TUMOR-LIKE GROWTH OF MOUSE EMBRYONIC STEM CELLS

BY

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THESIS

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ABSTRACT

Due to the capability of self-renewal and differentiation, embryonic stem cells (ESCs) are an invaluable tool for research in regenerative medicine. However, the main problem that hampers application of ESCs to practical stem cell therapies is that ESCs can grow as tumors when transplanted. To date, only one oncogene Eras is known to be responsible for tumorigenic growth of ESCs. Thus, the tumorigenic property of ESCs is poorly investigated.

By global gene expression profiling of mouse ESCs, surprisingly I found that the oncogene Gli2, a downstream target of the growth factor Sonic hedgehog (Shh), was expressed in undifferentiated mouse ESCs, which was further validated by semi-quantitative RT-PCR and immunofluorescent microscopy. It is well-known that the Gli2 protein is activated by Shh. However, interestingly, the Gli2 transcripts were expressed abundantly in mouse ESCs without endogenous Shh expression detected.

To test if expression of Gli2 in mouse ESCs is based on an autonomous mechanism or due to a signal(s) from the extracellular environment, mouse ESCs were cultured in either standard or chemically-defined serum-free conditions (CDSF) for 3 passages. Growth rates and expression levels of the transcription factor Oct3/4 (Pou5f1), a master regulator of pluripotency in mouse ESCs, were monitored. The mouse ESCs maintained in these conditions showed similar levels of Oct3/4 expression, but no endogenous Shh expression. However, intriguingly, the mouse ESCs maintained in CDSF conditions downregulated Eras and Gli2 expression, and exhibited a slower growth rate.

Next, the mouse ESCs cultured in these conditions were injected subcutaneously into NOD-SCID mice to test their tumorigenicity. The mouse ESCs maintained in standard
conditions generated a well-developed tumor in 4 weeks, whereas the mouse ESCs cultured in CDSF conditions failed to form a tumor up to 6 months. When CDSF culture was supplemented with the serum as a positive control, these mouse ESCs upregulated Gli2 expression, proliferated at the similar rate to the ones maintained in the normal conditions, and regained tumorigenicity.

Remarkably, when mouse ESCs were cultured under CDSF conditions supplemented with a pharmacological inhibitor of Gsk3β, they efficiently proliferated and developed into teratomas without upregulation of Eras and c-Myc, but had upregulated Gli2 expression. These findings indicate that Gli2 expression is tightly correlated with the incidence of tumor-like growth of mouse ESCs.

Taken together, these results indicate that Gli2 expression is associated with the tumorigenic property of mouse ESCs, which can serve as a novel marker. Further, we suggest that tumorigenicity in mouse ESCs can be manipulated without comprising the pluripotency of mouse ESCs by maintaining mouse ESCs in CDSF conditions. The tumorigenic growth of mouse ESCs is reversed by pharmacological inhibition of Gsk3β, which suggests that Gsk3β governs the tumor-like growth of mouse ESCs by means of a mechanism different from the one to support the pluripotency of ESCs.
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# TABLE OF CONTENTS

CHAPTER 1: Literature Review .........................................................1

CHAPTER 2: Short-term serum-free culture reveals that inhibition of Gsk3β induces the tumor-like growth of mouse embryonic stem cells..........................15

CHAPTER 3: Gli2 expression is associated with the tumorigenicity of mouse embryonic stem cells.................................................................44

CHAPTER 4: Conclusion .....................................................................67

REFERENCES ....................................................................................70
CHAPTER 1

Literature Review

Abstract

Embryonic stem cells (ESCs) are derived from preimplantation embryos and are capable of both long-term proliferation (self-renewal) and differentiation into cell types of all three germ layers (pluripotency). The self-renewal and pluripotency of ESCs are sustained by certain essential transcription factors. Intriguingly, the viral transduction of these transcription factors into differentiated adult somatic cells results in reprogramming of the developmental process that the somatic cells have undergone. Consequently, pluripotent cells similar to ESCs, termed induced pluripotent stem cells, can be artificially established from specialized cells. These two types of pluripotent stem cells (PSCs) have held the promise of providing customized tissue replacements as well as platforms for drug screening since they were first derived from human tissues and embryos. However, the heterogeneous nature of PSC cultures, which may reflect the plasticity of early embryonic cells, hampers the establishment of a definitive and reproducible culture microenvironment. In addition, the induction of PSC differentiation is dependent on random events and generates heterogeneous populations of specialized cells. Furthermore, PSCs, by definition, are able to generate benign tumors called teratomas, which consist of cell types of three germ layers. To prevent the growth of teratomas in therapeutic transplanted tissue replacements, it is necessary to establish techniques for efficiently manipulating cell fate decisions in PSCs and to understand the mechanism responsible for tumorigenesis in the stem cells. To our surprise, the
mechanism of teratoma formation from PSCs has received little attention to date. Thus, to understand better self-renewal, pluripotency and tumorigenesis in PSCs, this chapter will address the following three simple but overlooked questions:

1. Does every pluripotent stem cell possess identical self-renewal capability?
2. Are current standard culture conditions optimal for maintaining pluripotent stem cells?
3. Is the mechanism of their tumor-like growth known?

**Pluripotent Stem Cells Generate Heterogeneous Populations**

*Pluripotent Stem Cells*

Pluripotent stem cells (PSCs) are an excellent model to study mechanisms of cellular pluripotency and differentiation *in vitro* because of their capacity for self-renewal and their capability to become most kinds of specialized cells, including germ cells. The identification and characterization of a mouse strain that naturally develops testicular teratoma (Stevens, 1973; Stevens and Little, 1954) contributed to demonstrating that teratomas originate from PSCs (Solter, 2006). A benign teratoma, normally found in 1 out of 40,000 live births (Barksdale and Obokhare, 2009), is a “monstrous” tumor consisting of specialized cells derived from all three germ layers ectoderm, mesoderm and endoderm. The first PSCs, embryonic carcinoma cells (ECCs), were derived from malignant teratocarcinomas, which were experimentally generated by transplantation of peri-implantation embryos into the testes of host animals (Stevens, 1970). ECCs are
transplantable, in that they will develop into teratocarcinomas when transplanted. Because ECCs are pluripotent, the original study established an *in vitro* system to study the cell fate decision mechanism. Furthermore, this study indicated that there could be another kind of PSCs in early embryos that could be directly established by *in vitro* culture, but not by transplantation, of early embryos. During mouse preimplantation development, the first cell differentiation event gives rise to the pluripotent inner cell mass (ICM) and the lineage-committed trophectoderm. When cultured on embryonic fibroblasts, the ICM gives rise to pluripotent stem cells. Mouse embryonic stem cells (ESCs) were successfully derived in 1981 (Evans and Kaufman, 1981) and have been the primary model used to investigate mechanisms of cell fate decision. Similar PSCs were later established from primordial germ cells, namely embryonic germ cells (Matsui et al., 1992). These studies on mouse embryos paved the way for the derivation of embryonic stem and germ cells from human embryos (Shamblott et al., 1998; Thomson et al., 1998). The derivation of PSCs from human embryos shed light on regenerative medicine and helped to expand this field of research (Tanaka, 2010). ESCs have been derived from a variety of species (Tanaka, 2010). Studies on self-renewal and pluripotency using ESCs further enabled the establishment of other kinds of PSCs, including early primitive ectoderm-like stem cells (EPLCs; Rathjen et al., 1999) and epiblast-derived stem cells (EpiSCs; Brons et al., 2007; Tesar et al., 2007). Because EpiSCs are derived from, and EPLCs are thought to be equivalent to, cells of post-implantation embryos, their capabilities to generate differentiated cells are more restricted than those of ESCs (Hiratani et al., 2010). That is, embryonic development proceeds by restricting a cell’s ability to generate specialized cells. Therefore, a method to erase such acquired
restrictions in specialized cells was sought to restore differentiated cells to the pluripotent state. This was first achieved by transferring somatic cell nuclei into enucleated oocytes (Briggs and King, 1952; Campbell et al., 1996; Gurdon and Melton, 2008; Rideout et al., 2002; Wakayama et al., 1998). Intriguingly, recent studies have shown that delivering extra copies of four transcription factors that orchestrate self-renewal and pluripotency into differentiated cells results in the reprogramming of the specialized cells into PSCs, called induced pluripotent stem cells (iPSCs; Takahashi and Yamanaka, 2006). Since the successful derivation of iPSCs from human cells (Thomson et al., 1998; Takahashi et al., 2007; Yu et al., 2007), iPSCs have been considered to hold great potential for developing customized replacement tissues and for providing platforms for drug screening. However, cells differentiated from PSCs in vitro that have been transplanted into animal disease models (for example, Brederlau et al., 2006; Jomura et al., 2007; Kerr et al., 2003) tend to develop into teratomas due to residual populations of undifferentiated PSCs. Thus, a better understanding of extrinsic and intrinsic factors involved in cell fate decisions and tumorigenesis in PSCs is necessary to significantly improve iPSC-based stem cell therapy.

**Extrinsic Factors for Maintenance of Self-renewal**

The derivation of ESC lines from human and mouse embryos could not have been accomplished without feeder layers of embryonic fibroblasts. Although cultured ECCs do not require a layer of feeder cells for growth, both embryonic germ cell and iPSC cultures do. Interestingly, conditioned medium (CM) from embryonic fibroblasts was sufficient to support the culture of undifferentiated mouse ESCs in the absence of feeder layers (Smith and Hooper, 1983). Analysis of components in CM led to the identification of the
leukemia inhibitory factor (LIF) as a differentiation inhibitor (Smith et al., 1988; Williams et al., 1988). These studies laid the foundation for investigating the dependence of self-renewal and pluripotency of ESCs on other extrinsic factors. In addition to LIF, the maintenance of mouse ESC culture requires bone morphogenetic protein 4 (Bmp4; Ying et al., 2003), vitamin A (retinol and retinoic acid; Chen and Khillan, 2008; Chen and Khillan, 2010; Wang et al., 2008), threonine (Wang et al., 2009) and a decreased oxidation state (Yanes et al., 2010). The existence of another extrinsic factor independent from the LIF/Stat3 signal, namely ES cell renewal factor, has also been postulated (Dani et al., 1998). The supplementation of basal culture media with animal sera, such as fetal bovine serum (FBS), provides all of these extrinsic factors except LIF. Although human ESCs are similar to mouse ESCs with respect to their self-renewal and pluripotency, the extrinsic factors necessary for mouse ESC culture failed to support the culture of human ESCs. For example, the combination of LIF and serum could not support long-term self-renewal of human ESC lines (Bongso et al., 1994). Furthermore, Bmp4 promoted differentiation of human ESCs into trophoblasts (Xu et al., 2002), whereas long-term proliferation of these cells was maintained in the presence of Noggin, an antagonist of Bmp4 (Wang et al., 2005; Xu et al., 2005b). Instead, the maintenance of human ESC self-renewal and pluripotency mainly relies on basic fibroblast growth factor (bFGF; Xu et al., 2005a). In addition, members of the transforming growth factor β (TGFβ) superfamily, especially TGFβ, activin and Nodal, are essential for maintaining the pluripotency of human ESCs in combination with bFGF (Beattie et al., 2005; James et al., 2005; Vallier et al., 2005). Mouse and human iPSCs exhibit dependency on extrinsic factors similar to mouse and human ESCs, respectively. Mouse and rat EpiSCs are
dependent on activin and bFGF to sustain self-renewal and pluripotency, and thus human ESCs are more similar to these EpiSCs. These discrepancies are attributed to differences in development between mouse and human embryos, even though mouse and human ESCs have been derived from embryos at similar developmental stages. Very interestingly, it has been suggested that the reprogramming process makes human iPSCs more similar to mouse ESCs (Hanna et al., 2010). ECCs do not exhibit dependency on extrinsic factors, whereas the maintenance of embryonic germ cells requires LIF, bFGF and the c-Kit ligand, Steel factor (Matsui et al., 1991; Matsui et al., 1992). Thus, signals from these extrinsic factors may converge in maintaining the activity of a common set of intrinsic genetic factors that define cellular “stemness”.

**Intrinsic Factors to Maintain Self-renewal**

Maintenance of the self-renewal and pluripotency of mouse ESCs relies on the activity of the downstream target of the LIF signal, the Stat3 transcription factor (Matsuda et al., 1999; Niwa et al., 1998). However, key players further downstream of Stat3 are essential for these processes because the LIF/Stat3 signaling pathway is not required for the maintenance of pluripotent cells in developing embryos or for the self-renewal and pluripotency of human ESCs (Dani et al., 1998; Tanaka, 2009). This pathway may interact with the transcription factors Oct3/4/ Pou5f1 (Nichols et al., 1998; Niwa et al., 2000), Sox2 (Avilion et al., 2003; Masui et al., 2007), Nanog (Chambers et al., 2003; Mitsui et al., 2003), Klf4 (Li et al., 2005) and c-Myc (Cartwright et al., 2005). In a steady state, a balance of the relative expression levels of these genes is essential for fate decisions of mouse ESCs (Fujikura et al., 2002; Niwa et al., 2005). The genetic network
of these transcription factors and the expression of their downstream target genes have been elucidated by genomic approaches (Boyer et al., 2005; Ivanova et al., 2002; Loh et al., 2006; Matoba et al., 2006; Ramalho-Santos et al., 2002; Tanaka et al., 2002; Walker et al., 2007). These genomic approaches revealed that cellular pluripotency is characterized by the expression of a unique set of genes that suppress transcripts associated with cellular differentiation. Recently, the self-renewal of mouse ESCs was shown to be maintained by simple pharmacological inhibition of Erk, which is downstream of FGF receptors, and the inhibition of Gsk3β activity (Ying et al., 2008). Because mouse ESCs express Fgf4 (Wilder et al., 1997), these studies indicate that ESCs maintain self-renewal by competing against their own differentiation-inducing signals.

Mouse and human ESCs express Wnt (Lako et al., 2001; Nordin et al., 2008; Okoye et al., 2008), which is the biological inhibitor of Gsk3β, and the pharmacological inhibition of Gsk3β alone promotes self-renewal of both mouse and human ESCs (Sato et al., 2004) as well as derivation of ESC from the ICM (Umehara et al., 2007). However, exogenous Wnt promotes the differentiation of mouse (Lindsley et al., 2006) and human (Wang and Nakayama, 2009) ESCs. Thus, the role of Wnt in the self-renewal of ESCs requires further investigation. Finally, a comparison of global gene expression profiles of mouse ESCs of different genetic backgrounds, teratocarcinoma cells (ECCs) and embryonic germ cells showed that the expression of Rex1 was higher in cells with greater pluripotency (Sharova et al., 2007). The zinc-finger protein Rex1/ Zfp42 was originally identified as one of the genes whose expression was downregulated when the teratocarcinoma cell line F9 was induced to differentiate by retinoic acid (Hosler et al., 1989). However, the targeted knockout of Rex1 revealed that it is not required for the
maintenance of self-renewal (Masui et al., 2008). There are several genes expressed specifically in pluripotent embryonic cells at significant levels, which do not play any essential role in pluripotency (e.g., Esg1/ Dppa5; Amano et al., 2006; Tanaka et al., 2006; Western et al., 2005).

**Transcriptional Heterogeneity in Pluripotent Stem Cells**

One of the challenges in understanding the mechanism of self-renewal and pluripotency of PSCs is that cultured ESCs consist of cell populations that show fluctuating expression of genes. That is, a bulk preparation of ESCs may only show an averaged state of ESCs and thus obscure the presence of distinct ESC populations. Therefore, a better understanding of gene expression at the cellular level is critical. In fact, several groups have performed expression microarray analyses at the single-cell level and have revealed populations of cells that differ in their transcript profiles (Chiang and Melton, 2003; Crino et al., 1998; Kurimoto et al., 2006; Ramos et al., 2006; Tang et al., 2010). Several studies, including ours, have found that well-maintained mouse ESC cultures consist of a small percentage of cells that show fluctuating expression levels of genes such as Dppa3 (Stella/Pgc7; Hayashi et al., 2008; Payer et al., 2006), Nanog (Chambers et al., 2007; Singh et al., 2007), Pecam1 (Furusawa et al., 2004; Furusawa et al., 2006), Rex1 (Toyooka et al., 2008) and Zscan4 (Falco et al., 2007; Zalzman et al., 2010), or genes associated with cell differentiation, such as Brachyury/T (Suzuki et al., 2006a; Suzuki et al., 2006b), Rhox6/9 (Carter et al., 2008), Tcf15 and Twist2 (Tanaka et al., 2008). These genes are either downregulated (Nanog and Rex1) or expressed (the rest) in about one-tenth of cells in culture as a steady state (Tanaka, 2009). Mouse ESCs showing
fluctuating expression of *Nanog*, *Rex1*, *T*, *Dppa3* and *Zscan4* have been extensively characterized. When mouse ESCs were sorted according to expression levels of one of these genes and cultured separately, the resulting ESC populations eventually showed similar fluctuating expression of the gene. For example, when sorted *Zscan4*-positive and -negative subpopulations were replated and cultured separately, both subpopulations regained *Zscan4*-negative and -positive cells, respectively (Zalzman *et al.*, 2010). Each subpopulation possessed a unique differentiation potential. Thus, the heterogeneous nature of PSCs may reflect the plasticity of early embryonic cells (Hayashi *et al.*, 2008; Zalzman *et al.*, 2010). The underlying mechanism responsible for inducing the transcriptional heterogeneity in ESCs remains largely unknown. However, as will be discussed in the following sections, ESCs in culture may have received some signals from the microenvironment, such as the stiffness of culture dishes and serum components, which initiate the heterogeneous transcription of these genes.

**Impact of Culture Conditions on the Self-renewal of Pluripotent Stem Cells**

*Stiffness of a Culture Dish*

When LIF is supplied in the culture medium, mouse ESCs can be maintained on gelatin-coated plates without a layer of embryonic fibroblasts as feeders (Robertson, 1987). Similarly, human ESCs can be maintained on plates coated with Matrigel (a basement membrane preparation extracted from a murine Englebreth-Holm-Swarm sarcoma) independent of a feeder layer in a chemically defined culture medium. Interestingly, other extracellular matrix proteins elicit different responses from ESCs. For example, collagen
IA promotes the self-renewal of mouse ESCs (Furue et al., 2005), and fibronectin and laminin help decrease their differentiation potential (Hayashi et al., 2007; Hayashi et al., 2010). Collagen IV is an inducer of mesoderm lineages for both mouse and human ESCs (Schenke-Layland et al., 2007). Intriguingly, the analysis of Matrigel components has led to the discovery of synthetic polymers that can support the long-term self-renewal of human ESCs (Melkoumian et al., 2010; Rodin et al., 2010; Villa-Diaz et al., 2010).

Recently, it has become evident that cell fate decisions in stem cells are regulated by matrix elasticity or substrate stiffness (Discher et al., 2009). For example, synthetic soft substrates (Elasticity, E = ~1 kPa) that mimic the elasticity of the brain induced the differentiation of neurons from mesenchymal stem cells, whereas stiffer substrates (E = ~40 kPa) that mimic the elasticity of collagenous bone induced the differentiation of osteoblasts (Engler et al., 2006). In contrast, mouse ESCs are intrinsically soft and respond optimally to physical forces when cultured on substrates that match their intrinsic softness, which is 0.6 kPa (about 7000-fold softer than plastic culture dishes; Chowdhury et al., 2010). In culture conditions, mouse ESCs are grown on much harder substrates than any tissue in vivo. Remarkably, when mouse ESCs are plated on the soft substrate (0.6 kPa), they grew as uniformly round colonies without any noticeable differentiating colonies and are able to maintain the expression of markers for pluripotent cells, such as Oct3/4, alkaline phosphatase and Nanog (Chowdhury, Li, Wang and Tanaka submitted to PLoS ONE). Therefore, substrate stiffness is a critical extrinsic factor to sustain the self-renewal of mouse ESCs.

**Culture Conditions with Animal Serum**
Animal serum provides nutrients, hormones, growth factors, steroids and matrix proteins to cultured cells. It also contains remnants of plasma components used for the activation and processing of blood clots as well as other substances that do not normally pass through the endothelial barrier (Hewlett, 1991; Holliday, 1999; Sato et al., 2010). Despite the fact that animal serum is similar but not identical to the interstitial fluid (i.e., lymph) that surrounds cells in vivo, animal serum is preferred for cell culture because it significantly improves the growth of cells. However, animal serum is also known to negatively impact cells in culture (Sato, 1975). For example, complement in serum may inhibit cell growth; these components may be inactivated by heat (Robertson, 1987). In addition, serum promotes aneuploidy in cultured cells (Loo et al., 1987) that may contribute to the incidence of chromosomal instability in mouse ESCs (Rebuzzini et al., 2008). In fact, no cell types in vivo are exposed to serum for extended periods, except the ones in the vicinity of a wound where clotting has taken place (Barnes and Sato, 1980).

Because animal serum provides cell culture with many other uncharacterized components that may compromise the capability of PSCs to self-renew and differentiate, only qualified animal serum can be used for PSC culture (Robertson, 1987). Furthermore, animal products cannot be used to maintain human iPSCs for transplantation purposes (Ludwig et al., 2006b). Although attempts have been made to culture human ESCs in human serum, these cells exhibited extensive differentiation (Rajala et al., 2007).

Chemically defined culture is a preferable alternative, as it not only allows us to obtain more consistent results for better manipulation of PSC differentiation, but can also be applied to practical therapeutic uses for iPSCs.

**Serum-free Culture Conditions**
To eliminate the effects of unknown components in animal serum, chemically defined serum-free culture methods have been established for PSCs (Furue et al., 2005; Furue et al., 2008; Ludwig et al., 2006b; Ying et al., 2003). Typically, these defined culture media are composed of critical growth factors (e.g., LIF and Bmp4) and other factors present in animal sera, such as hormones (e.g., insulin and transferring), vitamins, fatty acids and minerals. In addition, a pre-mixed serum replacement that claims to include no animal serum components was introduced in 1998 (Cheng et al., 2004; Goldsborough et al., 1998). Although the exact components in the serum replacement cannot be disclosed by its patent (Price et al., 1998), the patent indicates that it contains at least albumin, amino acids, vitamins, transferrin, antioxidants, insulin, collagen precursors and some trace elements. In spite of the fact that the serum replacement successfully supported the growth of primate ESCs (e.g., Suemori et al., 2001), human ESCs cultured with this preparation indicated the presence of some BMP-like factors that induced the differentiation of trophoblasts (Xu et al., 2005b). The maintenance of the undifferentiated state of both mouse and human ESCs using defined culture media has been well documented (Hayashi et al., 2007; Ludwig et al., 2006a; Ludwig et al., 2006b; Ying et al., 2008), and the pluripotency of these mouse ESCs has been validated by their differentiation in vitro (Furue et al., 2005; Hayashi et al., 2007) and by the development of chimeric mice (Ying et al., 2003).

**Tumorigenesis in Pluripotent Stem Cells**
The ability of cells to grow as a teratoma after transplantation into a host animal is a hallmark of cellular pluripotency (see “1.1 Pluripotent stem cells”; Chambers and Smith, 2004; Damjanov and Andrews, 2007; Jaenisch and Young, 2008; Lensch and Ince, 2007; Solter, 2006). Testing this cellular ability requires no special techniques or equipment and reduces the use of experimental animals, and it is particularly useful and widely accepted for the validation of pluripotency in human PSCs (Yu and Thomson, 2008). However, this cellular ability is the major critical safety issue hampering the therapeutic application of human iPSCs (Yamanaka, 2009). According to Lawrenz et al. (2004), two mouse ESCs were sufficient to grow into a teratoma only when mixed with 2 x 10^6 non-tumorigenic fibroblasts (MRC-5) prior to transplantation into immunocompromised mice. To date, little is known about the tumorigenic property of PSCs, except that the oncogene Eras is responsible for the tumorigenic growth of mouse ESCs (Takahashi et al., 2003). It is interesting to note that Eras activates Akt (Takahashi et al., 2003) and that constitutive activation of Akt is sufficient to drive self-renewal of mouse and non-human primate ESCs (Watanabe et al., 2006). In addition, Akt mediates the inactivation of Gsk3β by insulin via phosphorylation (Bechard and Dalton, 2009; Cross et al., 1995; Wu and Pan, 2010). Gsk3β inhibits its downstream target c-Myc through β-catenin (Bechard and Dalton, 2009; He et al., 1998), so Eras may indirectly activate c-Myc, which is responsible for the self-renewal of mouse ESCs (Cartwright et al., 2005) and for tumorigenesis in mouse iPSCs (Nakagawa et al., 2010; Okita et al., 2007). However, this model may involve other uncharacterized gene products, as human ESCs do not express...
human ERAS (Kameda and Thomson, 2005; Tanaka et al., 2009) but develop into teratomas.
CHAPTER 2

Short-term serum-free culture reveals that inhibition of Gsk3\(\beta\) induces the tumor-like growth of mouse embryonic stem cells

Abstract

I am presenting evidence that the tumor-like growth of mouse embryonic stem cells ESCs is suppressed by short-term serum-free culture, which is reversed by pharmacological inhibition of Gsk3\(\beta\). Mouse ESCs maintained under standard conditions using fetal bovine serum (FBS) were cultured in a uniquely formulated chemically-defined serum-free CDSF medium, namely ESF7, for three passages before being subcutaneously transplanted into immunocompromised mice. Surprisingly, the mouse ESCs failed to produce teratomas for up to six months, whereas mouse ESCs maintained under standard conditions generated a well-developed teratoma in five weeks. Mouse ESCs cultured under CDSF conditions maintained the expression of Oct3/4, a master regulator of cellular pluripotency. In addition, when mouse ESCs were cultured under CDSF conditions supplemented with FBS, or when the cells were cultured under CDSF conditions followed by standard culture conditions, they consistently developed into teratomas. Thus, these results validate that the pluripotency of mouse ESCs was not compromised by CDSF conditions. Mouse ESCs cultured under CDSF conditions proliferated significantly more slowly than mouse ESCs cultured under standard conditions, and were reminiscent of Eras-null mouse ESCs. In fact, their slower proliferation was accompanied by the downregulation of Eras and c-Myc, which regulate the tumor-like growth of mouse ESCs. Remarkably, when mouse ESCs were cultured
under CDSF conditions supplemented with a pharmacological inhibitor of Gsk3β, they efficiently proliferated and developed into teratomas without upregulation of Eras and c-Myc, whereas mouse ESCs cultured under standard conditions expressed Eras and c-Myc. Although the role of Gsk3β in the self-renewal of ESCs has been established, it is suggested with these data that Gsk3β governs the tumor-like growth of mouse ESCs by means of a mechanism different from the one to support the pluripotency of ESCs.

**Introduction**

As summarized in Chapter 1 Literature Review, embryonic stem cells (ESCs; Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998) and induced pluripotent stem cells (iPSCs; Park et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007) are very promising tools for use in drug screening and customized tissue replacement (Gurdon and Melton, 2008) because they are capable of self-renewal that sustains pluripotency. The self-renewal and pluripotency of mouse stem cells ESCs and iPSCs are maintained by extrinsic factors, such as supplementing basal culture medium with leukemia inhibitory factor LIF (Niwa, 2007; Ohtsuka and Dalton, 2008; Smith et al., 1988; Tanaka, 2009; Williams et al., 1988) and fetal bovine serum (FBS). However, FBS also provides cultures with many other uncharacterized components that may affect the capability of ESCs and iPSCs to self-renew and differentiate. Undefined culture conditions using animal sera may have contributed to results finding contradictory roles of the Wnt signaling pathway in ESCs (Dravid et al., 2005; Lindsley et al., 2006; Sato et al., 2004; Ying et al., 2008). However, it is now firmly established that pharmacological
inhibition of glycogen synthase kinase 3β Gsk3β promotes the self-renewal of both mouse (Bechard and Dalton, 2009; Ying et al., 2008) and human ESCs (Sato et al., 2004), and derivation of mouse ESCs (Umehara et al., 2007).

To eliminate the effects of unknown components in animal sera as well as the contamination of animal products, chemically-defined serum-free culture methods have been established (Furue et al., 2005; Furue et al., 2008; Ludwig et al., 2006b; Ying et al., 2003). Commercially-made serum replacements that may contain animal products (Goldsborough et al., 1998) are often used to maintain ESC culture (e.g., Suemori et al., 2001), although the exact components cannot be disclosed by their patents (Furue et al., 2008). The maintenance of the undifferentiated state of mouse ESCs using defined culture media has been well documented (Hayashi et al., 2007; Ying et al., 2008). Furthermore, the pluripotency of these mouse ESCs has been validated by their differentiation in vitro (Furue et al., 2005; Hayashi et al., 2007) or by the development of chimeric mice (Ying et al., 2003).

Another way to validate the pluripotency of ESCs and iPSCs is to examine the ability of these cells to develop into tumors called teratomas after their transplantation into immunocompromised mice (Chambers and Smith, 2004; Jaenisch and Young, 2008; Solter, 2006). Such teratoma formation assays have validated the pluripotency of mouse ESCs maintained in the presence of a Gsk3β inhibitor (Sato et al., 2004; Umehara et al., 2007). This method, which requires no special technique or equipment and reduces the use of experimental animals, is particularly useful and widely accepted for the validation of pluripotency in human ESCs and iPSCs (Tanaka, 2009; Yu and Thomson, 2008). However, their tumor-like growth hampers the therapeutic application of human iPSCs.
Little is known about the inherent tumorigenic property of ESCs, except that the oncogene Eras regulates the tumor-like growth of mouse ESCs via activation of Akt1 (Takahashi et al., 2003), which may result in inactivation of Gsk3β (Bechard and Dalton, 2009; Cross et al., 1995; Wu and Pan, 2010). However, human ESCs do not express human ERAS (Kameda and Thomson, 2005; Tanaka et al., 2009), but grow into teratomas (Thomson et al., 1998). Therefore, the underlying mechanism involved in the tumor-like growth of ESCs remains unknown. On the other hand, mouse ESCs contribute to the development of normal chimeras, instead of forming teratomas, when mixed with mouse preimplantation embryos. This finding indicates that mouse ESCs require proper extrinsic signals or niches (Voog and Jones, 2010) to differentiate normally and to contribute to the development of chimeras. In contrast, mouse ESCs exhibit cell death when they are cultured without LIF (Duval et al., 2000; Furue et al., 2005). This result raises the question of whether mouse ESCs inherently possess the tumor-like property or are provided with extrinsic signals that promote their tumorigenesis. In this chapter, I present experimental evidence that short-term serum-free culture reduces the tumorigenicity of mouse ESCs, which is reversed by pharmacological inhibition of Gsk3β. It is suggested with these data that the activity of Gsk3β orchestrates the tumor-like growth of ESCs, which may support a novel mechanism independent from the one regulating the pluripotency of ESCs.
Materials and Methods

Cell culture

A mouse embryonic stem cell line of R1 (Nagy et al., 1993), which expresses EGFP driven by the Oct3/4 promoter (Viswanathan et al., 2003), was kindly provided by Dr. William L. Stanford, University of Toronto, Ontario, Canada (Walker et al., 2007). This mouse ESC line was thawed on feeders and maintained under standard (Tompers and Labosky, 2004) or chemically-defined serum-free CDSF (Furue et al., 2005) conditions at 37 °C, 5 % CO2. The exact number of passages that this mouse ESC line has gone through is unknown. However, when these ESCs were brought to our laboratory, they were passaged on feeders two more times and frozen as a stock. When these ESCs were thawed on feeders, more frozen stocks were made at passage 5-7. These stocks were used for the current study. Under standard conditions, mouse ESCs were maintained on 0.1 % gelatin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com)-coated tissue culture dishes in high glucose-Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 15 % fetal bovine serum (FBS; Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM GlutaMax I (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 units /ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich), 0.1 mM 2-Mercaptoethanol (Sigma-Aldrich), and 1,000 U/ml LIF (Millipore, Billerica, MA, http://www.millipore.com). When the mouse ESCs reached 80 % to 100 % confluence, they were routinely passaged at a ratio of 1:6 every two days using TrypLE™ Express (Invitrogen). The cells were discarded after being passaged 10 times onto gelatin-coated dishes. Approximately $1 \times 10^5$ /cm² mouse ESCs maintained under standard conditions were plated onto tissue culture dishes coated with
0.15 mg/ml type IA collagen (Nitta Gelatin Co., Osaka, Japan, http://www.nitta-gelatin.co.jp), which contain the ESF7 medium (Cell Science & Technology Institute Inc. Miyagi, Japan, http://www.cstimedia.com; Furue et al., 2005) supplemented with 1,000 U/ml LIF. This was counted as passage 1 under CDSF conditions. Mouse ESCs at passage 7-12 were used to start CDSF culture. Mouse ESCs grown under CDSF conditions were split every 3 or 4 days with 0.02 % EDTA Sigma-Aldrich. Similarly, mouse ESCs of W4 (129S6, purchased at passage 9; Taconic, Hudson, NY, http://www.taconic.com) were used to test CDSF conditions. When CDSF culture was supplemented with FBS, 0.02 % EDTA was used for passaging cells. The following serum lots were used to supplement CDSF culture: Lot. 1359246 and 726570, Invitrogen; Lot. L0228, Atlanta Biologicals (Lawrenceville, GA, http://www.atlantabio.com); Lot. A74B00Z, Gemini Bio-Products (West Sacramento, CA, http://www.gembio.com).

Images of cell morphology and fluorescence were taken under the same conditions using an inverted microscope equipped with an epi-fluorescence lamp (DMI4000B, Leica Microsystems, Wetzlar, Germany, http://www.leica-microsystems.com). To measure the frequency of cell doubling, the mouse ESCs were plated at 1x10^6 per one well of 6-well plates. Two days after plating, the number of cells was counted for the second and third passages. Cell doubling was calculated based on the following formula: [Cell doubling=log₂ (the number of cells 2 days after plating)/(1x10^6)]. Statistical tests were performed using the Mann-Whitney’s U-test.
Cell transplantation

At the fourth passage under standard or CDSF conditions, the mouse ESCs were trypsinized and counted. TrypLE™ Express was inactivated with an equal volume of 1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich). For this purpose, no culture medium with animal serum was used. One to two million cells were centrifuged at 1,000 g for 5 min and resuspended into 25 µl PBS, which was mixed with 25 µl of 0.3 mg/ml type I collagen. Mouse ESCs were kept on ice before being injected into NOD-SCID mice (the Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) subcutaneously. Animal health was monitored routinely until the diameter of the tumors reached several centimeters at which time the animals reached their end points and were euthanized. This procedure was approved by the Illinois Institutional Animal Care and Use Committee. The incidence of teratoma formation was statistically validated using the Fisher’s exact probability test.

Histology

Before dissecting the teratomas, pictures of the experimental animals were taken. The sizes of the longest axis of the teratomas were measured. Then, the teratomas were surgically dissected out, cut into smaller pieces, and fixed in 4 % paraformaldehyde (Sigma-Aldrich) at 4 ºC overnight, followed by dehydration and embedding in Paraplast plus (Sigma-Aldrich). Sections of 8 µm thickness were cut and processed for standard hematoxylin and eosin staining.
**RT-PCR**

A total of 1.6 µg of total RNA extracted from mouse ESCs cultured under each condition was used to synthesize the first cDNA strand as previously described (Tanaka et al., 2008; Tanaka et al., 2010). PCR mixtures were prepared using Phusion DNA polymerase (New England Biolab, Ipswich, MA, http://www.neb.com) according to the manufacturer’s instructions. The PCR conditions were as follows: initial denaturing at 98 °C for 1 min followed by 24 or 25 cycles of denaturing at 98 °C for 10 sec, annealing at 65 °C for 30 sec, extension at 72 °C for 30 sec, and a final extension at 72 °C for 7.5 min. The primer pairs have previously been described: Eras (Takahashi et al., 2003), Oct4 (Nichols et al., 1998), Esg1 and Efla (Tanaka et al., 2002), Sox2 (Masui et al., 2007), and c-Myc (Takahashi and Yamanaka, 2006).

**Screening of factors**

A day after mouse ESCs were plated under CDSF conditions, these cultures were supplemented with 10 nM retinol (Sigma-Aldrich) in 100 % EtOH with or without 1 nM retinol binding protein (RBP) from human urine (Sigma-Aldrich), 10 nM all-trans retinoic acid (Sigma-Aldrich), 10 ng/ml Bmp4 (R & D Systems, Minneapolis, MN, http://www.rndsystems.com), or 3µM Stemolecule™ CHIR99021 in DMSO (Stemgent, Cambridge, MA, http://www.stemgent.com). Before applying RBP to culture, buffer exchange was carried out with Amicon Ultra Millipore to remove a preservative. Mouse ESCs grown under these conditions were split with 0.02 % EDTA for 3 passages when
they reached confluence. At the fourth passage, these mouse ESCs were injected into NOD-SCID mice as described above.

**Results**

**Mouse Embryonic Stem Cells Reduced their Tumorigenicity but Maintained their Pluripotency under Chemically-defined Serum-free Culture**

To examine whether mouse ESCs are destined to grow as teratomas, mouse ESCs were cultured in chemically-defined serum-free medium with LIF (referred to as “CDSF”; Furue et al., 2005) for three passages (Fig. 2.1A and C) and subcutaneously transplanted them into non-obese diabetic mice with severe combined immunodeficiency disease (NOD-SCID) mice (Larochelle *et al.*, 1996). A uniquely formulated serum-free medium, ESF7 (see Materials and Methods), was used because components in this medium is fully disclosed (Furue *et al.*, 2005). This medium has been used to maintain mouse ESCs by other studies (e.g., (Hayashi *et al.*, 2007; Hayashi *et al.*, 2010). However, the pluripotency of mouse ESCs cultured under this medium has not been tested by teratoma formation assays. Surprisingly, these mouse ESCs failed to grow as teratomas (Fig. 2.1F-H), whereas mouse ESCs maintained under standard conditions (Fig. 2.1A and B) grew into a well-developed teratoma in 5 weeks (Fig. 2.1F, 2.2A-E and Fig. 2.3A). When mouse ESCs were cultured in CDSF supplemented with 15 % FBS (referred to as “CDSF+FBS”; Fig. 2.1A and D), they formed a well-developed teratoma in 5 weeks (Fig. 2.1G, 2.2F-J and Fig. 2.3B). Thus, tumorigenesis in mouse ESCs is not simply inhibited by CDSF conditions. When the injections were properly performed, we did not observe blood
coming out of the injection sites. Only properly performed injections were counted in the present study.

CDSF did not compromise the pluripotency of mouse ESCs per se for the following reasons. First, throughout the culture period (three passages, 9-12 days), the transcriptional activity of the master regulator of pluripotency, Oct3/4 (Pou5f1; Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990), was validated using a mouse ESC line that expresses the enhanced green fluorescent protein (EGFP) under the Oct3/4 promoter (Fig. 2.1B and C; Walker et al., 2007). Unless otherwise noted, this mouse ESC line was used throughout the present study. Furthermore, when the mouse ESC line was maintained in either CDSF+FBS (n=4) or CDSF for three passages and brought back to the standard medium for one or two passages (referred to as “CDSF-Standard”, n=4; Fig. 2.1A), EGFP expression was maintained (Fig. 2.1D and E) and the cells became teratomas (Fig. 2.1G and H, and 2.2F- O; p<0.00016, Table 2.1 and Fig. 2.3B and C). Similar results were obtained using four different lots of FBS. These data demonstrate that tumorigenesis in mouse ESCs can be suppressed by short-term culture in the serum-free medium. The plasticity of tumorigenicity in mouse ESCs appears to be unique; embryonic carcinomas F9 (Bernstine et al., 1973), which are also pluripotent stem cells, formed teratomas when cultured in CDSF and transplanted (Fig. 2.3D).
Mouse Embryonic Stem Cells Cultured under Serum-free Conditions Exhibited Longer Doubling Time while Maintained Expression of Genes Associated with Cellular Pluripotency

When NOD-SCID mice transplanted with mouse ESCs reached their end points, they were sacrificed and examined for teratomas. This procedure usually yielded teratomas of about 30 mm in diameter (bars in Fig. 2.4A). However, the number of days needed for the experimental animals to reach their end points varied (dots in Fig. 2.4A). Interestingly, it took 47 ± 3.1 days for mouse ESCs cultured in CDSF+FBS to grow into 30 mm teratomas, whereas it took 37 ± 1.8 days for mouse ESCs cultured in CDSF followed by standard conditions for two passages to reach 30 mm in diameter. However, when mouse ESCs cultured in CDSF were passaged only once into standard conditions, it took 76 days for these mouse ESCs to grow into a 30 mm teratoma. Although this sample size is too small to be statistically significant, it is suggested with these data that CDSF may even suppress the growth of teratomas.

Among the experimental NOD-SCID mice examined in this study, two out of the seven mice had mouse ESCs cultured in CDSF injected into one side of the animal and mouse ESCs cultured in media containing FBS injected into the other side. Therefore, the formation of teratomas from mouse ESCs cultured in CDSF could not be examined beyond the end point of the animals (Fig. 2.4A). However, in the other five animals, we were able to determine that transplanted mouse ESCs under CDSF did not generate teratomas for up to 6 months. This examination is ongoing.
At one injection site, we were able to identify a tiny mass of mouse ESCs that had been cultured under CDSF-Standard conditions one week after transplantation (Fig. 2.3E). The mouse ESCs in this mass had the appearance of cells undergoing initial differentiation (Fig. 2.5). However, we did not observe any cellular mass at the injection sites that were derived from mouse ESCs cultured in CDSF at either one week or six months after transplantation.

Mouse ESCs cultured in CDSF exhibited a significantly longer doubling time (~28 hrs) than ones cultured under standard conditions (~17 hrs, $p<0.005$) during the first two passages, whereas ones cultured in CDSF+FBS or under CDSF-Standard conditions took ~19 and ~20 hrs to divide, respectively (Fig. 2.4B, top). Similar results were obtained with another mouse ESC line, W4 (Auerbach et al., 2000; Raz et al., 1999) (data not shown). The differences in doubling times of mouse ESCs were evident as soon as two days after transfer to CDSF conditions (1.91 doublings ± S.E.M. of 0.0967 vs. 2.72 doublings ± S.E.M. of 0.0923 for the standard condition in 48 hrs, $p<0.005$). Despite the longer doubling time, mouse ESCs cultured in CDSF did proliferate steadily (Fig. 2.4B, bottom). Additionally, transcripts associated with cellular pluripotency, Sox2, Esg1/Dppa5 and Oct3/4, were expressed in mouse ESCs cultured in CDSF (Fig. 2.4C). Interestingly, Eras, which regulates the tumorigenic growth of mouse ESCs (Takahashi et al., 2003), was downregulated in these mouse ESCs (Fig. 2.4C), whereas it became upregulated when mouse ESCs were maintained in CDSF+FBS or under CDSF-Standard conditions (Fig. 2.4C). Collectively, the loss of tumor-like potential in mouse ESCs cultured under CDSF is associated with a slower growth rate and the reduced expression
of Eras. It is interesting to note that Eras-null mouse ESCs can contribute to the germline in chimeric animals but show significantly reduced growth rate (Takahashi et al., 2003).

**Pharmacological Inhibition of Gsk3β Reversed the Effect of Serum-free Culture on the Tumor-like Growth of Mouse Embryonic Stem Cells**

To identify a potential serum factor responsible for inducing the tumor-like growth in mouse ESCs, initially we focused on molecules known to sustain the self-renewing growth of mouse ESCs, such as LIF (Smith et al., 1988; Williams et al., 1988), Bmp4 (Ying et al., 2003), vitamin A derivatives (all-trans retinoic acid, RA; Wang et al., 2008) and retinol; (Chen and Khillan, 2008; Chen and Khillan, 2010), and simultaneous inhibition of Gsk3β and Erk (Ying et al., 2008). Pharmacological inhibition of Gsk3β alone promotes self-renewal of both mouse and human ESCs (Sato et al., 2004). We excluded LIF from screening because CDSF contains LIF (Furue et al., 2005). In the absence of LIF, mouse ESCs undergo differentiation or cell death (Duval et al., 2000; Furue et al., 2005). We also ruled out an inhibitor of Erk (Ying et al., 2008), which acts downstream of FGF receptors, because inhibition of Erk cannot promote the growth of mouse ESCs (Ying et al., 2008). Therefore, we focused on testing other molecules such as Bmp4, RA, retinol with or without retinol binding protein (RBP) (Soprano and Blaner, 1994), and an inhibitor of Gsk3β (CIHR99021; Ying et al., 2008).

Addition of RA in CDSF induced differentiation of mouse ESCs as evidenced by the reduced expression level of EGFP (Fig. 2.6A). Addition of retinol with or without RBP in CDSF did not induce differentiation of mouse ESCs (Fig. 2.6B and C), but failed to
accelerate their growth and to induce teratoma formation (Fig. 2.7A and B). In contrast, mouse ESCs cultured in CDSF with Bmp4 or the Gsk3β inhibitor maintained Oct3/4 expression (Fig. 2.6D and E), increased the number of cell doublings (Fig. 2.7A) and formed teratomas in 17 % or 67 % of transplantations by 7 months, respectively (Fig. 2.7B and C, and Fig. 2.3F and G). Also, W4 mouse ESCs exhibited similar phenotypic changes when maintained in CDSF with the Gsk3β inhibitor (Fig. 2.7B; the one with n.d. is from W4 cell line). Gsk3β is known to regulate the activity of the c-Myc protein in mouse ESCs (Bechard and Dalton, 2009; Cartwright et al., 2005). However, RT-PCR analysis showed that inhibition of Gsk3β did not result in upregulation of Eras and c-Myc in mouse ESCs cultured in CDSF, whereas Bmp4 induced upregulation of Eras and c-Myc (Fig. 2.7D).

Discussion

In this chapter, I present experimental evidence to suggest that short-term CDSF culture reduces the tumor-like growth of mouse ESCs, which is reversed by pharmacological inhibition of Gsk3β. The present study indicates that downstream of Gsk3β is primarily responsible for tumorigenesis in mouse ESCs, which may involve uncharacterized gene products. Although the exact mechanism currently remains unknown, our present study provides a basis for further study to establish the signaling pathway responsible for the tumor-like property of ESCs.

In general, serum provides hormones, growth factors, and steroids to cultured cells. It also contains remnants of plasma components used for the activation and processing of
blood clots and substances that do not normally pass through the endothelial barrier (Hewlett, 1991; Holliday, 1999; Sato et al., 2010). Therefore, serum is similar but not identical to the interstitial fluid (i.e., lymph) that surrounds cells in vivo (Sato, 1975). No cellular mass was observed at the injection sites, which were derived from mouse ESCs cultured in CDSF at either one week or six months after transplantation. Therefore, it is suggested with these data that interstitial fluid will not support the tumor-like growth of mouse ESCs.

Perhaps mouse ESCs cultured under short-term CDSF conditions became more susceptible to LIF and exhibited cell death after transplantation due to the absence of a continuous supply of LIF (Furue et al., 2005). However, because each mouse ESC line shows a different degree of LIF dependency (Raz et al., 1999), other mouse pluripotent stem cells cultured in the serum-free medium may exhibit a capability to continuously grow after transplantation, as observed in embryonic carcinomas (F9; Fig. 2.3D). In addition, the formation of teratomas became sporadic when 0.5 x 10^6 mouse ESCs cultured under standard conditions were transplanted for a period of 6 months. Collectively, this study demonstrated that one or more extrinsic factors or niche (Voog and Jones, 2010) plays an important role in the formation of a teratoma. This idea is further supported by the fact that two mouse ESCs were sufficient to grow into a teratoma only when mixed with 2 x 10^6 non-tumorigenic fibroblasts (MRC-5) prior to transplantation into immunocompromised mice (Lawrenz et al., 2004). Interestingly, the slow growth observed in Eras-null mouse ESCs became more evident when they were cultured without feeder cells (Takahashi et al., 2003).
These data indicate that animal sera contain one or more factors that inhibit the activity of the Gsk3β protein (Fig. 2.1 and 2.7). Gsk3β is involved in the canonical Wnt signaling pathway (Doble and Woodgett, 2003; Joje and Johnson, 2004; Wu and Pan, 2010) and interacts with other biologically important signaling pathways such as Phosphoinositide 3-kinase (PI3K)-Akt1 (Cross et al., 1995; Paling et al., 2004; Watanabe et al., 2006), Bmp4 (Itasaki and Hoppler, 2010) and hedgehog (Kim et al., 2009; Riobo et al., 2006; Zhang et al., 2005) signaling pathways. Although the secreted protein Wnt eventually inhibits the activity of Gsk3β, the role of Wnt in the maintenance of self-renewal and pluripotency of ESCs remains elusive (Dravid et al., 2005; Lindsley et al., 2006; Sato et al., 2004; Ying et al., 2008). On the other hand, pharmacological inhibition of Gsk3β supports the self-renewal and pluripotency of ESCs (Bechard and Dalton, 2009; Sato et al., 2004; Umehara et al., 2007; Ying et al., 2008). Now, the question is what Gsk3β upstream and downstream genes are active in self-renewing ESCs.

Both LIF-Stat3 (Matsuda et al., 1999; Niwa et al., 1998) and insulin pathways activate the PI3K-Akt1 signaling pathway (Bechard and Dalton, 2009; Paling et al., 2004), which mediates the inactivation of Gsk3β (Bechard and Dalton, 2009; Cross et al., 1995; Wu and Pan, 2010). However, CDSF includes LIF and insulin (Furue et al., 2005), and failed to support the tumor-like growth of mouse ESCs (Fig. 2.1 and 2.4). Thus, LIF and insulin are not upstream of Gsk3β. Because Eras activates Akt1 (Takahashi et al., 2003), the downregulation of Eras in mouse ESCs cultured in CDSF (Fig. 2.4C and 2.7D) may have resulted in the activation of Gsk3β. However, human ESCs do not express human ERAS but they do grow into teratomas (Kameda and Thomson, 2005; Tanaka et
al., 2009). Because this signaling cascade is not evolutionarily conserved, Eras is not upstream of Gsk3β.

Based on our results, Bmp4 poorly promoted proliferation and teratoma formation of mouse ESCs cultured under CDSF conditions (Fig. 2.7A and B). It is well known that the Bmp4 and Wnt signals interact with each other in many morphogenetic events, which could result in either synergistic or antagonistic effects depending on cell types (Itasaki and Hoppler, 2010). Because of the following two observations, we consider the effect of Bmp4 on Gsk3β or the tumor-like growth of mouse ESCs antagonistic or indirect. First, our RT-PCR results revealed that mouse ESCs cultured in CDSF supplemented with Bmp4 efficiently upregulated oncogenes Eras and c-Myc, which was not the case in mouse ESCs treated with the Gsk3β inhibitor (Fig. 2.7D). Second, in one set of these experiments, Bmp4 efficiently induced formation of teratomas when ESCs at the earlier passage number (passage 7) were used for the culture (see ii in Fig. 2.7B). In contrast, when mouse ESCs at passage 9 or later were cultured in CDSF with Bmp4 and transplanted, they sporadically developed into teratomas. Taken together, further investigation is required to determine upstream of Gsk3β in ESCs.

It is interesting to note that in concert with LIF, Bmp4 suppresses differentiation of the neural lineage in mouse ESCs maintained under serum-free conditions (Ying et al., 2003) supplemented with N2 and B27, which were originally developed to culture a neuroblastoma cell line (Bottenstein and Sato, 1979) and hippocampal neurons (Brewer et al., 1993), respectively. Mouse ESCs maintained under serum-free conditions supplemented with LIF, Bmp4, N2 and B27 are pluripotent and can contribute to the germline in chimeric animals (Ying et al., 2003). On the other hand, our current study
demonstrated that both the CDSF medium used in the present study, which contains LIF, and the CDSF medium supplemented with Bmp4 poorly sustained the tumor-like growth of mouse ESCs. Therefore, it is suggested with these data that the cellular pluripotency and the tumor-like growth of ESCs may be regulated by different mechanisms.

Gsk3β inhibits the activity of its target c-Myc (Bechard and Dalton, 2009; He et al., 1998), which is involved in the self-renewal of mouse ESCs (Cartwright et al., 2005) and responsible for an age-associated incidence of tumorigenesis in chimeric mice generated with mouse iPSCs (Nakagawa et al., 2010; Okita et al., 2007). In contrast, our RT-PCR results showed that mouse ESCs cultured under CDSF conditions with the Gsk3β inhibitor failed to significantly upregulate transcription of Eras and c-Myc (Fig. 2.7D) but efficiently developed into teratomas (Fig. 2.7B and C). Thus, Gsk3β downstream genes other than c-Myc may regulate the tumor-like growth of ESCs, which play independent roles from maintaining the self-renewal of ESCs. In addition, candidate Gsk3β downstream genes responsible for tumorigenesis in mouse ESCs may act independently from ERAS in human ESCs (Kameda and Thomson, 2005; Tanaka et al., 2009). Further study to identify the candidate genes and to test their roles in human ESCs and iPSCs will lead us to establishing a strategy to significantly improve the safety of human iPSCs.
Figure 2.1. The tumorigenicity of mouse embryonic stem cells can be reduced by short-term serum-free culture. (A): A mouse embryonic stem cell ESC line harboring an EGFP driven by the Oct3/4 promoter was maintained in either standard or chemically-defined serum-free (CDSF) medium as indicated. (B-E): Phase contrast (left) and fluorescence (right) images of the ESC line under the conditions indicated above are shown. Bars, 20 µm. (F-H): After the mouse ESCs were cultured as indicated, they were transplanted into NOD-SCID mice subcutaneously. Teratoma formation was observed by week 11. Bars, 1 cm.
<table>
<thead>
<tr>
<th>Ectoderm</th>
<th>Mesoderm</th>
<th>Endoderm</th>
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<tbody>
<tr>
<td>Neural tissue</td>
<td>Keratin pearl</td>
<td>Cartilage</td>
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Figure 2.2. Identification of three germ layers in teratomas. (A-E): Teratomas developed from mouse ESCs cultured under standard conditions. (F-J): Teratomas developed from mouse ESCs cultured in CDSF supplemented with 15% FBS (CDSF+FBS). (K-O): Teratomas developed from mouse ESCs cultured in CDSF followed by transfer to standard conditions (CDSF to Stand.) Ectoderm is represented by neural tissue and keratin pearl, mesoderm is represented by cartilage and striated muscle, and endoderm is represented by ciliated epithelium. Bars, 20 µm.
Figure 2.3. Anatomical images of NOD-SCID mice transplanted with mouse embryonic stem cells. (A-D, F and G): Teratomas developed from mouse ESCs and embryonic carcinomas (F9 in D) cultured under the conditions indicated are shown. Also, the number of weeks (wks) needed for the experimental animals to reach their end points are shown. Bars, 1 cm. CDSF+FBS, CDSF culture supplemented with fetal bovine serum; CDSF-Standard, CDSF conditions followed by standard conditions; CDSF (F9), embryonic carcinoma cells F9 maintained under CDSF conditions; CDSF+Bmp4, CDSF culture supplemented with Bmp4; CDSF+iGsk3β, CDSF culture supplemented with the Gsk3β inhibitor. (E): This animal was sacrificed one week after mouse ESCs cultured under CDSF conditions followed by standard conditions were transplanted. The rectangle indicates the area shown in the inset. Bar, 1 cm. The inset shows an enlarged image of a
Figure 2.3 (cont.)

tiny mass of the mouse ESCs depicted by dashed lines with a scale bar of 0.1 cm. See supplemental information Fig. 2 for detail.
Figure 2.4. Phenotypic changes observed in mouse embryonic stem cells cultured under CDSF conditions. (A): The sizes of the teratomas formed (orange bar, left axis) and the number of days required for the experimental NOD-SCID mice to reach their end points (blue stars, right axis) were compared among mouse ESCs cultured under the conditions indicated (see Fig. 2.1A). Parentheses indicate the number of biological replicates (i.e., mouse ESCs prepared at different passages) per culture condition. Standard errors of the means are indicated by bars. a: Only one passage in standard conditions followed CDSF culture. b: Two out of seven transplantations showed no sign of teratoma formation when paired with the standard and CDSF-Standard conditions, whereas five out of seven transplantations showed no sign of teratoma formation for 6 months. (B, top): Cell doublings were measured every 48 hours after plating 1x 10^6 cells per well onto 6-
well plates (see Materials and Methods for the formula). Only CDSF conditions produced statistically-significant differences compared to standard conditions. Parentheses indicate the number of biological replicates per condition. Standard errors of the mean are indicated by bars. (B, bottom): Phase contrast (top) and fluorescence (bottom) images are shown for the $Oct3/4::EGFP$ mouse ESC line (Fig. 2.1A) grown under the conditions indicated below 1 and 2 days after plating $0.1 \times 10^6$ cells per well in 6-well plates. Bars, 50 µm. (C): Abundance of each transcript indicated above was examined in mouse ESCs cultured under each condition on the right by 25 cycles of PCR. $Ef1\alpha$ was used as a reference.
Figure 2.5. Mouse embryonic stem cells exhibited initial differentiation as early as one week after transplantation when cultured under CDSF-standard conditions.

Mouse ESCs were cultured in CDSF for three passages followed by transfer to standard conditions for two passages prior to transplantation. (A): An epithelialized cellular mass has aggregates formed among the collagen fibers (stained pale pink) used to transplant the mouse ESCs. Rectangles indicate the areas shown in B, C and D. Bar, 500 µm. (B-D): Two types of cells are prominent, one of which is reminiscent of keratin pearls (B and D), and the other that resembles cartilage (C and D). Bars, 20 µm.
Figure 2.6. Identification of factors that support the tumor-like growth of mouse embryonic stem cells maintained under CDSF conditions. (A-E): Phase contrast (top) and fluorescence (bottom) images of mouse ESCs under CDSF conditions supplemented with each factor indicated above are shown. Bars, 20 µm. CDSF+RA, CDSF with retinoic acid; CDSF+RL, CDSF with retinol; CDSF+RL+RBP, CDSF with retinol and retinol binding protein; CDSF+Bmp4, CDSF with Bmp4; CDSF+iGsk3β, CDSF with the Gsk3β inhibitor.
Figure 2.7. Screening of factors responsible for the tumor-like growth of mouse embryonic stem cells. (A): Cumulative numbers of mouse ESCs were compared among ESCs cultured under each condition indicated for 3 passages. Cell counts were normalized to CDSF conditions. A value for CDSF conditions is normalized to 1. Parentheses indicate the number of biological replicates per condition. Standard errors of the mean are indicated by bars. +RA, CDSF with retinoic acid; +RL, CDSF with retinol; +RL+RBP, CDSF with retinol and retinol binding protein; +Bmp4, CDSF with Bmp4; +iGsk3β, CDSF with the Gsk3β inhibitor. (B): Total numbers of biological replicates that resulted in formation of teratomas were compared among mouse ESCs cultured under
each condition indicated. Orange and yellow boxes indicate the number of biological
replicates that developed into teratomas within 3 months and in more than 6 months,
respectively. Blue bars indicate the number of biological replicates that failed to form
teratomas for more than 6 months. Data for the Gsk3β inhibitor include results obtained
with R1 and W4 mouse ESCs. For those biological replicates indicated as (i), (ii) and (iii)
in the bar chart, the number of technical replicates, the number of days needed for the
experimental animals to reach their end points, and the sizes of the longest axis of the
teratomas are shown per technical replicate. Asterisks (*) indicate that the growth of the
teratoma is ongoing. n.d., not determined. (C, top): NOD-SCID mice that received
transplantation of mouse ESCs cultured in CDSF supplemented with Bmp4 (right) or the
Gsk3β inhibitor (left) are shown. Bars, 1 cm. (C, bottom): Representative histological
images are shown. The presence of three germ layers (see the legend for Fig. 2.2) is
evident in teratomas developed from mouse ESCs cultured in CDSF supplemented with
the Gsk3β inhibitor. Bars, 20 µm. (D): Abundance of each transcript indicated above was
examined in mouse ESCs cultured under each condition on the right by 24 cycles of PCR.
*Efla* was used as a reference.
Table 2.1. Effects of serum on the tumorigenicity of mouse embryonic stem cells.

<table>
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<th>Conditions</th>
<th># of Biological replicates</th>
<th>Teratomas formed</th>
<th>p-value</th>
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<td>p=0.0001554&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>With FBS</td>
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<td>4</td>
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<tr>
<td>CDSF to Stand.</td>
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<sup>a</sup>: Compared with conditions “with FBS”.
CHAPTER 3

Gli2 expression is associated with the tumorigenicity of mouse embryonic stem cells

Abstract

Here, I present experimental evidence that Gli2 expression is associated with the tumor-like growth of mouse ESCs. By global gene expression profiling of mouse ESCs, surprisingly I found that the oncogene Gli2, a downstream target of the growth factor Sonic hedgehog (Shh), was expressed in undifferentiated mouse ESCs, which was further validated by semi-quantitative RT-PCR and immunofluorescent microscopy. It is well-known that the Gli2 protein is activated by Shh. However, interestingly, the Gli2 transcripts were expressed abundantly in mouse ESCs without endogenous Shh expression detected. To test if expression of Gli2 in mouse ESCs is based on the autonomous mechanism or due to a signal(s) from the extracellular environment, mouse ESCs were cultured in either standard or CDSF conditions for 3 passages. Mouse ESCs maintained in these conditions showed similar levels of Oct3/4 expression. However, intriguingly, mouse ESCs maintained under CDSF conditions downregulated Gli2 as well as Eras, another oncogene responsible for the tumor-like growth of mouse ESCs, and reduced their capability to grow into teratomass. In contrast, a teratocarcinoma cell line F9 maintained expression of Gli2 and Eras expression as well as its capability to grow into teratomas even when F9 was cultured under CDSF conditions. When cultured under CDSF conditions supplemented with FBS or CDSF conditions brought back to standard conditions, mouse ESCs upregulated Gli2, and efficiently grew into teratomas.

Surprisingly, when an inhibitor of Gsk3β was added in CDSF conditions, mouse ESCs
enhanced their tumor-like growth, and upregulated *Gli2* expression. These findings indicate that *Gli2* expression is tightly correlated with the incidence of tumor-like growth of mouse ESCs.

**Introduction**

As described in Chapter 1 Literature Review, the underlying mechanisms of transcriptional heterogeneity as well as the tumor-like growth in mouse embryonic stem cells (ESCs) remain unknown. Without elucidating the molecular basis of these mechanisms, application of human iPSCs to the practical therapeutic use will be difficult. Toward a better understanding of the self-renewal of mouse ESCs, our laboratory has surveyed global gene expression profiles of mouse ESCs (Tanaka, 2009; Walker *et al.*, 2007) and compared them with other published data (Matoba *et al.*, 2006). Interestingly, this bioinformatical analysis has identified that the transcription factor *Gli2* is consistently highly expressed in undifferentiated mouse ESCs and becomes downregulated during their differentiation.

Originally, Gli1 was identified due to its amplification in human glioblastomas (Kinzler *et al.*, 1988). Vertebrates have two more homologues of Gli1, namely Gli2 and Gli3, which are also expressed in human glioblastomas (Clement *et al.*, 2007). Later, Gli proteins were identified as the orthologue of the zinc-finger transcription factor cubitus interrprpus Ci in Drosophila, which acts downstream of the Hedgehog Hh signaling pathway (Ingham and McMahon, 2001).
The gene encoding *Hedgehog Hh* was first discovered by screening Drosophila mutant larvae that exhibited defects in their body plan (Nusslein-Volhard and Wieschaus, 1980). Due to the appearance of mutant larvae having short and spiked cuticles, Nusslein-Volhard and Wieschaus named the gene responsible for this phenotype Hedgehog. Mouse Hh orthologs were cloned in 1993. Unlike Drosophila, the mouse has three orthologs of Hh, which are named after characters found in Nintendo “Sonic the hedgehog”, Desert hedgehog Dhh, Indian hedgehog Ihh and Sonic hedgehog Shh (Echelard *et al.*, 1993). Similarly, Hh orthologs were found in other vertebrates, such as rat (Roelink *et al.*, 1994) and human (Marigo *et al.*, 1995).

The Hh signal is transmitted through two transmembrane receptors, Patched Ptc and Smoothened Smo (Ingham and McMahon, 2001). Without the ligand Hh, Ptc binds to Smo and represses the activity of Smo (Taipale *et al.*, 2002). When Hh binds to Ptc, this results in repression of the Ptc activity, which subsequently promotes release of Smo. Then, Smo translocates into nuclei, activates the protein activity of the transcription factor Gli and regulates transcription of downstream targets of the Hh pathway (Stone *et al.*, 1996; Taipale *et al.*, 2002) (Fig. 3.2A).

Each Gli protein has a unique role in neurogenesis and skeletal patterning in a context dependent manner (Ruiz i Altaba *et al.*, 2007). Gli1 and Gli2 play the major role as transcriptional activators in ectopically activated Shh signals (Bai *et al.*, 2002) and Gli2 can be expressed in the absence of the Shh signal (Bai and Joyner, 2001; Ruiz i Altaba, 1998). Gli3 mostly acts as a transcriptional repressor (Ruiz i Altaba *et al.*, 2007). Transcriptional upregulation of *Gli1* and *Ptc* indicates that the Hh pathway is active (Shaw *et al.*, 2009).
The role of the Hh signal has been extensively investigated in neural stem cells (Ahn and Joyner, 2005; Balordi and Fishell, 2007; Palma et al., 2005) and hematopoietic stem cells (Dierks et al., 2008; Jiang et al., 2008). However, biological significance of the Hh function is less studied in embryonic stem cells. Recombinant mouse Shh proteins can stimulate the proliferation of mouse ESCs in concert with other pathways, such as epidermal growth factor (EGF) and Wnt (Heo et al., 2007), whereas recombinant human SHH proteins have failed to maintain the self-renewal of human ESCs (Wu et al., 2009). Therefore, the role that the Hh signal plays in ESCs remains elusive.

Materials and Methods

Microarray analysis

First, a mouse embryonic stem cell line, namely OVW4, which expresses the puromycin resistant gene under the Oct3/4 promoter (Yeom et al., 1996) was generated. Then, OVW4 was plated at 100 cells/cm² and maintaing under the presence or the absence of puromycin (2 μg/ml; Invivogen) for 5 days. Total RNA was extracted from three biological replicates of each condition, and subjected to Illimina microarray analysis. A list of differentially expressed genes with less than 0.05 of the false discovery rate (FDR) at the expression level of 200 or higher was generated. Then, the list of genes was compared with other published data (Matoba et al., 2006) by ConPath Navigator (DNA Chip Research Inc.).
Cell culture

The mouse embryonic stem cell lines (R1 and W4) were cultured under standard and CDSF conditions as described in Chapter 2. Teratocarcinoma cell lines of mouse (F9 and P19) and human (NTERA-2) were cultured under standard conditions for mouse ESCs without LIF, because they are not dependent on LIF. NTERA-2 cells (kindly provided by Dr. Fei Wang, UIUC) were expanded by cell scrapers. F9 and P19 were also cultured in CDSF conditions as the mouse ESCs. Human ESCs (H1; WiCell) were cultured on BD Metrigel™ (StemCell Technologies Inc)-coated 6-well tissue culture plate with mTeSR™1 (StemCell Technologies Inc) medium at 37 ºC, 5 % CO₂. When cells reached confluence, 1 mg/ml dispase (StemCell Technologies Inc) was used to incubate the cells at 37 ºC for 7 min, followed by 3 times washes with DMEM/F12 (Invitrogen). Cells were dislodged by 5 ml pipettes, centrifuged at 300 g for 5 min, and then resuspended with mTeSR™1 medium.

RT-PCR

RT-PCR was carried out as already described in Chapter 2. The primer sets used for this study are listed on Table 3.1.

Immunofluorescent microscopy

After fixation with 4% paraformaldehyde (Sigma) in PBS at room temperature for 15 min, mouse ESCs cultured on glass-bottom dishes (MatTek corporation) were washed with
PBS, and blocked with 0.1% Triton-X-100 at room temperature for 10 min. After similar PBS washes, these mouse ESCs were incubated with 10% Image-iT FX signal enhancer (Invitrogen) at room temperature for 30 min, followed by incubation with mouse anti-human Oct4 monoclonal antibody (Santa Cruz) and rabbit anti-human Gli2 polyclonal antibody (Abcam) diluted with 10% Image-iT FX signal enhancer at the 1:200 ratio at 4 °C overnight. After PBS washes, samples were incubated with goat Alexa Fluor 488 anti-mouse IgG and goat Alexa Fluor 568 anti-rabbit IgG diluted in 10% Image-iT in PBS at room temperature for 1 hour. Cellular nuclei were stained with Hoechst after similar PBS washes. These immunostained mouse ESCs were treated with ProLong Gold antifade reagent (Invitrogen). The confocal microscope LSM 700 was used.
Results

Undifferentiated Mouse and Human Embryonic Stem Cells Express Gli2

To understand the mechanism of cell fate decision in embryonic stem cells (ESCs), we have surveyed global gene expression profiles of mouse ESCs (Tanaka, 2009; Walker et al., 2007) and compared them with other published data (Matoba et al., 2006). Surprisingly, this bioinformatical analysis showed that the transcription factor Gli2 was consistently highly expressed in undifferentiated mouse ESCs and became downregulated during their differentiation, while the Gli3 expression pattern was opposite (Fig. 3.1A). When all-trans retinoic acid (RA) was supplemented in the culture medium without LIF to promote neural differentiation, Gli2 expression became upregulated, consistent with the role of Gli2 in neural development (Fig. 3.1B).

To validate our analysis of global gene expression profiles in mouse ESCs, we examined expression of Gli2, together with other genes downstream of Shh in undifferentiated and differentiated mouse ESCs (R1; Fig. 3.2B) and undifferentiated human ESCs (H1; Fig. 3.2C) by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Similar Gli2 and Gli3 expression patterns were confirmed in mouse ESCs (Fig. 3.2B). Expression of Gli1 and Ptc in undifferentiated ESCs indicates that Shh signaling was stimulated (Fig. 3.2B). Expression of genes downstream of Shh except Smo was downregulated when mouse ESCs were differentiated (Fig. 3.2B). In undifferentiated human ESCs, expression of GLI2 was also detected, but GLI3 exhibited a higher level of expression than in undifferentiated mouse ESCs (Fig. 3.2C). This discrepancy may be
attributed to the difference of culture conditions and the degree of cellular pluripotency between mouse and human ESCs. It is suggested with these results that Gli2 may be essential to sustain the undifferentiated state of ESCs. However, this suggestion is unlikely because Gli2-deficient embryos showed defects in body plans but not in cell differentiation per se (Mo et al., 1997).

To further validate our microarray analysis and test whether the Gli2 protein is present in undifferentiated mouse ESCs, immunofluorescent microscopy was conducted, because it is suggested with our array analysis that Gli2-positive and negative cells may exist among a population of undifferentiated mouse ESCs. Confocal microscopy was employed to remove ambiguity because mouse ESCs overlap within the colonies. The majority of the Gli2 protein was localized in nuclei which exhibited a patchy staining pattern, and that Gli2-positive mouse ESCs were also undifferentiated and Oct3/4-positive (Fig. 3.3). In addition, the mouse ESCs showing the higher level of Gli2 protein also showed the higher level of Oct3/4 protein (Fig. 3.3).

**Mouse ESCs Receives the Shh Signal from Culture Microenvironment**

Expression of Gli1 and Ptc indicates the activation of the Shh signal. Surprisingly, Gli1 and Ptc are abundantly expressed in undifferentiated mouse ESCs cultured under standard conditions, but there is no detectable endogenous Shh expression in mouse ESCs (Fig. 3.4 upper line). To investigate whether mouse ESCs receives some signals from the culture microenvironment, or autonomously express genes downstream of Shh, the same mouse ESCs were cultured in the chemically defined serum-free conditions (CDSF) as
described in Chapter 2. They were passaged 3 times under CDSF conditions before processed for RT-PCR. The CDSF medium we used does not contain any components that could induce Shh singling such as Vitamin A and TGFβ (Dennler et al., 2007; Riddle et al., 1993). Intriguingly, Gli2 became undetectable when mouse ESCs were cultured under CDSF conditions, whereas these mouse ESCs maintained a similar level of Oct3/4 expression to the ones under standard conditions (Fig. 3.4 bottom line). This result indicates that upregulation of Gli2 is triggered by some factors present in standard culture conditions. The downregulation of Gli2 expression was accompanied with downregulation of Eras (Fig. 3.4), which is responsible for the tumor-like growth of mouse ESCs (Takahashi et al., 2003). As discussed in Chapter 2, these mouse ESCs cultured under CDSF conditions failed to generate teratomas up to 6 months. Thus, these findings indicate that one or more factors in animal serum activated Gli2 in mouse ESCs, which make them tumorigenic. Furthermore, we found that supplementing CDSF conditions with recombinant mouse Shh (100 ng/ml or 500 ng/ml; Fig. 3.5A) failed to promote proliferation (Fig. 3.5B and C) and rescue the tumorigenicity of mouse ESCs (Fig. 3.5D), even though the expression of Gli2 is restored (data not shown), which led us to hypothesize that there are factors synergizing with Shh in the standard culture microenvironment, which trigger the Gli2 expression and tumor-like growth of mouse ESCs.
**Gli2 Expression may Serve as a Novel Marker of Tumorigenic Growth of Mouse ESCs**

To test whether expression of *Gli2* is associated with the tumor-like growth of pluripotent stem cells, teratocarcinoma cell lines of mouse (F9, testicular; P19, from postimplantation embryos) and human (NTERA-2, testicular), which are transplantable, malignant teratocarcinoma, were used. F9, P19 (Fig. 3.6) and NTERA-2 (data not shown) showed *Gli2* expression when cultured under standard conditions. Also F9 and P19 maintained expression of *Gli2* and *Eras* when cultured under CDSF conditions (Fig. 3.7), and that F9 cultured under CDSF conditions generated a well-developed teratoma within 4 weeks. Thus, CDSF conditions themselves have no negative effects on proliferation of cultured pluripotent stem cells. When mouse ESCs were cultured under CDSF conditions supplemented with FBS or CDSF conditions transferred back to standard conditions, the *Gli2* transcript level was maintained, which was associated with the incidence of teratoma formation (Fig. 3.8). Surprisingly, pharmacological inhibition of Gsk3β greatly activated *Gli2* expression in mouse ESCs cultured under CDSF conditions, and rescued their tumor-like growth (Fig. 3.9).

**Discussion**

In this report, it is demonstrated that *Gli2* encoding an oncogenic transcription factor downstream of Hh signaling is expressed in mouse and human ESCs. Mouse ESCs do not autonomously activate *Gli2* and Hh signaling. Extrinsic signals from culture microenvironment are responsible for the activation of the Hh pathway in mouse ESCs.
In concert with the data presented in Chapter 2, surprisingly, the transcriptional activity of Gli2 showed tight correlation with the tumorigenicity of mouse ESCs.

The hedgehog pathway governs specification of cell types, proliferation and survival of cells, and patterning and growth of embryos and adults (Ingham and McMahon, 2001). Three orthologs of Drosophila Hh in vertebrates have unique roles in embryonic development in a combinational manner. Dhh is expressed mostly in reproductive tissues, such as the testis and the ovary (Bitgood et al., 1996; Wijgerde et al., 2005; Yao et al., 2002). Dhh-null mice are viable, but mutant males are infertile (Bitgood et al., 1996). Ihh is specifically expressed in the primitive endoderm (Dyer et al., 2001), the gut (van den Brink, 2007), and the cartilage during bone formation (Vortkamp et al., 1996). Shh attracts much more attention because Shh is most broadly expressed. Shh is involved in embryogenesis, organogenesis and also stem cell maintenance in adults (Varjosalu and Taipale, 2008).

Approximately 25% of cancer-associated deaths are correlated with the disregulated activation of the Hh signal (Lum and Beachy, 2004). Aberrant expression of genes involved in the Hh signaling pathway has been implicated in tumorigenic transformation of normal cells (Wetmore, 2003). Furthermore, Holoprosencephaly (HPE), a defect of the forebrain and midface in humans, is associated with the heterozygous mutation in Shh (Roessler et al., 1996). Overexpression of Shh (Oro et al., 1997) or Gli2 (Grachtchouk et al., 2000) in the skin of transgenic mice induces development of basal cell carcinoma, the most common skin tumors in Caucasians.

Gli2 is the main modulator of Hh signaling (Bai et al., 2002). In addition, the activation of Gli2 requires some other cross regulatory mechanisms. For example, TGFβ
is an efficient transcriptional inducer of *Gli2* activation in a Smad-dependent manner (Dennler *et al.*, 2007). This finding explains why human ESCs cultured under mTeSR media, which is a chemically-defined serum-free medium including highly concentrated TGFβ, abundantly transcribes *GLI2*. Furthermore, SHH can crosstalk with extracellularly regulated kinase (ERK; Riobo *et al.*, 2006). Moreover, the SHH signal has synergistic interactions with FGFs, WNTs and BMPs during development (Bellusci *et al.*, 1997; Brewster *et al.*, 2000; Laufer *et al.*, 1994; Meyers and Martin, 1999; Munsterberg *et al.*, 1995; Murtaugh *et al.*, 1999; Yang and Niswander, 1995). This may explain why supplementing Shh alone could not rescue the tumorigenicity of mouse ESCs maintained under CDSF conditions.

The expression pattern of *Gli2* in undifferentiated and differentiated mouse ESCs is similar to ones in prostate cancer cells and epithelial cells (Thiyagarajan *et al.*, 2007). *Gli2* is aberrantly overexpressed in prostate cancer and tumor cells, whereas the expression level of *Gli2* is quite low in normal prostate cells. Knockdown of *Gli2* expression by short-hairpin (sh) RNA significantly reduced the growth rate of prostate cancer cells both in vitro and in vivo. Furthermore, overexpression of *Gli2* in prostate epithelial cells resulted in their accelerated growth and the cell cycle progression (Thiyagarajan *et al.*, 2007). Disregulated Shh expression often interacts with the insulin-like growth factor (IGF)/ phosphoinositide 3-kinase (PI3-kinase)/ Akt pathway, which induces a synergistic effect on the incidence of tumors (Rao *et al.*, 2004).
Figure 3.1. Global gene expression profiles showed Gli2 transcripts in mouse ESCs.

(A): Results obtained with Illumina mouse whole genome expression microarray (“Puro vs Ctrl”) were compared with a published data set (“Oct(-) Day 0” through “Day 6”;

Figure 3.1 (cont.)

Matoba, et al., 2006). (B): Expression microarray results (Walker et al., 2007) show that Gli2 expression is downregulated during differentiation of mouse ESCs LIF-, but upregulated by retinoic acid 1µM.
Figure 3.2. Confirmation of Gli2 expression pattern in mouse and human ESCs. (A): Shh signaling pathway. (B): Undifferentiated (Undif.) mouse ESCs (Oct3/4+) express genes downstream of Shh, but do not express Shh. “Dif.”, differentiated. (C): Human ESCs (H1; SOX2, NANOG & OCT4A+) express genes downstream of Shh. Ef1α and HPRT are loading controls. “RT-” for negative controls.
Figure 3.3. Co-expression of Oct3/4 and Gli2 in undifferentiated mouse ESCs. (A and B): immunocytochemistry of undifferentiated mouse ESCs W4 and R1 cell lines. (C): undifferentiated m ESCs W4 only immunostained with secondary antibodies serve as a negative control. Bars, 20µm. Gli2 protein is shown in red, Oct3/4 in green, nuclei in blue, and merged images are shown at the most right.
Figure 3.4. CDSF culture condition downregulated Gli2 expression in undifferentiated ESCs. Mouse ESCs cultured under chemically-defined serum-free conditions (CDSF) downregulated expression of Gli1, Gli2, Ptc and Eras compared to those cultured under standard conditions. Eflα is a loading control.
Figure 3.5. CDSF conditions supplemented with Shh failed to promote the proliferation and the tumor-like growth of mouse ESCs. (A and A’): phase contrast and fluoresce images of mouse ESC line that expresses enhanced green fluorescent protein (EGFP) under the Oct3/4 promoter, which was cultured under CDSF conditions supplemented with Shh 500 ng/ml. (B and C): Normalized cumulative cell counts after three passages under CDSF conditions or CDSF conditions with 100 ng/ml or 500 ng/ml Shh. (D): mouse ESCs cultured under CDSF conditions with Shh failed to generate teratomas up to 10 months. Bar, 1 cm.
Figure 3.6. Mouse teratocarcinoma cell lines F9 and P19 express genes downstream of Shh. F9 and P19 expressed genes downstream of the Shh pathway examined except Gli3 and Shh. Ef1α is a loading control. “RT-” for negative controls.
Figure 3.7. F9 and P19 cells cultured under CDSF conditions still maintain Gli2 expression. F9 and P19 cells cultured in CDSF conditions maintained the expression of Gli2 and Eras, but Gli1 and Ptc became undetectable, which indicates that the Shh pathway was not activated. Ef1α is a loading control.
Figure 3.8. *Gli2* expression is consistent with tumorigenicity of mouse ESCs. Genes involved in the Shh signaling pathway became upregulated in CDSF conditions supplemented with FBS (CDSF+FBS), and CDSF conditions brought back to standard conditions (CDSF to Stand.), when compared with CDSF conditions. *Eflα* is a loading control.
Figure 3.9. Upregulation of *Gli2* in mouse ESCs cultured under CDSF conditions supplemented with the Gsk3β inhibitor is associated with the incidence of teratoma formation. The tumor-like growth of mouse ESCs cultured under CDSF conditions supplemented with the Gsk3β inhibitor (CDSF+iGsk3β) is associated with upregulation of *Gli2*. *Ef1α* is a loading control.
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CHAPTER 4

Conclusion

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold great promise for regenerative medicine and tissue engineering because they have the capability to self-renew and differentiate into all of the tissues or organs in our body. However, there are hurdles that hamper application of ESCs and iPSCs for stem cell therapies, such as the heterogeneous nature of ESC culture and the tumor-like growth of ESCs and iPSCs. To prevent the growth of teratomas in therapeutic transplanted tissue replacements, it is necessary to establish methods for efficiently manipulating cell fate decisions in PSCs and to understand the mechanism responsible for tumorigenesis in the stem cells. To our surprise, the mechanism of teratoma formation from ESCs has received little attention to date. Thus, by using mouse ESCs as a model, our research group studies the mechanism of the tumor-like growth of ESCs. The goal of this research project is to eliminate the tumorigenic property of ESCs, which will make human induced pluripotent stem cells amenable to stem cell-based therapies.

In Chapter 2, I present experimental evidence to suggest that short-term CDSF culture suppresses the tumor-like growth of mouse ESCs, which is reversed by pharmacological inhibition of Gsk3β. The standard method to culture mouse ESCs is to provide cells with basal medium plus 15% fetal bovine serum (FBS), which contains a variety of unknown factors that can promote survival and growth of mouse ESCs. I found that mouse ESCs cultured under uniquely formulated chemically defined serum free (CDSF) conditions failed to form teratomas, whereas the ones cultured under standard conditions developed
into tumors. These data indicate that uncharacterized factors in serum may be responsible for the tumor-like growth of mouse ESCs. Although the pluripotency of mouse ESCs cultured under CDSF conditions needs to be evaluated by their germline transmission, the present data demonstrate that they still can differentiate into three germ layers in vitro (Furue et al., 2005) and retain the potential to grow as teratomas. The inhibitor of Gsk3β could efficiently promote the proliferation of mouse ESCs and rescue their tumor-like growth through upregulation of the oncogenic transcription factor \textit{Gli2}, but not \textit{Eras} and \textit{c-Myc}. Therefore, these data suggest that Gsk3β governs the tumor-like growth of mouse ESCs by means of a mechanism separate from the one to support the pluripotency of ESCs.

In Chapter 3, we demonstrated that \textit{Gli2}, a downstream target of the growth factor Sonic hedgehog (Shh), was expressed in undifferentiated mouse ESCs and became downregulated during differentiation. Although the Gli2 protein is activated by Shh, the \textit{Gli2} transcripts were expressed abundantly in mouse ESCs without endogenous \textit{Shh} expression detected. Furthermore, expression of \textit{Gli2} in mouse ESCs showed dependency on the presence of animal serum in culture. Surprisingly, the \textit{Gli2} expression pattern was tightly correlated with the tumorigenicity of mouse ESCs, such that when mouse ESCs showed \textit{Gli2} expression, they efficiently developed into teratomas. However, when \textit{Gli2} expression was downregulated, mouse ESCs still maintained pluripotency. Therefore, \textit{Gli2} expression is associated with the tumorigenic property of mouse ESCs, which can serve as a novel marker, but is dispensable for the self-renewal of mouse ESCs.

Multiple pathways may regulate the tumor-like growth of mouse ESCs. These data lay the foundation for further investigation to identify other signaling pathways
responsible for the tumor-like growth of mouse ESCs, which may be independent from
the mechanism of self-renewal. Genetic manipulation of mouse ESCs using \textit{Gli2} cDNA
will help determine its role in tumorigenesis in mouse ESCs. These findings have a great
impact on research of human ESCs and iPSCs and should be tested in these human
pluripotent stem cells. The first human ESCs-based stem cell therapy for spinal cord
injury was initiated by Geron Corporation last month (October, 2010). Geron is the only
company to date which received approval from the U.S. Food and Drug Administration
(FDA). Currently, the clinical trial is under the phase 1, which is to test the safety of the
treatment, and to regularly monitor signs of tumor growth. While we are waiting for the
safety result, the findings presented in this thesis may potentially lead to a solution for
efficient differentiation of human iPSCs to a desired cell type, while the tumor-like
property of human iPSCs is inhibited.
REFERENCES

Balordi, F. and G. Fishell (2007). Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. J Neurosci, 27, 5936-5947
Furusawa, T., K. Ohkoshi, C. Honda, S. Takahashi and T. Tokunaga (2004). Embryonic stem cells expressing both platelet endothelial cell adhesion molecule-1 and stage-specific embryonic antigen-1 differentiate predominantly into epiblast cells in a chimeric embryo. Biol Reprod, 70, 1452-1457


Kameda, T. and J. A. Thomson (2005). Human ERas gene has an upstream premature polyadenylation signal that results in a truncated, noncoding transcript. *Stem Cells*, 23, 1535-1540


