GENERATION, CHARACTERIZATION AND LEUKEMIA INHIBITORY FACTOR DEPENDENCY OF CANINE INDUCED PLURIPOTENT STEM CELL

By

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ABSTRACT

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More than five decades of research in dogs has provided fundamental breakthroughs in human and veterinary medicine. Stem cell transplant has been put forward as one potential means of treating both human and canine injury; however, the use of therapeutic cellular transplantation in dogs has been hampered by a lack of knowledge of the characteristics of canine stem cells and difficulties in reproducibly isolating viable pluripotent cells of dog origin. To remedy this situation, I began a series of experiments aimed at producing induced pluripotent stem cells of canine origin (ciPSCs), comparing the properties of ciPSCs to pluripotent cell types of other species, determining the capacity of ciPSCs to give rise to multiple types of somatic tissue, and defining conditions for their continued growth. To begin, multiple primary fibroblast cell populations were derived from tissue samples taken from live adult donor dogs. After characterization of primary lines to select those with the best growth properties, fibroblasts were converted to ciPSCs by infection with high titers of recombinant retroviruses encoding the pluripotency-associated transcription factors OCT4, SOX2, c-MYC, and KLF4. Infected cultures gave rise to colonies with the characteristics of pluripotent stem cell types within several weeks, and subcloned canine iPSCs lines were found to express genes and proteins characteristic of other mammalian pluripotent cells. Like iPSCs from other species, ciPSCs were also found to have silenced expression of the viral vectors used to induce pluripotency. Clonal ciPSC displayed

normal karyotypes and DNA fingerprinting analysis confirmed that iPSCs were a match for the genome of donor dogs. After a shift to culture conditions favoring differentiation, ciPSCs were observed to give rise to cells of ectodermal, mesodermal, and endodermal identity. Unlike iPSCs from many species, however, it was found that ciPSCs required the continued presence of leukemia inhibitory factor (LIF) to survive in vitro. To further elucidate the role of LIF-specific signaling pathways in maintaining ciPSC viability, we performed a series of experiments that revealed that activation of the LIF-specific JAK-STAT3 pathway was critical for preventing ciPSC death. In summary, the project performed to complete this dissertation has produced an efficient method for the derivation of pluripotent stem cells from the dog and has defined many of the molecular pathways required for their derivation and continued maintenance in vitro. This work serves as the foundation for the development of cell-based therapies for disease and injury in dogs with tremendous potential to inform our understanding of similar treatments in future human patients.

This dissertation is dedicated to my parents
for their love, endless support
and encouragement

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KEY TO SYMBOLS OR ABBREVIATIONS

AFP	alpha-fetoprotein
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2-deoxyuridine
cESC	canine embryonic stem cell
ciPSC	canine induced pluripotent stem cell
CSF	canine skin fibroblast
CTF	canine testicular fibroblast
DMSO	dimethyl sulfoxide
EB	embryoid body
ECC	embryonic carcinoma cell
EGF	epidermal growth factor
EMA	European Medicine Agency
ESC	embryonic stem cell
FBS	fetal bovine serum
FDA	Food and Drug Administration
H_2O_2	hydrogen peroxide
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
HLA	human leukocyte antigen
ICM	inner cell mass
IGF	insulin growth factor

iPSC	induced pluripotent stem cell
JAK	janus kinase
LIF	leukemia inhibitory factor
LIFR	receptor of leukemia inhibitory factor
MEF	mouse embryonic fibroblast
mESC	mouse embryonic stem cell
miPSC	mouse induced pluripotent stem cell
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
NES	nestin
NIH	National Institute of Health
OKSIM	OCT4, KLF4, SOX2, IRES and c-MYC
OKSM	OCT4, KLF4, SOX2 and c-MYC
p38MAPK	p38 mitogen-activated protein kinase
PBS	phosphate buffered saline
PI	propidium iodide
PSC	pluripotent stem cell
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SCI	spinal cord injury
SCNT	somatic cell nuclear transfer
SSEA	stage-specific embryonic antigen
STAT3	signal transducer and activator of transcription 3
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling

YFP	yellow fluorescent protein

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Regenerative Medicine

During the past decade we have witnessed the birth and exponential growth of the research field of regenerative medicine [1]. As described by the National Institutes of Health (NIH), the ultimate goal of regenerative medicine is to “replace, repair, and regenerate cells, tissues and organs in order to restore biological function that has been halted or compromised by injury or disease” [2]. A sine qua non requirement to achieve this goal is the availability of unlimited number cells of all types of the human body. Pluripotent stem cells (PSCs), having the capacity to self-renew and differentiate toward cell derivatives of the three-germ layers, are likely the most suitable for the task. The use of PSCs by multiple laboratories around the world over the last thirty years has facilitated the development of specific cell culture and differentiation techniques. The versatility of PSCs is such that they can develop into functional tissues and/or organs *in vitro*. This remarkable progress has brought the possibility of conducting preclinical experiments closer to reality.

One of the most well-characterized PSCs are embryonic stem cells (ESCs). These cells, isolated from mouse and human preimplantation embryos, provide the basis upon which therapeutic strategies for diseases, previously thought to be incurable, are developed, however among the many hurdles that must be addressed before this type of therapy arrives to the clinic, is the problem of tissue immune-compatibility [3-7].

Ideally, the cells should have identical human leukocyte antigen (HLA) types as the patient [8]. To address this issue, a variety of methods for generating autologous cells have been proposed, including somatic cell nuclear transfer (SCNT) into unfertilized oocytes and cell fusion. However, its low efficiency and generation of tetraploid cells respectively, have made their implementation problematic [9,10].

With the landmark discovery of induced pluripotent stem cell (iPSC) methodologies, mouse and human immuno-compatible cells can be produced in multiple laboratories using relatively simple protocols. An iPSC is a type of PSC generated by simply introducing a set of transcription factors, OCT-3/4, SOX2, KLF4 and c-MYC (OKSM or Yamanaka factors), into differentiated somatic cells [8]. iPSCs are morphologically similar to ESCs and share their differentiation potential as judged by teratoma formation and contribution to chimeric animals. To date, successful generation of iPSCs has been reported in species such as mouse, human, rat, rhesus monkey, cow and pig [8,11-14]. It is anticipated that within the next 5 to 10 years, new regenerative medicine treatments based on PSCs will reach the clinic, benefiting humans and animals alike.

1.2 The Dog as a Model for Translational Medicine

Governmental regulatory agencies, such as the Food and Drug Administration (FDA) in the USA and the European Medicine Agency (EMA) in the European Union, have begun requiring more stringent preclinical testing for PSC-based therapies. It is anticipated that when iPSC-derived cells are contemplated for use in human patients,

other animal species – in addition to rodents-should be considered as models for cell transplantation.

Lessons learned from canine medicine have extraordinary potential to inform our understanding of human diseases and uncover new therapeutic avenues for treatments. Compared to small animals such as rodents, dogs have a larger body size, a relatively long life span, organ relative positions, a diverse gene pool, and share many biochemical and pathological conditions with humans [15]. A large number of translational medicine studies have been performed in dogs quite successfully. More than five decades of research in dogs provided fundamental breakthroughs in human and veterinary medicine, particularly in the fields of bone marrow transplantation, metabolic diseases, neurological disorders, cancers and heart failure [15-17].

During the 1950's Dr. Norman Shumway performed seminal studies in dogs that culminated with the development of heart transplantation techniques that are today's' standard surgery practice in human [18]. A point example is the dog's heart ventricular physiology and pathology that reflects the human's more accurately than rodents. The dog's heart mirrors the human's time course of irreversible myocardial injury following ischemia, and it has facilitated the development of rescue treatments such as thrombolytic reperfusion [19]. These studies — among many others — established the foundation for current cardiovascular treatment guidelines during acute coronary syndromes [19].

Another unique feature of canine breeds is their genetic diversity, a product of thousands of years of breeding with human intervention, with the concomitant

development of mutations, many of them having a human equivalent, providing a great model to study human genetic disorders. To date, over 400 types of genetic diseases have been identified in dogs, half of them presenting similarity to those in human including cardiomyopathies, muscular dystrophy and prostate cancer [20,21]. An example of specific canine breed model that offers advantages over the mouse is the dog model of spinal cord injury (SCI). Up to 2% of the dogs admitted to the hospital arrive with SCI, 77% of them due to intervertebral disc diseases [22,23]. Chondrodystrophic dogs are particularly susceptible, suffering from SCI following spinal hyperesthesia, non-ambulatory hind limb paraparesis, and complete hind limb paralysis [22-25]. Surgical palliative treatment is a standard therapeutic option in veterinary medicine and protocols for cell transplantation have already been developed. It is expected that this model of SCI in particular will facilitate the testing of more ambitious strategies to cure SCI with a variety of cell types, including iPSCs [24,26].

The application of stem cells to treat conditions in the dog for which there are few, if any effective therapies, and that would ordinarily lead to life-long disability, or a significant impact on quality of life, would not only tremendously benefit the animal recipient, but would also provide us with knowledge to develop parallel treatments in human patients. As such, our long-term objective is to establish the platform for generation, differentiation and transplantation of canine iPSCs (ciPSCs) that would eventually allow us to establish the safety and efficacy of autologous iPSCs in a non-rodent model of human disease. Our short-term goal is to determine the specific requirements for derivation and maintenance of ciPSCs. This particular section of our work has already been published and subsequently replicated by others [27-30]. We

should mention that before our publication on ciPSCs in 2011, there was only one report describing ciPSC generation by Shimada et al [31,32]. However, the briefness and lack of details in their description of ciPSC generation and characterization rendered their work almost irrelevant. We also performed an in depth characterization of the growth factor dependency of ciPSCs and concluded that leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) are both required for maintaining the expression of pluripotency markers in ciPSCs. We also noticed that only LIF is essential for survival of ciPSCs [31]. This observation informed our subsequent experimental design. While our long-term goal remains unchanged, we realized that in order to succeed in differentiating ciPSCs for cell therapy purposes, LIF, the most important cell survival factor, must be removed from the media likely triggering cell death. In lieu of this unanticipated roadblock, we decided to carefully analyze the intracellular signaling pathways active in pluripotent ciPSCs in the presence or absence of growth factors, as well as the different mechanisms of cell death activated in ciPSCs.

1.3 Pluripotency and Stem Cells

Pluripotency is defined as “the capacity of individual cells to initiate all lineages of the mature organism in response to signals from the embryo or cell culture environment” [33]. Studies on cellular self-renewal and pluripotency date back to the work of Dr. Hans Driesch in 1895 on sea urchin embryos [34]. During the 1960s, Pieces et al firstly reported the isolation of embryonic carcinoma cells (ECC) from a testicular teratocarcinoma isolated from a mouse of the 129 strain [35]. ECCs could be induced to

differentiate spontaneously into multiple somatic cell lineages *in vitro,* and when injected into a host embryo, contribute to several tissues in the chimeric pups. However, frequent karyotyping abnormalities, loss of differentiation capability, uneven distribution of differentiated ECCs, and occasional lethality to fetuses due to uncontrollable tumor formation were frequently observed and limited their practical applications [35-38]. Nonetheless, ECCs provided crucial experimental data that would later inform how to derive ESCs from preimplantation embryos. In 1981, two independent groups reported the isolation of mouse ESCs (mESCs) using mouse embryonic fibroblasts (MEFs) as feeder layers or ECC-conditioned medium [39,40]. These cells shared some of the characteristics of ECCs, such as colony morphology, self-renewal capability, expression of cell surface antigens, gene expression profiles and capacity to differentiate into somatic cell types derived from three-germ layers *in vitro* and *in vivo*. More importantly, ESCs had normal karyotypes and better contributed to chimeras, quickly becoming an ideal cellular model of cell differentiation [39]. Another seminal breakthrough took place in 1998 when human ESC lines were established for the first time from human blastocysts, providing another essential tool to study human development [4]. Almost in parallel, several groups began to explore the possibility of using human ESCs (hESCs) in the context of regenerative medicine [4,41].

1.3.1 Mouse and Human Pluripotent Stem Cells

In the mouse, ESCs are derived from the inner cell mass (ICM) of pre-implantation blastocysts [39]. Under *in vitro* culture conditions, a colony of mESCs will form a dome-

shaped 3-D structure, a characteristic that set them apart from mouse ECCs. In terms of cell surface markers, mESCs display a type of glycosphingolipids called Stage-Specific Embryonic Antigens-1 (SSEA-1), originally identified in mouse preimplantation embryos [42]. At the gene and protein expression levels, mESCs express essential core of transcription factors — OCT4, SOX2 and NANOG — that regulate and maintain pluripotency [43]. The leukemia inhibitory factor (LIF), is specifically required to sustain the expression of the core transcription factors [44].

Human ESC lines were first isolated using procedures similar to the mouse. However, they have unique characteristics that make them different from mESCs [4]. Morphologically, they resemble cells from the epiblast in post-implantation blastocysts, unlike the mouse that are more ICM-like cells. They grow in a tightly adherent, flattened monolayer, instead of the typical mouse ESC dome-shaped colony. Finally, they show poor resistance to trypsin cell-dissociation treatments [45]. Similar to mESCs, hESCs express the same core of pluripotency-associated transcription factors, OCT4, SOX2 and NANOG. However, at the global transcription level, hESCs share only (depending on the study) between 13% to 55% of the transcripts expressed in mESCs [46]. In terms of pluripotency markers, hESCs express SSEA-3, SSEA-4 and tumor rejection antigens including TRA-1-60 and TRA-1-81, but not SSEA-1 [47,48]. Perhaps the most striking difference between mouse and hESCs is that the later require basic fibroblast growth factor (bFGF) instead of LIF as the main growth factor for pluripotency maintenance [4].

1.3.1.1 Induced Pluripotent Stem Cells

Mouse iPSCs (miPSCs) were initially derived in 2006 by Shinya Yamanaka's group by overexpressing exogenous *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM) in embryonic and adult fibroblasts [8]. In humans, the first report was published in 2007 by the same group, almost at the same time as Dr. James Thomson's group reported human iPSCs (hiPSCs)using different reprogramming factors, i.e. *OCT4*, *SOX2*, *LIN28* and *NANOG* [49,50]. Since then, modifications have been introduced to the original protocol to avoid the risk of tumorigenesis. Specifically, the proto-oncogene *c-Myc* was first replaced with *v-Myc* and subsequently dropped from the cocktail altogether [51]. In subsequent protocols, *Klf4* and *Sox2* were proven dispensable, albeit requiring the use of a specific type of target cell and to the detriment of efficiency of cell conversion into iPSCs [51,52]. The technique is robust and simple, allowing multiple laboratories around the world to replicate the results. Since first reported, a vast body of literature has been published describing an array of new genes and delivery methods capable of reprogramming cells into iPSCs, including the use of retrovirus, lentivirus, adenovirus, transposons, episomal vectors, mRNAs, and microRNAs [8,49,50,53-66]. The addition of small molecule inhibitors has been proven efficacious in conjunction with other reprogramming protocols. In the mouse, these include inhibitors targeting certain pathways (MEK inhibitor PD0325901 or glycogen synthase kinase3 inhibitor CHIR99021) or epigenetic modifiers (DNA methytransferase inhibitor 5'-AZA, histone deacetylase inhibitor valproic acid or trichostatin A) [67-69].

The culture conditions and growth factor requirements for iPSCs, once the initial conversion into iPSC takes place, are the same as that for ESCs, i.e. LIF for mouse and

bFGF for human. In the human, however, when PD0325901, CHIR99021 and forskolin were used along with the Yamanaka factors, LIF-dependent naïve iPSCs that resemble miPSCs have been reported [70].

Perhaps the most promising methods developed thus far are those that call for the use of small molecules only, bypassing the need for any type of foreign recombinant DNA or RNA. A recent study shows that mouse fibroblasts can be reprogrammed into iPSCs by simply exposing the cells to a cocktail of small molecule inhibitors without overexpressing any exogenous transcription factors [71]. The core inhibitor in this methodology is DZNep, which blocks histone methyltransferase EZH2, significantly enhancing the expression of Oct4 in mouse fibroblasts.

A variety of mouse and human somatic cells have been tested for the capacity to be reprogrammed, including embryonic and adult fibroblasts, neural stem cells, adipose-derived cells, cord blood cells, mesenchymal stem cells, B and T cells, and keratinocytes [8,49,50,52,61,72-75]. It appears that no somatic cell is incapable of reprogramming; however, some of them are more resistant to the process than others. For all practical purposes, dermal fibroblasts remain the main choice in both species.

Despite this increase in the number of reprogramming strategies, at the time of this writing, the original protocols described by Yamanaka and Thomson's groups using the culture conditions optimized for mESCs and hESCs continue to be the most reliable and the standard methods against which novel reprogramming schemes are tested.

1.3.1.2 Characterization of iPSCs

Similar to ESCs, molecular markers, specific gene expression profiles and differentiation potential are used to characterize iPSCs. Karyotype analysis is of particular importance in iPSCs, since they divide rapidly and there is a tendency for abnormal duplication and distribution of chromosomes that may cause tumorigenesis and a loss of differentiation capability. Microsatellite genomic sequencing assays are commonly used to verify the identity of the iPSCs.

Assessing the differentiation capability of iPSCs is as important as the presence of specific pluripotency-related markers. The most common *in vitro* method is embryoid body (EB) formation, in which iPSCs are cultured in non-adherent tissue culture plates in the absence of bFGF or LIF (human and mouse, respectively) for a short period, followed by two weeks of culture in tissue-cultured treated dishes in the presence of fetal bovine serum (FBS). Bona fide iPSCs should be capable of spontaneous differentiation and display markers representing the three germ layers, including ectoderm, mesoderm and endoderm [39]. The most common *in vivo* differentiation test is the teratoma formation assay. Initially used in mESC thirty years ago, the teratoma assay has become a routine test for human and mouse iPSCs [40]. Simply by injecting undifferentiated cells into immune compromised mice and allowing them to spontaneously grow and differentiate, 3-D structures representative of cells and tissues derived from the three germ layers can develop. The most informative differentiation test for miPSCs, though, is the chimera assay, in which undifferentiated cells are injected into a fertilized or, preferably, a tetraploid embryo, and further allowed to

develop to term in a surrogate female. The level of chimerism in the offspring is normally positively correlated with the pluripotency level of the injected cells [76-79].

1.3.2 Canine Pluripotent Stem Cells

The derivation of canine ESCs (cESCs) has been more difficult than previously thought, and only five groups have succeeded in establishing cESC or ESC-like cell lines from canine blastocysts. Some of the reported cell lines are no longer available [80-84]. All reports characterized canine ESC's pluripotency using molecular markers and *in vitro* differentiation [80-84]. However, only one, by Vaags et al, showed convincing *in vivo* differentiation results [82]. In their study, cESCs displayed mixed cell morphology with 3-D dome-shape colonies and monolayer-like colonies. The cells expressed the core pluripotency markers, including OCT4, SOX2 and NANOG, and the surface markers SSEA-3, SSEA-4, and TRA-1-60 but without SSEA-1 expression, similar to the markers expressed by hESCs. The cESCs were capable of differentiation toward cell derivatives of the three-germ layers *in vitro,* and ,more importantly, they were able to differentiate *in vivo* when injected into the kidney capsule of immune-deficient mice. cESCs have also been efficiently differentiated into specific cell lineages, including endothelial cells, cardiac myocytes, hepatocytes, neural stem cells, and endodermal cells. A unique feature of cESCs that sets them apart from human and mESCs is the requirement of growth factors LIF and bFGF to maintain pluripotency. While the signaling pathways of LIF and bFGF in the mouse and human, respectively, have been characterized (see following section), little is known about the synergistic

functions of the two factors applied simultaneously to cells *in vitro* for pluripotency and survival maintenance. There has been a description of crosstalk between the survival-associated signaling pathways regulated by LIF or bFGF: activation of both AKT and ERK1/2 can be triggered by LIF or bFGF. But in comparison, the activation of JAK-STAT3 signaling transduction axis is exclusively limited to the presence of LIF, not bFGF [85]. A more comprehensive characterization of LIF and bFGF pathways acting together in maintaining survival and pluripotency in ESCs/iPSCs is needed.

1.3.3 Growth Factors and Associated Signaling Pathways in PSCs

In 1988, Austin Smith's group reported that LIF was critical for maintenance of mouse ESC's self-renewal [44]. LIF is a member of IL-6 family. Its LIF receptor is a heteromeric complex composed of two types of transmembrane proteins, the LIF receptor (LIFR) and the gp130. In the presence of these two components, LIF binds to LIFR. And the LIF receptor-associated tyrosine kinase, Janus kinase (JAK), phosphorylates Y765/812/904/914 of the intracellular domain of gp130 and Y976/996/1023 of LIFR, which further recruits and phosphorylates the signal transducer and activator of transcription 3 (STAT3) [85]. Phosphorylated STAT3 targets and promotes the expression of a variety of genes associated with pluripotency and survival, including *c-Myc* and *Klf4*. Besides maintenance of mESCs in a highly undifferentiated state in culture through STAT3, LIF can also activate the PI3 kinase/AKT pathway. Phosphorylation of AKT proteins can modulate the function of numerous substrates, including the mammalian target of rapamycin (mTOR), and elicit proliferation and

suppression of cell death [85]. LIF is also able to robustly activate the Ras/ERK1/2 canonical signaling cascade, triggering the phosphorylation of a series of early transcription factors, including c-Jun and c-Fos, which are critical for maintaining cell viability and proliferation [85,86].

Striking differences exist on signaling pathways involved in pluripotency maintenance between the mouse and human ESCs and iPSCs. Unlike mESCs, the activation of STAT3 is dispensable for hESCs' pluripotency maintenance and survival, with bFGF required instead [87]. bFGF not only exerts its role on human PSCs directly, but indirectly through the feeder layer typically MEF, stimulating the release of activin-A (ActA) that in turn binds to the TGF-beta receptors in hESCs, triggering the activation of intracellular SMAD2/3 pathway. Phosphorylated SMAD2/3 positively modulates NANOG transcription, maintaining pluripotency [88]. In terms of cell survival, bFGF binds to its specific receptor and leads to the auto-phosphorylation and activation of PI3K/AKT and Ras/ERK1/2 signaling cascades, enhancing survival of hESCs [85,88]. The pro-survival role of bFGF via activating AKT and ERK1/2 pathways ubiquitously exists throughout all kinds of cell types [85].

1.3.4 Consequences of a Poor Understanding of Cell Survival in Canine PSCs

A fundamental aspect to consider when trying to understand survival of ESCs and iPSCs is whether these cells are undergoing any type of cell death that may be indicative of an inadequate *in vitro* culture system.

When cultured under normal conditions, hESCs undergo spontaneous apoptosis at a rate of 30%. This rate increases to 40% when hESCs are allowed to spontaneously differentiate in normoxic conditions [89,90]. Moreover, and in contrast with differentiated cells, both mouse embryos and mESCs cultured *in vitro* display hypersensitivity to DNA damage [91,92]. These observations support the notion that pluripotent cells generally seem to have to have a low tolerance to cellular stress and ultimately undergo cell death.

We have made the observation that ciPSCs seems to have an increased susceptibility to cell death when LIF is removed from the culture medium (described in Chapter 2). This observation, coupled with the fact that two major types of cell death — apoptosis and necrosis — were previously reported in pluripotent stem cells prompted us to investigate further the mechanisms involved, with the short term goal of increasing cell viability [93,94].

Apoptosis, also known as programmed cell death, is characterized by morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, and nuclear/DNA fragmentation [95]. Apoptosis can be triggered by a number of different stimuli, such as direct DNA damage, oxidative stress, upregulation of a death receptor, developmental programming (during embryonic development) or infection by a pathogen [95]. Depending on the stimuli and the molecular pathways involved, apoptosis can be mitochondrial- or receptor-mediated. In the mitochondrial pathway, the death stimulus induces the activity of the pro-apoptosis BCL-2 family proteins localized in the mitochondrial membrane, subsequently causing leakage of

cytochrome-C and activating the apoptosis effector caspase family of proteins. Caspase-9 is initially activated, and the cleavage of caspase-9 further triggers caspase-3 cleavage [96]. Caspase-3 is responsible for inducing endonucleases, which ultimately cause DNA fragmentation [96]. In the receptor-mediated apoptotic pathway, a 'death peptide' such as CD95-ligand binds to its receptor and specifically triggers the activity of downstream caspase-8 by cleaving it. Activated caspase-8 also cleaves caspase-3 directly and transduces the signal to activate members of the BCL-2 family of proteins to cause cell death through the mitochondrial pathway [96]. Both pathways share caspase-3 cleavage followed by DNA fragmentation. Caspase-8, however, is unique for the receptor-mediated apoptosis pathway.

Another type of cell death is necrosis, also called or non-programmed cell death. Compared with apoptosis, necrosis is characterized by swelling of the dying cells, rupture of the plasma membrane, and release of the cytoplasmic content into the extracellular environment. Necrosis-like cell death is commonly observed in many pathological conditions such as stroke, ischemia, and several neurodegenerative diseases [95]. It occurs when cellular injury is associated with a loss of ion homeostasis and drastic decreases in ATP levels. An essential feature of the necrosis is the loss of the cell membrane integrity. More recently, a growing body of evidence indicates that necrosis can occur under normal physiological conditions during development by regulated mechanisms as well [97].

In Chapter 3 we focus on both types of cell death, apoptosis and necrosis, in ciPSCs.

1.4 Rationale and Hypotheses

The clinical application of innovative, safe and efficient treatment options based on pluripotent stem cells depends upon the availability of reliable animal models. Autologous iPSCs generated and characterized in non-rodent models — more similar to human – can offer a better preclinical evaluation of safety and efficacy. The long-term goal of our study is to establish the platform for generation, maintenance, differentiation and transplantation of ciPSCs. Our short-term goal was to generate ciPSCs from canine somatic cells. We expected to harness the knowledge gained during the development of mouse and human ESCs and iPSCs and apply it towards our goal [8,50,82]. As we progressed toward these goals, we encountered roadblocks that challenged us to elaborate hypotheses to address such unknowns.

First we hypothesized that the ciPSCs can be generated based the similar reprogramming system for generating human or mouse iPSCs. We successfully achieved our first goal of deriving ciPSCs and proceeded with the implementation of direct differentiation strategies (Chapter 2). Unbeknown to us was the fact that dog ciPSCs were bFGF and LIF dependent, which would make differentiation more difficult than expected. As such we hypothesize that inactivation of a LIF-dependent/bFGF-independent pathway is solely responsible for the cell death of ciPSCs. Subsequently, we undertook studies on the pro-survival effect of LIF on ciPSCs and determined the activation status of LIF-associated pathways in ciPSCs (Chapter 3). These works

provide the foundation for future experiments aimed at developing canine cell replacement therapies described in more detail in Chapter 4.

CHAPTER 2

GENERATION OF LIF AND BFGF-DEPENDENT INDUCED PLURIPOTENT STEM CELLS FROM CANINE ADULT SOMATIC CELLS

2.1 Abstract

For more than fifty years, the dog has been used as a model for human diseases. Despite efforts made to develop canine embryonic stem cells, success has been elusive. Here, we report the generation of canine induced pluripotent stem cells (ciPSCs) from canine adult fibroblasts, which we accomplished by introducing human OCT4, SOX2, c-MYC, and KLF4. The resultant ciPSCs expressed critical pluripotency markers and showed evidence of silencing the viral vectors and normal karyotypes. Microsatellite analysis indicated that the ciPSCs had the same profile as the donor fibroblasts, but differed from cells taken from other dogs. Under culture conditions favoring differentiation, ciPSCs could form cell derivatives from the ectoderm, mesoderm, and endoderm. Further, ciPSCs required LIF and bFGF to survive, proliferate, and maintain pluripotency. Our results demonstrate an efficient method for deriving canine pluripotent stem cells, providing a powerful platform for the development of new models for regenerative medicine and for the study of the onset, progression, and treatment of human and canine genetic diseases.

2.2 Introduction

Embryonic stem cells (ESCs) were first reported in mice, then in nonhuman primates, humans, rats, and dogs [4,40,82,98,99]. ESCs have the capacity to renew themselves and to differentiate into all cell types found in adult bodies. While ESC availability has made possible new kinds of developmental and regenerative medicine studies, tissue rejection and immune-compatibility after transplantation remain as obstacles to their clinical use. Researchers have proposed several alternative methods of reprogramming somatic cells to solve this problem, including somatic cell nuclear transfer (SCNT) into unfertilized oocytes and somatic cell fusion with ESCs to attain pluripotency [9,10]. However, a lack of reliable sources of oocytes and the generation of tetraploid cells, respectively, have made their implementation in humans problematic [100]. Success in deriving induced pluripotent stem cells (iPSCs) using a set of transcription factors — such as OCT3/4, SOX2, KLF4, and c-MYC (Yamanaka factors), or OCT4, SOX2, NANOG and LIN28 — into differentiated somatic cells may address the immune rejection problem [8,50]. iPSCs are similar to ESCs in morphology, proliferation, and pluripotency. Successful generation of iPSCs has been reported for mice, humans, rats, monkeys, and pigs [8,12,13,101]. While the use of iPSCs in basic research is moving forward, their use as a therapeutic tool remains a challenge, mostly due to the lack of appropriate animal models for testing their efficacy and safety.

For more than thirty years, the dog has provided a valuable model for human diseases, particularly in the study and implementation of cell-based therapy protocols

[102]. Over 400 dog breeds show a high prevalence of more complex multigenic diseases [21,103]. Approximately 58% of dog genetic diseases resemble the specific human diseases caused by mutations in the same gene [20,21]. Also, dogs share a variety of biochemical and physiological characteristics with humans; their physiologies, disease presentations, and clinical responses often parallel those of humans better than do those of rodents [21,82]. This underscores the dog's importance as a reliable preclinical model for testing the feasibility of regenerative medicine and tissue engineering approaches to treat its own diseases and those of man.

The distinct reproductive physiology and embryonic development of dogs and the difficulty of deriving their ESCs has blocked the establishment of the canine model for further regenerative medicine studies. The lack of well-defined methods for maturing and fertilizing canine oocytes in vitro has narrowed the choices for harvesting ESCs from natural canine blastocysts [80,104,105]. Only one group has successfully established a bona fide canine ESC line. The scarcity of published data is likely due to poor understanding of canine preimplantation embryonic development and canine embryo culture conditions [80,81]. Recently, a report on the derivation of induced ESC-like cells described the source of donor cells as embryonic fibroblasts. The evidence demonstrating complete reprogramming to pluripotency in such cells is succinct, making the results — while promising — incomplete [106]. We still need an efficient, safe and well-described method for generating canine iPSCs (ciPSCs).

Here, we report the production of iPSCs from adult canine cells using a method like that described for human and mouse iPSCs [8,107,108]. We systematically showed

the degree of pluripotency of the generated lines, explored their capacity for stable maintenance, and assayed their ability to form embryoid bodies (EBs) and to differentiate into multiple cell lineages. We also noticed that the ciPSCs demonstrated dependency on both leukemia growth factor (LIF) and basic fibroblast growth factor (bFGF) to maintain self-renewal. The ciPSC lines described here reveal similarities and differences between canines and other species and reveal ciPSCs as a unique new tool for future application to, and understanding of, analogous conditions in humans.

2.3 Material and Methods

2.3.1 Derivation of Canine Fibroblasts and Cell Culture

Fibroblasts (CTFs) were derived from the testicle of a seven-month-old German shorthair pointer undergoing routine castration at the Veterinary Medical Center at Michigan State University. The testis was minced and incubated in trypsin (Gibco, Carlsbad, CA) at 37ºC for one hour. Then, shredded tissues were centrifuged, minced again, and subsequently cultured with fibroblast medium (DMEM containing 10% fetal bovine serum (FBS)) at 37ºC with 5% CO2 [107]. We replaced the culture medium every 24 hours. All ciPSCs were generated from CTFs older than passage two.

We maintained ciPSCs on the feeder layer of mitomycin-treated or irradiated mouse embryonic fibroblasts (MEFs) with ciPSC medium, which consisted of DMEM/F-

12 (Gibco, Carlsbad, CA) supplemented with 15% (v/v) knockout serum (Gibco, Carlsbad, CA), 0.1 mM MEM nonessential amino acid solution (Sigma, St. Louis, MO), 1 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.075 mM β-mercaptoethanol, 4 ng/mL human bFGF (Invitrogen, Carlsbad, CA), and 10 ng/mL human LIF (Millipore, Billerica, MA). Colonies with compact ES-like cells were mechanically isolated and subcultured onto new MEFs every four to six days using glass Pasteur pipettes.

2.3.2 Virus Construction and Production

We produced and concentrated recombinant OKSIM lentivirus, as previously described [107,108]. Canine fibroblasts were assessed for infection efficiency with recombinant lentivirus using a pSIN-EF1a-YFP reporter gene. We rated lentiviral infection by quantifying the percentage of yellow-fluorescent cells determined to be identical in infectivity to human fibroblasts. Concentrated OKSIM lentivirus was directly titered by infecting canine fibroblasts followed by immunostaining for OCT4 gene product at 72 hours. The OKSIM viral titer was approximately 3X105/mL, and 0. 5 mL (in triplicate) was used to infect $2.5X10^5$ canine cells for iPSC production.

2.3.3 Immunocytochemistry Assay

The immunocytochemistry assay protocol was mostly as described in previous reports [6,107,108]. Table 2.1 lists details about the primary and secondary antibodies

used for some proteins. After washing the cells with phosphate-buffered saline (PBS), we then stained the nuclei by rinsing the cells with PBS containing Hoechst 33342 (1μg/mL) for 15 minutes.

2.3.4 RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

RNA was isolated and purified using the NucleoSpin RNA XS Total RNA Isolation Kit (Macherey-Nagle, Bethlehem, PA), following the manufacturer's instructions. We performed the RT-PCRs as previously described [107,108]. Table 2.2 lists the primers used.

2.3.5 Bisulfite Genome Sequencing

Approximately 20,000 cells from ciPSC colonies or CTFs were collected and kept at -80°C until needed. We extracted canine genomic DNA using the ReadyAmp Genomic Kit (Promega, Madison, WI) and conducted bisulfite mutagenesis using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to the manufacturers' instructions. Bisulfited DNA was eluted in 20 μL elution buffer and subjected to two rounds of PCR (35 cycles each) with primer pairs for canine OCT4 and NANOG promoters. Primers were designed based on randomly chosen sequences localized at the OCT4 and NANOG promoters close to the initiators [109-111] (Table. 2.3). We

verified PCR products on a 2% agarose gel. We ligated PCR products into the pTOPO 10 Vector System (Invitrogen, Carlsbad, CA) and randomly chose more than ten clones from each cell line to sequence.

2.3.6 Karyotyping Analysis

Twenty G-banded metaphase cells were subjected to cytogenetic analysis for each cell line. Cell Line Genetics (Madison, WI) performed standard G-banding karyotype analysis.

2.3.7 Microsatellite Assay

We used the following tetranucleotide microsatellite markers, each located on a separate autosome, for genotype analysis: FH2054, FH2165, FH2233, FH2313, and FH2324. We obtained primer sequences for these markers from Mellersh et al. [112]; and the allele frequencies, derived from over 1000 dogs from 28 dog breeds, from Irion et al. [113]. Amplified fragments were fluorescently labeled with 6-FAM using chimeric primers and a labeled M13 primer [114]. We amplified all markers in 25 µL reactions under the following conditions: 50 mM KCl, 10 mM Tris (pH 8.3 at 20ºC), 1.5 mM MgCl2, 100 µM dNTPs, 0.1 µM M13 and reverse primers, 0.01 µM chimeric primer, 10–100 ng DNA, and 0.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA). Reactions were cycled under the following conditions: 1 min, 94°C, 2 min 59°C, and 3 min 72°C,

for 50 cycles. Amplification was verified by imaging agarose gels on a Typhoon scanner (Amersham Biosciences, Piscataway, NJ), and performed high-resolution fragment analysis on an ABI PRISM 3130 Genetic Analyzer at the Michigan State University Research Technology Support Facility. We calculated the probability that the samples derived from an unrelated dog genome that, by chance, had identical allele sizes with the CTF-derived cell lines using the allele frequencies obtained from Irion et al. (taking into account the size of the M13 tail for the comparisons) [113]. To produce a conservative probability, we assumed that the allele size between our data and that of Irion et al. could be one repeat unit off, so we used the most frequent allele of the three possible alleles (the determined allele size, plus or minus one repeat unit) from Irion et al. for each calculation [113].

2.3.8 EB Formation

We isolated ciPSC colonies from the MEF and transferred them to ciPSC medium without bFGF or human LIF in 35x10 mm Petri dishes. After five days in suspension, we transferred the EBs to tissue culture dishes coated with 0.1% gelatin (Sigma, St. Louis, MO, St. Louis, MO), culturing them using the same medium without growth factors, with 5% FBS (Gemini, West Sacramento, CA) and 10% serum replacement. The culture medium for suspension and subsequent spontaneous differentiation was partially changed daily. We cultured the attached EBs in the differentiation media for at least three weeks.

2.3.9 Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay

We washed cells with PBS and fixed them in 4% paraformaldehyde for 15 minutes. We performed TUNEL assays using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) following manufacturer instructions. As positive control, cells were treated with RQ1 DNase (Promega, Madison, WI, 10 IU/mL). After washing in PBS, we counterstained all nuclei with Hoechst 33342 (1µg/mL) for ten minutes at room temperature.

2.3.10 5-Bromo-2-Deoxyuridine (BrdU) Incorporation Assay

We cultured cells overnight with 30 µg/mL of BrdU before immunostaining. We described the BrdU incorporation assay protocol in a previously published report [6]. The nuclei were counterstained with Hoechst 33342 (1µg/mL) for five minutes at room temperature.

Table 2.1 Antibodies for immunocytochemistry assay.

Antigen	catalog#	Isotype	Manufactor	Concentration
OCT4	SC8628	Goat IgG	Santa Cruz Biotechnology	1:300
SOX2	AB5603	Rabbit IgG	Abcam	1:500
NANOG	SC33759	Rabbit IgG	Santa Cruz Biotechnology	1:500
LIN-28	67266	Rabbit IgG	Santa Cruz Biotechnology	1:250
SSEA-4	MC813-70	Mouse IgG	Abcam	1:500
TRA-1-60	09-0009	Mouse IgG	Stemgent	1:250
Fibronectin	HFN7. 1	Mouse IgG	Abcam	1:500
TUJ1	14545	Mouse IgG	Abcam	1:7000
Vimentin	AMF17B	Mouse IgG	Developmental Studies Hybridoma Bank	1:250
AFP	SC8108	Goat IgG	Santa Cruz Biotechnology	1:500
Cy-3 Goat anti-Mouse IgG + IgM	09-0038	N/A	Stemgent	1:1000

Table 2.1 (cont'd)

Alexa 488 Donkey anti-mouse IgG	A21207	N/A	Invitrogen	1:1000
Alexa 488 Donkey anti-goat IgG	A11055	N/A	Invitrogen	1:1000

Table 2.2 Primers designed for qRT-PCR.

primers		sequence
RPL13	forward	GGAGAAGGCCAGAGTCATCACA
	reverse	TTTGCCCTGATGCCAAAAAG
OCT	forward	ACGATCAAGCAGTGACTATTCG
	reverse	GAGGGACTGAGGAGTAGAGCGT
NANOG	forward	CTAGGGACCCTTCTCCAATGC
	reverse	CATTGGCAAGGATGCAGGAT
TERT	forward	TTACAGAGCATAGGAATCAGACAACTC
	reverse	GGTGTCTCCTGACCTCTGCTTCT
SOX2	forward	AACCCCAAGATGCACAACTC
	reverse	CGGGGCCGGTATTTATAATC
c-MYC	forward	GGACGCGCAGAGGCTCTACC
	reverse	GGTTTCCACTCTCCGGAGGAG
LIN-28	forward	CCACCCCAGCCCAAGAA
	reverse	CAGTGGACACGAGGCTACCA
SOCS3	forward	CGAGAAGATCCCTCTGGTGTTG
	reverse	TTTCTCGTAGGAGTCCAGGTG

Table 2.2 (cont'd)

GBX2	forward	AAGGCTGGCAATGCCAATT
	reverse	TGACTTCTGATAGCGAACCTGC
FOXD3	forward	GCAGAGCCCGCAGAAGAAG
	reverse	GGGAAGCGGTTGCTAATGAA
O-K	forward	TCTCCCATGCATTCAAACG
	reverse	GTGGAGAAAGATGGGAGCAG
NESTIN	forward	AGCCCTACTTCCCTCTCCTT
	reverse	CTGAAGTGTGGGCGGGATGGGG
NEFL	forward	GGAAACTCTTGGAAGGTGAGGA
	reverse	TAACCCACCATAGGCAGATCG
CD34	forward	CCTACAACAGCACCAGCCTTGT
	reverse	CCGGAACATTTGATTTCTCCCT
GATA2	forward	GCCACTGACCATGAAGAAGGAA
	reverse	ACAGCTCCTCAAAGCACTCTGC
CXCR4	forward	ACTCCATGAAGGAACCCTGCTT
	reverse	TGCCCACTATGCCAGTCAAGA
AFP	forward	CTGAAAACCCTCTTGAATGCCA
	reverse	TTTCTGGAAGAGGCCACAGCT
CDX2	forward	CCAAGTGAAAACCAGGACGAA
	reverse	CGGATGGTGATGTAACGACTGT

Table 2.3 Primers designed for canine NANOG and OCT4 promoter regions in bisulfite genomic sequencing. “out” or “in” stands for that the primers were designed for the amplification of the outer or inner region in nested genomic PCR. “F” or “R” stands for the forward or reverse primers.

dNANOG1outF	GTATTTTTGATTTTAAAGGATGGA
dNANOG1outR	AAAACCTCCACATATAAAAAATAAA
dNANOG1in F	TAGAAATATTTAATTGTGGGGTT
dNANOG1in R	CATATAAAAAATAAAAAAAAAACAAAAT
dOCT41out F	ATATAGGGAGGAGTGTTTAGGTTA
dOCT41in F	GAGGAGTGTTTAGGTTATTTTAT
dOCT41in R	CTCAACACCTCTCTCCCTCC
dOCT41out R	AAAAACTCTCCTAAAAACTACTCAA
dOCT42out F	AGGTTAGTGGGTGGGATTGG
dOCT42in F	AGGTGTTGAGTAGTTTTTAGGAGA
dOCT42in R	ACTCCCACCTAAAATCCACAATA
dOCT42out R	CCTTAAAACAACAACCCCACTC

2.4 Results

2.4.1 Generation of ciPSCs

We derived CTFs from canine testicular tissue, as described (Figure 2.1A). The infection efficiency of recombinant lentivirus was initially examined in CTFs and canine skin fibroblasts (CSF) from an old (> ten passages) canine fibroblast line derived from another dog, using a yellow fluorescent protein (YFP) reporter vector. Infection efficiency, shown by YFP, was over 75% in both CTFs and CSFs (Figure 2.2). The CTFs and CSFs were then infected by lentivirus OKSIM which had been used previously to generate human iPSC lines [107]. We confirmed successful introduction of OKSIM 72 hours postinfection by immunostaining for OCT4 and SOX2 transgenes; 40% of the target cells carried the virus (Figure 2.3). To understand the best conditions for reprogramming, we added different concentrations of LIF (1 ng/mL or 10 ng/mL) or bFGF (0.4 ng/mL or 4 ng/mL). No ESC-like colonies were observed when using LIF or bFGF alone ten days postinfection (Figure 2.4). However, when both LIF and bFGF were supplied, we observed ESC-like colonies on day six to eight postinfection (Figure 2.1 B and Figure 2.5 F). From two independent infections, two (DI-A1 and DI-A2) and five (DI-B1, DI-B2, DI-B3, DI-B4, and DI-B5) cell lines were derived and passed to new MEFs (Figure 2.1 C-D). Three to four days after the first passage, the morphology of the colonies in all cell lines resembled human ESCs (Figure 2.1 D-F; Figure 2.5 A-E). All

seven cell lines proliferated at similar rates and required subculturing at 1:6 dilution ratios every five days. We chose the DI-B2 iPSC line to characterize growth rate. The ciPSC doubling time at passage five (P5) took 27 hours, compared with the CTFs at P5, which doubled in 43 hours. Beyond that ciPSCs require both LIF and bFGF, these results demonstrate that ciPSC can be generated and maintained using a protocol similar to the one used to derive human iPSCs.

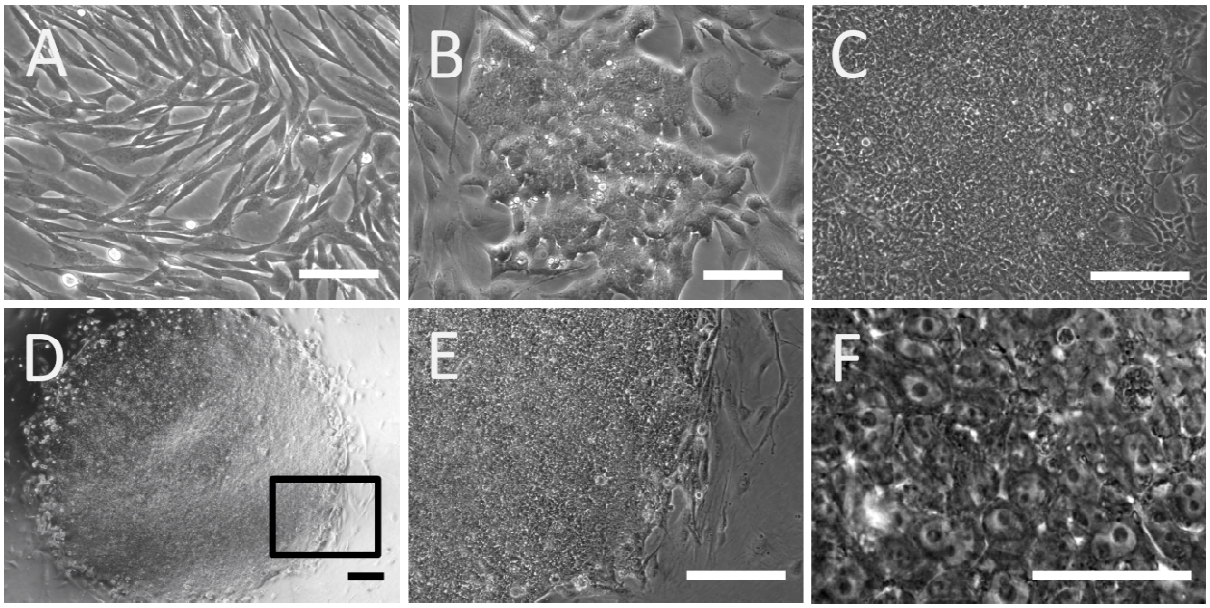


Figure 2.1: **Induction of ciPSCs from adult canine testicular fibroblasts.** (A) Input CTFs; (B) a typical first-observed ciPSC colony on day 6 after lentiviral-mediated transduction; (C) ciPSC colony on day 9 after viral transduction; (D) ciPSC colony (DI-A2) after being passaged on the feeder layer of MEFs; (E) ciPSC colony on MEF with 10X objective; (F) ciPSCs with 40X objective. (Scale bar: 100 μm for A and B; 250 μm for C, D and E; and 25 μm for F)

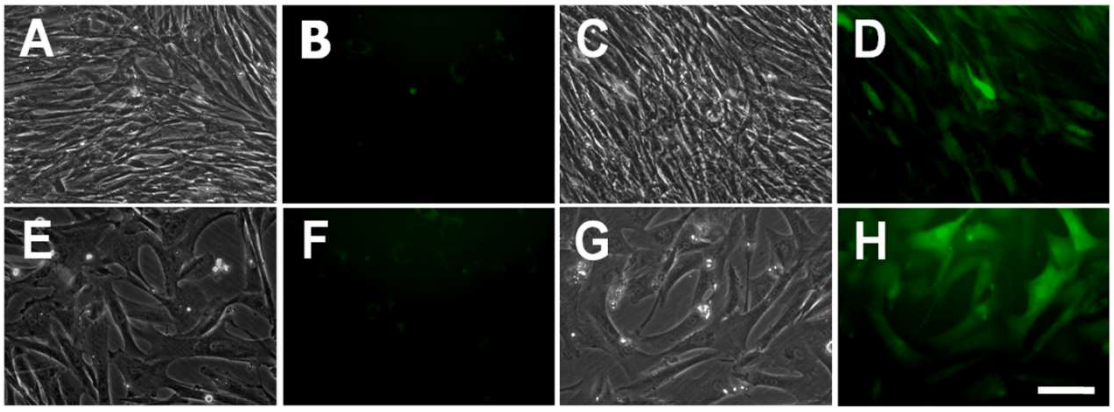


Figure 2.2: **Lentiviral infected canine fibroblasts show YFP expression.** (*A-B*): Uninfected CSFs, (*C-D*): infected CSFs, (*E-F*): uninfected CTFs, (*G-H*): infected CTFs. (Scale bar: 100 μm)

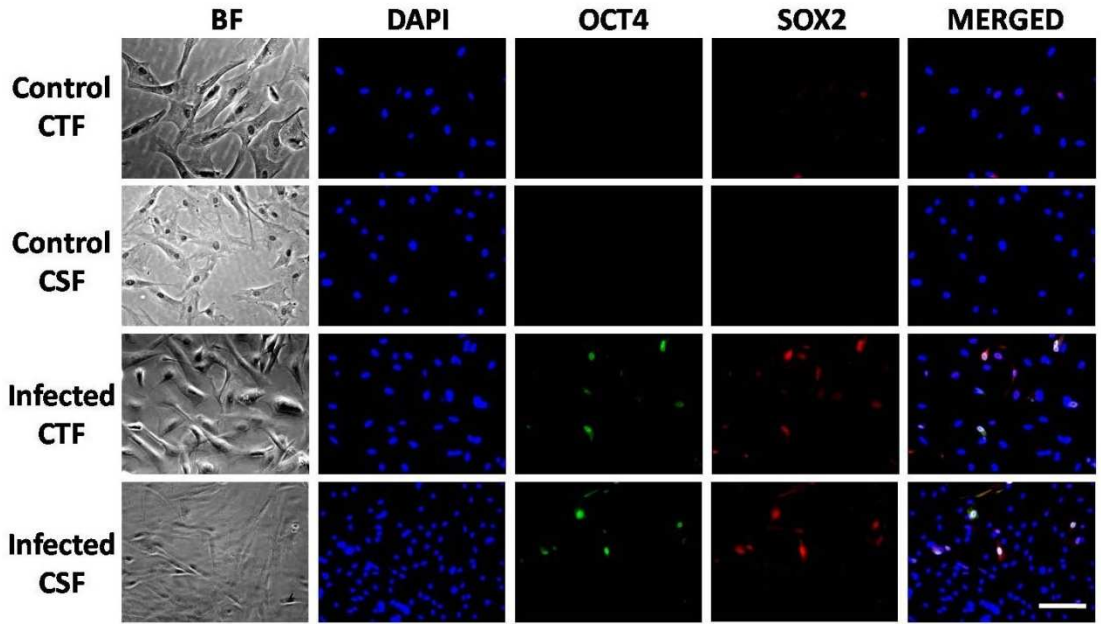


Figure 2.3: **Immunocytochemistry of human OCT4 and SOX2 after transduction.** CTFs and CSFs on day 3 after viral-transduction partially express introduced genes (human OCT4 and SOX2), while the uninfected CTFs and CSFs remain negative after immunostaining. The DNA was labeled by DAPI staining. (Scale bar: 250 μm)

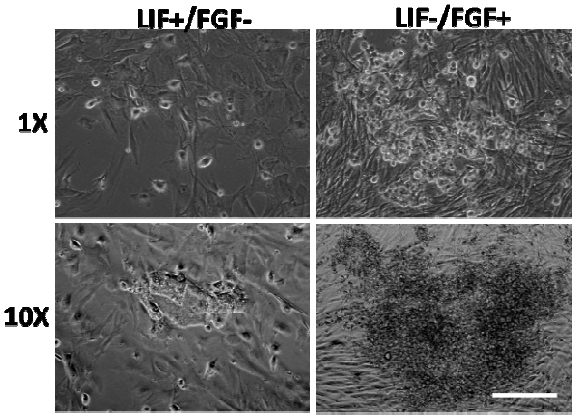


Figure 2.4: **LIF and bFGF dependency of ciPSCs.** Morphology of the canine donor cells on day 10 after viral infection based on different treatments of growth factors. The cells were cultured respectively with human LIF (LIF+) or bFGF (FGF+) in concentrations of 1X (10 ng/mL for human LIF and 4 ng/mL for bFGF) or 10X (100 ng/mL for human LIF and 40 ng/mL for bFGF. Scale bar: 250 μm)

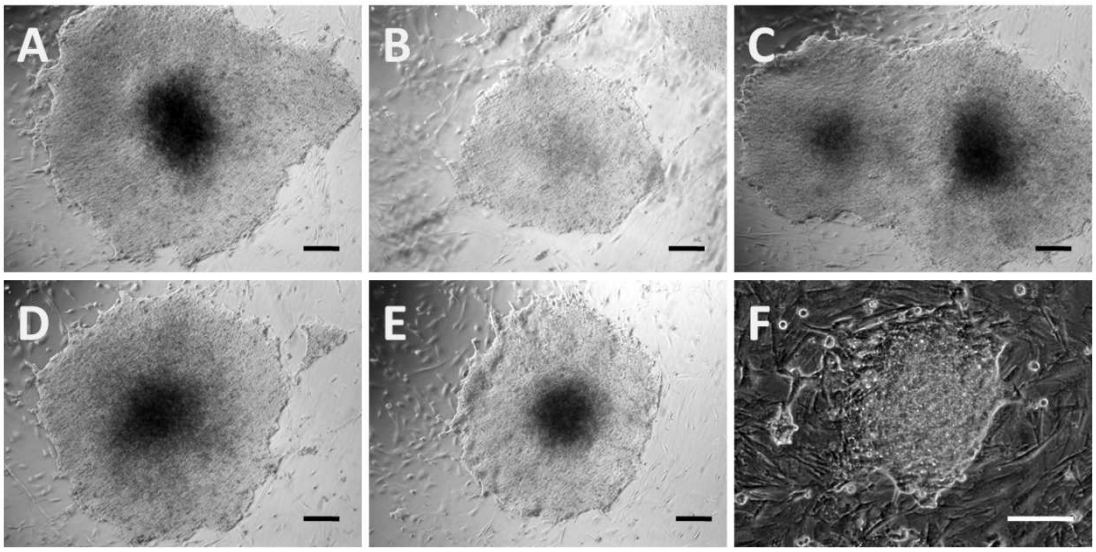


Figure 2.5:**The ciPSCs derived from the second batch of donor fibroblasts.** (A-E) The typical ciPSC colonies from cell line DI-B1 to DI-B5 at P1; (F) The first colony of cell line DI-B2 on day 9 post viral transduction. (Scale bar: 100 μm)

2.4.2 Immunocytochemistry Assay

The expression of pluripotency-associated transcription factors OCT4, SOX2, NANOG, and LIN28 was positively displayed in ciPSC colonies; they were also positive for carbohydrate antigens TRA-1-60 and SSEA-4 (Figure 2.6 A-D; Fig. 2.7). In contrast, the parental CTF cells expressed fibroblast markers, including fibronectin and vimentin, while pluripotency markers were not detected (Figure 2.6 E; Figure 2.7).

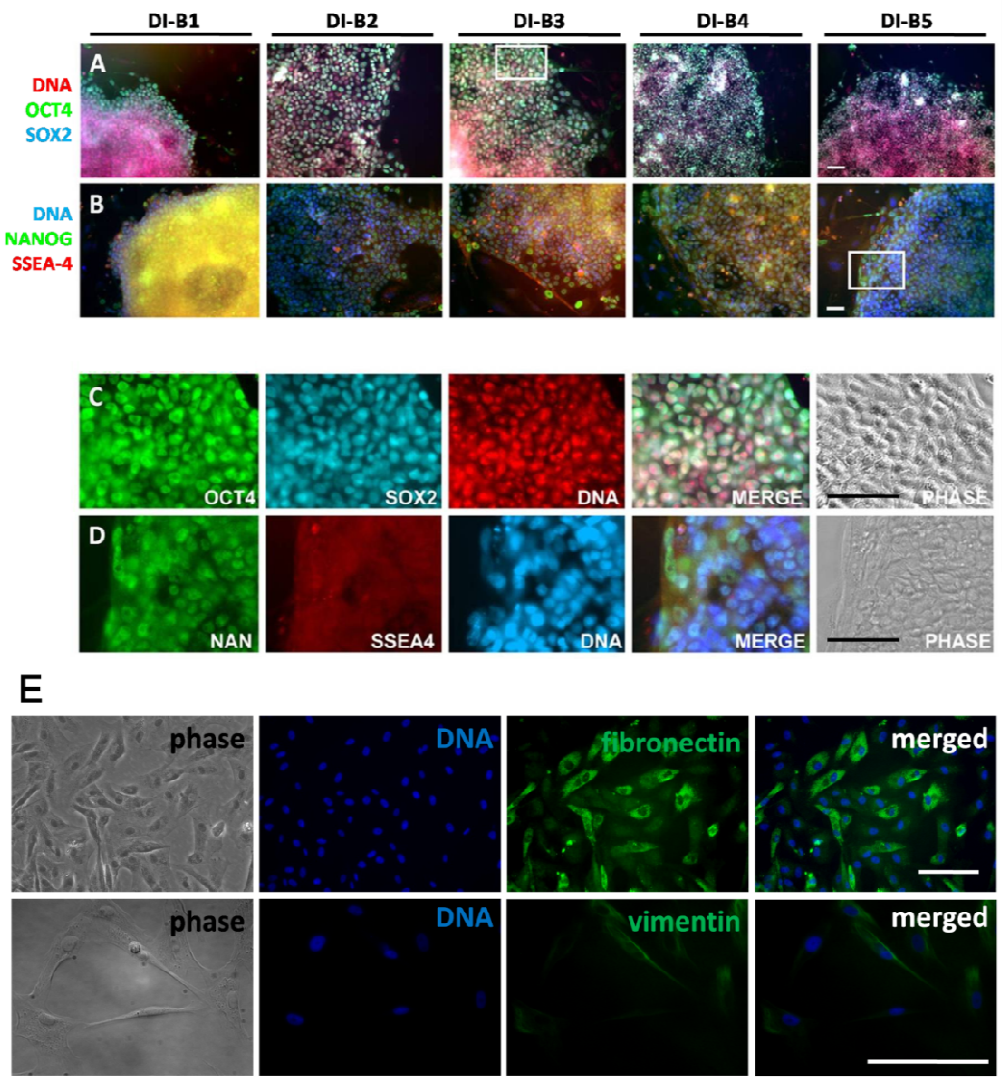


Figure 2.6: **Immunocytochemistry of ciPSCs.** (A–D) Showing immunofluorescent staining of pluripotent cell markers OCT4, SOX2 (line A), NANOG, and SSEA-4 (line B) in five cell lines cultured on MEFs (line A and line B, from left to right: DI-B1, DI-B2, DI-B3, DI-B4 and DI-B5). Localizations of nuclei were visualized by staining with propidium iodide (lines A and C) and DAPI (lines B and D). Localizations of representative cells in lines C and D were chosen, respectively, from the frames in lines A and B. (E) CTFs express fibroblast markers, including fibronectin (upper line) and vimentin (lower line). (Scale bar: 100 μm for A–D; 250 μm for E)

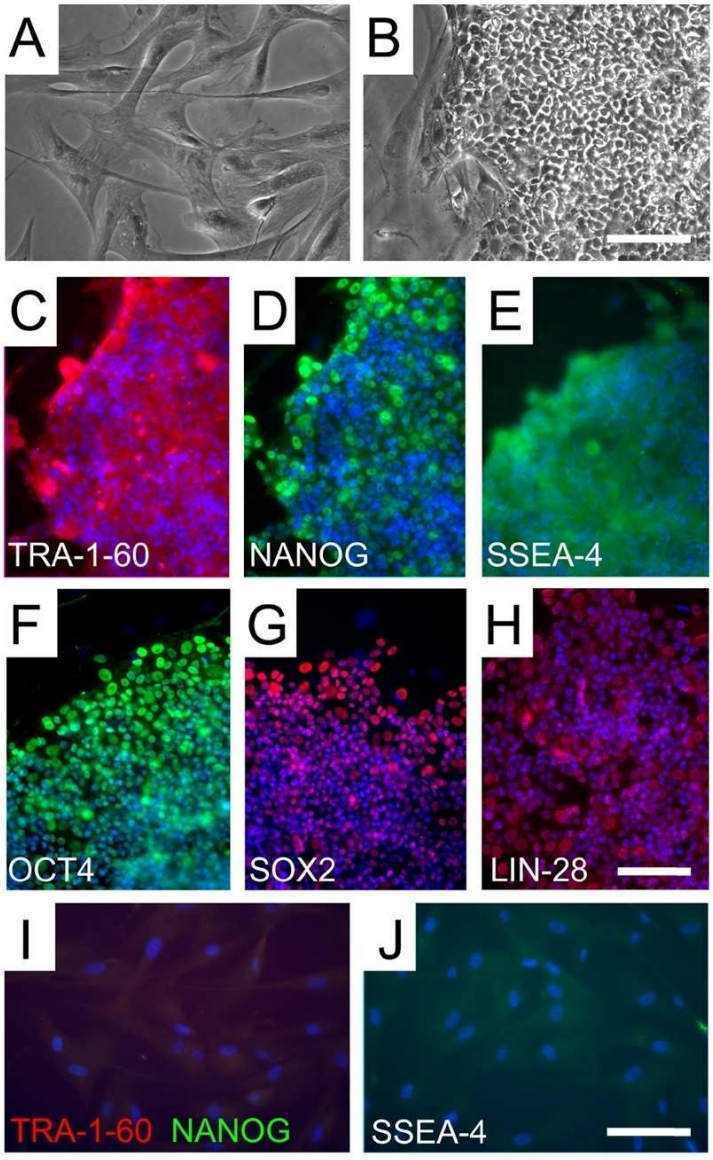


Figure 2.7: **Immunocytochemistry of DI-A1, DI-A2 and CTFs**. (A) Phase-contrast image of canine fibroblasts used for ciPSC generation at P3. (B) Phase-contrast image of ciPSCs at P7. (C-H) Immunocytochemistry of pluripotency markers in ciPSCs as labeled. The pluripotency markers include: (C) TRA-1-60, (D) NANOG, (E) SSEA-4, (F) OCT4, (G) SOX2, and (H) LIN-28. (I-J) Examples showing that pluripotency markers are

not expressed in input canine fibroblasts. DNA was labeled by DAPI staining and shown in blue. (Scale bar: 250 μm)

2.4. 3 Pluripotency Gene Expression and Epigenetics

We examined the expression of pluripotency genes in ciPSCs by qRT-PCR assay. Canine-specific pluripotency genes (OCT4, NANOG, TERT, and FOXD3) were robustly expressed in all ciPSC lines, but not in CSFs or CTFs ($P<0.05$, Figure 2.8A). However, the levels of OCT4, TERT, and FOXD3 in DI-B1 to B5 were significantly higher than in DI-A1 and DI-A2. Also, the fold change of NANOG expression in DI-B1 was comparatively lower than in other ciPSC lines ($P<0.05$). To confirm the specificity of canine gene amplification, primers for canine OCT4 were used in qRT-PCR for human H9 ESCs; no PCR products were detected (Figure 2.9). To confirm the silence of viral vectors, we compared transgene expression in ciPSCs to CTFs harvested two days after viral transduction (Figure 2.8 B). Forward and reverse primers were designed for the intersection between viral OCT4 and KLF4 (O-K). The result indicated that DI-B1 to B5 expressed transgenes negligibly compared to infected CTFs, which displayed 13,000-fold higher transgene expression ($P<0.05$). DI-A1 and DI-A2 had higher transgene expression (4,000-fold and 100-fold, respectively) than DI-B1, suggesting that the vectors were not shut down in DI-A1 and DI-A2. We further evaluated the expression of other canine pluripotency genes, (including SOX2, c-MYC, LIN-28,

SOCS3, STAT3, and GBX2) in CTFs and in DI-B1, DI-B2, and DI-B3 cell lines. Except for LIN-28 and STAT3 in the DI-B1 cell line, we found significantly higher gene expressions in ciPSCs than in CTFs (Figure 2.8 C).

We further investigated the CpG dinucleotide methylation status in one canine NANOG regulatory region and two OCT4 regulatory regions (regions 1 and 2) by bisulfite genomic sequencing. We selected ciPSCs DI-A1, DI-A2, DI-B1, and DI-B5 to compare with CTFs. Results showed demethylated NANOG promoters in DI-A2 and DI-B5, while DI-A1 and DI-B1 maintained the same level as CTFs. However, OCT4 methylation status in ciPSCs maintained the same level as CTFs or even increased (Fig. 2.10). These results indicate that, at least for the residues investigated, the DNA methylation level for the OCT4 gene does not always correlate with the gene expression observed.

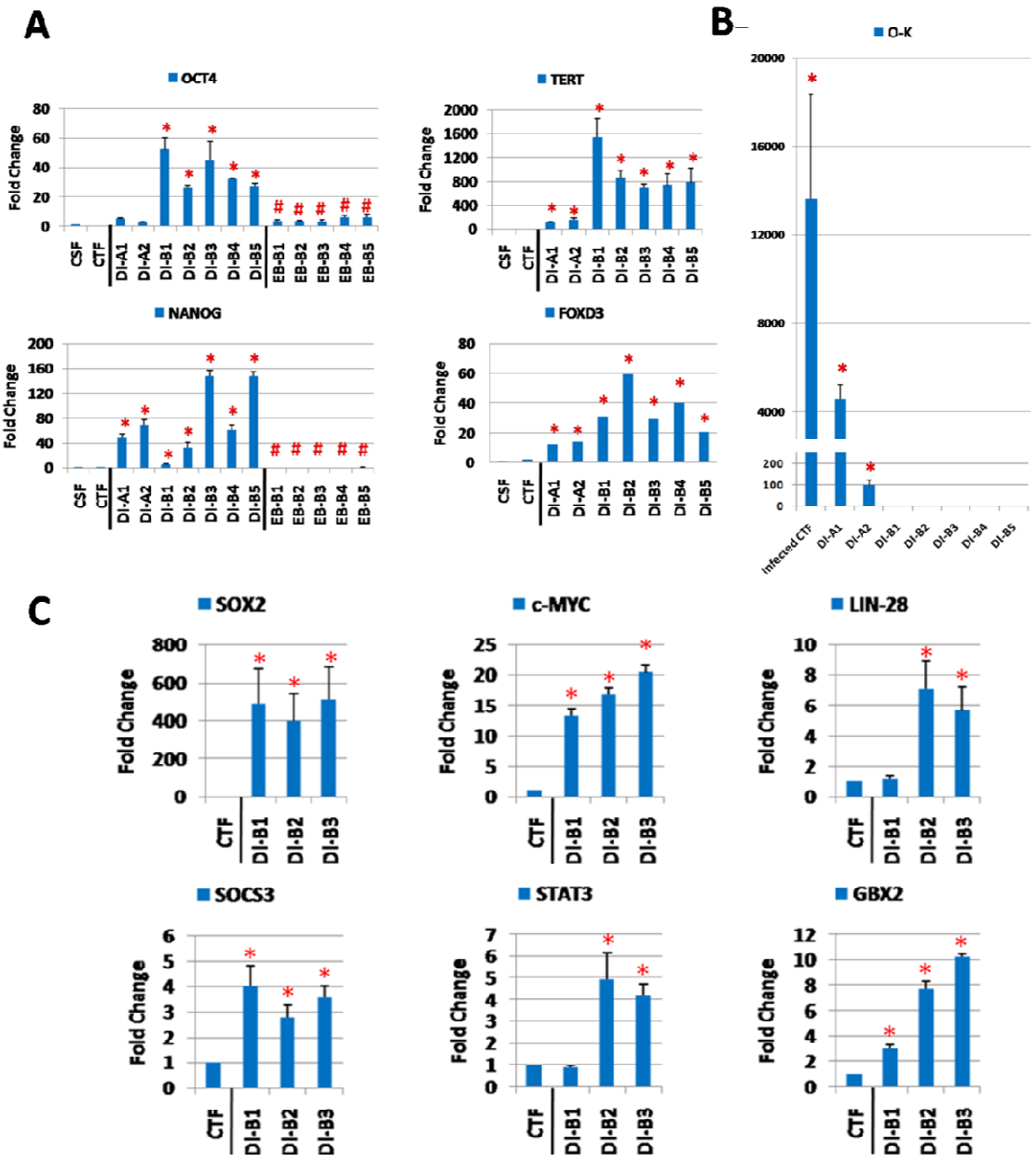


Figure 2.8: **Gene expression of ciPSCs.** (A) qRT-PCR analysis of relative transcript amounts of pluripotency-associated genes in CSF, CTF, all seven ciPSC lines, and all five cell lines from EBs (OCT4 and NANOG only). Pluripotency-associated genes include canine OCT4, NANOG, TERT, and FOXD3. Values in the *y* axis represent fold changes relative to canine RPL13 expression. The gene expression in CTF and ciPSC

Figure 2.8 (cont'd)

lines is relative to that in CSF (*: P<0.05), and the expression in EB cells is relative to their ciPSC lines respectively (#: P<0.05). (B) qRT-PCR analysis of relative transcript amounts of the transgene sequence in CSF, CTF, and all seven ciPSC lines. The transcripts of transgenes are represented by amplification of the intersection between hOCT4 and hKLF4 within the transgene. The *y* axis stands for fold changes relative to canine RPL13 expression. (C) qRT-PCR analysis of relative transcripts amount of pluripotency-associated genes in CTF, DI-B1, DI-B2 and DI-B3. Values in the y axis represent fold change relative to canine RPL13 expression (*: P<0.05).

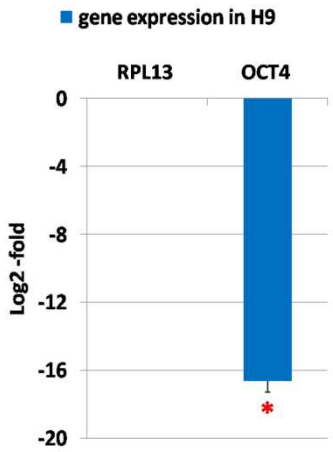


Figure 2.9: **Validation of specificity of canine OCT4 primers for ciPSCs.** qRT-PCR analysis of relative transcripts amount of canine RPL13 and OCT4 in human ESC H9. The y axis represents the fold change (Log2) relative to RPL13. * : $p<0.05$.

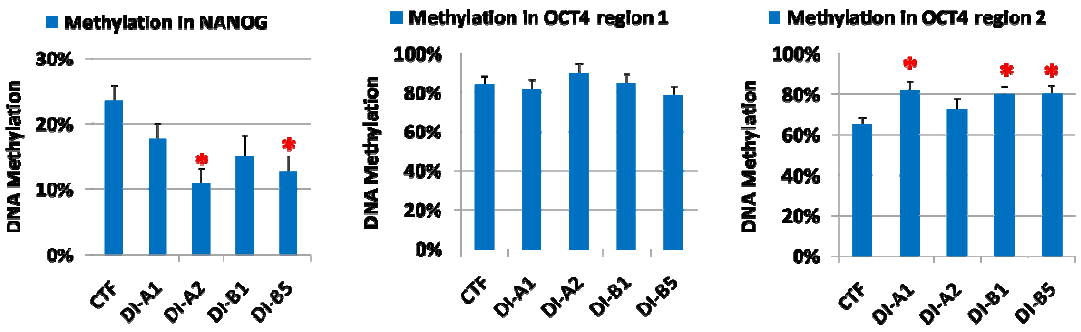


Figure 2.10: Epigenetics analysis of ciPSCs. Bisulfate genomic sequencing for DNA methylation in the promoter regions of canine NANOG and OCT4 within CTFs and ciPSC lines DI-A1, DI-A2, DI-B1 and DI-B5. The percentages of methylation in four ciPSC lines are compared with that in CTF by proc GLM from SAS. Error bars stand for the standard errors of each column. * : $P<0.05$.

2.4.4 Karyotype Analysis

We randomly chose DI-A1, DI-A2, DI-B2, and DI-B5 for karyotype analysis. Results indicated that all ciPSC lines had normal karyotypes (Fig. S8). Specifically, ciPSCs with normal karyotypes among all the G-banded ciPSCs had ratios of 17/17 (DI-A1, P4), 14/16 (DI-A2, P3), 8/10 (DI-B2, P4), and 9/10 (DI-B5, P5). Cells with abnormal karyotype were mostly considered a culture artifact.

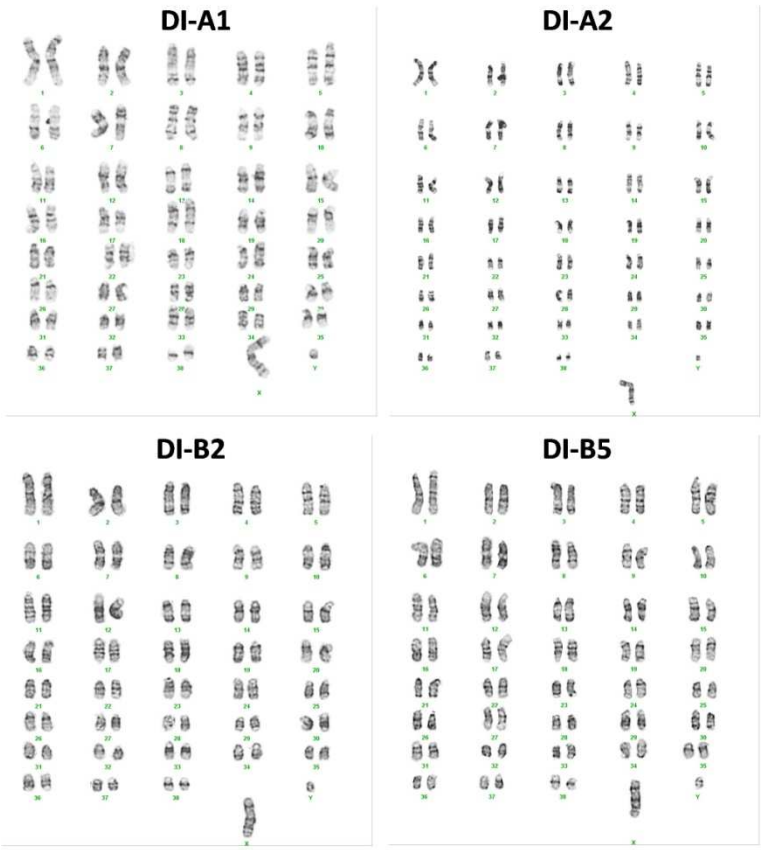


Figure 2.11: Karyotype analysis of ciPSCs. G-banding chromosomes of DI-A1 (P4), DI-A2 (P3), DI-B2 (P4) and DI-B5 (P5) demonstrate the normal male karyotypes.

2.4.5 Microsatellite Analysis

To confirm that ciPSC lines derived from the original fibroblast line, we examined five canine microsatellites. All ciPSC lines displayed the same alleles as parental CTFs but differed from CSFs with different origins, indicating that ciPSCs and CTFs were equal but different from CSFs in identity (Table 2.4). The probability that CTFs and derived cell lines were not from the same dog was less than 1.9×10^{-8}.

Table 2.4 Genotypes for the iPSC cells using five canine tetranucleotide repeat microsatellites. The allele sizes of the microsatellite markers in canine skin fibroblasts (CSF), canine testicular fibroblasts (CTF), and all ciPSCs (DI-A1, A2, B1, B2, B3, B4 and B5) are listed.

Sample Markers	CSF	CTF	DI-A1	DI-A2	DI-B1	DI-B2	DI-B3	DI-B4	DI-B5
FH2054	157, 162	150, 166[a,b]	150, 166	150, 166	150, 166	150, 165	N/A	150, 165	150, 166
FH2165	386, 394	453, 470	453, 470	453, 470	453, 470	453, 470	453, 470	453, 470	453, 470
FH2233[c]	359, 367	281, 351, 409	281, 351, 409	281, 351, 409	281, 351, 409	281, 351, 408	281, 351, 409	281, 351, 409	351, N/A
FH2313	269, 272	293, 307	293, 307	293, 307	292, 306	292, 306	292, 306	292, 306	292, 306
FH2324	253, 262	253, 257	253, 257	253, 257	253, 257	253, 257	253, 257	253, 257	253, 257

Table 2.4 (cont'd)

a. Allele sizes are shown without the M13 tail used to label the amplicons so that direct comparisons can be made with the allele frequency data contained in Irion et al [113].

b. Sizes are rounded to the nearest whole number. Single base differences among allele sizes are deemed to represent the same allele.

c. This marker showed three alleles in all cell lines except CSF. The three alleles are caused by a duplication ("copy number polymorphism", or CNP) that contains the FH2233 marker [115].

2.4.6 *In vitro* Differentiation

To evaluate the capability of differentiation *in vitro*, we induced ciPSC lines to differentiate using the EB formation assay (Figure 2.12 A). Cells derived from plated EBs on day 20 post-differentiation were analyzed and found positive for the presence of cell derivatives from the three germ layers, including β-III neuron-specific tubulin (TUJ1) for the ectoderm, vimentin for the mesoderm, and alpha-fetoprotein (AFP) for the endoderm (Figure 2.12 B) [49,82]. Using qRT-PCR, we also found that differentiated ciPSCs silenced the canine OCT4 and NANOG ($P<0.05$, Figure 2.8 A). Differentiation-related genes in EB cells derived from DI-B2, DI-B3, and DI-B5 ciPSCs — i.e. ectoderm (NESTIN and NEFL), mesoderm (CD34 and GATA2), and endoderm (CXCR4 and AFP) — were upregulated ($P<0.05$, Figure 2.12 C). Interestingly, we observed large multinuclear cells resembling giant cells from the trophectoderm in differentiated cells (Figure 2.13). We therefore evaluated the expression of trophoblast marker CDX2, which was highly expressed in EB cells but not in the original fibroblasts or undifferentiated ciPSCs ($P<0.05$, Figure 2.12 C). These results demonstrate that the vast majority of our ciPSC lines could differentiate into the three germ layers and express lineage-specific markers.

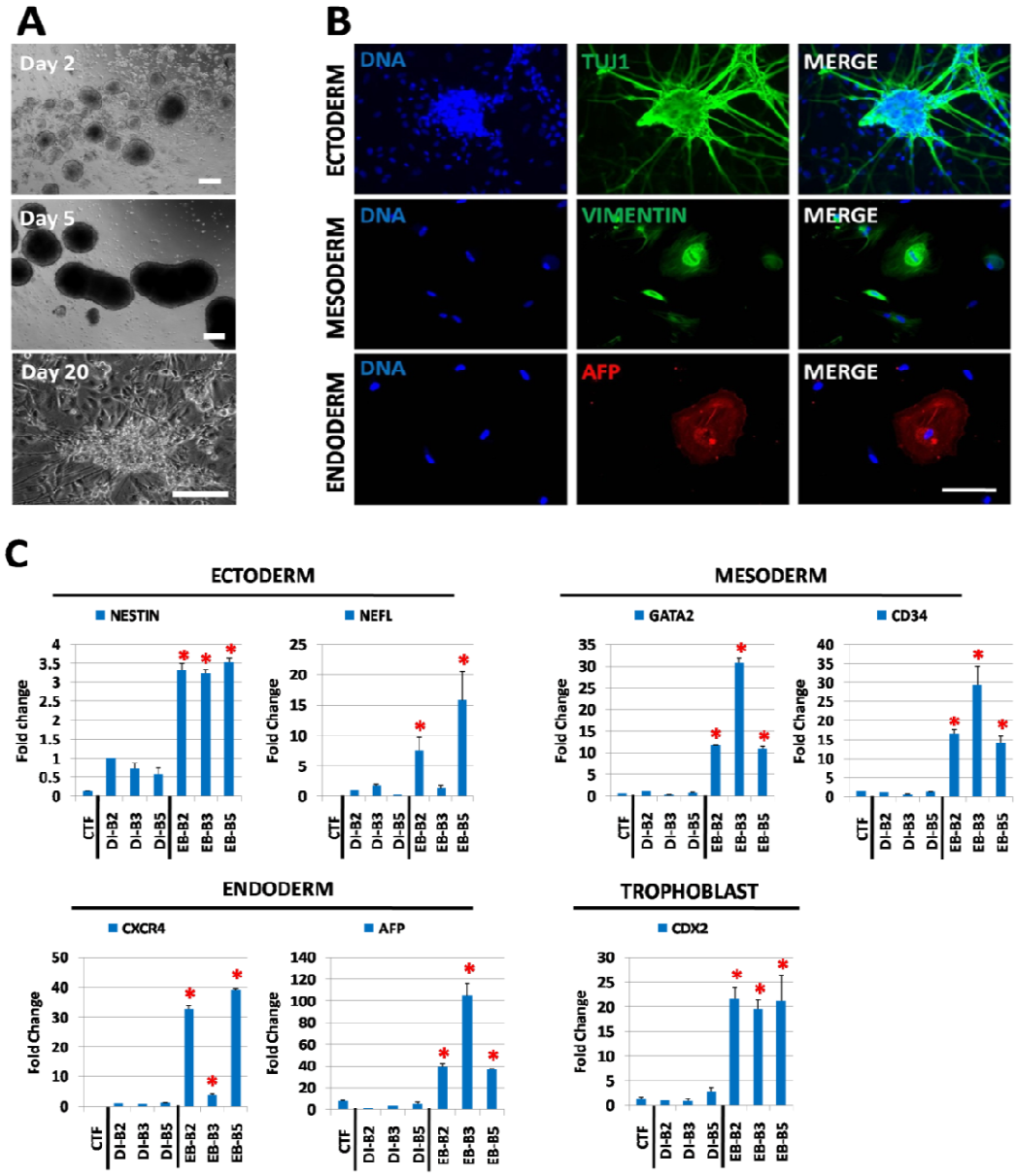


Figure 2.12: Differentiation of ciPSCs into EBs. (A) The morphology of floating and attached EBs. Pictures represent the EBs on days 2, 5, and 20 after isolation of ciPSC colonies for EB formation culture. (B) Ectoderm, mesoderm, and endoderm cell derivatives are respectively marked by TUJ1, vimentin, and AFP. (C) qRT-PCR analysis of relative transcript amounts of differentiation genes in CTF; the three ciPSC lines DI-

Figure 2.12 (cont'd)

B2, DI-B3, and DI-B5; and the EBs from these three ciPSC lines. Differentiation genes include NESTIN and NEFL (representing ectoderm and CD34), and GATA2 (representing mesoderm and CXCR4), AFP (representing endoderm), and CDX2 (representing trophoblast cells). Values in the *y* axis represent fold changes relative to canine RPL13 expression. (Scale bar: 250 μm for A and B)

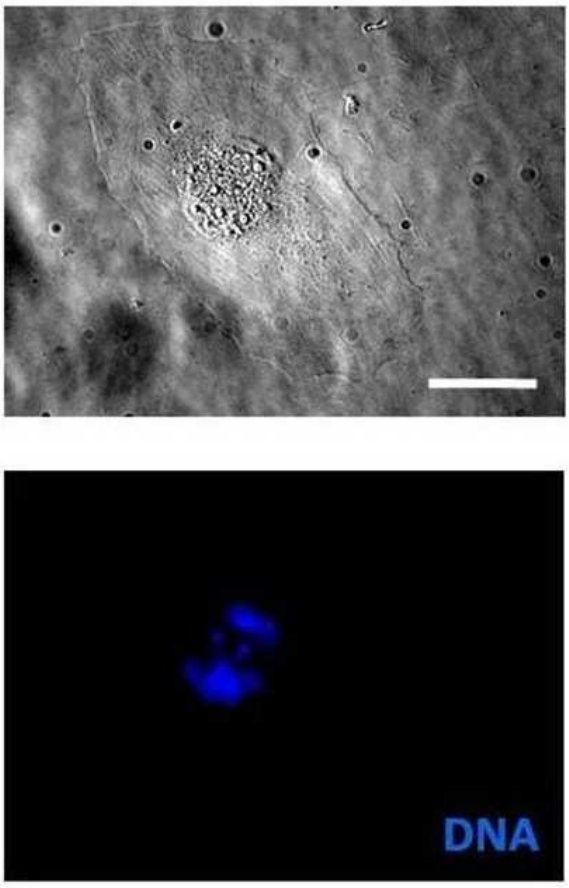


Figure 2.13: Morphology and DNA staining of ciPSC-differentiated trophoblast cell-like cell. (Scale bar: 100 μm)

2.4.7 LIF and bFGF Dependency

We examined the dependency of growth factors during ciPSC maintenance and found that, when LIF or bFGF were independently withdrawn from the culture medium, ciPSCs did not maintain their undifferentiated morphology (Figure 2.14 A, Figure 2.15, $P<0.05$). To investigate the role of LIF and bFGF in maintaining self-renewal, we cultured ciPSC on Matrigel-coated plates (Invitrogen, Carlsbad, CA, Carlsbad, CA)with MEF-conditioned ciPSC media supplemented with only LIF (LIF+/FGF-) or bFGF (LIF-/bFGF+) or both (LIF+/FGF+). TUNEL assays demonstrated that, while no difference existed in the percentage of apoptotic cells in the LIF+/FGF- and LIF+/FGF+ treatments, the percentage in the LIF-/bFGF+ cells was significantly higher (Figure 2.14B, $P<0.05$ Using BrdU incorporation assay we also determined that LIF+/FGF+ ciPSC exhibited the highest proliferation rates (Figure 2.14 C, $P<0.05$). To test the effects of LIF and bFGF on pluripotency maintenance — measured by NANOG expression levels — we cultured ciPSCs for seven days and immunostained them (Figure 2.14 D). Results indicated that removing either LIF or bFGF is sufficient to lose the pluripotency marker NANOG, suggesting that ciPSCs need both LIF and bFGF to maintain self-renewal. Our data indicate that withdrawing LIF also triggers signs of apoptosis, while bFGF is associated with proliferation of undifferentiated ciPSCs (Figure 2.14 E).

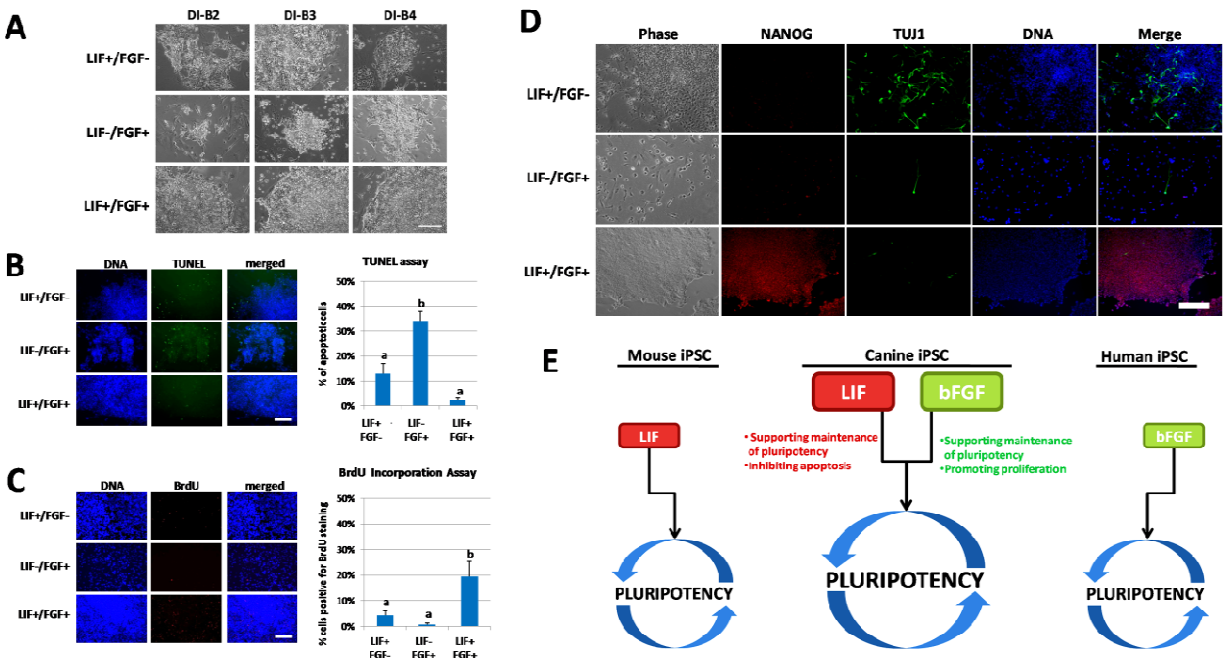


Figure 2.14: **Role of LIF or bFGF in survival, proliferation, and pluripotency maintenance of ciPSCs.** (A) Morphology of ciPSCs from line DI-B2, DI-B3, and DI-B4 on day 6 without passaging when cultured with human LIF only (LIF+/FGF-), bFGF only (LIF-/bFGF+), and both human LIF and bFGF (LIF+/FGF+). (B) TUNEL assay in ciPSCs when cultured with LIF+/FGF-, LIF-/bFGF+, or LIF+/FGF+ for 4 days. Quantification results were analyzed by PROC GLM from SAS. Values in y axis represent the percentage of apoptotic cells among the total cells. (C) BrdU incorporation assay for ciPSCs cultured with supplement of LIF+/FGF-, LIF-/bFGF+, or LIF+/FGF+ for 4 days. BrdU+ cells were counted as the cells with de novo synthesized DNA. The quantification results were analyzed by PROC GLM from SAS. Values in y axis represent the percentage of BrdU+ cells among the total cells. (D) Immunofluorescent staining of pluripotency marker NANOG and differentiation marker TUJ1 in ciPSCs cultured for 7 days with LIF+/FGF-, LIF-/bFGF+, or LIF+/FGF+. (E) The potential functions of LIF and

Figure 2.14 (cont'd)

bFGF during pluripotency maintenance of ciPSCs. Withdrawal of either LIF or bFGF, which resembles mouse or human ESC culture conditions, causes spontaneous differentiation and cell death or slowdown of proliferation. Pluripotency of ciPSCs can be maintained with both LIF and bFGF present in the culture medium. (Scale bar: 250 μm)

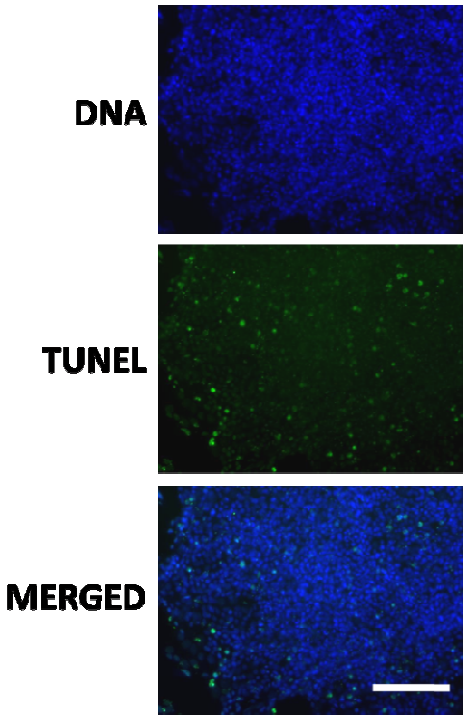


Figure 2.15: **TUNEL assay for the DNase-treated ciPSCs as the positive control.** Green cells represent the apoptotic cells. (Scale bar: 250μm)

2.5 Discussion

This study demonstrated that canine somatic cells isolated from an adult animal can be dedifferentiated into pluripotent cells. Following the strategy described for humans, we successfully induced fibroblasts to become pluripotent cells by transduction of four transcription factors — OCT4, KLF4, SOX2, and c-MYC (OKSIM) [107,108]. We successfully expanded and characterized seven ciPSC lines: DI-A1, DI-A2, and DI-B1 to B5. Like human and mouse ESCs, the proliferation of ciPSCs required co-culturing with MEFs [8,50]. Surprisingly, the generation of ciPSCs required the presence of both LIF and bFGF. We also found that ciPSCs, like their human counterparts, expressed many pluripotency-associated factors — including OCT4, SOX2, NANOG, TRA-1-60, TERT, FOXD3, and SSEA-4 [4,82,116] — while silencing the OKSIM transgene in most ciPSC lines.

The cell line used to derive our ciPSCs, CTF, was isolated from the testicle of an adult dog. Therefore, in an effort to rule out the possibility that the original cells were already pluripotent, we compared the gene expression profile of a set of pluripotency-associated genes with that of another canine cell line isolated from the skin of a different animal (CSF). At the time of these experiments, the CSF line was more than ten passages old. Our qRT-PCR results showed that the expression of pluripotency genes in CTFs was negligible and as low as in CSFs. Further, the morphology of CTFs had all the characteristics of a typical fibroblast, consistent with the expression of the proteins fibronectin and vimentin. While we cannot completely rule out the possible presence of a germ-line-derived cell within the culture of CTFs, our results indicate that, at the time

of OKSIM infection, the cells were not pluripotent and were most likely stromal fibroblasts.

We found that the DI-A1 and DI-A2 ciPSC lines expressed lower levels of NANOG than the other ciPSC lines. This could be due to the OKSIM transgene remaining expressed, indicating incomplete reprogramming [117]. We also considered failure to derive EBs in these two lines as evidence of incomplete reprogramming [118].

At present, there is no report on the methylation status of canine pluripotency genes. Our bisulfite genome sequencing showed that the NANOG promoter was demethylated in the DI-A2 and DI-B5 cell lines. However, the methylation status of OCT4 was similar in CTFs and ciPSCs — or even more methylated in ciPSCs. Interestingly, our results were similar to data recently published suggesting that murine iPSCs maintained methylation signature characteristics similar to their differentiated donor cells in OCT4 and NANOG regulatory regions [119]. Although a more comprehensive epigenetic analysis for ciPSCs and CTFs is needed, our results suggest that the epigenetic status of ciPSCs may be similar but not identical to the donor fibroblasts and that, while the epigenetic memory of donor fibroblasts remains intact in some residues, it may not alter the overall characteristics of the ciPSCs derived from them. Additional regulatory factors enhancing epigenetic reprogramming might be necessary to help optimize the current reprogramming system, such as the use of microRNAs and small molecules [68,120,121].

Differentiation potential is one feature critical to determining the utility of pluripotent stem cells for regenerative medicine. Immunocytochemical and qRT-PCR analyses of

EBs from the DI-B1 to B5 ciPSCs found significantly increased expressions of markers for cell derivatives of the three germ layers and significantly downregulated pluripotency gene expression. Also noteworthy, cells appeared that resembled trophectoderm cells, with upregulated expression of trophoblast marker CDX2, a feature similar to that reported in pig iPSCs [101]. Why porcine and canine pluripotent cells produce cells with features of extra-embryonic tissues while human and mouse cell do not, remains unresolved.

To understand the requirement of growth factors, we attempted to culture ciPSCs with media used for mouse or human ESCs or iPSCs [8,50]. Unlike mouse or human ESCs, which required LIF or bFGF, respectively, for survival, removing LIF or bFGF caused, respectively, the loss of pluripotency markers and apoptosis or the loss of pluripotency markers and the slowdown of proliferation (Figure2.14E). The role of LIF in self-renewal maintenance was widely reported in the mouse ESCs [85]. In the presence of LIF receptors (LIFR), LIF supports pluripotency by activating the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway [85]. In dogs, LIFR was reportedly expressed in kidney cells; these canine cells responded to human LIF by further activating the JAK/STAT3 pathway [82,85,122]. The requirement of LIF for ciPSC culture also agrees with the culture conditions reported for canine ESCs [80,82]. Interestingly, we noticed that absence of LIF triggers severe apoptosis. Previous reports have indicated an anti-apoptotic role for LIF when culturing primordial germ cells, oligodendrocytes, and cardiomyocytes, but the mechanism governing this was not yet understood [123-125]. Human ESCs, recognized as pluripotent cells in the epiblast stage, and mouse epiblast stem cells reportedly depend on bFGF but do not react with

LIF [70,88]. We speculate that bFGF may act in ciPSCs through similar signaling pathways, i.e. , stimulating MEFs to synthesize activin A — which, in turn, activates Smad2/3 and promotes NANOG expression — and activating the FGF/ERK pathway, thus promoting proliferation [85,88]. Naïve mouse ESCs are described as comparable to cells from the blastocyst inner cell mass (ICM) and are LIF/STAT3-pathway-dependent [85]. Since ciPSCs present dual-factor dependency, it will be necessary to determine the position of ciPSCs in the “pluripotency map” and to clarify their apparent ICM/epiblast concomitant state. A better understanding of ciPSC pluripotency regulation may enhance our understanding of the molecular mechanisms responsible for the transition from ICM to epiblast cells.

The physiologies, anatomies, disease presentations, and clinical responses of dogs and humans are very similar, making the dog a very promising model for human disease research [102]. Among approximately 400 known hereditary canine diseases, over half have equivalent human diseases, including retinal diseases, epilepsy, narcolepsy, cardiomyopathies, muscular dystrophy, and such malignant tumors as prostate cancer [102,126]. In terms of stem cell kinetics — e. g. hematopoietic stem cells -and responsiveness to cytokines, the dogs are more biologically comparable with humans than mice, making the dog the most commonly used species for early transplantation research in human regenerative medicine [102]. However, until now, approaches that involve deriving natural canine pluripotent stem cells have been poorly explored. The successful establishment of a robust ciPSC derivation and culture system offers a novel template for human regenerative medicine studies. It will help us to understand and treat human diseases, including those of genetic origin. Our further

finding, about dual growth factor dependency in ciPSCs, provides a new opportunity to understand mechanisms of self-renewal maintenance.

CHAPTER 3

ROLE OF LEUKEMIA INHIBITORY FACTOR (LIF) DURING CULTURE OF CANINE INDUCED PLURIPOTENT STEM CELLS

3.1 Abstract

Our previous work presented evidence that canine induced pluripotent cells (ciPSCs) are simultaneously dependent on both basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF), and that in the absence of LIF ciPSC colonies do poorly. LIF is required for survival of ciPSCs during the early stages of differentiation. Considering that LIF function is also required to maintain pluripotency, the efficiency of ciPSCs in vitro differentiation, when compared to species such as mouse and human, is diminished. Here we report the pathways activated by LIF that promote cell survival in ciPSCs. We found that JAK-STAT3 is the pathway exclusively activated by LIF but not bFGF in ciPSCs. Downregulation of JAK-STAT3 by removal of LIF from the culture triggers apoptosis and DNA fragmentation in a caspase-3-dependent manner. Elucidation of the pathways involved during culture of undifferentiated ciPSCs, will help develop novel cell differentiation strategies leading to a more efficient derivation of cells for preclinical studies in regenerative medicine.

3.2 Introduction

The dog is a valuable large animal model for human preclinical studies, in particular for cell therapy related-studies that require in-depth monitoring of transplanted cells for safety and efficacy [102]. Progress towards the implementation of cell therapies using pluripotent stem cells (PSCs) has been slow, in part due to the lack of adequate animal models. Two recent reports described the derivation and characterization of canine ESCs (cESCs) capable of differentiation into cell derivatives of the three-germ layers [80-82]. Subsequently, a number of different groups, including ours, successfully generated and characterized canine iPSCs (ciPSCs) from adult fibroblasts and adipose tissue-derived cells [30,32,127]. These cells require both leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) to proliferate and maintain their pluripotent state [30,32,127]. We have also shown that LIF, but not bFGF, withdrawal from ciPSC culture induces apoptosis [31].

Our long-term research goal is to establish a robust platform for ciPSC generation, differentiation and transplantation. This, in turn, will allow us to evaluate the safety and efficacy of autologous iPSCs in the canine model. Establishing detailed protocols for ciPSC differentiation is critical for achieving our goal. Differentiation of PSCs toward a specific somatic cell lineage requires the removal of specific growth factors that support pluripotency from the culture medium. In the case of ciPSCs these include LIF and bFGF. We showed that LIF is capable of not only preventing differentiation of ciPSC, but maintaining cell viability as well. Attempts to differentiate ciPSCs for two weeks by removing bFGF-only failed to robustly upregulate gene

expression of differentiation-related genes (see Appendix A). Specifically, the expression of alpha-fetoprotein (*AFP,* an early hepatocyte differentiation marker) and nestin (*NES*, a marker of early neural differentiation) were unaffected or significantly downregulated when compared to the undifferentiated ciPSCs on day 0.

In agreement with our previous results and the results of others, LIF must be removed from ciPSC culture medium to allow spontaneous differentiation to proceed [30,32,127]; however, the abrupt removal of LIF triggers cell death, decreasing the final yield of differentiated cells. The purpose of this work is to uncover the molecular pathways involved in ciPSC death after LIF removal.

Studies performed in mouse ESCs (mESCs) have demonstrated that LIF is required to maintain cell pluripotency, survival and proliferation. LIF binds to the cell membrane LIF receptor which then activates the tyrosine kinase Janus kinase (JAK) enzyme, activating three branches of signal transduction pathways associated with survival: STAT3, AKT, and ERK1/2 signaling cascades. Activated JAK phosphorylates the receptor of LIF to recruit and phosphorylate the Signal Transducers and Activators of Transcription 3 (STAT3) [85]. Activated STAT3 targets and promotes the expression a list of genes that are critical for pluripotency and survival. Activation of the canonical AKT and ERK1/2 signaling cascades by LIF also supports pluripotency, proliferation, and survival [88]. Unlike mESCs, human ESCs are dependent on bFGF for pluripotency maintenance by activating SMAD2/3 and eventually stimulating NANOG expression in the presence of the feeder cells [88]. However, bFGF also activates ERK1/2andAKT

pathways to promote self-renewal [88]. Therefore, JAK-STAT3 pathway is specifically activated by LIF, but not bFGF.

Critical for understanding cell death in ciPSCs is determining which pathway is the primary mechanism responsible for the demise of the cells. Two major types of cell death, apoptosis and necrosis, have been reported in pluripotent stem cells [93,94]. Cells dying by apoptosis activate caspase-3 that in turn activates endonucleases that fragment the DNA [95,96]. Apoptosis can be mitochondrial- and receptor-mediated apoptosis. Caspase-8 cleavage is a unique marker of the receptor-mediated pathway [96]. Necrosis is identified by morphological changes such as swelling of the dying cell, rupture of the plasma membrane, and release of the cytoplasmic content into the extracellular environment, as well as a loss of the cell membrane integrity [95,97].

Considering the signaling transduction activated by LIF and the fact that ciPSC cell death is specifically caused by withdrawal of LIF, we hypothesize that inactivation of a LIF-dependent/bFGF-independent pathway is solely responsible for the cell death of ciPSCs. To test this hypothesis, we first determined the effect of LIF on the activation status of LIF-associated pathways in ciPSCs cultured in the presence of LIF and/or bFGF. Subsequently, we determined the effect of inactivation of LIF-dependent pathway on cell viability. We assessed both apoptosis and necrosis in ciPSCs cultured in the presence of LIF and bFGF. Our results revealed that LIF withdrawal causes inactivation of JAK-STAT3 pathway and induces death by apoptosis.

3.3 Material and Methods

3.3.1 Cell Culture

Mouse embryonic fibroblasts (MEFs) were used as feeder layers to maintain ciPSCs as previously reported [127]. MEFs were expanded with fibroblast medium (DMEM containing 10% fetal bovine serum (FBS)) at 37ºC with 5% CO2. Culture medium was replaced every 24 hours. MEFs were mitotically inactivated using fibroblast culture medium containing 10 µg/mL mitomycin C (Sigma, St. Louis, MO) for 4 hours and then seeded in density of $2x10^4$ cells/cm^2 prior to the co-culture with ciPSCs.

Once ciPSC colonies were isolated, they were re-seeded on top of MEFs with iPSC medium i.e. DMEM/F-12 (Gibco, Carlsbad, CA) supplemented with 15% (v/v) knockout serum (Gibco, Carlsbad, CA), 0.1 mM MEM nonessential amino acid solution (Sigma, St. Louis, MO), 1 mM L-glutamine (Invitrogen, Carlsbad, CA), 0. 075 mM β-mercaptoethanol, 4 ng/mL human bFGF (Invitrogen, Carlsbad, CA), and/or 10 ng/mL human LIF (Millipore, Billerica, MA) [127]. Colonies with compact ES cell-like morphology were manually isolated and passaged onto new MEFs every five days using glass Pasteur pipettes.

3.3.2 Western Blotting Assay

ciPSCs were cultured in different media or treated with different small molecule inhibitors and further harvested as indicated in the experimental design in the *Results* section. All ciPSCs for western blotting assays were cultured on Matrigel-coated plates and maintained in culture medium that was previously conditioned by the feeder cells for 24 hours. Cell samples were collected using the Corning Costar cell scraper (Sigma, St. Louis, MO) without trypsin. Cells were lysed in RIPA buffer and kept at –80℃ until use. Protein concentration was determined by BCA assay according to the manufacturer's instruction from BCA assay kit (Thermo scientific, Rockford, IL). Thawed samples were boiled for 5 minutes and loaded into 10% SDS-PAGE for protein electrophoresis, and resolved polypeptides were transferred onto PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 5% nonfat dry milk in phosphate buffered saline (PBS, Sigma Aldrich, St. Louis, MO)–0. 1% Tween (Sigma, St. Louis, MO) for 30 minutes at room temperature and incubated overnight at 4℃ with primary antibody. On the second day, the membranes were incubated for one hour with a horseradish peroxidase–labeled secondary antibody. Immunoreactivity was detected by Amersham ECL western blotting detection system according to manufacturer's instructions (GE Healthcare, Buckinghamshire, UK) and developed using Amersham Hyperfilm^{TM} MP (GE Healthcare, Buchinghamshire, UK). Three biological replicates were done per each protein analyzed. See Table 3. 1for the complete lists of antibodies used.

3.3.3 Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay

ciPSCs were cultured and harvested as indicated in the experiment designs in the *Results* section. To collect each sample, ciPSCs were dissociated in to single cells by trypsin treatment, pelleted, washed with PBS and fixed in 4% paraformaldehyde for 15 minutes. TUNEL assays were performed according to the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) following manufacturer instructions. As positive control, cells were treated with DNase (Promega, Madison) as recommended in the TUNEL kit instructions. After washing in PBS, we counterstained all nuclei with propidium iodide (PI) (50 µg/mL) for 30 minutes at 37ºC. The stained cells were stored in 4^{o}C until subjected to flow cytometry assay to quantify the percentage of TUNEL positive cells. Three biological replicates have been done for each treatment.

3.3.4 Propidium Iodide Staining for Unfixed Cells

ciPSCs were cultured and further stained by PI, as indicated in the experimental design in the *Results* section. To stain the samples, ciPSCs in culture were treated with 4 mg/mL of PI for 5 minutes. Then the cells were immediately washed once with PBS, trypsinized, and washed again with PBS and immediately subjected to flow cytometry assay to calculate the percentage of PI-positive cells. As control we used cells exposed to 5mM of hydroxyl peroxide (H_2O_2) for 24 hours. Three biological replicates were

completed for each treatment. Stained cells were immediately subjected to flow cytometry assay to count PI positive cells. Three biological replicates were done for each treatment.

3.3.5 Flow Cytometry Assay

All cells were trypsinized and transferred to flow buffer consisting of PBS. All assays were performed using LSRII Flow cytometer (BD Biosciences, San Jose, California) and analyzed using Diva v. 6 (BD Biosciences, San Jose, California) with the assistance of Dr. Louis King at the flow cytometry core of Michigan State University.

3.3.6 Statistical Analysis

SAS software (SAS Institute, Cary, NC) was used to analyze the data. We performed ANOVA using PROC GLM, considering treatment as an independent variable and using Tukey's adjustment as a *post hoc* test to compare means. Probability values (P) <0.05 were considered significant.

Table 3.1 –Antibodies used for western blotting assay

Antigen	catalog#	Isotype	Manufactor	Concentration
Phosphorylated STAT3 (tyr705)	#9131	Rabbit IgG	Cell signaling technology	1:1000
STAT3 (k-15)	sc-483	Rabbit IgG	Santa Cruz	1:1000
Phosphorylated AKT (ser437)	#9271	Rabbit IgG	Cell signaling technology	1:1000
AKT	#9272	Rabbit IgG	Cell signaling technology	1:1000
Phosphorylated ERK1/2 (thy202/204)	#4370	Rabbit IgG	Cell signaling technology	1:1000
ERK1/2 (137F5)	#4695	Rabbit IgG	Cell signaling technology	1:1000
Caspase-3 (H-277)	sc-7148	Rabbit IgG	Santa Cruz	1:1000
Cleaved caspase-3 (Asp175)	#9664	Rabbit IgG	Cell signaling technology	1:1000
Caspase-8 (S-19)	sc-6135	Goat IgG	Santa Cruz	1:1000
Cleaved caspase-8 (Asp387)	#8529P	Rabbit IgG	Cell signaling technology	1:1000
Beta-actin	#3700	Mouse IgG	Cell signaling technology	1:1000
Goat anti-rabbit IgG-HRP	sc-2004	N/A	Santa Cruz	1:3000

Table 3.1 (cont'd)

Goat anti-mouse IgG-HRP	sc-2005	N/A	Santa Cruz	1:3000
Donkey anti-goat IgG-HRP	sc-2020	N/A	Santa Cruz	1:3000

3.4 Results

3.4.1 Elucidate Signaling Transduction Pathways in ciPSCs Cultured in the Presence of LIF and/or bFGF.

As indicated above, LIF initiates a cascade controlling three signal transduction pathways associated with survival: STAT3, AKT, and ERK1/2 [85]. Of note, the JAK-STAT3 signaling axis is exclusively activated by LIF whereas the AKT, and ERK1/2 pathways are activated by both LIF and bFGF as well as other growth factors present in the culture medium [85]. Therefore, we hypothesized that like mESCs, following the removal of LIF from the culture medium but maintaining bFGF, would inactivate JAK-STAT3 pathway in ciPSCs, while the JAK-AKT or JAK-ERK1/2 pathways would remain active. To test our hypothesis we evaluated the phosphorylation status of STAT3, AKT, and ERK1/2. ciPSCs were cultured in media containing different growth factor combinations (LIF+/bFGF+, LIF+/bFGF-, LIF-/bFGF+ and LIF-/bFGF-) for three days. Cells were harvested and proteins were isolated for western blotting assays (Fig. 3.1). Compared to the sample of ciPSCs cultured with both growth factors (LIF+/bFGF+), only the removal of LIF (LIF-/bFGF+ and LIF-/bFGF-) reduced the phosphorylation of STAT3. Phosphorylation of either AKT or ERK1/2 was consistently maintained in all groups and was not affected by the presence or absence of any growth factor. This data indicates that LIF removal only inactivates the JAK-STAT3 pathway, but not AKT or ERK1/2 in ciPSCs.

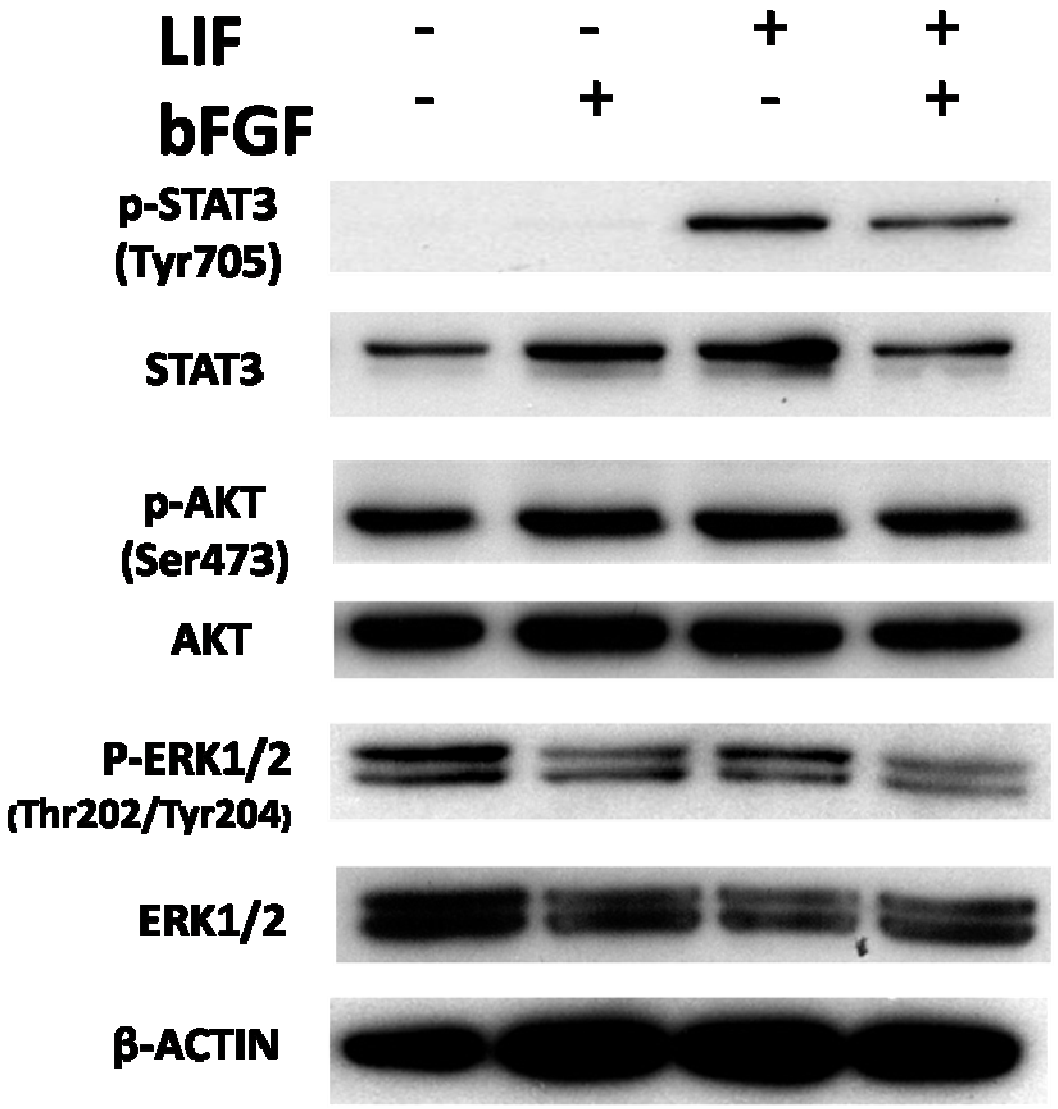


Figure 3.1: **Phosphorylation status of LIF-associated signaling pathways in ciPSCs maintained in the presence/absence of LIF/bFGF for three days.** Protein candidates for analysis are listed on the left, including p-STAT3, STAT3, p-AKT, AKT, p-ERK1/2, ERK1/2. β-actin was used as control. The"+" and "-" on the top indicate the presence and absence of LIF or bFGF.

3.4.2 Functional Analysis of LIF-Responsive Pathways in ciPSCs.

To further elucidate the role of individual LIF-responsive pathways on ciPSCs survival, we compared the effects of drugs known to specifically inhibit phosphorylation of STAT3, AKT, or ERK1/2. To test the JAK-STAT3 pathway, we used JAK inhibitor I (JAKi) to inactivate JAK activity and NSC74859 (STAT3i) to inhibit STAT3 activation directly and MK2206 (AKTi) and PD184352 (ERKi) to inhibit AKT and ERK1/2 phosphorylation respectively. Prior to applying the inhibitors and evaluating cell death, we sought to determine the minimum concentration of each small molecule inhibitor that was sufficient to inactivate the target protein without affecting the other pathways analyzed. The minimum effective concentration of each inhibitor was first determined by culturing ciPSCs in LIF+bFGF with each inhibitor (or dimethyl sulfoxide (DMSO) vehicle alone) at different concentrations for 24 hours. After treatment, proteins were extracted and subjected to western blotting to evaluate the phosphorylation of target proteins.

As indicated in Fig. 3.2, JAKi was tested at concentrations ranging from 10 nM to 10 μM. One micromolar was the lowest concentration for JAKi to block the phosphorylation of the STAT3 protein with no apparent effect on the phosphorylation of AKT and ERK1/2. Using the same process, the optimal concentration determined for STAT3i was 500 μM, for AKTi, 10 μM, and ERKi, 1 μM.

To determine the effect of the individual pathways on cell survival, ciPSCs were cultured in LIF+bFGF in the presence of each of the inhibitors or vehicle alone for 24 hours and assayed at the end of this time. These cultures were compared to parallel cultures of ciPSCs grown in each of the growth factor combinations (LIF+/bFGF+,

LIF+/bFGF-, LIF-/bFGF+, LIF-/bFGF) for 3 days and assayed on day 0, day 1, day 2 and day 3. All groups of cells were evaluated for survival and the mechanism of cell death (i.e. apoptosis or necrosis). Apoptosis was assessed by morphological changes, DNA damage (TUNEL assay and comet assay) and caspase-3/8 cleavage using western blot. Necrosis was assessed by morphological examinations and measure of cell membrane integrity using propidium iodide staining of unfixed cells.

As predicted by earlier experiments using different growth factor combinations, only cells cultured in the absence of LIF (LIF-/bFGF+ and LIF-/bFGF-) displayed morphological signs of cell death as indicated by a loss of colony compactness and the emergence of phase-bright pyknotic cells. In the inhibitor groups, only treatment of JAKi or STAT3i resulted in cells with morphological indicators of cells death essentially identical to LIF-withdrawn cells. Vehicle controls or AKTi or ERKi cells showed no morphological indicators of cell death (Fig. 3.3). These results using morphological indicators alone reinforce the idea that the blocking the JAK-STAT3 pathway triggers cell death in ciPSCs.

Cell death by apoptosis was further evaluated in all groups by assessing DNA damage by TUNEL assay. For the growth factor supplement groups, results demonstrated that LIF withdrawal (LIF-/bFGF+ and LIF-/bFGF-) significantly increased the percentage of TUNEL-positive cells from day 0 to day 3 (Fig. 3.4). In agreement with morphological indicators, in the inhibitor treatment groups, only the JAKi and STAT3i cultures displayed a significant increase of TUNEL-positive cells. We observed that the

TUNEL-positive cell rate in ciPSCs treated with JAKi was significantly lower than that in STAT3i treated group, the source of this difference remains to be elucidated.

We then evaluated caspase-3 and caspase-8 cleavage in these cells (Fig. 3.5). In agreement with the TUNEL results, only the absence of LIF in the growth factor supplement groups or the JAKi or STAT3i cultures in the inhibitor treatment groups induced caspase-3 cleavage. Moreover, the level of caspase-3 cleavage in JAKi treated group was lower than that in STAT3i treated group, as indicated by the less intense band. We did not observe caspase-8 cleavage under any culture condition or treatment, suggesting that although LIF removal or direct JAK-STAT3 inhibition induced caspase-3 activation, the caspase-8 pathway remained inactive.

Finally, to evaluate necrosis, unfixed ciPSCs were maintained in the same conditions or treatments as described above and subjected to propidium iodide staining to evaluate cell membrane integrity. The absence of either growth factor or the treatment of any inhibitor did not induce a significant increase of propidium iodide-positive cell numbers (Fig. 3.6). These results that necrosis is not a primary or "acute" cause of cell death in the transition of ciPSCs to differentiated cells.

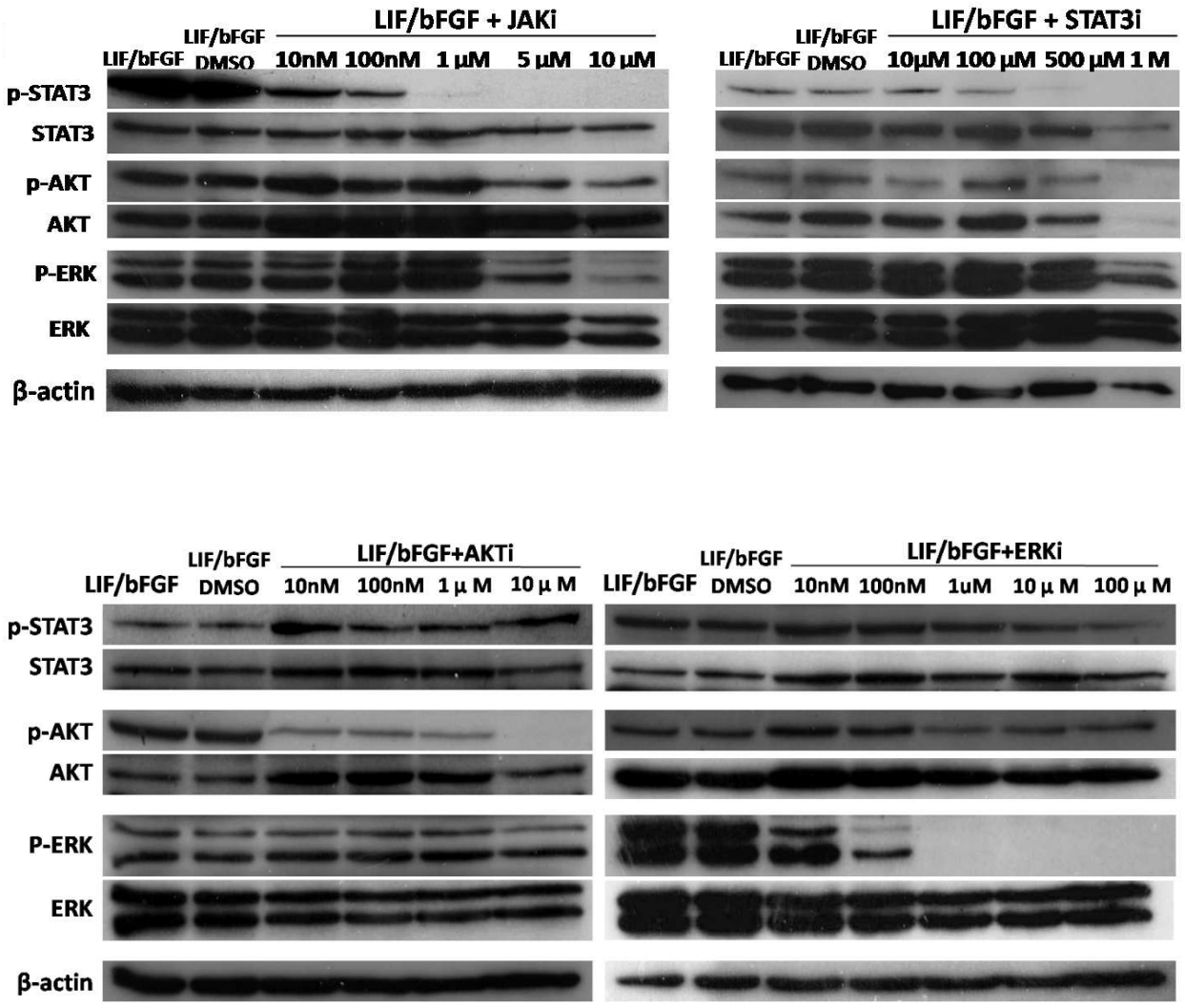
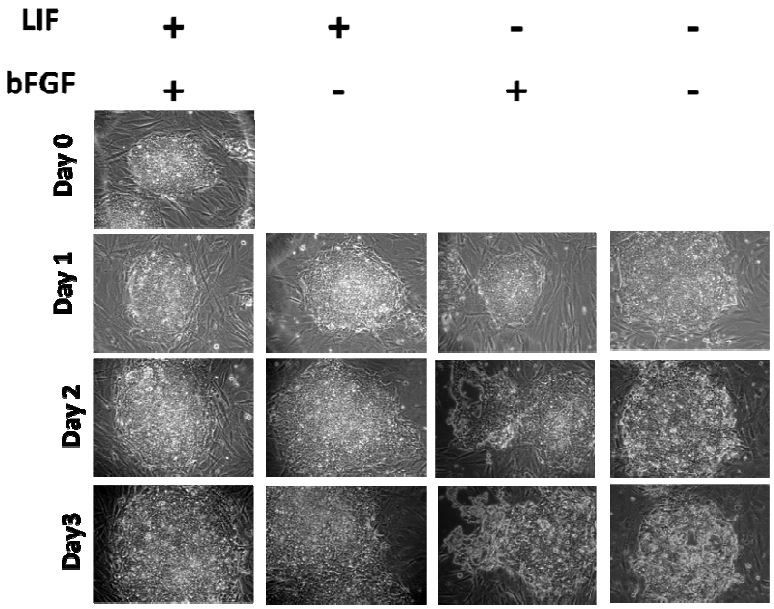


Figure 3.2: **Effects of JAK, STAT3, AKT and ERK1/2 inhibitor (JAKi, STAT3i, AKTi and ERKi) on activities of different signaling transduction proteins in ciPSCs.** Western blotting assays of protein factors of LIF and/or bFGF associated signaling pathways within ciPSCs collected after 24 hour-treatment of inhibitor with concentration gradients. The protein factors include phosphorylated (p-) STAT3, STAT3, p-AKT, AKT, p-ERK1/2, and ERK1/2. β-actin was applied as reference protein. The concentrations for each inhibitor are labeled on the top.

A



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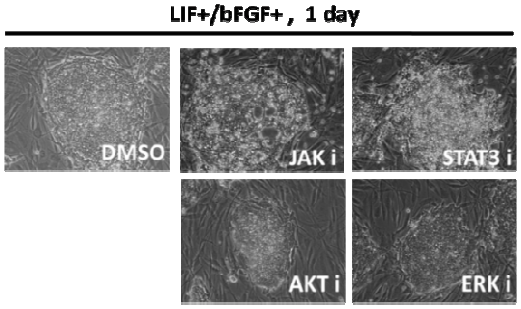


Figure 3.3: **Morphological changes of ciPSCs treated with protein inhibitors.** ciPSCs were maintained under culture conditions with different growth factor supplements on day 0, day 1, day 2 and day 3 (panel A), or treated with specific inhibitors, JAKi, STAT3i, AKTi, or ERKi for 24 hours (Panel B) DMSO was used as control. The supplement of growth factors are labeled on the top in panel A and "+" and "-" indicate the presence and absence of LIF or bFGF.

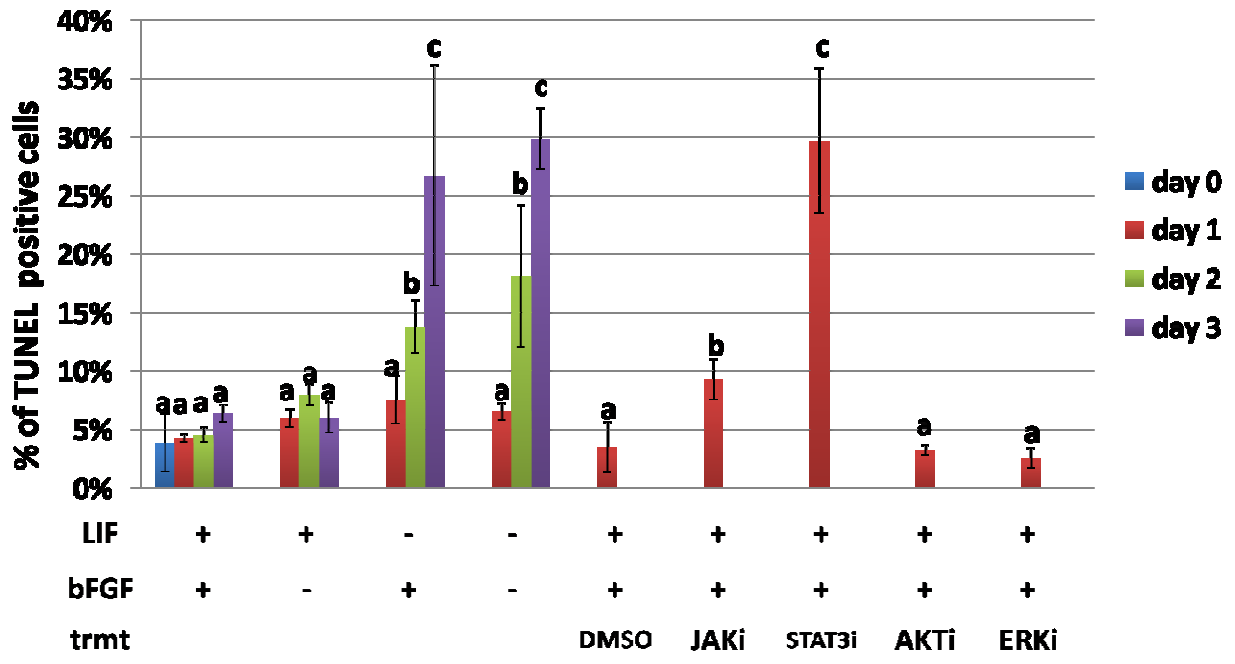


Figure 3.4: **Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay indicating the DNA damage of ciPSCs.** x-axis indicates the ciPSCs maintained in LIF+/bFGF+ medium, LIF+/bFGF- medium, LIF-/bFGF+ medium and LIF-/bFGF- medium collected on day 0, day 1, day 2 and day 3, as well as the ciPSCs maintained in LIF+/bFGF+ medium treated with DMSO, JAK inhibitor (JAKi,1 μM), STAT3 inhibitor (STAT3i, 500 μM), AKT inhibitor (AKTi, 10 μM), and ERK1/2 inhibitor (ERKi, 1 μM) for 24 hours. The "+" and "-" indicate the presence and absence of LIF or bFGF. The y-axis indicates the percentage of TUNEL positive cells. And different colors are used in columns to indicate the ciPSCs collected on different times. Different letters (a, b, and c) indicate $P < 0.05$.

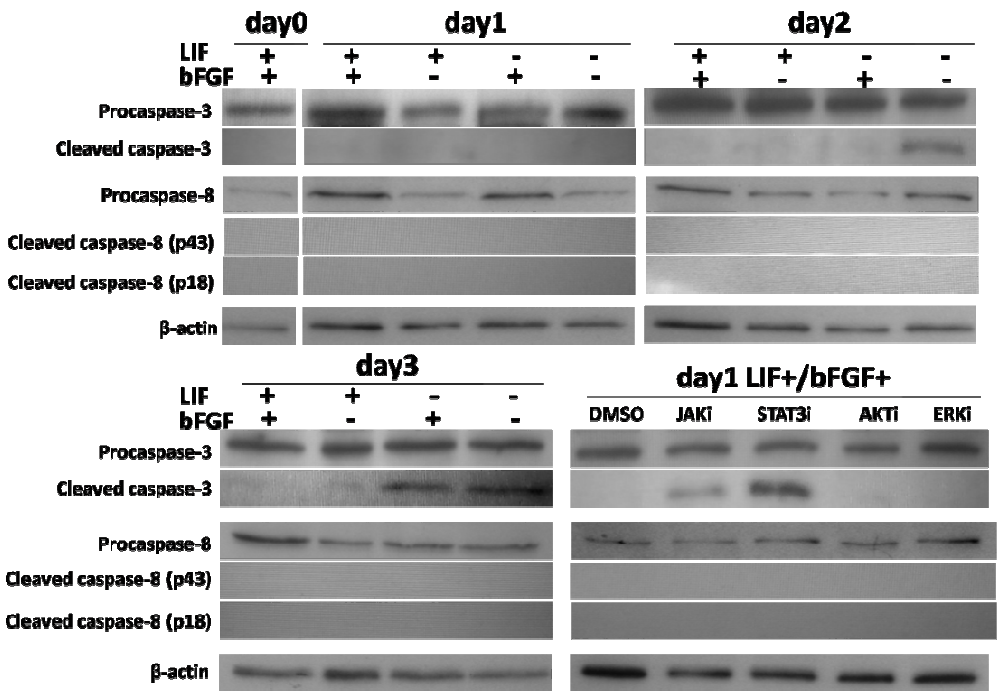


Figure 3.5: **Western blotting assays indicating the caspase-3/8 activation in ciPSCs.** ciPSCs maintained in LIF+/bFGF+ medium, LIF+/bFGF- medium, LIF-/bFGF+ medium and LIF-/bFGF- medium collected on day 0, day 1, day 2 and day 3, as well as the ciPSC maintained in LIF+/bFGF+ medium treated with DMSO, JAK inhibitor (JAKi,1 μM), STAT3 inhibitor (STAT3i, 500 μM), AKT inhibitor (AKTi, 10 μM), and ERK1/2 inhibitor (ERKi, 1 μM) for 24 hours. Protein candidates for analysis are listed on the left, including procaspase-3, cleaved caspase-3 (activated caspase-3), procaspase-8, and cleaved caspase-8 (activated caspase-8, including two subunits p43 and p18). β-actin was used as control. The "+" and "-" indicate the presence and absence of LIF or bFGF.

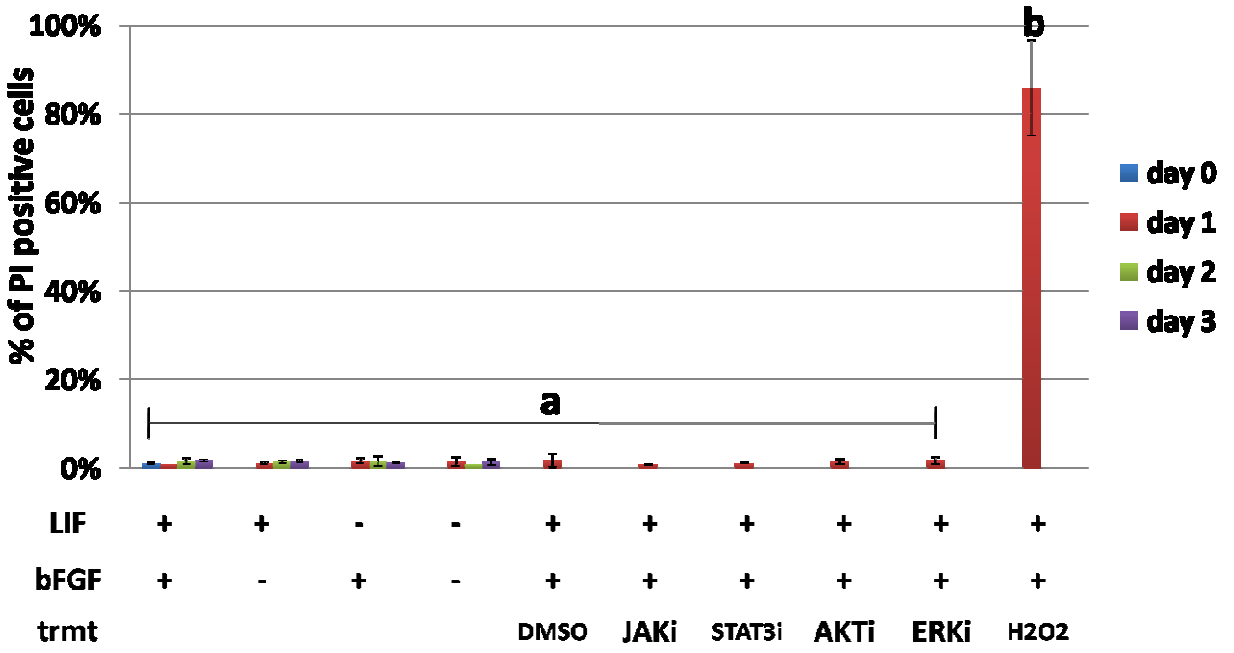


Figure 3.6: Propidium iodide (PI) staining assay in ciPSCs. x-axis indicates the ciPSCs maintained in medium that contained LIF+/bFGF+, LIF+/bFGF-,LIF-/bFGF+ or LIF-/bFGF-. Cells were collected for analysis on day0, 1, 2 and 3, as well as ciPSCs maintained in LIF+/bFGF+ medium treated with DMSO, JAK inhibitor (JAKi,1 μM), STAT3 inhibitor (STAT3i, 500 μM), AKT inhibitor (AKTi, 10 μM), or ERK1/2 inhibitor (ERKi, 1 μM) for 24 hours. An extra group of H_2O_2 treatment was included as the positive control that is listed on the right of the x-axis. The "+" and "-" indicate the presence and absence of LIF or bFGF. The y-axis indicates the percentage of PI positive cells. Different colors are used in columns to indicate the ciPSCs collected at different times. Different letters (a and b) indicate $P < 0.05$.

3.5 Discussion

The development of novel animal models for regenerative medicine experiments that require the use of stem cells are highly needed. The canine offers the opportunity to expand our knowledge beyond rodent models, and has been used as a template for human medicine for almost five decades [128]. We have recently reported the derivation of canine iPSCs that in combination with canine homologs of human disease could significantly inform our understanding of the disease pathogenesis and the potential of new treatments.

ciPSCs have some unique features that make them different from those of mouse and human. The most striking is that they require both LIF and bFGF to maintain pluripotency and proliferate, implying that there are different signaling pathways involved with pluripotency regulation [28,30,32,82,127]. We found that the removal of LIF triggered cell death in ciPSCs and therefore we focused on determining the mechanism by which LIF regulates the survival of ciPSCs.

LIF plays a key role in mouse PSC cultures as described in the introduction to this chapter. Unlike the mouse, human PSCs are dependent of bFGF, not LIF. The activation of ERK1/2 and AKT pathways by bFGF supports cell proliferation and survival as well [88]. Therefore, among the three signaling transduction pathways activated by LIF, only LIF-JAK-STAT3 is exclusively activated by the presence of LIF in mESCs.

Our results confirmed the exclusivity of this pathway with regard to LIF responsiveness in canine cells. Using western (protein blot) analysis, we observed that

only in the absence of LIF (LIF-/bFGF+ and LIF-/bFGF-) was there a loss in phosphorylation of STAT3 without affecting the phosphorylation of AKT or ERK1/2 in ciPSCs, providing strong evidence that the activation of STAT3 pathway in ciPSCs is dependent on the presence of LIF. Notably, AKT and ERK1/2 phosphorylation appeared unchanging in any growth factor combination. AKT and ERK1/2 pathways have been shown to be activated by factors produced by feeder cells such as insulin growth factor 1 (IGF-1), epidermal growth factor (EGF) and activin-A supporting pluripotency and survival of PSCs via AKT and ERK1/2 signaling cascades, but feeder cells were present in all treatments and therefore neither pathway appeared directly responsive to either LIF or bFGF, the phosphorylation of these proteins did not change and do not directly impact survival after growth factor withdrawal [85,129-131]. We should point out that it remains possible that indirect effects of growth factors acting on, and produced by, feeder cells, could mask a small direct effect of LIF on the ciPSC ERK1/2 and AKT pathways.

Small molecule inhibitors were used to block the activity of the specific LIF-associated pathways to evaluate their roles in cell survival. Drug-inhibited cultures were compared to ciPSCs cultured in different growth factor combinations to determine the extent and "type" of cell death induced. As predicted, only cultures with either LIF removed for 3 days or cultures with drug-based inhibition of the JAK-STAT3 pathway for 1 day displayed cytotoxicity. PI uptake indicative of cell membrane compromise and necrotic cell death were not observed under any condition, however, in cultures displaying cells with morphological indications of cell death, several features of apoptosis (DNA fragmentation as indicated by TUNEL assay and caspase-3 cleavage

revealed by western blot) were observed. Interestingly, we noted that JAKi treatment induced lower TUNEL-positive cell rate (9.27% in average) than the STAT3i treatment (29.67% in average), which reflects a lower apoptosis rate when treated by JAKi. The same pattern was repeated in the caspase-3 cleavage assay, as indicated by a band of cleaved caspase-3 protein with lower intensity in ciPSCs treated with the JAKi. It was previously reported that inactivation of JAK enhances NANOG expression through epigenetic regulation in mESCs and in ESCs, escalation of NANOG expression results in the inhibition of differentiation and an increase in cell survival via escalation the HSPA1A expression, a NANOG target [132,133]. It is possible that the increase of NANOG expression caused by the inhibition of JAK activity represses to certain extent, apoptosis in ciPSC.

We also noted that no significant change was observed in caspase-8 cleavage and or cell membrane integrity under any treatment conditions. This result revealed that the cell death triggered by LIF withdrawal or inhibition of the JAK-STAT3 signaling pathway is not activated through a death receptor pathway. When LIF is removed from the culture media, apoptosis appears to be the overwhelming mechanism of cell death in ciPSC making their transition from a pluripotent to a differentiated state.

The importance of STAT3 in the survival of pluripotent stem cell has been previously reported. One explanation of this effect is the activation of p38 mitogen-activated protein kinase (p38MAPK) [134,135]. LIF withdrawal during mESC culture may induce the inactivation of STAT3, which subsequently fails to inhibit the activity of the p38MAPK protein. If the expression of anti-apoptosis factor *BCL-2* cannot be up-

regulated in time, p38MAPK protein can trigger cell death in mESCs [135]. Furthermore, our results of caspase-8 activation and PI staining show no difference among all the groups in ciPSCs reinforcing the idea that cell death in ciPSCs is not due to receptor-mediated apoptosis or necrosis. It is reasonable to speculate that the requirement for LIF to maintain survival is mainly due to a culture system for ciPSCs that still requires optimization to remove stressful stimuli. LIF and the subsequent activation of JAK-STAT3 pathway compensates for these chronic stressors, permitting survival and growth.

Apoptosis is a typical cellular stress response during *in vitro* cell culture. It has been reported that embryonic stem cells are hypersensitive to apoptosis triggered by DNA damage due to mismatch repair, as a mechanism that may contribute to reduction of the mutational load in the progenitor population [92]. We also evaluated the survival of ciPSCs based on comet assay, a more sensitive assay to evaluate DNA fragmentation based on the DNA electrophoresis of live cells (see Appendix B). We observed that compared to the healthy canine fibroblast control, ciPSCs cultured in the presence or absence of either growth factor or by the treatment by either inhibitor demonstrated some indications of DNA damage. This data implies that the ciPSCs cultured even under conditions that we currently consider "optimal", may be accumulating stress and DNA damage. Removal of LIF lowers the cells' ability to respond to these accumulated stressors, further accelerating DNA fragmentation, and ultimately triggering apoptosis. The existence of stressors in the ciPSC culture environment is not certain, but previous reports have revealed sources of culture stress for other cell types. Osmolarity of the culture media is an important parameter to consider with dog cells. When flushing

canine blastocysts from the uterus using regular flushing media with an osmolarity of 270-310 mOsmol/L (which has a similar osmolarity of our current ciPSC culture medium) significant shrinkage the embryos is observed, whereas the use of a buffer with lower osmolarity corrected this problem [136]. This suggests that our current culture medium may be a source of hyperosmotic stress to ciPSCs. Another possible explanation is the potential negative effect from β-mercaptoethanol since it was originally added to the recipe for mouse ECCs due to its positive effect on cell-cloning efficiency, and it was subsequently applied to mouse and human ESC cultures [137]. Interestingly, a recent study on chemically defined medium for human ESC culture has demonstrated that β-mercaptoethanol is toxic for human ESCs. It was apparently added to reduce the variability generated by albumin that was also part of the original recipe. By removing albumin and β-mercaptoethanol from the media, human ESC cultures performed much better. More work must be done to optimize the culture conditions and test this hypothesis among many others.

In summary, this study demonstrates that LIF is critical for ciPSC survival and that the action of LIF is overwhelmingly through the JAK-STAT3 pathway. This information was instrumental to an improved characterization and differentiation of ciPSCs and moved us closer to practical solutions for their application to veterinary medicine.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The canine model stands out as a valuable pre-clinical model because of its similarity to humans in terms of pathology, physiology, biochemistry, body size, life span, genetic diversity, and anatomy [15]. Stem cells, especially pluripotent stem cells (PSCs), are one of the most important components of future regenerative medicine strategies. iPSCs in particular are the ideal source of cells for autologous tissue replacement. Multiple differentiation protocols have demonstrated that functional cells of many phenotypes can be produced *in vitro* from iPSCs, and experiments in rodents have shown compelling data arguing in favor of using iPSC-derived cells and tissues. However, there is limited knowledge on the use of iPSCs for therapeutic transplantation in larger animals such as dogs.

The therapeutic use of stem cells in dogs is currently being explored; particularly the use of mesenchymal stromal cells (MSCs). Pluripotent stem cells such as iPSCs could especially benefit dogs suffering from diseases lacking effective therapies or causing life-long disability, impacting quality of life. These treatments could also promote the development of parallel treatments in human. For many conditions, treatment of dogs with reprogrammed autologous stem cells may be critical to eventually implementing such therapies in humans [82].

Our understanding of PSCs from dogs (and most non-rodent species) and the molecular foundation of their self-renewal were far from complete when my studies on ciPSCs started in 2009. My research project was initiated with the long-term objective of producing and characterizing induced pluripotent stem cells from canine somatic cells for future application in the treatment of injury or disease in dogs. This work will contribute to a better understanding of cellular reprogramming and stem cell biology and will help to address human and animal health issues in a non-rodent system.

4.1 Generation and Characterization of ciPSCs

To accomplish this ambitious goal, it was necessary to first develop a method for reprogramming canine somatic cells to pluripotency and to characterize such ciPSCs. Since iPSC technologies were in their relative infancy when the project began, the success at obtaining high-quality ciPSC lines was not trivial. Nonetheless, ciPSC lines that had normal phenotype and karyotype, and displayed conventional pluripotency markers were successfully derived. In addition, ciPSCs could be differentiated *in vitro* into cell derivatives of the three-germ layers. The main challenge was the significant loss of cells when attempting to differentiate ciPSCs, as discussed further below.

While the feasibility of reprogramming canine somatic cells to cells with essential characteristics of pluripotency was validated, like iPSCs from most domesticated species, ciPSCs could not produce teratomas following introduction into immune-compromised mice. The causes of this phenomenon still remain to be elucidated.

For most domestic and companion species, iPSC and ESC derivation remains an expensive and labor-intensive process and as a consequence, there are only a handful of published studies. A literature search for iPSCs from felids produced a single report of iPSCs from the snow leopard, Panthera uncia [138]. A similar search for cow- and horse-derived cells likewise yielded two reports from the same group for the cow and two from independent groups for the horse [139-142]. Canine iPSCs have been reported more often although one recurring theme of all of these reports is that canine pluripotent cells tended to form either poor teratomas or none at all [12,28,29,31,32,143,144]. For canine ESCs specifically, only one study reported teratomas, and the resulting tumors were small and of poor quality [82]. The data published in *Stem Cells and Development* (data in Chapter 2) and presented in this thesis agrees with the literature, in which canine cells, with apparently all of the properties of pluripotency, had difficulty forming teratomas in immune-compromised mice [27,31,32]. The only domesticated species that has repeatedly shown high-quality teratomas from ESCs or iPSCs is the pig [11,145-153]. Must be noted though that sustained expression of viral transgenes is required for maintenance of the pluripotent phenotype, suggesting that porcine iPSCs may also differ from mouse and human iPSCs [145].

Scarcity of reports does not necessarily means that there is low interest on developing iPSCs from domesticated and companion species. It is possible that multiple attempts at iPSC production may have been performed in different laboratories around the world, only to be halted at a later stage, because of poorly developed *in vitro* culture conditions and/or failure to demonstrate teratoma formation. We speculate that the

absence of teratomas from iPSCs of most domestic species most likely arises from some fundamental incompatibility in physiology of the mouse that prevents the proliferation of non-mouse cells. These incompatibilities could be at the level of growth factors, cell adhesion and extracellular matrices, neo-vascularization, sub-clinical pathogens, or even something as ordinary as body temperature. In short, caution must be exercised when assuming that because rodent and primate cells can grow in the body of a mouse, cells from other species will do as well.

It is often mentioned that because a given line of reprogrammed cells, displaying all of the hallmarks of pluripotency, are incapable of forming teratomas, therefore they do not fit the current definition of "pluripotent cells", and they have little intrinsic value in either basic research or translational medicine. The data presented in this thesis and the results of experiments still in progress suggest that this is not the case. Despite not forming teratomas in mice, the ciPSCs are capable of giving rise to a number of stable cell lineages, critical for the development of novel therapies, with tremendous potential value to veterinary and human medicine.

It has been uncovered by us that one characteristic of ciPSCs which differ from human or mouse PSCs in that they are dependent on the presence of two exogenous growth factors — LIF and bFGF — to maintain pluripotency and survival. It was found that LIF is critical for maintaining both survival and pluripotency, while bFGF appeared to be required only to maintain pluripotency. As indicated above, the removal of LIF from the culture medium triggered substantial cell death and presented a significant obstacle for the eventual use of ciPSCs as a source of differentiated cell types for

therapeutic applications. As a consequence, a better understanding of the molecular mechanisms of both growth factors in the transition from pluripotency to differentiated state became a major focus of my research as described in Chapter 3.

4.2 Elucidating the Roles of Growth Factors in ciPSC Maintenance

Experiments aiming at understanding the role of LIF in maintaining the survival of ciPSCs and elucidating the LIF-dependent signaling pathways critical to ciPSC maintenance were my first priority. Using drugs known to inhibit specific components of the LIF-associated signaling cascades, it was found that the withdrawal of LIF led to inactivation of a critical signaling pathway known as the JAK-STAT3 pathway, but had negligible impact on two other known LIF-associated signaling pathways, the JAK-AKT and JAK-ERK1/2 pathways. In addition, as with ciPSCs maintained without LIF, inhibition of the LIF-JAK-STAT3 pathway in ciPSCs triggered caspase-3 activation, DNA damage, and eventual cell death by apoptosis. As indicated in the discussion section in Chapter 3, there are a number of publications showing that the JAK-STAT3 pathway is protective against multiple cell stressors, suggesting that inactivation of JAK-STAT3 in the presence of some unknown stressful components of the ciPSC culture environment was responsible for the rapid cell loss [154]. From this it is hypothesized first, that inhibiting the activity of the stress-induced pro-apoptosis effector such as the p38 mitogen-activated protein kinase, or optimizing the current ciPSC culture condition could prevent the ciPSCs from death in the absence of LIF; and second a very slow withdrawal of LIF coupled with the simultaneous and gradual addition of components

predicted to lower cell stress could improve the efficiency of differentiation. Although outside of the scope of my thesis, the later of the two were evaluated.

Beside the studies on LIF, a second set of experiments must be done focusing on the role of bFGF in the maintenance of pluripotency in ciPSCs. It was found that both LIF and bFGF were required to maintain the expression of pluripotency markers such as NANOG in ciPSCs. Dual-growth factor dependency is not necessary for human or mouse PSCs and it is also distinctive from other recently-defined classifications of PSC lines such as LIF-dependent, ICM-derived "naïve" ES cells or bFGF-dependent, epiblast-derived "primed" ESCs. Naïve and primed ESCs present distinct regulatory mechanisms for maintaining pluripotency and inhibiting differentiation [33]. ciPSCs displayed a monolayer morphology and the canine NANOG promoter contained a SMAD2/3 consensus binding sequence that would appear to indicate that they are closer to "primed" PSCs which is dependent on bFGF related signaling transduction; however, ciPSCs do not appear to fit perfectly into either category [155]. By describing the molecular regulatory pathways maintaining pluripotency in ciPSC, it will be able to determine where these cells lay in the pluripotency map, which will eventually facilitate the development of the efficient protocols for ciPSC derivation, maintenance and differentiation toward the desired somatic cell type of choice.

4.3 Reprogrammed Cells in Animal and Human Medicine

It is hoped that the project initiated in pursuit of my doctorate will eventually lead to the development of new treatment options for a variety of diseases and injuries, such as spinal cord injury. Chondrodystrophic canine breeds such as Dachshunds have particular susceptibility to spinal cord injury and represent just one of the potential beneficiaries of this type of research as described above. It was able to produce neuronal spheres from ciPSCs by spontaneous or directed differentiation. Can the development of reliable protocols for the further differentiation of ciPSC-derived neurons to CNS subtypes such as motor neurons and oligodendrocytes be far behind? Will ciPSC-derived MSCs delivered to the affected tissues reduce inflammation and promote the regeneration of local neuronal progenitor cells, leading to the repair the injured spinal cord? The results of my research are the foundation upon which new and more challenging questions like these could be answered in the context of pathological conditions afflicting real patients, dogs and human alike.

The results presented suggest that because iPSCs from different species are likely to display their own unique set of properties — such as a dependency on specific levels and combinations of growth factors — it is likely that the derivation and use of reprogrammed cells in the veterinary clinic will be more complex than previously thought. Despite these obstacles, the potential benefits to be realized by the use of these cell types to treat injury and disease in dogs, and thereby provide valuable lessons for the future use of cellular reprogramming to treat human patients, will more than justify the labor-intensive nature of the research described in this thesis.

APPENDICES

APPENDIX A

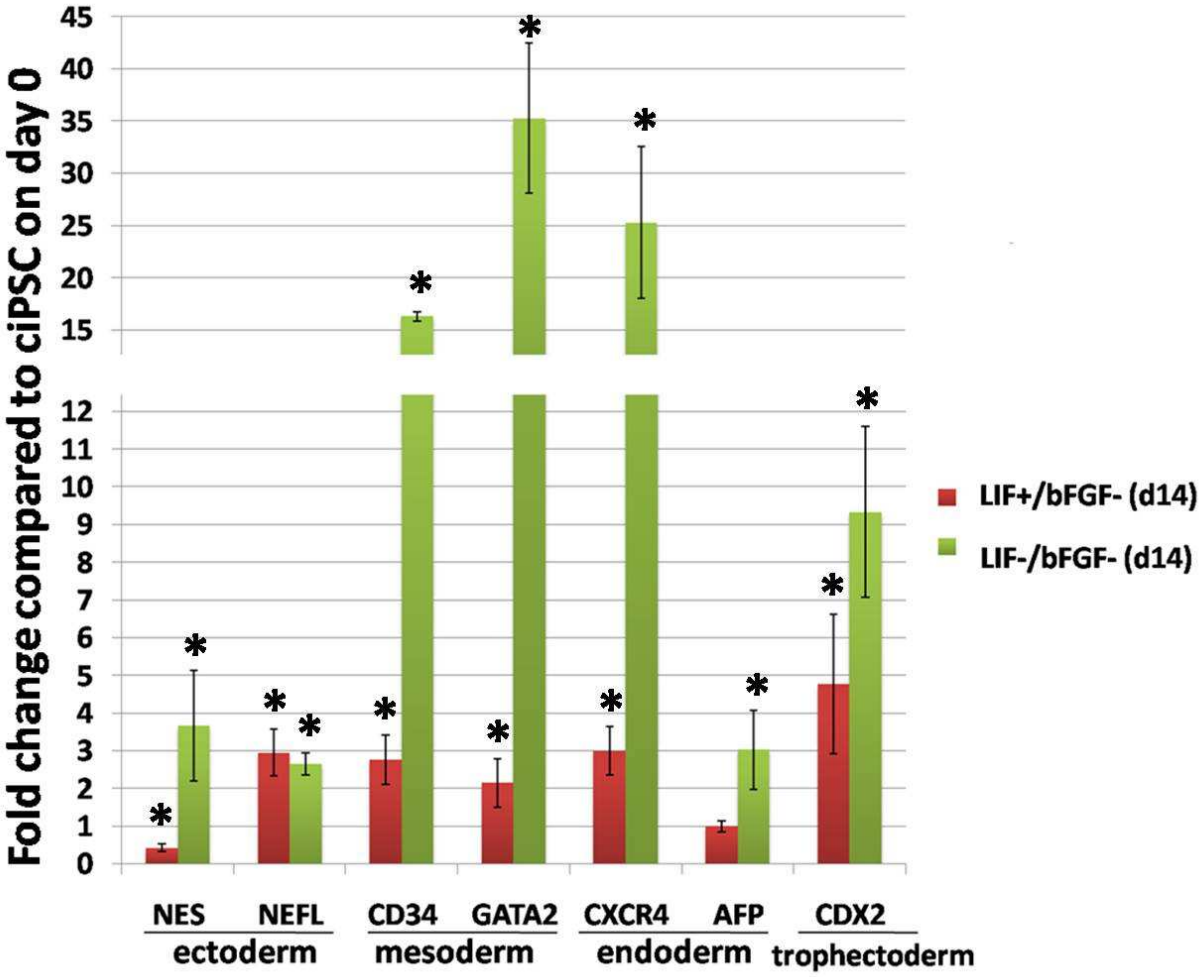


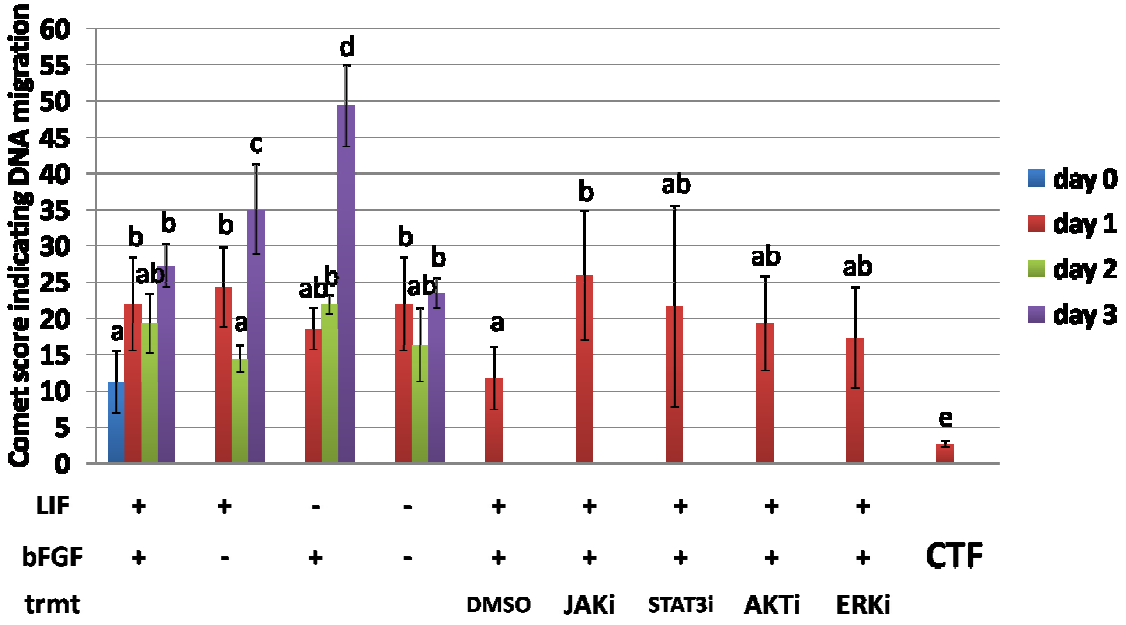
Figure A1: Gene expression of differentiation markers in ciPSCs cultured in the presence or absence of LIF. qRT-PCR analysis of relative transcript amounts for germ layer-specific genes in ciPSCs (DI-B3) differentiated in the presence of LIF (LIF+/bFGF-) or without the presence of LIF (LIF-/bFGF-) for 14 days. Differentiation genes include canine nestin (*NES*) and *NEFL* for ectoderm, *CD34* and *GATA2* for mesoderm, *CXCR4* and *AFP* for endoderm, and *CDX2* for trophectoderm. The primers for amplifying the

Figure A1 (cont'd)

genes above were listed in Table 2.2 in Chapter 2. Values in the *y* axis represent fold change of gene expression in differentiated ciPSCs relative to that in undifferentiated ciPSCs on day 0, and the gene expression is relative to canine *GAPDH*. * indicates significant difference ($P<0.05$) of gene expression level in differentiated ciPSCs on day 14 compared to the undifferentiated ciPSCs on day 0.

APPENDIX B

A



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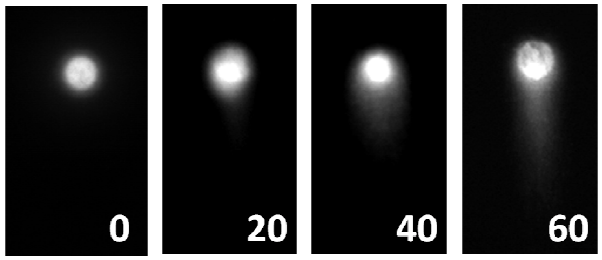

Figure A2: Comet assay indicating the extent of DNA damage in ciPSCs cultured under different conditions. A. ciPSCs maintained in LIF+/bFGF+ medium,

Figure A2 (cont'd)

LIF+/bFGF- medium, LIF-/bFGF+ medium and LIF-/bFGF-medium collected on day0, day 1, day 2 and day 3, as well as the ciPSC maintained in LIF+/bFGF+ medium treated with DMSO, JAK inhibitor (JAKi,1 μM), STAT3 inhibitor (STAT3i, 500 μM), AKT inhibitor (AKTi, 10 μM), and ERK1/2 inhibitor (ERKi, 1 μM) for 24 hours. Canine testicular fibroblasts (CTF) were applied as control. The y-axis on the left and right panels indicates the comet scores. Different letters (a, b, c, d and e) indicate $P<0.05$. **B.** The typical representatives of ciPSCs with different comet scores generated by program Comet Assay IV. The numbers (0, 20, 40, and 60) indicate the comet scores of the cell representative displayed in each picture.

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