowths.

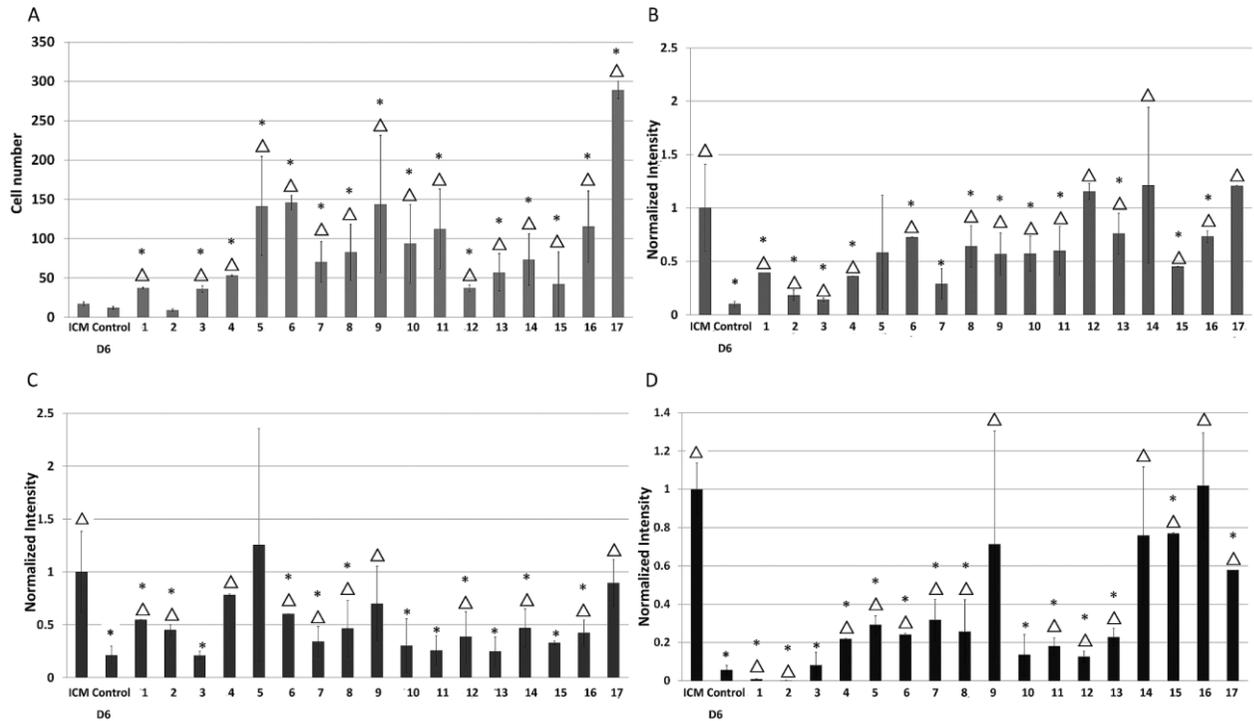


Figure 2.5. Characterization of inner cell mass (ICM) outgrowths cultured in vitro for 6 days with combinations of growth factors (FGF2, hLIF, fLIF) at different concentrations and inhibitors (GSK3i and MEKi). Combinations are detailed in Table 2.1. Positive controls were ICM in expanded blastocysts on Day 0 (ICM). Other control groups were ICM outgrowths cultured for 6 days without growth factor supplementation (Control D6). A) Cell numbers in ICM outgrowths. Relative fluorescence intensity in cat ICM outgrowths of: B) NANOG; C) POU5F1; and D) SOX2. Fluorescence intensity values were relative to the control ICM. Values were expressed as mean \pm standard deviation

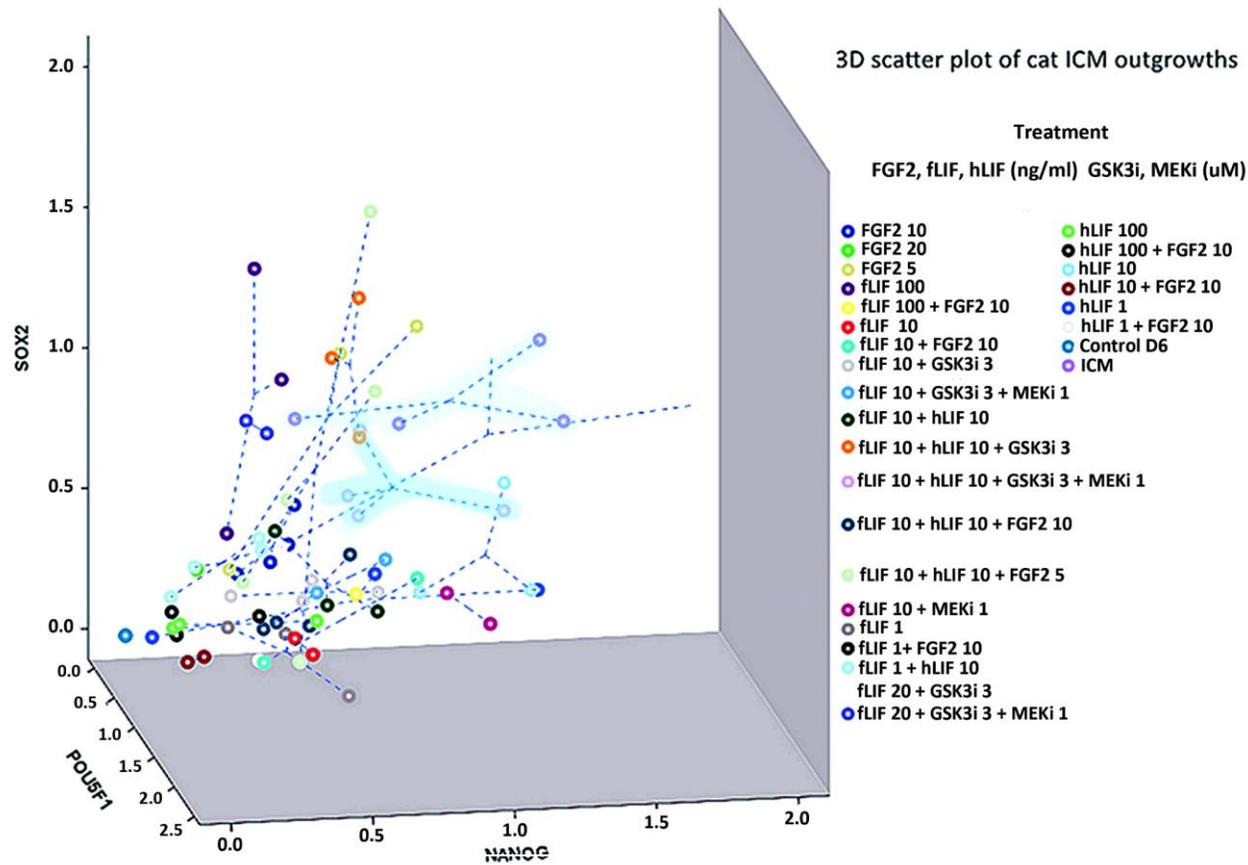


Figure 2.6. Scatter plot of individual ICM outgrowths positioned by relative intensities of proteins. Individuals in ICM group and selected treatment group No. 17 (fLIF 10 ng/ml + hLIF 10 ng/ml + GSK3i 3 μ M + MEKi 1 μ M) with smallest Euclidean mean are highlighted by blue shading.

Propagation of embryonic cells with feline and human LIF plus GSK3i and MEKi

Through 10 passages, colonies exhibited dome to flat shape morphologies (Fig. 2.7A) indicating that cells were stabilized. Genotyping of cells that formed colonies confirmed derivation from cat ICM outgrowths and not from mEFs used as feeder cells (Fig. 2.7B). More than 80% of cells from colonies (24/28, 19/23, 17/21) had normal chromosome numbers ($N = 38$, Fig. 2.7C).

All three proteins were present in embryonic cells after 10 passages, whereas NANOG and POU5F1 was detectable, but with lower intensity (Fig. 2.7D, Fig. 2.8B). The overall transcript profiles between ICM and embryonic cell passage 10 were similar ($P > 0.05$) (Fig. 2.8A, Appendix Fig. 1). Messenger RNAs of *NANOG*, *POU5F1*, *SOX2*, *FGF2 receptor*, *MEK* and *ERK* in embryonic cells remained at comparable levels to ICM controls. *KLF4*, *CMYC* and *LIF-receptor* decreased whereas *STAT3* increased (Fig. 2.8A; Appendix Fig. 1). Messenger RNA levels of *Activin A-receptor*, *BMP2-receptor* and *SMAD4* were unchanged compared to ICM (Fig. 2.8A, Appendix Fig. 1). However, intensity levels of NANOG and POU5F1 were decreased ($P < 0.05$) compared to the ICM control (Fig. 2.8B). Embryonic cells generated from expanded ICM maintained many pluripotency-related features analogous to original cells.

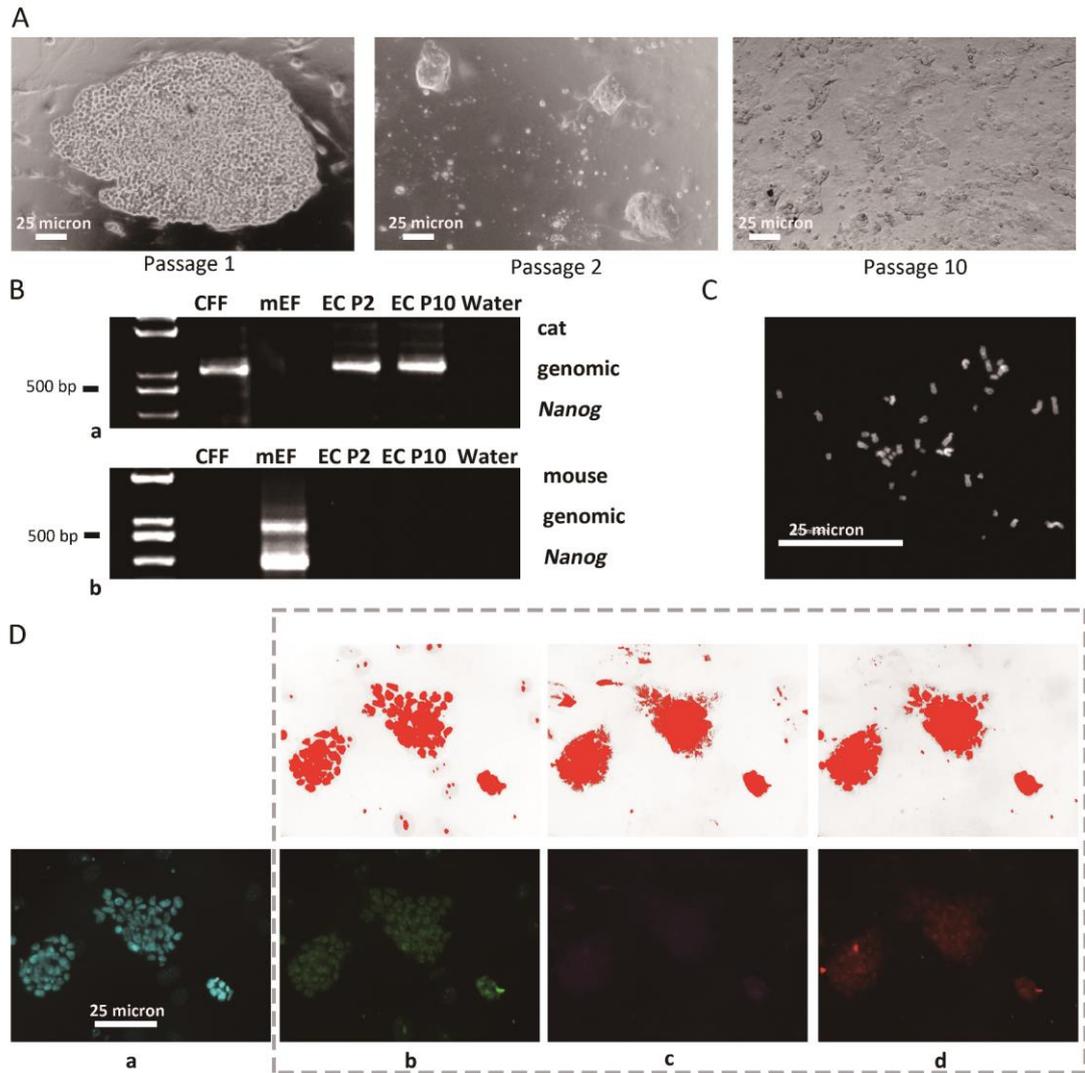


Figure 2.7. Characteristics of ICM outgrowths and derived cells at different passages. A) Morphologies of embryonic cells at different passages, passage 1 (a), passage 2 (b), passage 10 (c). B) Genotyping for cat fetal fibroblast (CFF), mouse embryonic fibroblast (mEF) and cat embryonic cell at Passage 2 (EC P2) and Passage 10 (EC P10). C) Karyotype of cat embryonic cells. D) Expression of major pluripotency markers in colonies at Passage 10, chromatin (a), NANOG (b), POU5F1 (c), SOX2 (d) (N = 10 colonies per cell line). Quantified areas in ImageJ are displayed above fluorescence images.

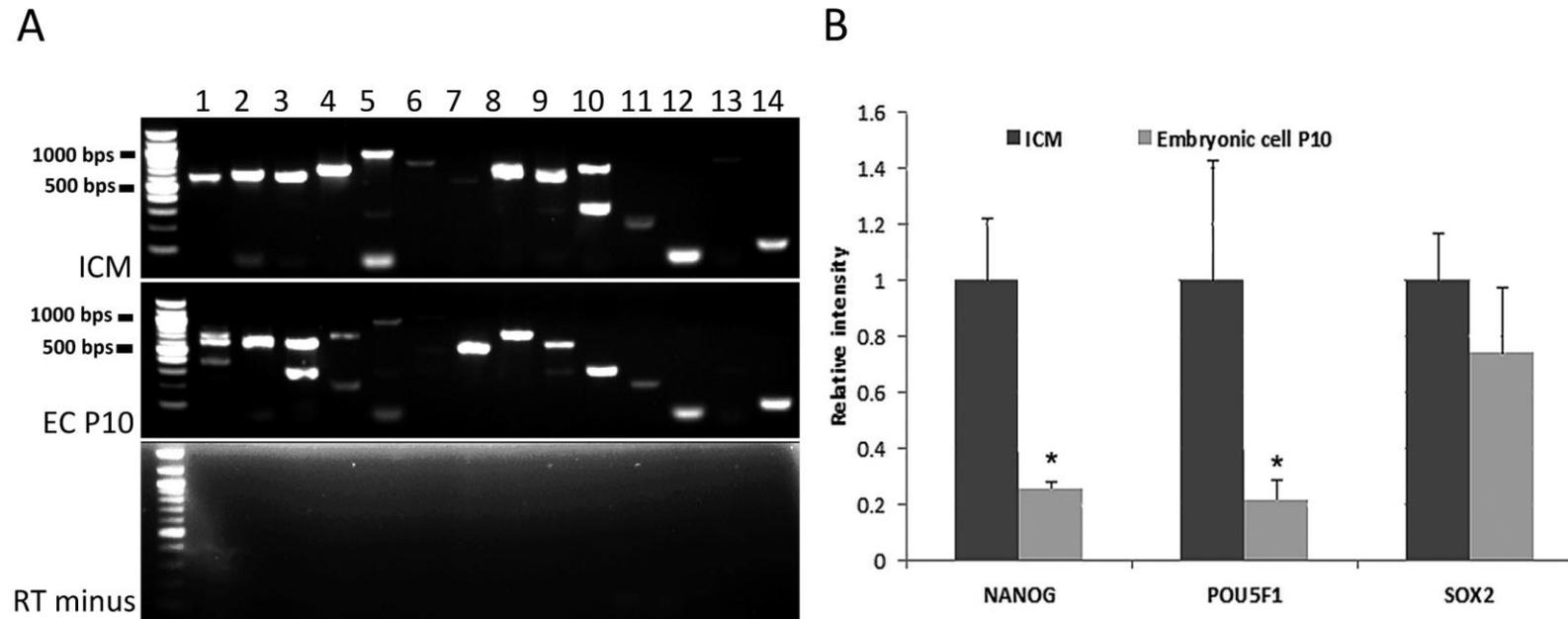


Figure 2.8. Gene expression in ICM outgrowths and derived cells after 10 passages (EC P10). A) RT-PCR detection of transcript expression for pluripotent and signaling components, lanes were numbered as follows, DNA ladder, *NANOG* (1), *POU5F1* (2), *SOX2* (3), *KLF4* (4), *CMYC* (5), *LIF-receptor* (6), *STAT3* (7), *FGF2-receptor* (8), *MEK* (9), *ERK* (10), *Activin A-receptor* (11), *BMP2-receptor* (12) and *SMAD4* (13). *RPL17* (14) was used as a reference gene, and cat ICM cDNA was used as a positive control. B) Relative intensities of immuno-staining for NANOG, POU5F1, and SOX2 in EC P10 compare to ICM; * above the bars indicates a difference ($P < 0.05$) from ICM.

2.5 Discussion

All the proteins markers were stained in nucleus, like mouse and human (Tesar PJ, 2005; Wang *et al.*, 2012), which agreed with their roles in regulating gene expressions during early embryo development. My hypothesis was validated by the decreased NANOG, POU5F1, SOX2 and cell numbers. The presence and enrichment of these three proteins in the beginning of the culture, and their elimination in the end of culture proved that they could be used as markers to assess pluripotency. Collective results suggested ICM cells in blastocysts neither remained pluripotent nor proliferative after 6 days of culturing in medium without cytokine supplementation. It helped setting up the time end point to assess the effect of cytokines in maintaining cell protein markers and cell proliferation. Low levels of *LIF-receptor* and *STAT3* expressions in cat ICMs may convey a modest activity of LIF/STAT3 signaling, resulting in inconsistent levels of phosphorylated STAT3, which is stimulating NANOG, POU5F1, and SOX2 expressions (Do *et al.*, 2013). The absence of detectable transcript for *BMP2-receptor* and *SMAD4*, and decreased levels of *LIF-receptor* as well as *STAT3* in the cat ICM indicated pluripotency regulatory machinery that appeared unique to this model system (James *et al.*, 2005).

As measured in the mouse and human (Tesar, 2005; Tesar *et al.*, 2007; Guo *et al.*, 2016), LIF sustained NANOG, POU5F1 and SOX2 in the cat ICM. Yet, the impact of LIF was insufficient to sustain transcription factors at levels similar to that observed in the ICM control. This is consistent with what has been previously reported (Gomez *et al.*, 2010; Yu *et al.*, 2008). However, we observed variable

effectiveness between human LIF and feline LIF on transcription factor levels. The two proteins human LIF and feline LIF have similar amino acid composition (164/180, respectively; 91.1% sequence identity). Given the fact that human LIF can work at close efficiency to mouse LIF in maintaining mouse ESCs (with sequence identity ~90%) (Youngblood *et al.*, 2014), human and feline LIF are assumed to have similar effect on cat ICM outgrowths. Yet, the responses of NANOG, POU5F1 and SOX2 were different in the treatment groups with feline LIF and human LIF. The reason is not clear and will require further investigations. Low levels of *LIF-receptor* and *STAT3* expressions in cat ICMs may convey a modest activity of LIF/STAT3 signaling, resulting in inconsistent levels of phosphorylated STAT3, which is stimulating NANOG, POU5F1, and SOX2 expressions. LIF/STAT3 pathway might not be the major mechanism maintaining pluripotency in the cat. Other pathways could be playing a regulatory role, for example, an inhibitor of GSK3 or a WNT3 analogue, both of the latter known as supportive to maintaining mouse ESC in the absence of exogenous LIF (Sato *et al.*, 2004). FGF2 supplementation alone also was unable to maintain NANOG, POU5F1 and SOX2 expressions. This was in contrast to studies of the human ESC (Eiselleova *et al.*, 2009; Thomson *et al.*, 1998) showing that FGF2 alone would support ESCs at a stable pluripotent state. We also observed a trend of increasing cell number, and decreasing transcription factors in ICM outgrowths, with ascending FGF2 concentration. It is likely that FGF2 might be serving as a proliferation enhancer and a pluripotency repressor within the cat ICM. This could be consistent with what has been observed in mouse ESCs where FGF2 is

known to stimulate active MEK1 suppressing the expression of Nanog (Santostefano *et al.*, 2012; Sarabipour *et al.*, 2016). Single factor treatments lead to variable responses. It is known that overexpressing OCT4 or SOX2 will lead to cell fate decision (OCT4 - mesendoderm lineage, SOX2 - neural ectodermal lineage) (Thomson *et al.*, 2011). The changing pattern of OCT4 and SOX2 was observed in our culture system, which implied an unstable balance among NANOG, POU5F1 and SOX2 within a given ICM outgrowth, and might lead to a loss of pluripotent state (Thomson *et al.*, 2011).

To better illustrate the capacity of each treatment to collectively sustain production of all three protein markers, relative intensities of each in individual ICM outgrowths were plotted three-dimensionally (Fig. 8). One treatment in particular was highly similar to the ICM control, 10 ng/ml feline LIF plus 10 ng/ml human LIF plus 3 μ M GSK3i plus 1 μ M MEKi (Treatment 17) giving the shortest Euclidean mean. This combination then was selected for further propagation of embryonic cells derived from ICM outgrowths.

Based on initial findings, we tested multiple cytokine combinations on cat ICM outgrowths over a 6-day culture. To stabilize the balance of pluripotency, we supplemented the culture medium with GSK3 and MEK inhibitors. Some treatments (No. 4, 5, 9, 12, 14, 16, 17) maintained pluripotency markers at levels similar to the control ICM group. Nonetheless, we did not prove our original assumption that supplementations that resulted in the highest similarity in NANOG, POU5F1 and SOX2 levels would produce normal, extended pluripotency and proliferation. The

impact of adding cytokines targeting different pathways resulted in enhanced cell proliferation and transcription factor response, yet in no case supporting NANOG, POU5F1 and SOX2 at a normal level. However, the use of a Euclidean means assessment assisted in identifying those treatment groups producing a result approximating the ICM control. The most promising results were generated with the supplementation of LIF combined with the GSK3 and MEK inhibitors. Specifically, we demonstrated that adding the MEK inhibitor promoted the rapid proliferation of cat embryonic cells. Similar to what has been observed in rodents (Czechanski *et al.*, 2014; Li *et al.*, 2008); inhibiting MEK/ERK pathway did not compromise ESC growth. The morphology of the resulting cell colonies remained consistent from passage 2 to 10; implying decreased levels NANOG, POU5F1 and SOX2 did not lead to differentiation. The good expansion of stabilized embryonic cells then enabled us to evaluate certain pluripotent features, including the maintenance of transcription factors and signaling components supporting pluripotency. The combined cytokine treatment has boosted *STAT3* which is essential in pluripotency stabilization (Do *et al.*, 2013). Our observation of decreased *KLF4*, *CMYC* and *LIF-receptor* transcripts in cultured embryonic cells suggested that cell status may have been compromised. Furthermore, reductions in NANOG, POU5F1 and SOX2 intensity by passage 10 showed that the protein expressions were not directly equalized to rising mRNA levels. Mouse studies have conclusively demonstrated the need for sustained levels of NANOG, POU5F1 and SOX2 proteins to promote pluripotency (Rodda *et al.*, 2005). By contrast, our observation in cat embryonic cells

probably was indicating a loss of pluripotency with advanced passages.

Consequently, we cannot conclude that our selected cytokine combinations had the capacity to sustain embryonic cells to a state analogous to ESCs reported in other studies (Czechanski *et al.*, 2014; Li *et al.*, 2008; Guo *et al.*, 2016).

Our cat model confirms that there is complexity in regulating NANOG-POU5F1-SOX2 circuitry, not only due to the doses need to target hundreds of ESC hallmark genes (Wang *et al.*, 2012), but also to their positive/negative feedback loop in regulating each other (Rodda *et al.*, 2005). Certainly, the dynamism we observed in the cat ESC system indicated that there was no simple cytokine formula for perpetuating cell pluripotency. Deviations in signaling pathways and cellular fate may be related to natural differences among species in modes of embryo development preimplantation. This already is apparent from the notable disparities in timing and signaling requirement in mouse versus human (Czechanski *et al.*, 2014; Guo *et al.*, 2016). Whereas a mouse blastocyst implants at approximately embryonic Day 4.5 (E4.5) (Schrode *et al.*, 2013), the human counterpart embeds at E 7-10 (Wilcox *et al.*, 1999). Then more specifically in terms of mechanisms, the human epiblast experiences a highly expressed transforming growth factor- β (TGF β) pathway (Blakeley *et al.*, 2015) (e.g., nodal) that, if inhibited, down-regulates the core pluripotency factor NANOG (James *et al.*, 2005). However, this identical manipulation in the mouse has no discernible influence on the same metrics (James *et al.*, 2005), again demonstrating a remarkable species variation in pluripotency regulation. Therefore, given that the cat embryo has a comparatively long

preimplantation embryo development period and that the cat blastocyst proceeds to gastrulation before implantation (at E13-14) (Knospe 2002), it probably explains why the mouse protocol for sustaining pluripotency in embryonic cells was not translatable to the cat.

Our findings help to explain the loss of pluripotency observed in previous studies attempting to create viable cat ESCs (Gomez *et al.*, 2010; Yu *et al.*, 2008). We demonstrated that, unlike for the mouse and human, LIF or FGF2 solely could not support NANOG, POU5F1 and SOX2 expressions. Those findings plus the absence of detectable transcript for *BMP2-receptor* and *SMAD4*, and decreased levels of *LIF-receptor* as well as *STAT3* in the cat ICM indicated pluripotency regulatory machinery that appeared unique to this model system. To sustain pluripotency in cat ESCs, the nature of cat early embryos needs to be further investigated. For instance, the dynamics of LIF- receptor, STAT3, and the Interleukin 6 (IL6) family need to be drawn, and the control elements on these factors have to be revealed. The relation between featured signaling pathways (LIF/IL6/STAT3, FGF2/MEK/ERK, etc.) and embryonic stage (4-cell, morulae, inner cell mass) would contribute to a deeper understanding of pluripotency. Furthermore, time-lapse transcriptome and proteome analysis in cat embryos up to blastocysts might be necessary to finely tune the culture conditions.

2.6 Acknowledgement

I thank Dr Brent Whitaker (Animal Rescue Inc.) and Dr Keiko Antoku, and

their staff for providing domestic cat ovaries. This research was supported by a JoGayle Howard Graduate Fellowship in Felid Reproduction to me and a Smithsonian-University of Maryland seed grant for supplies and equipment.

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Chapter 3 Reprogramming cat fetal fibroblasts using inducible transcription factors

3.1 Abstract

Pluripotent stem cells have great value, both in clinical and basic research, and for endangered species conservation. In the cat model, establishment of induced pluripotent stem cells (iPSC) have several advantages over the embryonic stem cell (ESC), 1) fibroblasts are readily available in contrast to the limited supply of embryos, and 2) expression of transcription factors can be manipulated to reprogram and maintain the cells in a pluripotent state. In our study, efficiency of induction using five transcription factors (*NANOG*, *POU5F1*, *SOX2*, *KLF4* and *CMYC*) was assessed. A tet-on system was integrated into cat fetal fibroblasts to control the transgene expression. Cells were cultured in DMEM/F12 supplemented with LIF and 2i (GSK3 and MEK inhibitors). Forced expression of the transgenes led to increased expression of endogenous *NANOG*, *POU5F1*, *SOX2*, and acquisition of stem cell characteristics. Colonies with a dome shape and clear border were selected after two weeks of treatment. A portion of these colonies stained positive for alkaline phosphatase (0.002% per fibroblasts seeded). After silencing the transgenes by removal of doxycycline, the stem cell characteristics were sustained independently. While detectable, the relative intensities of *NANOG*, *POU5F1*, and *SOX2* following immuno-staining were lower than that in inner cell mass (0.2, 0.19, and 0.45 vs. 1.0, respectively) and remained at similar levels over 40 days of culture. The reprogrammed cells proliferated rapidly and

could be expanded over 10 passages and form embryoid bodies. Colonies had mixed staining patterns for alkaline phosphatase, transcription factors, and morphologies. Expression levels of differentiation markers increased in iPSCs over time, implying that while some of the colonies were pluripotent-like, other colonies might undergo differentiation.

3.2 Introduction

Induced pluripotent stem cells (iPSCs) are considered indistinguishable from embryonic stem cells, in regard to their capacity of self-renewal and directed differentiation (Okita *et al.* 2007 and Maherali *et al.* 2007). The process of transforming differentiated somatic cells into undifferentiated pluripotent stem cells is defined as reprogramming, which was first accomplished by forced expression of four transcription factors (POU domain, class 5, transcription factor 1 (POU5F1 aka OCT4), Sex determining region Y box 2 (SOX2), Kruppel-like factor 4 (KLF4) and myelocytomatosis proto-oncogene protein (CMYC)) in mouse fibroblasts (Takahashi and Yamanaka 2006). The establishment of iPSC lines in mouse and human, by different research groups shows that this method to produce stem cells is highly repeatable (Haase *et al.*, 2009; Hockemeyer *et al.*, 2008; Lu *et al.*, 2011; Nakagawa *et al.*, 2008). While only limited success has been reported in domestic and companion animals, such as dogs, pigs, cattle, and horses (Brenton *et al.*, 2013; Chakritbudsabong *et al.*, 2017; Talluri *et al.*, 2015; Whitworth *et al.*, 2012), development of these techniques in non-rodent species is highly desirable for use in clinical, and basic

research. In the cat model, establishment of induced pluripotent stem cells (iPSC) has several advantages over the embryonic stem cell (ESC), 1) fibroblasts are readily available in contrast to the limited supply of embryos, 2) expression of transcription factors can be manipulated to reprogram and maintain the cells in a pluripotent state, and 3) individual specific lines can be made.

The domestic cat is an invaluable model in biomedical research and conservation biology for which it serves as a model for the feline family (Johnson *et al.* 2006) and has been used in studies for reproduction (Hobbs *et al.* 2012). Partial success in reprogramming cells from wild felids has been reported in which five transcription factors including Nanog homeobox (NANOG) were used to generate the potential iPSCs (Verma *et al.* 2012 and 2013). This achievement relied on the high identity of transcription factors between human and cat and the conserved roles of the core transcription factors, NANOG, POU5F1, and SOX2, in acquiring pluripotency (Breton *et al.* 2013; Haase *et al.*, 2009; Talluri *et al.*, 2015; Takahashi and Yamanaka, 2006). The challenge with the wild feline studies was that two of the transgenes, human KLF4 and CMYC, couldn't be silenced (Verma *et al.* 2012 and 2013). In mouse and human continued expression of exogenous genes is not required for maintenance of iPSCs after reprogramming (Okita *et al.* 2008 and Soares *et al.* 2015). While these factors were required during initial reprogramming (Takahashi *et al.* 2007), continued expression of KLF4 (Hu *et al.* 2015) and CMYC (Lin *et al.* 2012) may increase tumorigenesis during differentiation, thus limiting the potential utilization of iPSCs for regenerative medicine. Generally, the transgenes are prone to systemic post-

transcriptional gene silencing and DNA methylation (Jaenisch *et al.* 1981 and Dalakouras *et al.* 2011). The reason why human KLF4 and CMYC remained active in the wild feline is unknown, but this issue needs to be overcome for successful application of iPSC to clinical and conservation uses.

We proposed using a drug-inducible system with reverse tetracycline-transactivator (rtTA) (Hockemeyer *et al.* 2008e) that would control the transgene expression efficiently. In our study, rtTA-M2, a mutant of rtTA which has increased stability and inducibility in the presence of doxycycline was used. This inducible activator was expected to have reduced background expression in the absence of doxycycline (tetracycline analog) (Urlinger *et al.*, 2000). Moreover, we have identified a growth factor and inhibitor combination that maintains pluripotency-related proteins for ten passages in feline ESC-like cells (Chapter 2). This combination will aid in the generation of iPSCs.

3.3 Materials and methods

Approval from an Institutional Animal Care and Use Committee was not required as domestic cat gravid uteri, ovaries, and testes were obtained from local veterinarians following routine owner requested ovariohysterectomies or orchiectomies. These are exempt studies under Title 9, Code of Federal Regulations, Subchapter A- Animal Welfare, Part 1 to 3. Tissues from second-trimester fetuses were processed on the same day of collection. Cells were isolated and cultured as described for mouse embryonic fibroblasts in previous studies (Nagy A *et al.*, 2002). After 48 hours of culturing, cells

were 100% confluent and were frozen for future usage. Frozen cat fetal fibroblasts (CFFs) were recovered and seeded 24 hours before transduction.

Oocytes were collected and matured for 24 hours with FSH and LH, then fertilized by motile sperms at 1×10^6 /ml for 24 hours. Embryos were cultured for 7-9 days until expanded blastocyst stage at 38.5 °C, 6% CO₂. Inner cell mass (ICM) cells were obtained by manual dissection from in vitro produced blastocysts as previously described (Chapter 2).

Transduction of CFFs

Lenti-X™ 293T cells were maintained following the manufacturer's instructions (Clontech). The 293T cells (80% confluent) were split 1,2 into 10 cm tissue culture plates 24 hours prior to transfection and transfected with plasmids containing human *NANOG*; *POU5F1*; *SOX2*; *KLF4* and *CMYC* (Addgene). Briefly, 5µg of plasmid of pFUW-M2rtTA or pFUW-tetO-transcription factor along with pCMV-dR8.2 dvpr and pCMV-VSV-G at a ratio of 2,2,1 was mixed with 15 µl PolyJet™ in vitro DNA transfection reagent following the manufacturer's instructions (SignaGen Laboratories, Addgene, <https://www.addgene.org/viral-vectors/lentivirus/lenti-guide> and <https://www.addgene.org/tetracyclin>). After 12 hours of transfection, medium for 293T cells was replaced by 10ml Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) with 20% HyClone defined fetal bovine serum (DFBS, GE Healthcare Life Sciences), and 1 mM L-Glutamine (Life Technologies). Medium from each plate was collected after 48 hours of incubation with transfected 293T cells and centrifuged

at 500g for 8 minutes. CFFs around 40% confluent were transduced using a modified protocol for reprogramming human somatic cells (Hockemeyer *et al.* 2008). Ten ml of the combined viral prep supernatant was mixed with 4 µg/ml hexadimethrine bromide (Sigma) and added to each 10cm plate of CFFs for 24 hours. On day 0, CFFs were transduced by virus mix for rtTA and human NANOG; on day 1, CFFs were transduced by virus mix for human POU5F1 and SOX2; on day 2, CFFs were transduced by virus mix for human KLF4 and CMYC. On day 3, medium for CFFs was replaced by 10 ml DMEM with 10% FBS and 1mM L-Glutamine. Doxycycline 5 µg/ml was added from day 4. Medium was replaced every two days until the first colony with a clear border was observed. CFFs were incubated with 10 ml of StemFlex medium (ThermoFisher Scientific) with feline LIF 10ng/ml, human LIF 10ng/ml, GSK3 inhibitor 3 µM, MEK inhibitor 1 µM, ROCK inhibitor 10 µM (Chapter 2) and doxycycline 5 µg/ml for 28 days. Mouse embryonic fibroblasts (MEFs) were transduced and culture using the same procedures as a positive control. High alkaline phosphatase activity is considered a characteristic of stem cells and is useful for initial screening of potential iPSC. Transduced CFFs and MEFs were stained every two days from day 10 to 32 for alkaline phosphatase activity using alkaline phosphatase (AP) live stain (Thermo Fisher).

Expanded culture of potential iPSCs

Colonies with a dome shape and clear edge were selected for isolation by a glass scalpel (Verma *et al.* 2013), placed on mitomycin-inactivated CFFs (Gomez *et al.* 2010). Colonies were allowed to attach to the feeder cells for three days, then cells

were disassociated by Accutase (Innovative Cell Technologies) and seeded onto inactivated CEFs. Cells were maintained in a humidified incubator (Thermo Fisher Scientific) (37°C, 5% CO₂ in air) until colonies occupied 40 – 50 % of the well surface area (every two to three days). Colonies were then disassociated by Accutase and split (ratio of 1,3 to 1,4). Doxycycline was withdrawn from passage 2. Cells were expanded until passage 10. Colonies then were disassociated with Accutase and one million cells were suspended in 2 ml medium, and each hanging drop was of 25µl cell suspension. Embryoid bodies were collected from Day 2 from the hanging-drop and cultured in petri dishes in StemFlex medium without growth factors, but with 10µM retinoid acid, for six days (Appendix Figure 3).

Fluorescent Immunocytochemistry (ICC)

Expression of protein markers of pluripotency in the putative iPSC was assessed using immunocytochemistry. Cells were fixed by 4% paraformaldehyde (PFA) in PBS at room temperature (10 minutes) and then rinsed twice with 1x DPBS (5 minutes, Sigma). Fixed cells were permeabilized 0.2% Triton X-100 and 0.1% Tween 20 in PBS at room temperature (10 minutes), then rinsed twice in TBS with 0.05% Tween 20 (5 minutes). Cells were rinsed with PBS, and then blocked with Maxblock Blocking Medium (Active Motif) at 4°C (overnight). Cells were incubated with the primary antibodies (anti-human NANOG [500-P236; Peprotech], anti-human POU5F1 [MAB4401; Millipore] and anti-human SOX2 [SC1002; Millipore] at 1.5 µg/ml) in Maxbind Staining Medium (Active Motif) at 4°C (overnight) with gentle rocking. Cells were rinsed with Maxwash Washing Medium (5 minutes, Active Motif) three

times. Cells were then incubated with the secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG [H+L], Alexa Fluor 647 goat anti-mouse IgG1, Alexa Fluor 594 goat anti-mouse IgG2a (Thermo Fisher Scientific) at 1 $\mu\text{g/ml}$] in Maxwash Washing Medium at room temperature (1 hour, in the dark) with gentle rocking. Cells were rinsed with Maxwash Washing Medium four times (5 minutes each). Hoechst 33342 (Invitrogen) was added during the third wash at 1 $\mu\text{g/ml}$ to stain nuclei. Imaging was done using a Leica DMI 6000 inverted fluorescent microscope. Fluorescence intensities were analyzed using ImageJ for semi-quantification of proteins (NANOG, POU5F1 and SOX2). Intensities of each stained proteins were normalized to the average intensity in ICMs in Day 7 to 9 blastocysts as positive controls (Nestle *et al.*, 2012). Two negative controls were used 1) non-transfected CFFs cultured in the same StemFlex medium with the same supplementations and 2) immunostaining with secondary antibody only (Appendix figure 4). Transduction rate was calculated using ImageJ, by the ratio of cell number with green fluorescent protein staining to total cell number with nucleus staining.

RT-PCR and RT-qPCR analysis

For reverse transcription-polymerase chain reaction (RT-PCR) and RT-quantitative PCR (RT-qPCR) analysis, cells were lysed using extraction buffer (PicoPure RNA isolating kit, Applied Biosystems). RNAs were extracted with DNA removal treatment on columns following the manufacturer's instruction (PicoPure RNA isolation kit, Applied Biosystems).

Complimentary DNA was generated by reverse transcription of 50 ng of RNA

using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). For transgene expression analysis, cDNA samples were amplified using GoTaq Green Master Mix (Promega). For endogenous gene expression analysis, cDNA samples (3 biological replicates for each passage) were amplified using QuantiFast SYBR Green Master Mix (Qiagen) in a BioRad CFX96 real-time PCR system. Genes selected for amplification and primer sequences are shown in Appendix Table 1. The quantitative PCR amplification was performed for 5 minutes at 95°C followed by 40 cycles for 10 seconds at 95°C, 30 seconds at 60°C. Triplicate of Cq values were analyzed by Comparative Cq method ($2^{-\Delta\Delta CT}$) to reference gene RPL17, which was validated in a previous study (Penning *et al.*, 2007). Reverse transcription minus controls (from RNA products in iPSC at passage 3, 6 and 10) were included in the reactions. Each PCR product (20 ng) was amplified using a Big Dye Terminator V3.1 cycle sequencing kit (following manufacturer's instruction; Applied Biosystem™) and were sequenced using a 96-capillary 3730xl DNA Analyzer (Applied Biosystem™). PubMed Gene and Genome were the reference databases of sequences (Pontius *et al.* 2007 and Irizarry *et al.* 2012).

For genotyping, genomic DNA (3 biological replicates) was isolated using a Purelink Genomic DNA Mini Kit (Thermo Fisher Scientific) from CFFs, transduced CFFS and iPSCs. Genomic DNA samples (10 ng/10 µl reaction system) were amplified using AmpliTaq Gold 360 Master Mix (Applied Biosystems) in an Eppendorf thermocycler. CFF transduced with transcription factors were used as positive controls, CFFs were used as negative controls.

Karyotyping

Chromosome spreads (n = 29, 38, 25 for each cell line at passage 10, respectively) prepared as previously described (Priscila *et al.* 2009). Chromosomes were stained using Hoechst 33342 (Invitrogen) at 1 µg/ml with subsequent imaging using a Leica DMI 6000 inverted fluorescent microscope (Latt *et al.* 1975).

Statistical analysis

Values presented for staining intensity are means ± S.D. Protein levels are normalized to the mean of ICM proteins. Transcriptional expressions are normalized to RPL17 expression (ΔCq), and then relative to a reference ($\Delta\Delta Cq$). Data were analyzed using a generalized linear mixed model in SAS University Edition with statistical significance set at $P < 0.05$.

3.4 Results

The transduction efficiency by pseudo lenti virus was over 90% in CFFs (Fig. 3.1A), the majority of cells were stained positive for NANOG, POU5F1 and SOX2. The first colony stained AP positive in cat appeared on day 13 of doxycycline induction, a week later than mouse colony formed (Fig. 3.1B). Only 10% of colonies were AP positive. The accumulation of AP positive colonies in MEFs was faster than in CFFs. The overall colony formation rate was 0.002% (8 out of 5×10^5 cells seeded) for CFFs, much lower than mouse (0.02%, 101 out of 5×10^5 cells seeded) (Fig. 3.1C). The colony morphologies were similar between mouse and cat.

Within 3 days after withdrawal of doxycycline at passage 2, the transgenes were

silenced (Fig 3.2). This result was validated by quantitative PCR for transgene expressions in pooled iPSC samples from each passage. Colonies selected under doxycycline were dissociated into single cells and daughter colonies were formed without doxycycline (Fig. 3.3A). Cells proliferated rapidly such that colonies needed to be passaged every two to three days. Morphologies of colonies were similar in different passages. These colonies were stained positive for NANOG, POU5F1 and SOX2 (Fig. 3.3A), yet the levels of the three proteins were much lower than those in ICM ($P < 0.05$, Fig. 3.3B), except for SOX2 in iPSC passage 6 and 10. The intensity levels of individual proteins were different between colonies (Fig. 3.3A). Furthermore, AP positive levels varied between different colonies in the same passage, around 50% of colonies stained negative for AP (Fig. 3.3A).

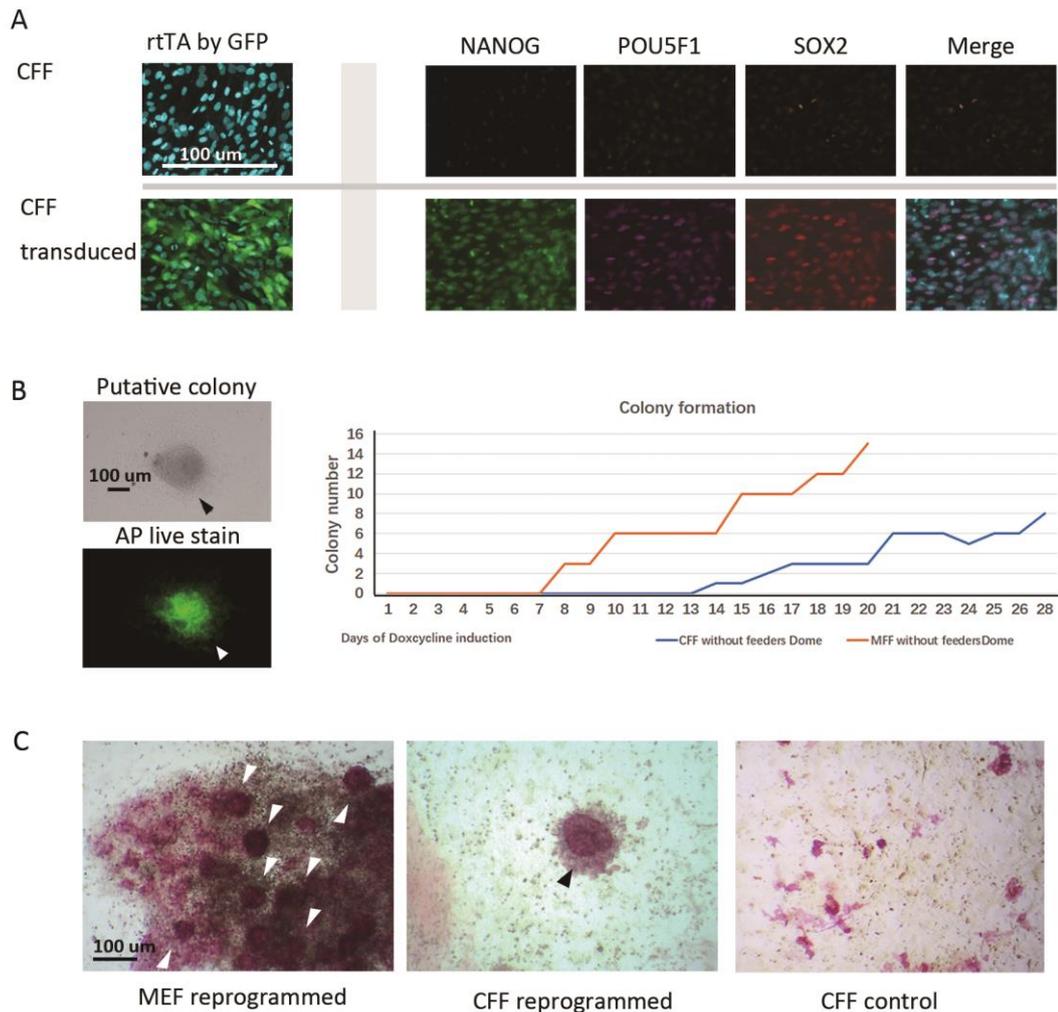


Figure 3.1. A) The expression of rtTA was confirmed by tet-on GFP in control CFFs and transduced CFFs, control CFFs and transduced CFFs (without tet-on GFP integration) after 7 days of Doxycycline induction were stained for NANOG, POU5F1 and SOX2 (human and cat proteins were not distinguishable); B) The numbers of AP live positive colonies in transduced mEFs and CFFs along time; C) AP stain for transduced mEFs and CFFs after 28 days of Doxycycline induction. Arrows pointed putative colonies.

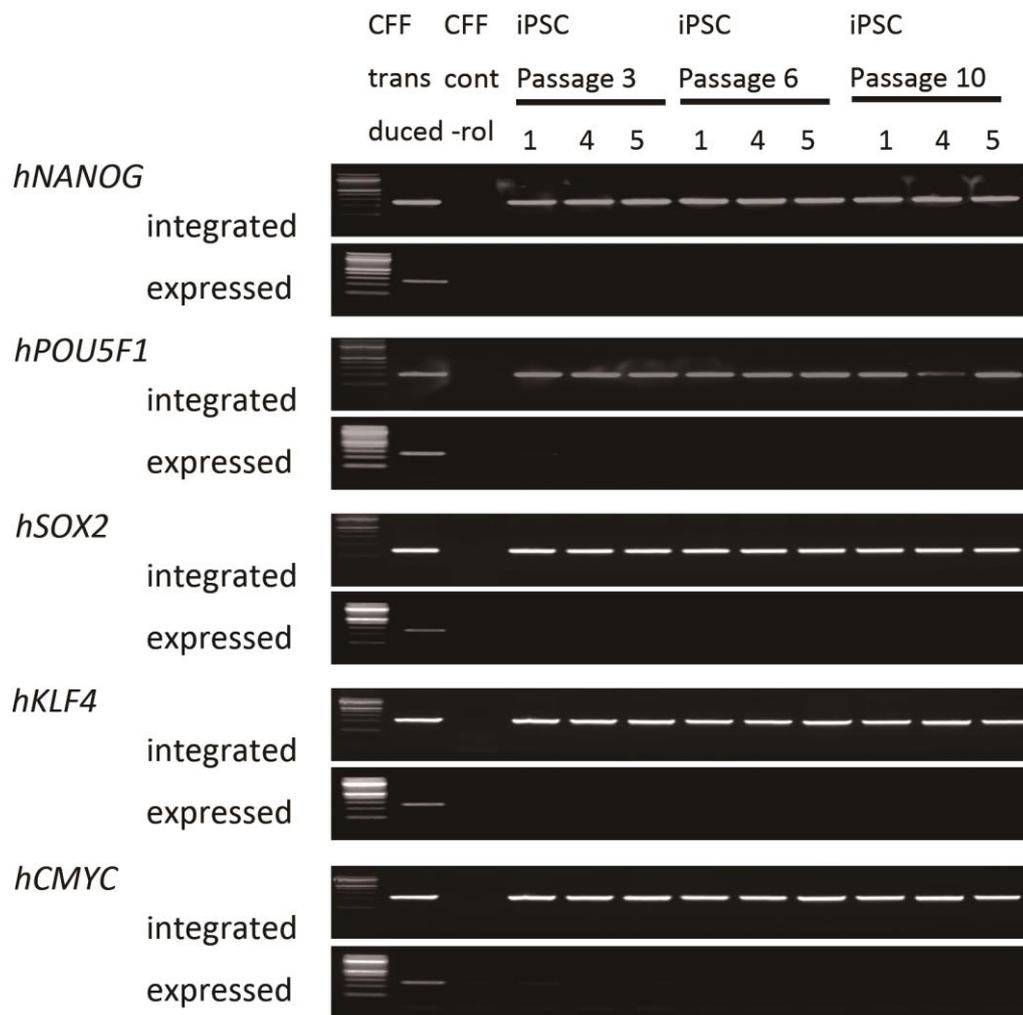


Figure 3.2 Genomic PCR for human transgenes integrations, RT-PCR for human transgene expressions in cat iPSCs in different passages without Doxycycline induction. CFFs transduced were positive controls, and CFFs before transduction were negative controls.

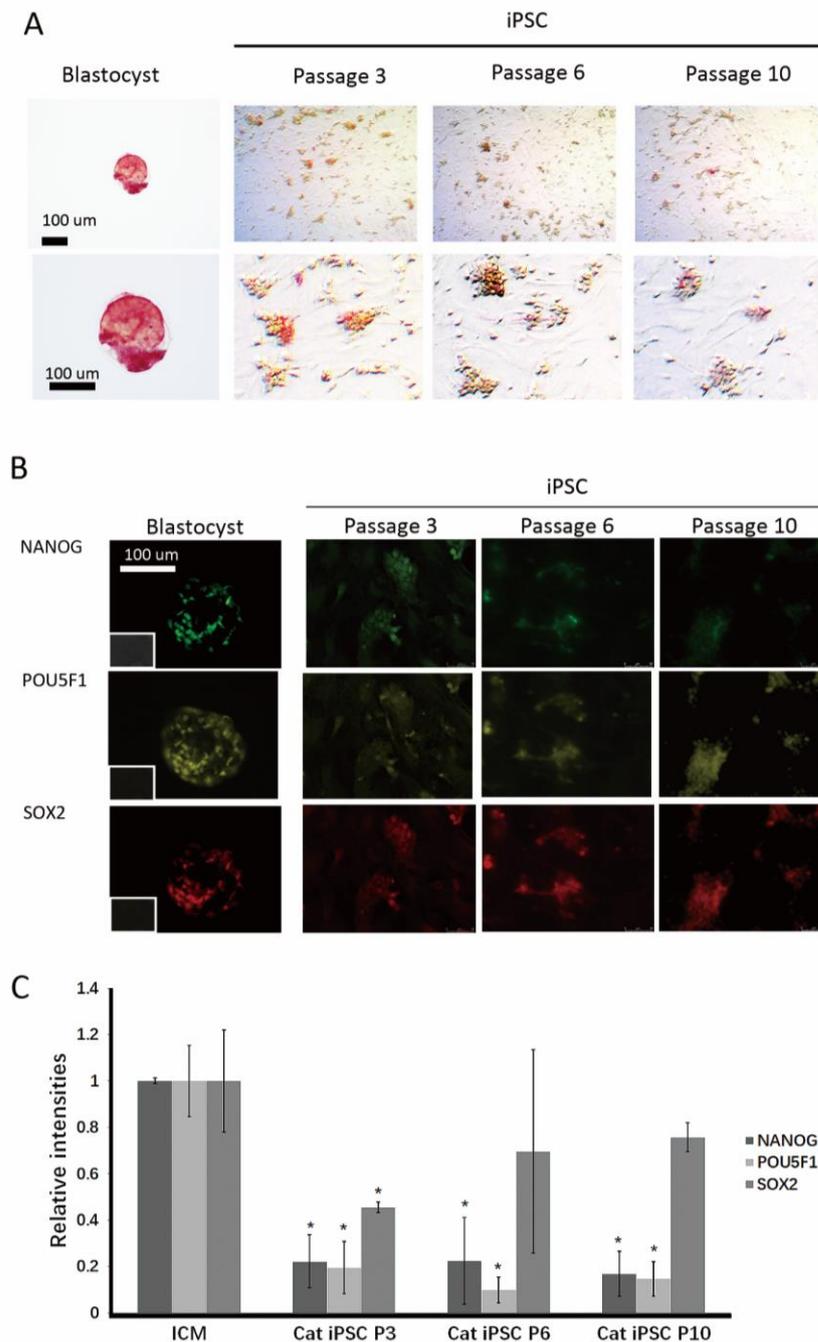


Figure 3.3. A) AP stain, B) ICC stain for NANOG, POU5F1 and SOX2 in cat blastocysts, cat iPSC passage 3, 6 and 10; C) Relative quantification of NANOG, POU5F1, SOX2 levels by ICC in cat blastocysts, cat iPSC passage 3, 6 and 10. Fluorescence intensity values were relative to the average of control ICM. Left bottom corner images on blastocyst images were negative control without primary antibodies. Full size negative controls can be found in Appendix 4. Values were expressed as mean \pm standard deviation (blastocysts=4-5, iPSC biological replicate =3, 5 colonies in each biological replicate). * $P < 0.05$, compare to cat ICM in blastocysts.

The transcription patterns in CFFs and CFF-iPSCs were different (Appendix Fig. 2). While *NANOG*, *POU5F1* at passage 3 and 6 remained similar in iPSCs ($P>0.05$), in contrast to *POU5F1* at passage 10, *SOX2* (pluripotency markers) increased ($P<0.05$). *CMYC* decreased in iPSCs ($P<0.05$). *SOX17* (endoderm markers), *Brachyury*, *FOXF1* (mesoderm markers), and *PAX6* (ectoderm marker) increased in all iPSCs ($P<0.05$). *SOX1* (ectoderm markers) increased at passage 3 and 6 ($P<0.05$) (Fig. 3.4 A). *KLF4* and *REX1* (naïve state markers) expression varied between different cell lines at different passages, so the change was not significant ($P>0.05$) (Fig. 3.4A). *FGF5* (primed state marker) and *GATA6* did not change significantly.

When iPSC transcript amounts were compared relative to expression in ICM, *NANOG* was decreased in all cell lines regardless of passage number ($P<0.05$) (Fig. 3.4B). The apparent increase in *POU5F1* and *SOX2* in all three biological replicates regardless of passages was not significant ($P>0.05$) (Fig. 3.4B). *KLF4* and *CMYC* levels did not change significantly ($P>0.05$) (Fig. 3.4B). *REX1* decreased in all three iPSC cell lines ($P<0.05$) (Fig. 3.4B). Differentiation markers *SOX1* decreased in iPSC passage 10, *GATA6* decreased in all iPSCs ($P<0.05$) (Fig. 3.4B). There was obvious variation in differentiation markers. There was no detectable *Brachyury* (primed state and mesoderm marker) in ICM. Regarding the increased *Brachyury* compared to CFFs (which had low levels of *Brachyury*), it was clear that *Brachyury* was increased in iPSCs. The other differentiation markers, *FOXF1*, *SOX17* and *PAX6*, which represented mesoderm, endoderm and ectoderm lineages respectively, were not changed significantly ($P>0.05$) (Fig. 3.4B). There was one cell line which at passage

3 showed overall low levels in lineage markers ($P < 0.05$), and similar levels of pluripotency markers to later passages ($P > 0.05$). However, levels of lineage markers had increased by passage 6 (Fig. 3.4B), like the other two lines.

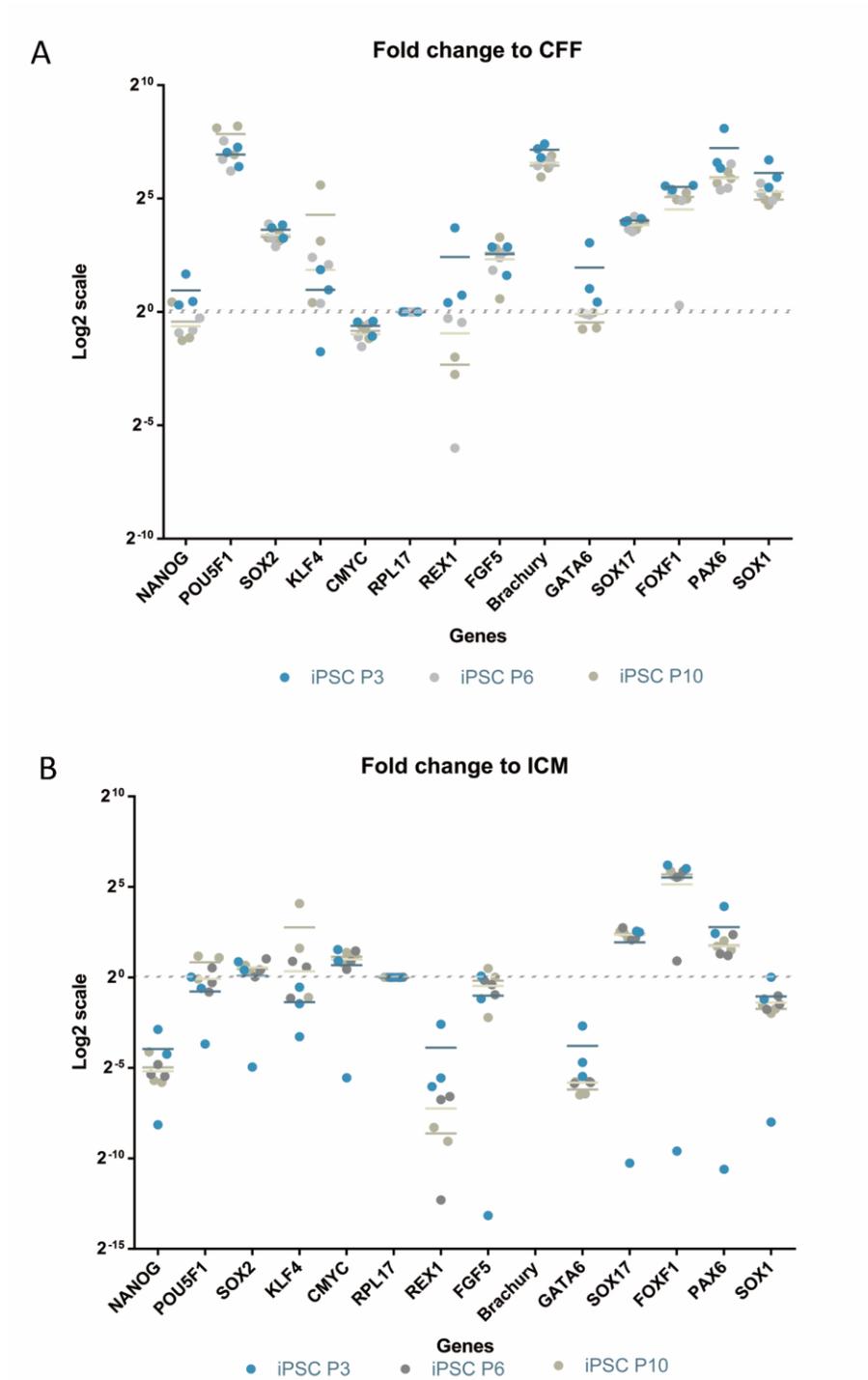


Figure 3.4 A) Gene fold changes ($2^{-\Delta\Delta Cq}$) to CFFs in iPSCs at different passages (N=3 biological replicates); B) Gene fold changes ($2^{-\Delta\Delta Cq}$) to ICM in iPSCs at different passages (N=3 biological replicates).

Cat iPSCs at passage 10 could form embryoid body-like cell aggregates after 1 day of culture in hanging drops (Appendix Fig. 3). Pluripotency markers *NANOG*, and *POU5F1* and *SOX2* were decreased ($P<0.05$) in Day 6 EBs, compared to its origin iPSC at passage 10 (Fig. 3.5A). *KLF4* did not change significantly ($P>0.05$), when each cell line showed various changes. *FGF5* and *CMYC* were increased in all EBs ($P<0.05$) (Fig. 3.5A). *REX1* was significantly lower ($P<0.05$) in all the EBs (Fig.3.5A). *PAX6* (ectoderm) and *FOXF1* (mesoderm) remained similar ($P>0.05$). The other lineage markers, *Brachyury*, *GATA6*, *SOX17*, and *SOX1* declined ($P<0.05$) (Fig. 3.5A).

When compared relative to ICM expression, lineage markers increased in EBs, including *SOX17* ($P=0.05$), *FOXF1* ($P<0.05$), and *PAX6* ($P<0.05$), which were similar to what has been observed in late passage iPSCs (Fig. 3.4 B). Like the comparison to iPSCs, pluripotency markers *NANOG*, *POU5F1*, and *SOX2* declined ($P<0.05$). However, *KLF4* and *CMYC* rose significantly ($P<0.05$) (Fig. 3.5B). *REX1* (naive state marker) was significantly lower ($P<0.05$). *FGF5* was at reduced levels ($P<0.05$) as well. *GATA6* and *SOX1* were much lower ($P<0.05$) than that in ICM, while *Brachyury* was detected in EB, comparable to iPSC passage 10, but not in ICM.

The percentage of normal karyotypes (N=38 chromosomes) ranged from 87% to 95% for the three cell lines tested.

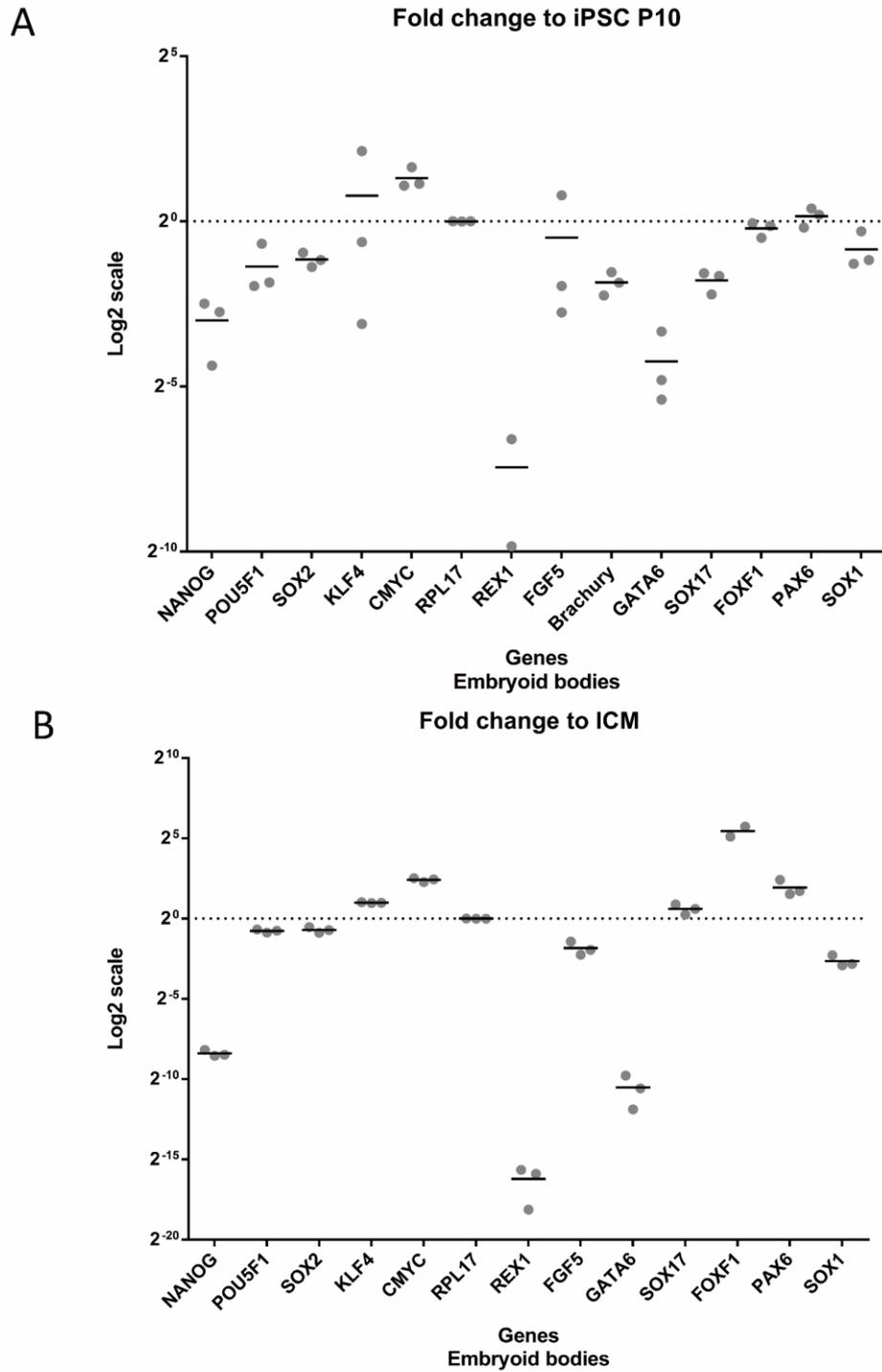


Figure 3.5 A) Gene fold changes ($2^{-\Delta\Delta Cq}$) to iPSC P10 in EBs (N=3 biological replicates); B) Gene fold changes ($2^{-\Delta\Delta Cq}$) to ICM in EBs (N=3 biological replicates).

3.5 Discussion

The generation of iPSCs in endangered species could have great therapeutic potential (Beevers *et al.* 2013; Lam *et al.*, 2014, Verma *et al.* 2013). However, because exogenous *KLF4* and *CMYC* remained active, successful establishment of fully reprogrammed iPSC in feline cells has not been achieved (Verma *et al.*, 2012 and 2013).

In this study, a lenti-viral based, drug inducible system (Hockemeyer *et al.*, 2008) was used to generate potential iPSCs in the domestic cat in a more regulated manner. The control element rtTA and human transgenes, *NANOG*, *POU5F1*, *SOX2*, *KLF4* and *CMYC* were successfully integrated into the cat fibroblast genome using pseudo-lenti viral transduction. This enabled controlled expression of human transcription factors in CFFs using doxycycline. While colony formation rate for reprogrammed CFFs was slower and at a lower efficiency than that of the MEF control (0.002% versus 0.02%, respectively), the colony formation rate was acceptable and similar to those obtained in canine studies (0.007%, Whitworth *et al.*, 2012). Notably, unlike previous reports in the cat, colonies sharing partial pluripotent features could be maintained after the transgenes were silenced. Analysis of transcription markers in iPSCs found disparities between transcripts and protein expression levels for pluripotency markers. For example, *POUF1* and *SOX2* had higher transcriptional levels and lower protein levels compared to ICM. Although not as extreme, other disparities were observed (Appendix Table 2), e.g., *NANOG* which had varying levels of transcript and protein in the CFF, iPSC and ICM. Such disparities between transcript and protein levels have been previously reported in goat embryos (He *et al.*, 2006). Taken together, the

iPSCs generated from this system shared partial features of its embryonic counterpart ICM, yet different from what has been generated from mouse and humans (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007).

Comparing to what has been observed and validated in mouse, there were several issues to be addressed for this feline study. First of all, the reprogramming efficiency might be compromised in the cat, The reprogramming efficiency is relatively low in cat compared to mouse in this study (0.002% versus 0.02%); however, the amino acid sequence identities for the pluripotency transcription factors are similar for cat and mouse as compared to human (Pontius *et al.* 2007; Irizarry *et al.*, 2012; Genebank, Pubmed). As the reprogramming efficiency for mouse in this study is similar to that in mouse studies using homologous transgenes (Takahashi and Yamanaka, 2006), this suggests that the minor difference in amino acid sequence did not compromise reprogramming in the cat and it may be that other factors that are affecting reprogramming efficiency in the cat.

Secondly, pluripotency maintenance was insufficient in this system. After isolation of colonies that stained positive for AP, cells were allowed to expand on feeders, but AP levels started to vary in the daughter colonies. As alkaline phosphatase is a useful marker for selecting colonies that could exhibit other pluripotency features (Lu *et al.*, 2011), this indicated that from early passage the pluripotency state was not stable. Within the time of culture, cat iPSC lines had already started to differentiate, which may explain why EBs did not express increased lineage markers compared to iPSC at passage 10. Notably, *CMYC* increased in EBs (fig 3.5). The increase of *CMYC*

might be a cellular response to enhance self-renewal and block differentiation during EB culture (Sato *et al.*, 2004). Although *CMYC* is dispensable in generating mouse and human iPSCs (Nakagawa *et al.*, 2008), it was mainly used as an enhancer in this procedure (Araki *et al.*, 2011, Verma *et al.* 2012). It is known that *CMYC* is context-dependent in cell fate determination in progenitor cells (Wilson *et al.*, 2004). As *REXI* was declining from early to late iPSC lines in our study, while *CMYC* was high and *FGF5*, *Brachyury* and other lineage markers were increasing, the role of *CMYC* in reprogramming and EB generation in the cat is not clear.

One possibility affecting these issues is that the culture supplementation (LIF, GSK3i and MEKi), which was adequate for mouse ESCs and iPSCs (Czechanski *et al.*, 2014; Takahashi and Yamanaka, 2006), but was not adequate for maintaining pluripotency in the cat and further improvements are needed (Chapter 2). In all, it is encouraging that pluripotent-like colonies could be generated in this drug-inducible system using human transcription factors. The colonies, though in an unstable state still showed partial features of pluripotency.

3.6 Acknowledgement

I thank Drs. Julie Maul, Joy Lewis and Kathleen Howard (Spay Now, Laurel), and their staff for providing domestic cat reproductive tracts. This research was supported by a JoGayle Howard Graduate Fellowship in Felid Reproduction to me and a Smithsonian-University of Maryland seed grant for supplies and equipment.

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Chapter 4 Summary and Future directions

4.1 Summary

This project explored methods to improve the derivation of pluripotent stem cells in the domestic cat, as a model for endangered felids. Two approaches were taken, the first was to assess effect of cytokine combinations in maintaining NANOG, POU5F1 and SOX2, as well as features of cellular pluripotency in ICM-derived embryonic cells, and the second was induction of pluripotency by forced expression of exogenous transcription factors in the same culturing supplementation formulated in the first study.

In the first study on embryonic stem cells derivation, NANOG, POU5F1 and SOX2 were affirmed as pluripotency markers in cat embryos and its derivatives. Using RT-PCR, the presence of transcripts for genes in the LIF/STAT3, and FGF/MEK/ERK signaling pathways in cat ICM were validated. Therefore, growths factors targeting these two pathways were selected and assessed in sustaining pluripotency markers. As single growth factor supplementation alone could not maintain all three proteins at their original levels as in ICM, combinations of growth factors and selected inhibitors (GSK3 inhibitor and MEK inhibitor-2i) were evaluated for their capacity of sustaining NANOG, POU5F1 and SOX2. Similar with mouse ESCs, LIF, with 2i was the most effective cocktail in preserving these proteins. Colonies with morphology like mouse ESCs were generated from blastocyst outgrowths, sharing some of the pluripotent stem cell characteristics, including rapid self-renewal. However, decreased levels of

NANOG, POU5F1 and SOX2 implied that the cell state was not identical to in vivo counterparts (ICM).

In the second study, pluripotency was acquired by forced expression of exogenous transcription factors in cat fetal fibroblasts. Owing to the beneficial effects observed in Study 1, a LIF and 2i combination was used to aid in reprogramming and colony expansion. As observed in cat embryonic cells, partial characteristics of pluripotency were sustained, in the form of fast proliferation, NANOG, POU5F1, and SOX2 expression, as well as alkaline phosphatase, although at lower levels than would be expected in fully reprogrammed cells. Notably, unlike previous reports in the cat, colonies sharing partial pluripotent features could be maintained after the transgenes were silenced. In addition to the protein and transcript markers for pluripotency, lineage marker dynamics were examined in ICM, iPSCs, and embryoid bodies. The outcome suggested the cells generated with LIF and 2i had developed beyond the undifferentiated stage of ICM in expanded blastocyst. Collective results not only challenged the efficacy of the cytokines combinations LIF and 2i in maintaining feline pluripotency, but also suggest the future direction of such research, in elucidating major mechanism in pluripotency regulation in cats.

4.2 Future directions

4.2.1 Investigating signaling pathways that regulate pluripotency in cats

It is known that STAT3 signaling is highly active in mouse pre-implantation embryos (Do *et al.*, 2013). In mouse oocytes, STAT3 is highly expressed and its

phosphorylation is necessary for pluripotency regulation. At 4-cell stage, STAT3 becomes phosphorylated and translocates to nucleus (Do *et al.*, 2013). STAT3 phosphorylation firstly depends on LIF at 4-cell stage in all mouse strains. As embryos develop to blastocysts, this modification starts to rely on various members in Interleukin 6 (IL6) family, including LIF, Cardiotrophin-1 (CT1), and IL6, in different mouse strains (Do *et al.*, 2013; Kabayashi *et al.*, 2014; Shen *et al.*, 2009). STAT3-null or STAT3-dephosphorylated embryos arrest before the pre-implantation stage (Do *et al.*, 2013; Yin *et al.*, 2014). The study on small molecules tested on bovine embryos also agreed that STAT3 activation has a positive impact on blastocyst formation (Meng *et al.*, 2015), and no *LIF-receptor* transcripts were detected at the morula to blastocyst transition, which suggested there is another factor activating STAT3 (Eckert and Niemann, 1998). These studies showed that STAT3 is the core mediator in early embryo from different species, but LIF is not the only factor that regulates its phosphorylation. After implantation, the signaling pathway that regulates embryonic development switches to FGF/MEK and Nodal in mouse (Bessonnard *et al.*, 2014; Doughton *et al.*, 2014; Ghimire *et al.*, 2015). If the activation of FGF/MEK is inhibited in mouse embryos, the hypoblast formation can be suppressed, which indicates a blockage of development (Nicholas *et al.*, 2009). Again, these facts were observed in bovine embryos (Modan *et al.*, 2005). Notably, inhibition of FGF/MEK did not alter the segregation of epiblast and hypoblast in human embryos (Kuijk *et al.*, 2012). Species variance in signaling regulating development/pluripotency must be taken into account.

In chapter 2, PCR result showed that *STAT3* transcript was low in ICM from expanded blastocysts, while *MEK* and *ERK* transcripts were high. This suggested that cells are in a later stage of cat blastocyst development, and that the reliance switch from *STAT3* to *MEK/ERK* may have already occurred. This finding also implied that there could be other pathways involved, yet not identified. Even if the assessed cytokine combinations sustained partial pluripotent markers, results suggest that right timing of applying LIF and 2i to suppress development or maintain pluripotency may have been missed. A more thorough validation could be conducted with quantitative PCR for the transcripts and immunoassays (e.g. ICC, western blot) for receptor and pathway mediator proteins. In all, knowledge of what signaling controls cat early embryo development at different stages is critical. Approaches include, but are not limited to, RNA-sequence analysis, signaling pathway time-lapse tracking, and gene-engineering in cat embryos and cell lines obtained. These approaches are discussed in detail below with special consideration to the limited availability of cat embryos.

RNA-sequence analysis, has been used to track the transcriptome change from ICM to ESCs in mouse (Tang *et al.*, 2010). This research group has uncovered expression dynamics for over 2500 genes, with 6331 transcript variants in total. These indicated a notable amount of alternative splicing. In the same study, a correlated change of epigenetic regulator and micro RNAs have been revealed in the same cell population, which allows the identification of genes that directly regulate transcription factor POU5F1. This analysis only required 20 single cells from the embryos to generate the whole transcriptome (Tang *et al.*, 2009). The sensitivity and reliability of the data are

encouraging for cat embryological studies, since the cat embryo production is more challenging compared to the mouse model, and assessment of embryo quality is still inadequate. The strategy for utilizing such technology could be single-cell comparison for transcriptome pattern change from 4-cell to expanded blastocyst, and single-cell comparisons for transcriptome difference regarding specification in morula and blastocysts. Once such dataset is established, there are several ways to improve the data interpretation, 1) comparing the data to what has been published in mouse and human studies (Yan *et al.*, 2013), to locate genes upregulated and down-regulated specifically in cats, including long noncoding RNAs; 2) applying the time-expression clustering pattern (Tong *et al.*, 2010) from mouse to cat dataset, to find the gene clusters that share the similar pattern during cell specification, especially the cluster resembling specific signaling pathways; 3) coupling with cell state surface markers to resolve heterogeneity in the embryos (Wilson *et al.*, 2015); and 4) combining with chromatin immunoprecipitation sequence (Laurent *et al.*, 2015), to reveal the modifiers on gene regulatory elements (Li *et al.*, 2017). These analyses will help identify potential candidates for gene engineering, and comparatively define cell states in cat blastocysts (Rossant and Tam, 2009). It is noteworthy that there are technical errors that may be mistaken as extensive biological heterogeneity, especially when the biological variation is obvious in cat embryos produced in vitro, due to the uncontrolled parental resources. To confirm that a transcript is truly heterogeneous in a specific population of cat embryos, orthogonal validation using digital drop-let PCR and quantitative PCR would be essential (Wang *et al.*, 2014), which requires additional

set of precious samples. In addition to this challenge, the database of cat genome and its transcriptome is still in need of improvement, particularly for genetic studies in random bred cat embryos (Li *et al.*, 2016; Montague *et al.*, 2014).

Time-lapse signaling pathway analysis by fluorescence anisotropy (Marchand *et al.*, 2001) is another approach that would add to the RNA-sequence analysis in the cat model. For example, IL6 family has high evolutionary plasticity (Hsu *et al.*, 2015), consisting of ciliary neurotrophic factor, cardiotrophin-like cytokine (CLC), CT1, IL6, IL11, LIF, IL-27 and oncostatin M (OSM), which can be labelled by fluorescent dyes. When bound, anisotropy could be detected. They are clustered into one family because the receptor of each cytokine contains two (IL-6 and IL-11) or one molecule (all other cytokines) of the signaling receptor and a subunit gp130 (Matsuo *et al.*, 2014). After activation Janus kinase on the receptor, STAT3 will be phosphorylated. Thus, tracking the receptor fluctuation and IL6 family production in the embryos may lead to the discovery of the cytokines associated with cell specification in morula and blastocysts. The limitation in this approach is the selection of candidate signaling pathway is restricted to what has been validated in mouse and human, which may overlook the signaling pathways specific for cats. Fortunately, there is a novel protein analysis method that may compensate for this limitation, an integrated native mass spectrometry and top-down proteomics method (Li *et al.*, 2018). It allows the macromolecules analyzed and structured in one single experiment, which minimizes the sample size, also the sensitivity is around 5 ppm which allows average protein concentrations as low as 10 pM. Using clustering analysis and ortholog identification,

the active signaling could be identified beyond the current understanding for cat embryos.

After the identification of signaling pathways in cat embryos, the validation of signaling function could rely on gene-engineered embryos. To interfere with the normal embryo development before cell specification as little as possible, a condition knock out at morula stage could be used. The traditional Cre-flox system (Hoess *et al.*, 1984) requires intensive selection of embryos bearing spontaneous homologous recombination, which cannot be fulfilled with limited amount of cat embryos specimens. A method using small interfering RNA has been generated recently (Ikeda *et al.*, 2018). It includes a simple lipofection method to transfect siRNA into 8-16-cell stage embryos using zona removal and the well-of-the-well culture system. This approach is more straightforward and can affect the embryos at desired time point. The reported knockdown efficiency was 35-88%, which may not be perfect. The limited number of cat embryos could restrict the use of such an approach, however with improved embryo production, this would be a valid evaluation of indispensable pathways for development. The development and transcripts of interfered embryos will be assessed to determine if the gene is critical in regulating cell specification and differentiation in blastocysts.

Once the signaling pathway are determined, formulation of cytokines that maintain pluripotency in cat embryos becomes much easier and more accurate.

4.2.2 Investigating cytokine effect with generated cell lines

While the embryo experiment takes time to produce an answer, there can be alternative way to bulk screen known cytokines and small chemical molecules using cell lines. In chapter 3, the transduction efficiency was over 90%, yet the putative colony formation was low (0.002%). One of the major reasons could be that the cytokine combination used was not optimized for cat pluripotency maintenance. The transcript and protein analysis in the cells generated implied that the daughter colonies advanced to a direction towards differentiation. Based on the previous studies in mouse reprogramming (Zunder *et al.*, 2015), there can be several sub-populations in the reprogrammed CFFs, NANOG^{low}POU5F1^{low} partial reprogrammed cells, fibroblast-like cells, ESC-like Nanog^{high}Sox2^{high} cells, and mesendoderm-like Nanog^{low}Sox2^{low}Lin28^{high} cells. The co-existence of such populations allows selection of two major groups of supplementations, cytokines that maintain pluripotency in ESC-like Nanog^{high}Sox2^{high} cells, small chemicals that enhance pluripotency NANOG^{low}POU5F1^{low} partial reprogrammed cells, which together result in increased number of ESC-like colonies and stabilization of pluripotency in generated colonies.

A successful chemical/cytokine reverse screening campaign must meet three requirements, 1) a group of robust markers, which allows to distinguish between potent and less potent molecules, 2) a quantitative assessment for candidate molecules to evaluate their efficiency, and (3) an experimental strategy for elucidating the connection of molecule and its target. In this study, the levels of NANOG, POU5F1 were good markers representing pluripotent status. Cells can be engineered with two

reporter proteins, e.g. green fluorescence proteins and yellow fluorescence protein following NANOG, POU5F1 genes, respectively. The subpopulation may be sorted by the levels of co-expressions of the fluorescence proteins (Hayashi *et al.*, 2011), and provide several populations with one or two fluorophores. The cells with high levels of both fluorophores are considered ESC-like cells and treated with different combinations of cytokines that are known effective in mouse and human ESC, including but not limited to LIF, IL6, OSM, CT1, GSK3 inhibitor, MEK inhibitor, ERK inhibitor, MAP2/3K inhibitors (Thorold *et al.*, 2014). The endpoint evaluation would be the homogeneity of colonies continuously expressing high levels of NANOG and POU5F1. The other population would be more of a mix of NANOG^{low}POU5F1^{low}, and NANOG/POU5F1^{low}, partial reprogrammed cells. This group can be used to select small chemicals that enhance pluripotency, and the endpoint assessment would be the efficiency of converting cells to NANOG^{high}POU5F1^{high} population. The pool of small chemicals is a combined library data set based on previous study in mouse (Hou *et al.*, 2013).

During the process of optimizing reprogramming, a profiling of cell surface proteins (Collier *et al.*, 2017) can be generated by comparing NANOG^{high}POU5F1^{high} to the other population. Surface proteins identified may be useful to track pluripotency dynamic in cat embryos. After all, the cytokine/small chemicals need to be validated on embryo-origin pluripotent stem cells.

Back to the accomplishment in this dissertation, the generation of embryonic and reprogrammed cells has set a foundation of cell state markers, cytokine selection and

genome manipulation in the cat. The establishment of fast-proliferating cell lines have provided materials to optimize the condition for cat pluripotency regulation. With the proposed research above, a deeper understanding of pluripotency regulation in cat is anticipated, with the discovery of molecules that eventually provide the conditions that allow generation of ESCs and iPSCs for the feline family.

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Appendix

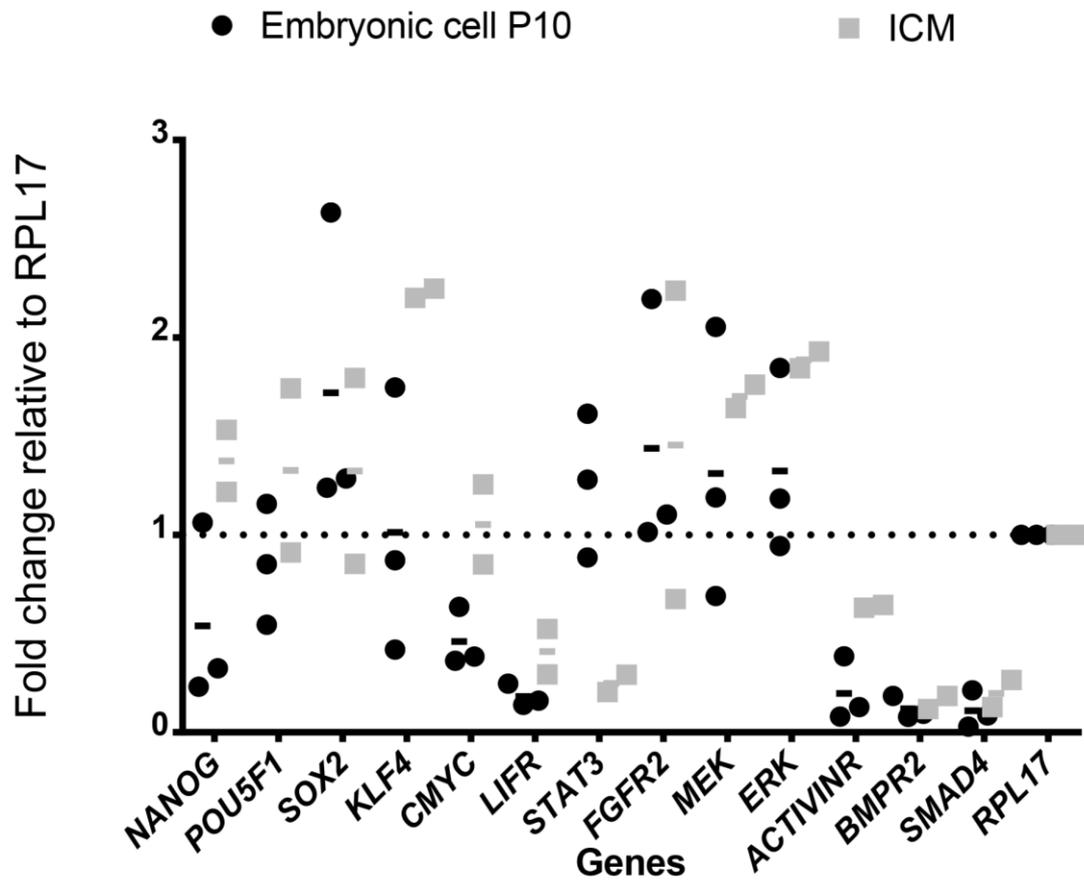
Appendix Table 1 Primers, target genes and anticipated products.

RT-qPCR target		forward primer	reverse primer	product (bp)	
cat	KLF4	CTGACGAACTGACCAGGCAT	TCTTCTTGGCAGTGTGGGTC	161	
	POU5F1	GCCCGAAAGAGAAAAGCGAAC	AACCACACTCGGACCACATC	149	
	NANOG	CTCTCCTCTTCCTTCCT	AGCTGGGTCTGAGAGAA	171	
	SOX2	CTACAGCATGATGCAGGACCAG	TGCTGCGAGTAGGACATGCT	180	
	CMYC	GCAGCGACTCTGAGGAAGAA	AGGTTTGCTGTGGCCTCC	131	
	REX1	AGGCATCTCCTCGTTCATGC	AAACGTTTTCCGCACCCTTC	149	
	FGF5	CGGATGGCAAAGTCAATGGC	TCGGTAAATTTGGCACTTGCAT	160	
	PAX6	TTCCGAATTCTGCAGACCC	GTCTGATGGAGCCAGTCTCG	119	
	SOX1	CAAGATGCACAACCTCGGAGATC	TCCTTCTTGAGCAGCGTCTTGGTC	177	
	Brachyury	TGTCCAACGGGCTGGGAGCTC	GTCCACGAGGCTATGAGGAGG	187	
	FOXF1	TACTCGTACATCGCGCTCAT	TGGTCCAGTAGTGGCCCTTG	220	
	SOX17	CAGTGACGACCAGAGCCAGC	GCGTTCATCGCCGCCGGATGC	195	
	GATA6	CCTGTGTGCAATGCTTGTGG	GAGCAAGCTTTTGACTTATTAT	128	
	RT-PCR				
cat	NANOG	GAATACGGATCCAGCTCAGCC	GTCTGGTTGCCCATATTGGAA	600	
	POU5F1	GGGGGATGACGTACTGTGG	ATTGCAGAACCACACTCGGA	629	
	SOX2	GAACCCCAAGATGCACAACCTC	CTGGAGTGGGAGGAAGAGGTAA	600	
	KLF4	ACCTGCAGCTTCAGCTATCC	GCTCTTGGTAATGGAGCGGT	685	
	CMYC	ACTCGGTGCAGCCCTATTTTC	TGCCACTGTCCAACCTTAGCC	948	
	LIF-R	GGTACCATGCCATCCATCTT	TTCATTTCCCCACCCACAT	756	
	STAT3	GGTGTACAGATTACGTGGGCT	TCAATGAATGGTGTACACAGA	529	
	FGF2-R	AGCAGGAACATCGCATTGGA	AGAAACCTGTCTCCGCAAGG	692	
	MEK	GTTTGGCGTATCTCCGGGAA	GGCAGTCACTCCCGAATTGT	603	
	ERK	AGTGCACCTACTGCTTACCG	CGTCATCCCAGGTGTTTCCA	284	
	ACTIVIN-AR	TGAGTGTACATTGCCGGAG	TGACTCAGCTACCTTAGGGCT	188	
	BMP2-R	CATTCCAAATCCAGCACCGC	GGTAGCTGCTTTCTCTTGTGC	740	
	SMAD4	TTAAACTCGGACCGGGACTC	TGATGGCTGCCCTTCAAAGT	756	
	RPL17	CTCTGGTCATTGAGCACATCC	TCAATGTGGCAGGGAGAGC	109	
			CAAATTCTCCTGCCAGTGACTT		
		NANOG-WPRE	G	CATAGCGTAAAAGGAGCAACAT	180
			GCCCTCACTTCACTGCACTGTA		
		POU5F1-WPRE	CT	CATAGCGTAAAAGGAGCAACAT	180
		SOX2-WPRE	CCGGCACGGCCATTAACGGC	CATAGCGTAAAAGGAGCAACAT	200
	KLF4-WPRE	CGCTCAGATGAACTGACCAGG	CATAGCGTAAAAGGAGCAACAT	150	
	CMYC-WPRE	CATACATCCTGTCCGTCCAA	CATAGCGTAAAAGGAGCAACAT	150	
genomic PCR					
		TGGAGCCAAGGTCTGAAGCTCA	AGTGCGTGCCCCTAGTGCCAGCAG		
mouse	NANOG	TTGTC	ATGG	561	

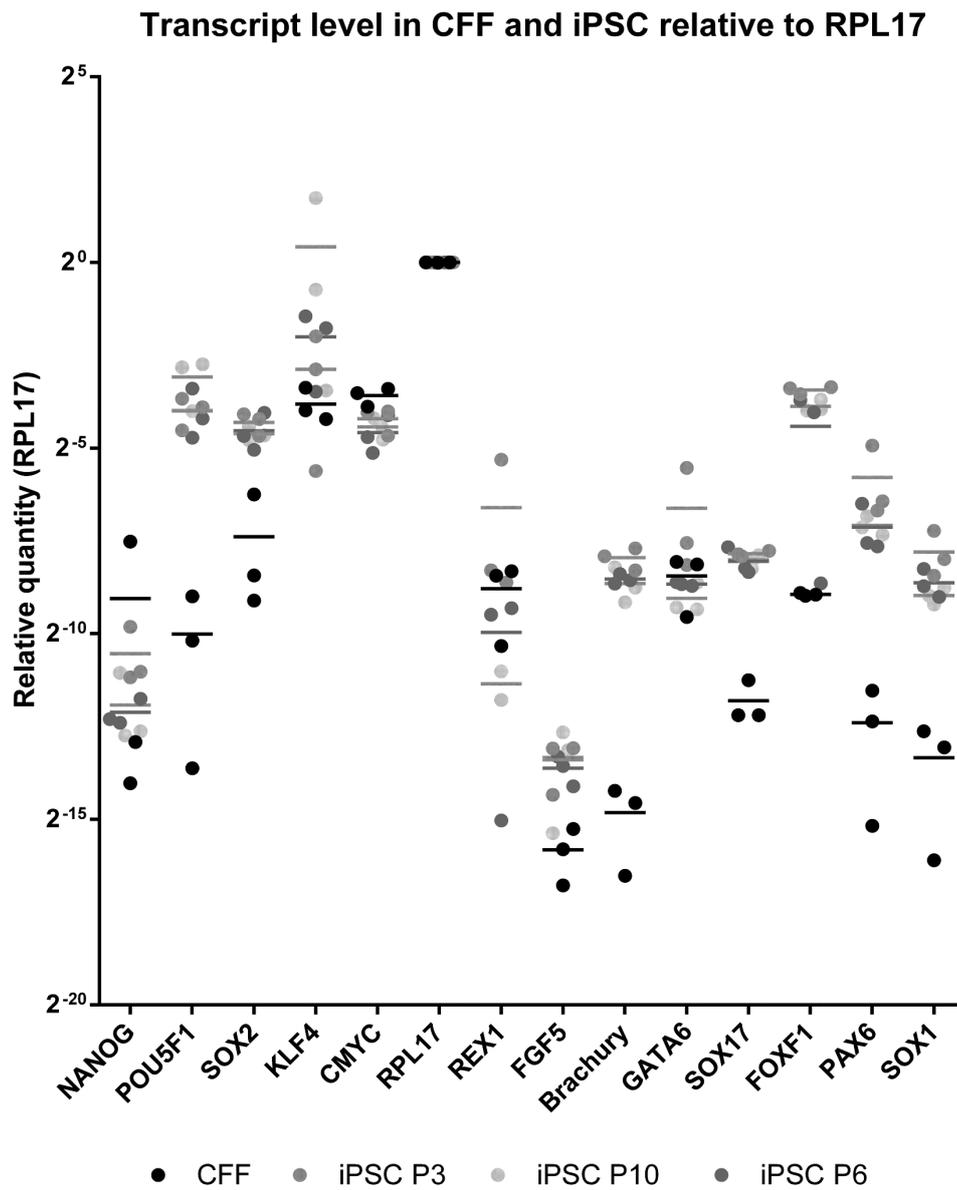
		GCGAGATCGTGACCTGGCTGAA	ACTTGGCCGTTTGCTGTATCACACT
cat	NANOG	GTCGGACG	G
			660

Appendix Table 2 P values for relative fold change

Genes	iPSC P3 to CFF	iPSC P6 to CFF	iPSC P10 to CFF	iPSC P3 to ICM	iPSC P6 to ICM	iPSC P10 to ICM	EB to iPSC	EB to ICM
NANOG	0.308	0.889	0.953	0.000	0.000	0.000	0.000	0.000
POU5F1	0.118	0.122	0.006	0.728	0.999	0.276	0.007	0.000
SOX2	0.002	0.005	0.007	0.999	0.728	0.822	0.000	0.001
KLF4	1.000	0.994	0.348	0.998	1.000	0.429	0.623	0.000
CMYC	0.047	0.007	0.014	0.819	0.510	0.353	0.010	0.000
REX1	0.434	0.998	0.988	0.000	0.000	0.000	0.000	0.000
FGF5	0.164	0.286	0.140	0.486	0.841	0.983	0.601	0.000
Brachyury	0.000	0.002	0.004	N/A	N/A	N/A	0.000	N/A
GATA6	0.319	1.000	0.998	0.000	0.000	0.000	0.000	0.000
SOX17	0.000	0.000	0.000	0.298	0.091	0.080	0.000	0.050
FOXF1	0.003	0.100	0.016	0.204	0.394	0.141	0.150	0.001
PAX6	0.044	0.595	0.572	0.333	0.889	0.876	0.419	0.020
SOX1	0.004	0.078	0.188	0.139	0.071	0.041	0.026	0.000

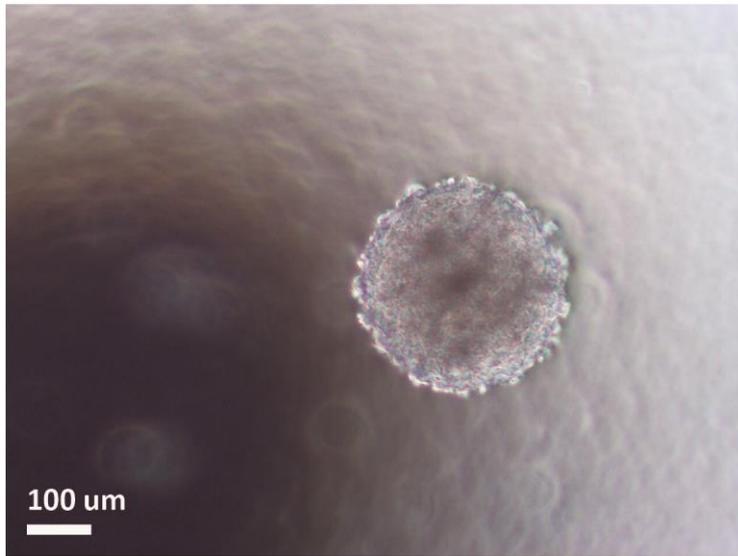


Appendix Figure 1. Relative quantification of transcripts *NANOG*, *POU5F1*, *SOX2*, *KLF4*, *CMYC*, *LIF*-receptor, *STAT3*, *FGF2*-receptor, *MEK*, *ERK*, *Activin A*-receptor; *BMP2*-receptor and *SMAD4*. *RPL17* was used as a reference gene. No significant difference between inner cell mass and embryonic cell passage 10 ($P > 0.05$).

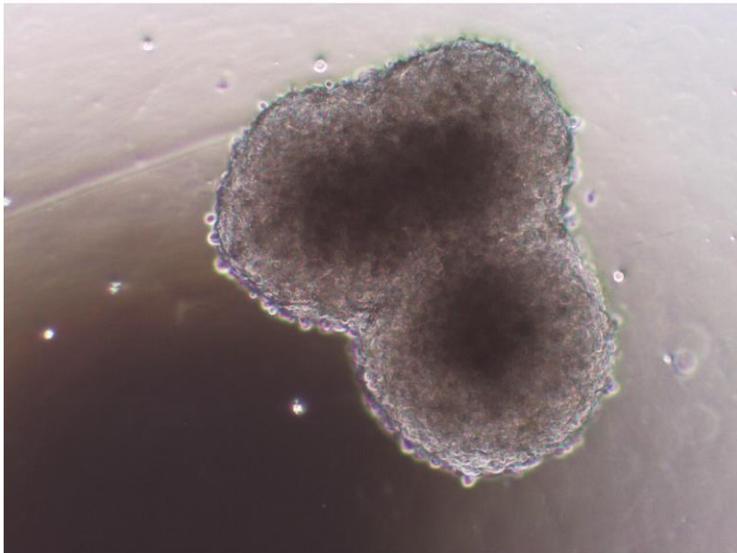


Appendix Figure 2. Relative quantification of transcript in CFFs and iPSCs at different passages for transcripts *NANOG*, *POU5F1*, *SOX2*, *KLF4*, *CMYC*, *REX1*, *FGF5*, *FGF2*, *Brachyury*, *GATA6*, *SOX17*, *FOXF1*, *PAX* and *SOX1*. *RPL17* was used as a reference gene, quantity = $2^{-\Delta Cq}$. N= 3 biological replicates.

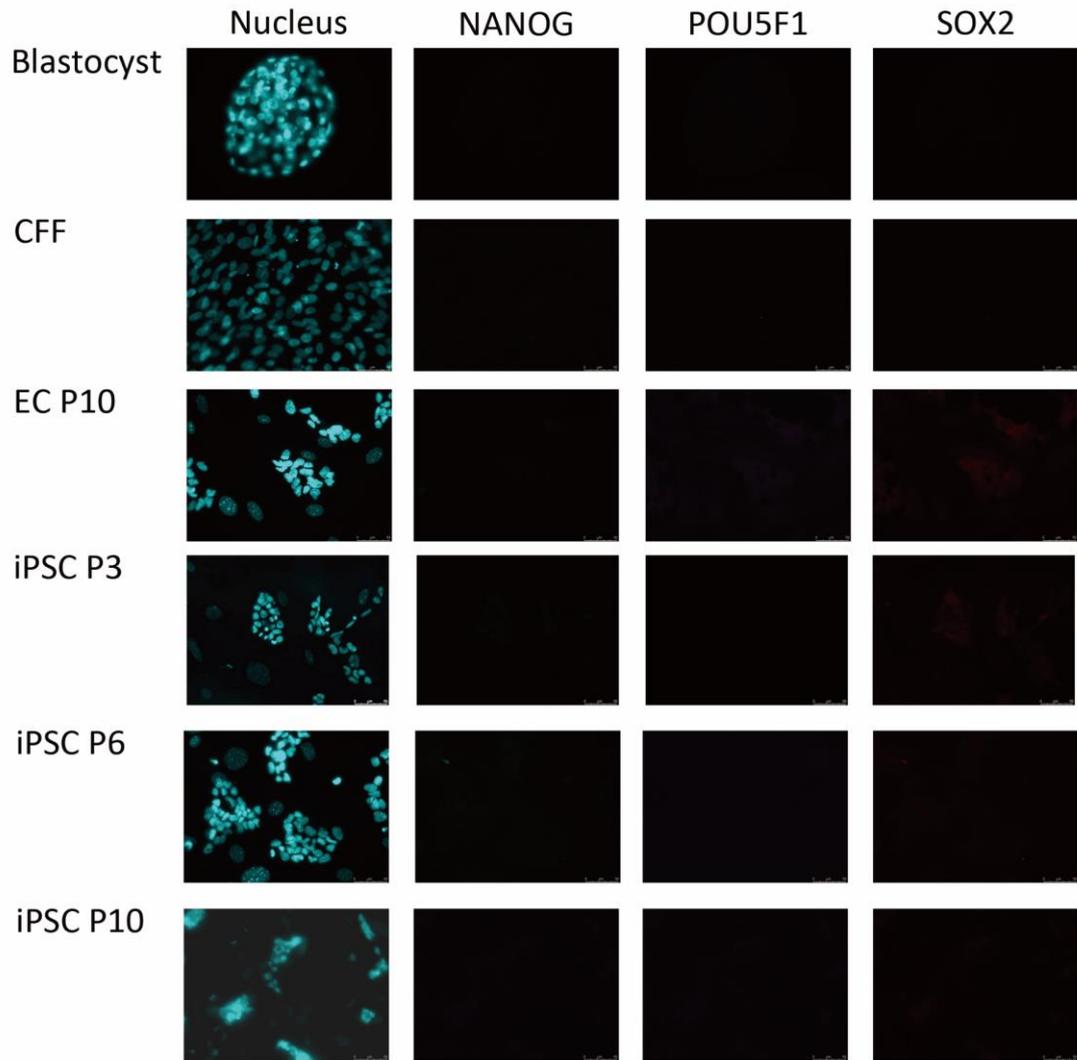
A



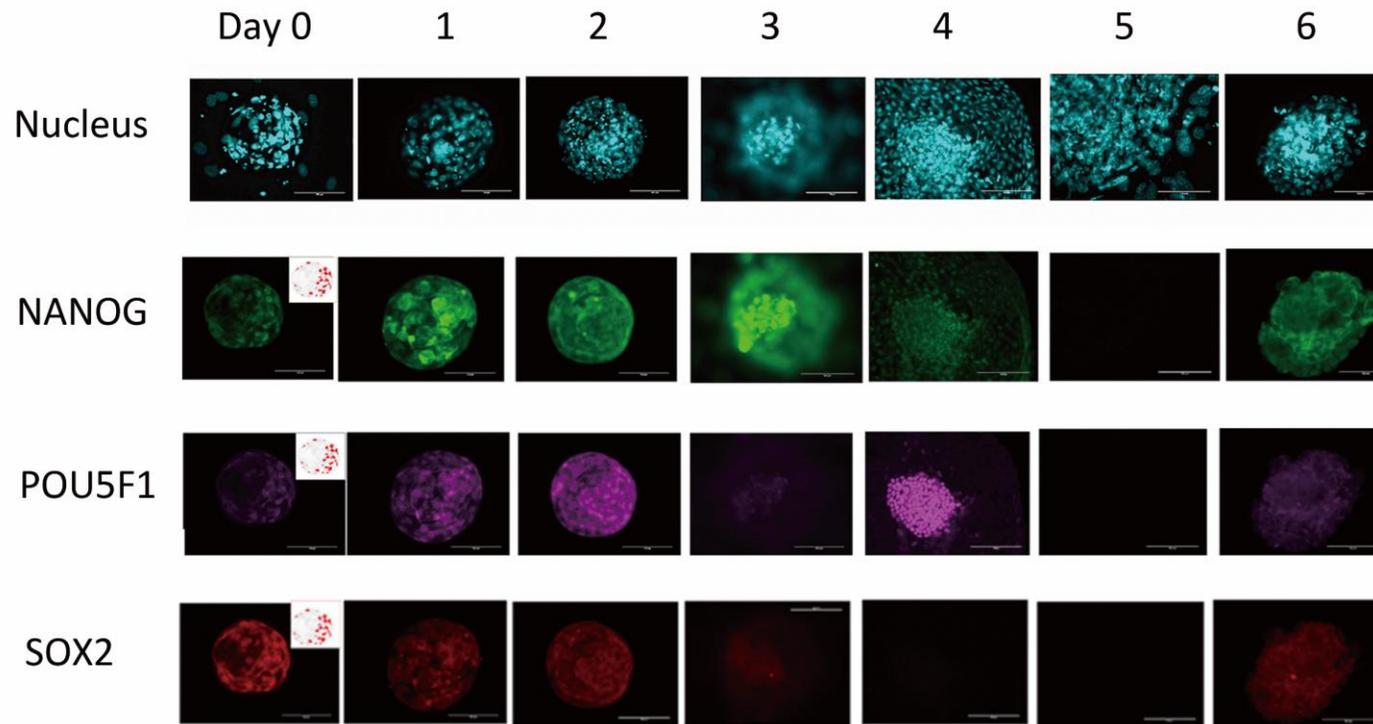
B



Appendix Figure 3. Embryoid body culture from iPSC at passage 10, A) Embryoid bodies were formed in hanging drops with 5000 cells; B) embryoid bodies were transferred to petri dishes and cultured with retinoic acid for six days.



Appendix Figure 4. Cells stained without primary antibodies, but with all three secondary antibodies and Hoechst 33324 at 1 $\mu\text{g}/\text{ml}$ in immunocytochemistry.



Appendix Figure 5. Blastocysts were cultured without cytokine supplementations for 6 days. ICC was used to assess cell number, NANOG, POU5F1 and SOX2 expression in ICM outgrowths.

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