Short-Term Serum-Free Culture Reveals that Inhibition of Gsk3β Induces the Tumor-Like Growth of Mouse Embryonic Stem Cells

Yanzhen Li, Tamaki Yokohama-Tamaki, Tetsuya S. Tanaka*

Department of Animal Sciences, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America

Abstract

Here, we present evidence that the tumor-like growth of mouse embryonic stem cells (mESCs) is suppressed by short-term serum-free culture, which is reversed by pharmacological inhibition of Gsk3β. 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 mESCs failed to produce teratomas for up to six months, whereas mESCs maintained under standard conditions generated well-developed teratomas in five weeks. Mouse ESCs cultured under CDSF conditions maintained the expression of Oct3/4, Nanog, Sox2 and SSEA1, and differentiated into germ cells in vivo. In addition, when mESCs 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 mESCs was not compromised by CDSF conditions. Mouse ESCs cultured under CDSF conditions proliferated significantly more slowly than mESCs cultured under standard conditions, and were reminiscent of Eras-null mESCs. In fact, their slower proliferation was accompanied by the downregulation of Eras and c-Myc, which regulates the tumor-like growth of mESCs. Remarkably, when mESCs 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 mESCs 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 mESCs by means of a mechanism different from the one to support the pluripotency of ESCs.

Introduction

Embryonic stem cells (ESCs) [1,2,3] and induced pluripotent stem cells (iPSCs) [4,5,6,7] are very promising tools for use in drug screening and customized tissue replacement [8] 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) [9,10,11,12,13] and fetal bovine serum (FBS). FBS further facilitates their self-renewal by offering other factors, such as bone morphogenetic protein 4 (Bmp4) [14], retinooids [15,16,17], threonine [18] and glutathione [19]. 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 [20,21,22,23]. However, it is now firmly established that pharmacological inhibition of glycogen synthase kinase 3β (Gsk3β) promotes the self-renewal of both mouse [22,24] and human ESCs [23], and derivation of mouse ESCs [25].

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 [14,26,27,28]. Typically, defined culture media are composed of critical growth factors (e.g., LIF and/or Bmp4) and other factors present in animal sera, such as hormones (e.g., insulin and transferrin), vitamins, fatty acids and minerals. Commercially-made serum replacements that may contain these components in animal sera [29] are often used to maintain ESC culture (e.g., [30]), although the exact components cannot be disclosed by their patents [28]. The maintenance of the undifferentiated state of mouse ESCs (mESCs) using defined culture media has been well documented [31,32]. Furthermore, the pluripotency of these mESCs has been validated by their differentiation in vivo [26,32] or by the development of chimeric mice [14].

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 [33,34,35]. Such teratoma formation assays have validated the pluripotency of mESCs maintained in the presence of a Gsk3β inhibitor [23,25]. 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 [13,36]. However, their tumor-like growth hampers the therapeutic application of human iPSCs [37]. Little is known about the inherent tumorigenic property of ESCs, except that the oncogene 

**Results**

**Mouse embryonic stem cells reduced their tumorigenicity but maintained their pluripotency under chemically-defined serum-free culture**

To examine whether mESCs are determined to grow as teratomas, we cultured mESCs in chemically-defined serum-free medium with LIF (referred to as “CDSF”) [26] for three passages (Fig. 1A, 1C) and subcutaneously transplanted them into non-obese diabetic mice with severe combined immunodeficiency disease (NOD-SCID mice) [45]. We used a uniquely formulated serum-free medium, ESF7 (see Materials and Methods), because components in this medium is fully disclosed [26]. This medium has been used to maintain mESCs by other studies (e.g., [46,47]). However, the pluripotency of mESCs cultured under this medium has not been tested by teratoma formation assays. Surprisingly, these mESCs failed to grow as teratomas (Fig. 1F-1H), whereas mESCs maintained under standard conditions (Fig. 1A, 1B) grew into a well-developed teratoma in 5 weeks (Fig. 1F, 2A-2E, and supporting information Fig. S1A). When mESCs were cultured in CDSF supplemented with 15% FBS (referred to as “CDSF+FBS”; Fig. 1A, 1D), they formed a well-developed teratoma in 5 weeks (Fig. 1G, 2F-2J, and supporting information Fig. S1B). Thus, tumorigenesis in mESCs 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.

We conclude that CDSF did not compromise the pluripotency of mESCs 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) [48,49,50], was validated using a mESC line that expresses the enhanced green fluorescent protein (EGFP) under the Oct3/4 promoter [51] (Fig. 1B, 1C). Unless otherwise noted, this mESC line was used throughout the present study. Next, immunofluorescence microscopy confirmed that the mESC line cultured under CDSF conditions maintained the expression of Nanog, Sox2, and SSEA1 (Fig. 3A-3C). In addition, when the mESC line cultured under CDSF conditions was aggregated with wild-type morulae, resulting chimeric blastocysts exhibited green fluorescence in the inner cell mass (Fig. 3E). Eleven days after these blastocysts were transferred to pseudopregnant females, fluorescent cells were detected in a nascent male gonad of an embryo at embryonic day 13.5 (Fig. 3F, 3G).

**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 mESCs 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. 4A). However, the number of days needed for the experimental animals to reach their end points varied (stars in Fig. 4A). Interestingly, it took 47±3.1 days for mESCs cultured in CDSF+FBS to grow into 30 mm teratomas, whereas it took 37±1.8 days for mESCs cultured in CDSF followed by standard conditions for two passages to reach 30 mm in diameter. However, when mESCs cultured in CDSF were passaged only once into standard conditions, it took 76 days for these mESCs 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 mESCs cultured in CDSF injected into one side of the animal and mESCs cultured in media containing FBS injected into the other side. Therefore, the formation of teratomas from mESCs cultured in CDSF could not be examined beyond the end point of the animals (Fig. 4A). However, in the other five animals, we were able to determine that transplanted mESCs under CDSF did not generate teratomas for up to 6 months.

At one injection site, we were able to identify a tiny mass of mESCs that had been cultured under CDSF-Standard conditions one week after transplantation (supporting information Fig. S1E). The mESCs in this mass had the appearance of cells undergoing initial differentiation (supporting information Fig. S2). However, we did not observe any cellular mass at the injection sites that were derived from mESCs 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. 4B, top). Similar results were obtained with another mESC line, W4 [53,54] (data not shown). The differences in doubling times of mESCs 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, mESCs cultured in CDSF did proliferate steadily (Fig. 4B, bottom). Additionally, transcripts associated with cellular pluripotency, Sox2, Esg1/Dppa5 and Oct3/4, were expressed in mESCs cultured in CDSF (Fig. 4C). Interestingly, Eras, which regulates the tumorigenic growth of mESCs [38], was downregulated in these mESCs (Fig. 4C), whereas it became upregulated when mESCs were maintained in CDSF+FBS or under CDSF-Standard conditions (Fig. 4C, see also supporting information Fig. S5). Collectively, the loss of tumor-like potential in mESCs cultured under CDSF is associated with a slower growth rate and the reduced expression of Eras. It is interesting to note that Eras-null mESCs can contribute to the germline in chimeric animals but show significantly reduced growth rate [38].

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 mESCs, initially we focused on molecules known to sustain the self-renewing growth of mESCs, such as LIF [9,10], Bmp4 [14], vitamin A derivatives (all-trans retinoic acid, RA [15], and retinol [16,17]), and simultaneous inhibition of Erk and Gsk3β [31]. Pharmacological inhibition of Gsk3β alone promotes self-renewal of both mouse and human ESCs [23]. We excluded LIF from screening because CDSF contains LIF [26]. In the absence of LIF, mESCs undergo differentiation or cell death [26,44]. We also ruled out an inhibitor of Erk [31], which acts downstream of FGFR receptors, because inhibition of Erk cannot promote the growth of mESCs [31]. Therefore, we focused on testing other molecules such as Bmp4, RA, retinol with or without retinol binding protein (RBP) [55], and an inhibitor of Gsk3β (CIHR99021) [31].

Addition of RA in CDSF induced differentiation of mESCs as evidenced by the reduced expression level of EGFP (supporting information Fig. S3A). Addition of retinol with or without RBP in CDSF did not induce differentiation of mESCs (supporting information Fig. S3B, S3C), but failed to accelerate their growth and to induce teratoma formation (Fig. 5A, 5B). In contrast, mESCs cultured in CDSF with Bmp4 or the Gsk3β inhibitor maintained Oct3/4 expression (supporting information Fig. S3D, S3E), increased the number of cell doublings (Fig. 5A) and formed teratomas in 17% or 67% of transplantations by 7 months, respectively (Fig. 5B, 5C and supporting information Fig. S1F).
When cultured in other established CDSF media supplemented with N2 [56], B27 [57], and either Bmp4 and LIF [14], or pharmacological inhibitors of Erk and Gsk3β [22], mESCs grew into teratomas more efficiently (see “N2B27-BL” and “N2B27-2i” in supporting information Table S1 and Fig. S4). Also, W4 mESCs exhibited similar phenotypic changes when maintained in CDSF with the Gsk3β inhibitor (Fig. 5B). Gsk3β is known to regulate the activity of the c-Myc protein in mESCs [24,58]. However, RT-PCR analysis showed that inhibition of Gsk3β did not result in upregulation of Eras and c-Myc in mESCs cultured in CDSF, whereas Bmp4 induced upregulation of c-Myc (Fig. 5D and supporting information Fig. S5).

**Discussion**

In this report, we present experimental evidence to suggest that short-term CDSF culture reduces the tumor-like growth of mESCs, which is reversed by pharmacological inhibition of Gsk3β.
Our present study indicates that downstream of Gsk3\(\beta\) is primarily responsible for tumorigenesis in mESCs, 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 [59,60,61]. Therefore, serum is similar but not identical to the interstitial fluid (i.e., lymph) that surrounds cells in vivo [62]. We were unable to observe any cellular mass at the injection sites that were derived from mESCs cultured in CDSF at either one week or six months after transplantation. Therefore, it is suggested with our data that interstitial fluid will not support the tumor-like growth of mESCs.

Perhaps mESCs 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 [26]. However, because each mESC line shows a different degree of LIF dependency [54], other mouse pluripotent stem cells cultured in the serum-free medium may exhibit a capability to continuously grow after transplantation, as we have seen in embryonic carcinomas (F9; supporting information Fig. S1D) and germline-incompetent mESCs, D3 (data not shown). In addition, we found that the formation of teratomas became sporadic when we transplanted \(0.5 \times 10^6\) mESCs cultured under standard conditions for a period of 6 months. Collectively, our study demonstrated that one or more extrinsic factors or niche [43] plays an important role in the formation of a teratoma. This idea is further supported by the fact that two mESCs were sufficiently able to grow into a teratoma only when mixed with \(2 \times 10^6\) non-tumorigenic fibroblasts (MRC-5) prior to transplantation into immunocompromised mice [63]. Interestingly, the slow growth observed in Eras-null mESCs became more evident when they were cultured without feeder cells [38].

Our present data indicate that animal sera contain one or more factors that inhibit the activity of the Gsk3\(\beta\) protein (Fig. 1, 4 and 5). Gsk3\(\beta\) is involved in the canonical Wnt signaling pathway [64,65,66] and interacts with other biologically important signaling pathways such as phosphoinositide 3-kinase (PI3K)-Akt1 [40,67,68], Bmp4 [69] and hedgehog [70,71,72] signaling pathways. Although the secreted protein Wnt eventually inhibits the activity of Gsk3\(\beta\), the role of Wnt in the maintenance of self-renewal and pluripotency of ESCs remains elusive [20,21,22,23].
On the other hand, pharmacological inhibition of Gsk3β supports the self-renewal and pluripotency of ESCs [22,23,24,25]. Now, the question is what Gsk3β upstream and downstream genes are in self-renewing ESCs. Both LIF-Stat3 [73,74] and insulin pathways activate the PI3K-Akt1 signaling pathway [24,68], which mediates the inactivation of Gsk3β [24,40,64]. However, CDSF includes LIF and insulin [26], and failed to support the tumor-like growth of mESCs (Fig. 1 and 4). Thus, LIF and insulin are not the upstream of Gsk3β.

Based on our results, Bmp4 poorly promoted proliferation and teratoma formation of mESCs cultured under CDSF conditions (Fig. 5A, 5B). 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 [69]. Because of the following two observations, we consider the effect of Bmp4 on Gsk3β or the tumor-like growth of mESCs antagonistic or indirect. First, our RT-PCR results revealed that mESCs cultured in CDSF supplemented with Bmp4 upregulated c-Myc, which was not the case in mESCs treated with the Gsk3β inhibitor (Fig. 5D and supporting information Fig. S5). Second, in one set of our 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. 5B). In contrast, when mESCs at passage 9 or later...
were cultured in CDSF with Bmp4 and transplanted, they sporadically developed into teratomas. Further investigation is required to determine the upstream of Gsk3β in ESCs.

We showed that a CDSF medium supplemented with Bmp4 and LIF supported the proliferation of mESCs that maintained transcriptional activity of Oct3/4 (supporting information Fig. S3D). In contrast, Hayashi et al. demonstrated that a CDSF medium supplemented with Bmp4 supported the differentiation of trophoblasts from mESCs [47]. However, Hayashi et al. used a basal CDSF medium that lacked oleic acid and LIF, and plated mESCs on laminin-coated dishes, but not on collagen Iα-coated dishes. In addition, the presence of LIF under these CDSF conditions inhibited the differentiation of trophoblasts [47]. Therefore, it is likely that the different chemical composition of a basal medium contributed to the differentiation of trophoblasts when Bmp4 was supplemented in the culture.

It is interesting to note that in concert with LIF, Bmp4 suppresses differentiation of the neural lineage in mESCs maintained under serum-free conditions [14] supplemented with N2 and B27, which were originally developed to culture a neuroblastoma cell line [56] and hippocampal neurons [57], respectively. Mouse ESCs maintained under serum-free conditions supplemented with LIF, Bmp4, N2 and B27 are pluripotent and can contribute to the germline in chimera mice [14], and grew into teratomas (supporting information Table S1 and Fig. S4). On the other hand, we showed that both the CDSF medium used in the present study, which contains LIF, and the CDSF medium supplemented with Bmp4 poorly sustained the tumour-like growth of mESCs. Taken together, it is suggested with these data that the cellular pluripotency and the tumor-like growth of ESCs may be regulated by different mechanisms, and that extrinsic factors play significant roles in cell fate decisions of mESCs. It is interesting to investigate whether ESCs need unique substrate stiffness to grow into teratomas, because our previous study showed that stiffer substrates promoted differentiation of mESCs [75].

Gsk3β inhibits the activity of its target c-Myc [24,76], which is involved in the self-renewal of mESCs [58] and responsible for an age-associated incidence of tumorigenesis in chimeric mice generated with mouse iPSCs [77,78]. In contrast, our RT-PCR data that the cellular pluripotency and the tumor-like growth of mESCs. Taken together, it is suggested with these data that the cellular pluripotency and the tumor-like growth of ESCs may be regulated by different mechanisms, and that extrinsic factors play significant roles in cell fate decisions of mESCs. It is interesting to investigate whether ESCs need unique substrate stiffness to grow into teratomas, because our previous study showed that stiffer substrates promoted differentiation of mESCs [75].

Materials and Methods

Ethics statement

Use of animals was approved by the Illinois Institutional Animal Care and Use Committee (protocol # 10093, approved on 6/18/10).

Cell culture

A mouse embryonic stem cell (mESC) line of R1 [79], which expresses EGFP under the Oct3/4 promoter [80], was kindly provided by Dr. William L. Stanford [51]. This Oct3/4::EGFP mESC line was thawed on feeders and maintained under standard [81] or chemically-defined serum-free (CDSF) [26] conditions at 37°C, 5% CO2. The exact number of passages that this mESC line has gone through is unknown. However, when these ESCs were brought to our laboratory, they were passed 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, mESCs 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 U/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 mESCs 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 1×107/cm2 mESCs 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.csimedia.com) [26] 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, mESCs of W4 (129S6, purchased at passage 9; Taconic, Hudson, NY, http://www.taconic.com) and D3 (129S2/SvPas, CRL-1934, ATCC, Manassas, VA) were used to test CDSF conditions. When CDSF culture was supplemented with FBS, 0.02% EDTA was used for passaging cells. Following serum lots were used to supplement CDSF culture: Lot. 1359246 and 276570, Invitrogen; Lot. L0228, Atlanta Biologicals (Lawrenceville, GA, http://www.atlantabio.com); Lot. A7480Z, 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 epifluorescence lamp (DMI4000B, Leica Microsystems, Wetzlar, Germany, http://www.leica-microsystems.com) [82,83]. To measure the frequency of cell doubling, the mESCs were plated at 1×10^5 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)/(1×10^5)]. Statistical tests were performed using the Mann-Whitney’s U-test.

To compare the incidence of teratoma formation, another basal culture medium was prepared by mixing the NeurobasalTM medium supplemented with B27 (Invitrogen) 1:1 with DMEM/F12 (Invitrogen) supplemented with N2 (Invitrogen) and 50 µg/ml bovine serum albumin as described already [84]. The resulting basal medium was supplemented with either 10 ng/ml LIF and 10 ng/ml Bmp4 (R & D systems) [14], or 1 µM Stemolecule TM PD0325901 and 3 µM Stemolecule TM CHIR99021 (2; Stemgent, Cambridge, MA, https://www.stemgent.com/) [22], and used to culture mESCs for three passages, which needed seven days before subcutaneous injection into NOD-SCID mice.
Cell transplantation

At the fourth passage under standard or CDSF conditions, the mESCs 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 A 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 teratomas was compared among different CDSF conditions. 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 the different CDSF conditions indicated. 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+Gsk3β, CDSF culture supplemented with the Gsk3β inhibitor. (E): This animal was sacrificed one week after mESCs 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 tiny mass of the mESCs depicted by dashed lines with a scale bar of 0.1 cm. See supporting information Fig. S2 for detail.

Supporting Information

Figure S1 Anatomical images of NOD-SCID mice transplanted with mouse embryonic stem cells. (A-D, F and G): Teratomas developed from mouse embryonic stem cells (mESCs) 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+Gsk3β, CDSF culture supplemented with the Gsk3β inhibitor. (E): This animal was sacrificed one week after mESCs 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 tiny mass of the mESCs depicted by dashed lines with a scale bar of 0.1 cm. See supporting information Fig. S2 for detail.

Figure S2 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 mESCs. 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 S3 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 mESCs 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+Gsk3β, CDSF with the Gsk3β inhibitor.

Figure S4 The incidence of teratoma formation was compared among different CDSF conditions. 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 bars, right axis) were compared among mESCs cultured under the conditions indicated. Parentheses...
indicate the number of biological replicates (i.e., mESCs prepared at different passages) per culture condition. Standard errors of the means are indicated by bars. Two transplantations for ESF7 showed no sign of teratoma formation when paired with the standard conditions. Standard, Standard conditions; CDSF, CDSF conditions; BL, other established CDSF conditions supplemented with N2, B27, Bmp4 and LIF; 21, other established CDSF conditions supplemented with N2, B27, and pharmacological inhibitors of Erk and Gsk3β.

Figure S5 Expression levels of markers were compared among different culture conditions. Abundance of each transcript indicated above was examined in mESCs cultured under each condition on the right by 19, 21 and 23 cycles of PCR (indicated by triangles). EF1α was used as a reference. White arrowheads indicate the PCR product of e-Myc.

References
1. Evans MJ, Kaufman MB (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154–156.
2. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78: 7634–7638.
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282: 1145–1147.
4. Takahashi K, Yamanaka S (2006) Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126: 663–676.
5. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, et al. (2007) Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. Science 318: 1917–1920.
6. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichioka T, et al. (2007) Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell 131: 861–872.
7. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451: 141–146.
8. Gurdon JB, Melton DA (2008) Nuclear reprogramming in cells. Science 322: 1011–1015.
9. Williams RL, Hilton DJ, Pease S, Willson TA, Stewert CL, et al. (1988) Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336: 684–687.
10. Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, et al. (1988) Differentiation of embryonic stem cell lines by purified polypeptides. Nature 336: 683–690.
11. Niwa H (2007) How is pluripotency determined and maintained? Development 134: 635–646.
12. Ohnaka S, Daloisio S (2008) Molecular and biological properties of pluripotent embryonic stem cells. Gene Ther 15: 74–81.
13. Tanaka TS (2009) Transcriptional heterogeneity in mouse embryonic stem cells. Reprod Fertil Dev 21: 67–75.
14. Ying QL, Nichols J, Chambers I, Smith A (2003) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115: 281–292.
15. Wang R, Liang J, Yu HM, Liang H, Shi YJ, et al. (2008) Retinoic acid maintains self-renewal of embryonic stem cells by collaboration with STAT3. Cell 115: 281–292.
16. Ohtsuka S, Dalton S (2008) Molecular and biological properties of pluripotent embryonic stem cells. Gene Ther 15: 74–81.
17. Chen L, Khllan JS (2008) Promotion of feeder-independent self-renewal of embryonic stem cells by retinoic acid (vitamin A). Stem Cells 26: 1838–1846.
18. Chen L, Khllan JS (2010) A novel signaling by vitamin A/retinol promotes self-renewal of mouse embryonic stem cells by activating PKR/Akt signaling pathway via insulin-like growth factor-I receptor. Stem Cells 28: 57–63.
19. Wang J, Alexander P, Wu L, Hammer R, Cleaver O, et al. (2009) Dependence of mouse embryonic stem cells on threonine catabolism. Science 325: 435–439.
20. Yanes O, Clark J, Wong DM, Patti GJ, Sanchez-Ruiz A, et al. (2010) Metabolic oxidation regulates embryonic stem cell differentiation. Nat Chem Biol 6: 411–417.
21. Lindley RC, Gill JG, Kyba M, Murphy TI, Murphy KM (2006) Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Dev Biol 293: 373–376.
22. Dravid G, Ye Z, Hammond H, Chen G, Pyle A, et al. (2005) Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem Cells 23: 1489–1501.

Table S1 Effects of serum on the tumorigenicity of mouse embryonic stem cells.

| Serum | Effect |
|-------|--------|
| None  | +      |
| 10%   | -      |

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Author Contributions
Conceived and designed the experiments: YL, TST. Performed the experiments: YL, TYT TST. Analyzed the data: YL, TST. Contributed reagents/materials/analysis tools: TST. Wrote the paper: YL, TST.
67. Watanabe S, Umehara H, Murayama K, Okabe M, Kimura T, et al. (2006) BMP4 induction of trophoblast from mouse embryonic stem cells in defined culture conditions on laminin. In Vitro Cell Dev Biol Anim 46: 416–430.

68. Scholer HR, Ruppert S, Suzuki N, Chowdhury K, Gross P (1990) New type of POU domain in germ line-specific protein Oct-4. Nature 344: 433–439.

69. Oikawa K, Okazawa H, Okuda A, Sakai M, Muramatsu M, et al. (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 60: 461–472.

70. Walker E, Ohishi M, Davey RE, Zhang W, Cassar PA, et al. (2007) Prediction and testing of novel transcriptional networks regulating embryonic stem cell self-renewal and commitment. Stem Cell 1: 71–86.

71. Auerbach W, Dunmore JH, Fairchild-Huntress V, Fang Q, Auerbach AB, et al. (2000) Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. Biotechniques 29: 1024–1028, 1030, 1032.

72. Walther-Kelly CA, Castello-Perez, P, Levy DE (1999) Essential role of STAT3 for embryonic stem cell pluripotency. Proc Natl Acad Sci U S A 96: 2846–2851.

73. Soprano DR, Blaner WS, eds (1994) Plasma retinol-binding protein. 2nd ed. New York: Academic Press. pp 391–396.

74. Hayashi Y, Furue MK, Okamoto T, Ohnuma K, Miyoshi Y, et al. (2007) Integrins regulate mouse embryonic stem cell self-renewal. Stem Cells 25: 3003–3015.

75. Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, et al. (2005) LIF/IL-6 and Gsk3 inhibition induces teratoma formation. J Biol Chem 279: 48063–48070.