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Self-Renewal, Pluripotency and Tumorigenesis in Pluripotent Stem Cells Revisited

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1. Introduction

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 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, in order to better understand 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 tumorigenesis an inherent feature of cellular pluripotency?

Accumulating experimental evidence, including our recent studies using mouse ESCs as a model, indicates that the self-renewal of PSCs can be easily compromised by extrinsic factors in the culture microenvironment that can turn the stem cells tumorigenic. Thus, the safety of PSC-based therapy may be significantly improved by more careful manipulation and definition of the cellular microenvironment.

2. Pluripotent stem cells generate heterogeneous populations

2.1 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 & Little, 1954; Stevens, 1973) contributed to demonstrating that teratomas originate from PSCs (Solter, 2006). A benign teratoma, normally found in 1 out of 40,000 live births (Barksdale & 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 (Martin, 1981; Evans & 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 (Thomson *et al.*, 1998; Shamblott *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 in order to restore differentiated cells to the pluripotent state. This was first achieved by transferring somatic cell nuclei into enucleated oocytes (Briggs & King, 1952; Campbell *et al.*, 1996; Wakayama *et al.*, 1998; Rideout *et al.*, 2002; Gurdon & Melton, 2008). 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 & Yamanaka, 2006). Since the successful derivation of iPSCs from human cells (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, Kerr *et al.*, 2003; Brederlau *et al.*, 2006; Jomura *et al.*,

2007) 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.

2.2 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 & 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 & Khillan, 2008; Wang *et al.*, 2008; Chen & Khillan, 2010), 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".

2.3 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 (Niwa *et al.*, 1998;

Matsuda *et al.*, 1999). 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 (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002; Tanaka *et al.*, 2002; Boyer *et al.*, 2005; Loh *et al.*, 2006; Matoba *et al.*, 2006; 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 (Nordin *et al.*, 2008; Lako *et al.*, 2001; 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 ESCs from the ICM (Umehara *et al.*, 2007). However, exogenous Wnt promotes the differentiation of mouse (Lindsley *et al.*, 2006) and human (Wang & 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*; Western *et al.*, 2005; Amano *et al.*, 2006; Tanaka *et al.*, 2006).

2.4 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 (Crino *et al.*, 1998; Chiang & Melton, 2003; 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*; Payer *et al.*, 2006; Hayashi

et al., 2008), *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 (Fig. 1; 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

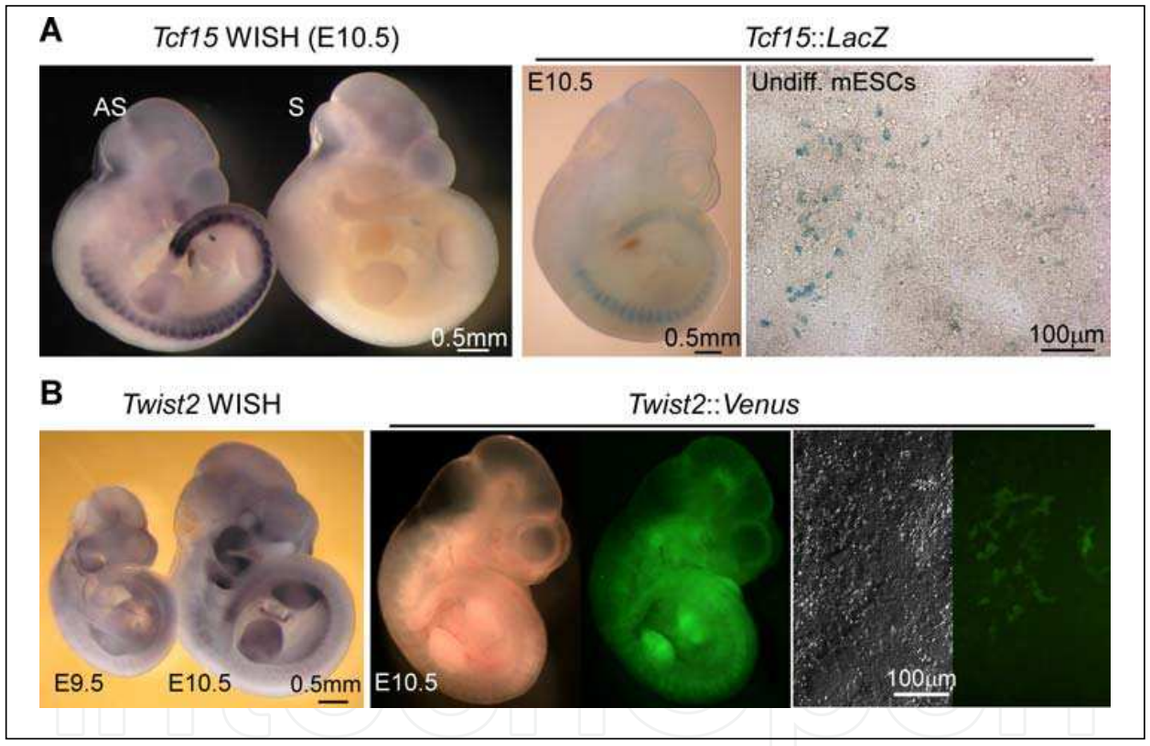

Fig. 1. **Standard culture of mouse embryonic stem cells (ESCs) exhibit fluctuating expression of genes** (modified from Tanaka *et al*., 2008). (**A**, **left**) The *Tcf15* expression pattern in a 10.5 days post-conception (d.p.c.) embryo shown by whole-mount *in situ* hybridization (WISH). S, sense (negative) control. (**A**, **right**) Expression of a reporter (*LacZ*) under the *Tcf15* promoter in a 10.5 d.p.c. embryo derived solely from the mouse ESCs by tetraploid aggregation and in undifferentiated mouse ESCs plated on gelatin-coated dishes (Undiff. mESCs). (**B**, **left**) *Twist2* expression patterns in 9.5 and 10.5 d.p.c. embryos examined as in A. (**B**, **right**) Expression of a fluorescent reporter (*Venus*) under the *Twist2* promoter in a 10.5 d.p.c. embryo derived solely from mouse ESCs and in undifferentiated mouse ESCs.

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.

3. Impacts of culture conditions on the self-renewal of pluripotent stem cells

3.1 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

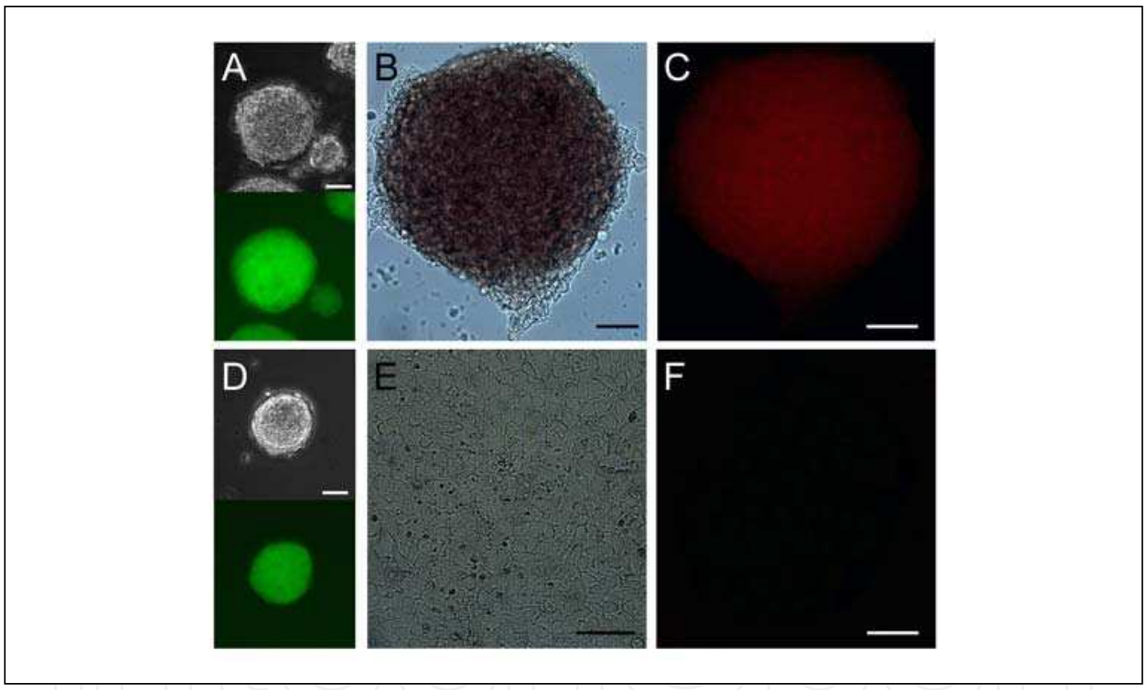

Fig. 2. **Soft substrates promote mouse embryonic stem cell self-renewal.** Mouse ESCs were plated on substrates that have the same stiffness as mouse ESCs (**A-C**) or on glass-bottomed dishes (**D-F**) and maintained under standard culture conditions with LIF (**A & D**) or without LIF for 5 days (**B**, **C**, **E & F**). Bars, 50 μm. (**A & D**) In the presence of LIF, mouse ESCs typically formed round colonies (**top**) on collagen type IA-coated surfaces and maintained *Oct3/4* expression, indicated by the enhanced green fluorescent protein (EGFP) driven by the *Oct3/4* promoter (*Oct3/4::EGFP*, **bottom**). (**B & C**) Mouse ESCs on soft substrates without LIF for 5 days formed round colonies that maintained active alkaline phosphatase (**B**) and the expression of Nanog (**C**). (**E & F**) Mouse ESCs on a glass-bottomed dish without LIF for 5 days exhibited appearance of differentiated cells with no detectable alkaline phosphatase activity (**E**) or Nanog expression (**F**).

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, we found that 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*. To investigate the effect of soft substrates on the self-renewal of mouse ESCs, we plated a mouse ESC line expressing enhanced green fluorescent protein (EGFP) under the *Oct3/4* promoter (**Fig. 2A & D**; Walker *et al*., 2007) on either soft substrates or glass-bottomed dishes in the absence of LIF for 5 days. Remarkably, mouse ESCs on the soft substrate grew as uniformly round colonies without any noticeable differentiating colonies (see **Fig. 2E**) and were able to maintain the expression of markers for pluripotent cells: *Oct3/4* (data not shown), alkaline phosphatase (**Fig. 2B**) and Nanog (**Fig. 2C**). Mouse ESCs cultured on a glass-bottomed dish fully differentiated and downregulated these markers (**Fig. 2E & F**). Therefore, these results strongly indicate that substrate stiffness is a critical extrinsic factor to sustain the self-renewal of mouse ESCs (Chowdhury *et al*., 2010).

3.2 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 & 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.

3.3 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 (Ying *et al.*, 2003; Furue *et al.*, 2005; Ludwig *et al.*, 2006a; Ludwig *et al.*, 2006b; Furue *et al.*, 2008). 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 transferrin), vitamins, fatty acids and minerals. In addition, a pre-mixed serum replacement that claims to include no animal serum components was introduced in 1998 (Goldsborough *et al.*, 1998; Cheng *et al.*, 2004). 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 (Ludwig *et al.*, 2006a; Ludwig *et al.*, 2006b; Hayashi *et al.*, 2007; 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).

4. Tumorigenesis in pluripotent stem cells

4.1 Intrinsic factors involved in tumorigenesis

The ability of cells to grow as a teratoma after transplantation into a host animal is a hallmark of cellular pluripotency (see **"2.1 Pluripotent stem cells"**; Chambers & Smith, 2004; Solter, 2006; Jaenisch & Young, 2008; Damjanov & Andrews, 2007; Lensch & Ince, 2007). 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 & 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 able to grow into a teratoma only when mixed with 2×10^6 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 tumor-like 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 & Dalton, 2009; Wu & Pan, 2010; Cross *et al.*, 1995). Gsk3β inhibits its downstream target c-Myc through β-catenin (He *et al.*, 1998; Bechard & Dalton, 2009), 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 (Okita *et al.*, 2007; Nakagawa *et al.*, 2010). However, this model may involve other uncharacterized gene products, as human ESCs do not express human *ERAS* (Kameda & Thomson, 2005; Tanaka *et al.*, 2009) but develop into teratomas.

4.2 Extrinsic factors responsible for tumorigenesis

Interestingly, mouse PSCs contribute to the development of normal chimeras, instead of forming teratomas, when mixed with mouse preimplantation embryos (Bradley *et al.*, 1984; Auerbach *et al.*, 2000; Polo *et al.*, 2010). Thus, mouse PSCs may require proper extrinsic signals or niches (Voog & Jones, 2010) to differentiate normally and to contribute to the development of chimeras. The fact that mouse PSCs behave differently when exposed to different microenvironments raises the question of whether PSCs are inherently tumorigenic or are provided with extrinsic signals *in vitro* that promote tumor-like growth. To address this question, we transferred mouse ESCs maintained under standard conditions (**Fig. 3A**) using fetal bovine serum (FBS) into chemically defined serum-free (CDSF) conditions (**Fig. 3B**).

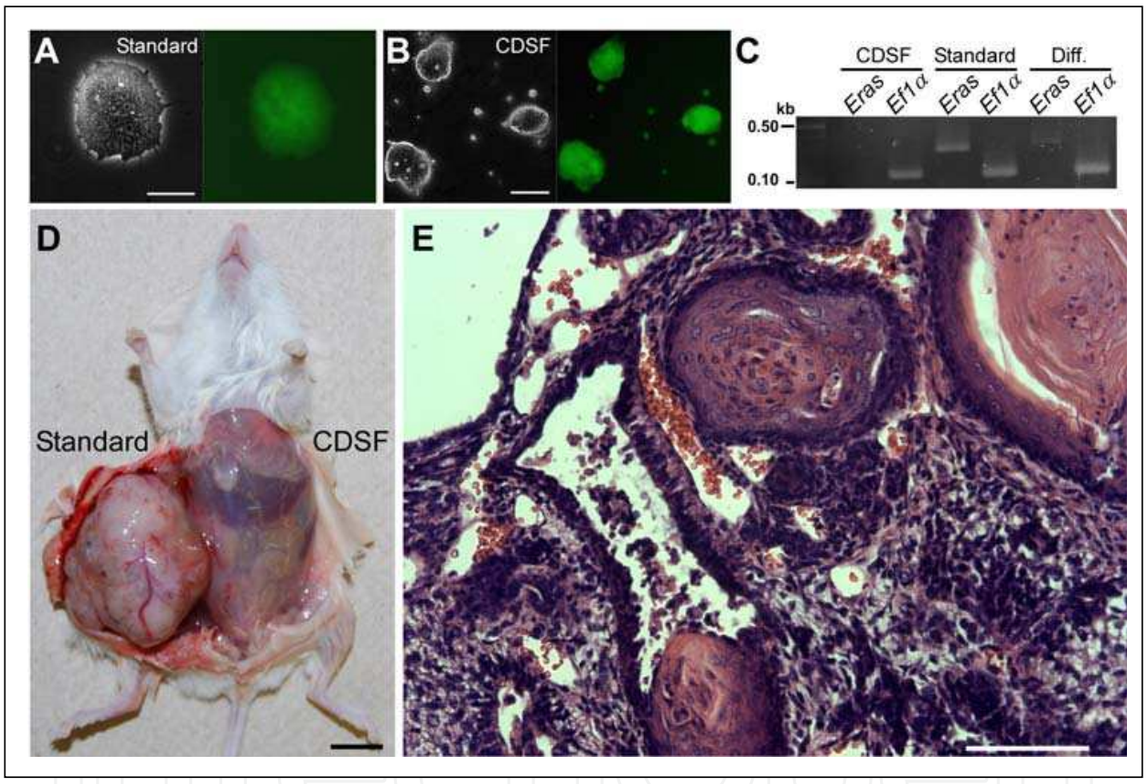

Fig. 3. **Mouse embryonic stem cells gain tumorigenicity from animal serum.** (**A**) A mouse ESC line that harbors an EGFP reporter driven by the *Oct3/4* promoter (right) was maintained under standard conditions using fetal bovine serum (FBS). Bar, 50 μm. (**B**) The same ESC line shown in (A) was plated on a collagen IA-coated plate and cultured under chemically defined serum-free (CDSF) conditions. The transcriptional activity of *Oct3/4* is evidenced by the green fluorescence (right). Bar, 50 μm. (**C**) Expression of *Eras* was examined in mouse ESCs cultured under the indicated conditions. Diff., ESC differentiation was induced by the withdrawal of LIF for 5 days. *Ef1a* is shown as a control. (**D**) 1 x 10^6 cells maintained under each indicated condition were transplanted subcutaneously into NOD-SCID mice, and their growth was monitored for 11 weeks. Bar, 1 cm. (**E**) Histological image of a teratoma consisting of a variety of specialized cells. Bar, 100 μm.

These ESCs were maintained under CDSF conditions for three passages before being subcutaneously transplanted into immunocompromised mice. Surprisingly, the ESCs failed to produce teratomas for up to six months, whereas mouse ESCs maintained under standard conditions generated well-developed teratomas within five weeks (**Fig. 3D & E**). 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. The tumorigenic plasticity of mouse ESCs appears to be unique; ECCs (F9; Bernstine *et al.*, 1973) cultured in CDSF formed teratomas when transplanted (data not shown). Because serum is different from interstitial fluid (i.e., lymph), it is suggested with our present data that interstitial fluid will not provide tumorigenicity. Mouse ESCs cultured under CDSF conditions proliferated significantly more slowly than mouse ESCs cultured under standard conditions. Their slower proliferation was accompanied by the downregulation of *Eras* (**Fig. 3C**), which is responsible for the tumorigenicity of mouse ESCs. However, mouse ESCs cultured under CDSF conditions maintained the expression of transcripts associated with cellular pluripotency, *Oct3/4* (**Fig. 3B**), *Sox2* and *Esg1* (data not shown; see "**2.3 Intrinsic factors to maintain self-renewal**"). These results indicate that the tumorigenicity of mouse ESCs is reduced without compromising the pluripotency by short-term serum-free culture (Li & Tanaka, submitted). Perhaps these mouse ESCs exhibited cell death after transplantation due to the absence of a continuous supply of LIF (Furue *et al.*, 2005), even though mouse ESCs express their own LIF transcripts (Shen & Leder, 1992). Because the effect of long-term serum-free culture on tumorigenesis in mouse ESCs has not yet been evaluated, we cannot rule out the possibility that undifferentiated mouse ESCs that have adapted to long-term serum-free culture may regain tumorigenic properties.

5. Conclusion

Here we present experimental evidence to suggest that soft substrates promote mouse ESC self-renewal and that short-term serum-free culture reduces the tumorigenicity of mouse ESCs. The underlying mechanisms involved in the cell-substrate interaction and tumorigenesis in mouse ESCs are currently unknown. However, these studies using mouse ESCs provide a basis for further study and help establish simple strategies to significantly enhance the control of differentiation and increase the safety of human iPSCs.

6. Acknowledgement

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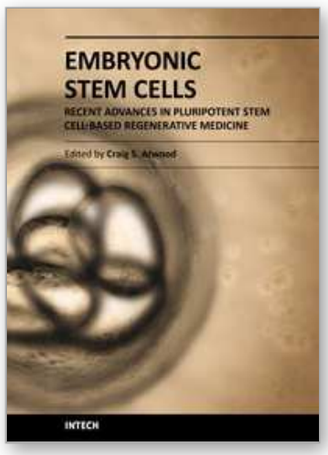

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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