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The Growth Factor Environment Defines Distinct Pluripotent Ground States in Novel Blastocyst-Derived Stem Cells

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SUMMARY

Pluripotent stem cell lines can be derived from blastocyst embryos, which yield embryonic stem cell lines (ES cells), as well as the postimplantation epiblast, which gives rise to epiblast stem cell lines (EpiSCs). Remarkably, ES cells and EpiSCs display profound differences in the combination of growth factors that maintain their pluripotent state. Molecular and functional differences between these two stem cell types demonstrate that the tissue of origin and/or the growth factor milieu may be important determinants of the stem cell identity. We explored how developmental stage of the tissue of origin and culture growth factor conditions affect the stem cell pluripotent state. Our findings indicate that novel stem cell lines, with unique functional and molecular properties, can be generated from murine blastocyst embryos. We demonstrate that the culture growth factor environment and cell-cell interaction play a critical role in defining several unique and stable stem cell ground states.

INTRODUCTION

Following fertilization, the totipotent zygote undergoes rapid cleavage divisions to form a preimplantation blastocyst embryo, a hollow sphere in which two different cell types can be identified. An outer layer of trophectoderm cells encloses a small group of pluripotent cells known as the inner cell mass (ICM), from which the embryo proper will develop. At implantation, the ICM forms the extraembryonic endoderm and the epiblast, consisting of pluripotent cells that give rise to all embryonic germ layers. It was the pioneering work of Martin and Evans that demonstrated that cells in the ICM can be propagated indefinitely in vitro in a stable pluripotent state as embryonic stem (ES) cells, while maintaining the ability to generate all tissues of the adult body (Evans and Kaufman, 1981; Martin, 1981; Martin and Evans, 1975). Like their murine counterparts, human ES cells can be propagated indefinitely in vitro and demonstrate the ability to generate derivatives of all three germ layers (Thomson et al., 1998). Human ES cells were long thought to be equivalent to murine ES (mES) cells, despite clear morphological differences and different growth factor requirements between these two ES cell types.

The recent derivation of epiblast stem cells (EpiSCs) from postimplantation epiblasts provides a new perspective on the nature of human ES cells (Brons et al., 2007; Tesar et al., 2007). At the molecular level EpiSCs are much more similar to human ES cells than mES cells. EpiSCs display a flattened two-dimensional (2D) colony morphology, which is also characteristic for human ES cells, and are maintained under similar growth factor conditions. The close match between EpiSCs and human ES cells suggests a functional similarity between these cells. EpiSCs display many characteristic hallmarks of pluripotent stem cells such as the expression of Oct4, Sox2, and Nanog and the ability to generate derivatives of all three germ layers during both in vitro differentiation and in vivo teratoma formation. But interestingly, EpiSCs fail to contribute to chimera formation when injected into recipient blastocysts.

The above comparisons of mES cells, human ES cells, and EpiSCs illustrate that stem cell pluripotency is not a fixed ground state but is strongly influenced by developmental and environmental context. Distinct pluripotent stem cell lines with unique functional characteristics can be derived from different parts of the embryo and under different growth factor conditions. For example, the functional differences in developmental potential between mES cells and EpiSCs may reflect the tissue of origin

from which the stem cell line is initially derived, inner cell mass versus epiblast, or they may be a consequence of their different culture conditions. After all, mES cells require a combination of Leukemia Inhibitory Factor (LIF) and Bone Morphogenetic Protein 4 (BMP4) to maintain their undifferentiated state (Ying et al., 2003), while the factors that support murine EpiSC or human ES cell self-renewal are a combination of bFGF, ActivinA, or TGFβ and activation of the Wnt signaling pathway (Brons et al., 2007; Carpenter et al., 2004; Denning et al., 2006; Mallon et al., 2006; Rosler et al., 2004; Tesar et al., 2007; Xu et al., 2005).

To dissect the effect of the growth factor milieu and the developmental age of the tissue of origin on the stem cell pluripotent state, we derived novel stem cell lines from murine blastocyst embryos in culture conditions previously applied to derivation of EpiSCs from epiblast stage embryos. We designated these cells FAB-SCs for b*F*GF, *A*ctivin, and *B*IO-derived stem cells. We demonstrate that FAB-SCs are molecularly and functionally distinct from both ES cells and EpiSCs. FAB-SCs express common molecular markers of stem cell pluripotency, Oct4, Nanog, and Sox2, but unexpectedly, the cells fail to pass hallmark tests of pluripotent differentiation such as in vitro embryoid body formation, teratoma formation, or contribution to embryonic development upon blastocyst transplantation. However, brief (transient) stimulation of FAB-SCs with LIF and BMP4 induces the potential to generate teratomas and give germline contribution in chimeric mice. Our study provides new insights into the role of growth factor environment in reprogramming of the stem cell pluripotent state and identifies an unexpected role for cell-cell adhesion in this process.

RESULTS

Derivation and Characterization of Blastocyst Stem Cells

To analyze the role of the developmental stage of the embryo on the developmental potential of embryo-derived stem cells, we explored whether novel cell lines could be derived from blastocysts under similar growth factor conditions as previously described for EpiSC- and human ES cell cultures (bFGF, ActivinA, and BIO). Initial experiments using matrigel as substrate were unsuccessful, but when instead blastocysts were hatched on MEFs in the presence of bFGF, ActivinA, and BIO and a blocking antibody against murine LIF, we were able to derive stable cell lines with novel properties, which we designated FAB-SCs, for bFGF, Activin, and BIO-derived stem cells. If the zona pellucida was left intact, only 10% of the blastocysts hatched and upon trypsinization and passaging of the ICM outgrowths, a third of the hatched blastocysts yielded stable cultures that were homogenous in appearance (n = 184). Removal of the zona pellucida prior to plating of the embryos improved the derivation frequency significantly, as 80% of the embryos demonstrated robust ICM outgrowth and upon passaging 30% of the original blastocysts yielded stable FAB-SC lines (n = 99). It is interesting to note that under FAB-SC conditions ICM expansion was noticeable within 2 days after plating. In contrast, when blastocysts are plated under mES cell conditions, ICM expansion is delayed and occurs several days later. The difference in ICM outgrowth is not due to differences in cell proliferation rates since FAB-SCs and mES cell proliferation rates are similar (not shown). Instead, the delay in ICM outgrowth under ES cell conditions may indicate that ES cell lines are derived from a small subpopulation of cells within the ICM while FAB-SC conditions allow the entire ICM to expand. Alternatively, the delayed ICM outgrowth in ES cell conditions may reflect a pause in cell proliferation associated with epigenetic reprogramming events that are essential for the derivation of mES cells (Kaji et al., 2006). While speculative, this latter option would imply that such reprogramming does not occur under FAB-SC conditions.

FAB-SCs Share Features with EpiSCs and mES Cells Yet Are Distinct from Both

Unlike mES cell colonies, which have a characteristic three-dimensional appearance of tight shiny colonies, FAB-SCs grew as monolayer colonies reminiscent of EpiSCs (Figure 1A). Q-PCR analysis of Oct4, Sox2, and Nanog demonstrated that all three pluripotency transcription factors were expressed in FAB-SC lines as well as in traditional mES cells (Figure 1B). We further confirmed the homogeneous expression and nuclear localization of these transcription factors using immunohistochemistry (Figure 1C). In addition, we confirmed the expression of the cell-surface marker SSEA1 on the FAB-SCs (not shown), further demonstrating that the FAB-SC cultures homogeneously express molecular hallmarks of pluripotent cells. Although FAB-SCs are derived on a feeder layer of MEFs, in the presence of a blocking antibody to LIF, we cannot formally exclude that very low residual levels of LIF signaling are required for FAB-SC derivation. However, established FAB-SC lines can be maintained on gelatin or Matrigel coated dishes in serum-free media in the absence of LIF or BMP4, with sustained expression of pluripotent markers, demonstrating that these growth factors are not required for the maintenance of these cells. Finally, as we will demonstrate below, stimulation of FAB-SCs with LIF and BMP4 induces profound permanent phenotypic changes in these cells, arguing that FAB-SCs do not experience these growth factors during their derivation.

FAB-SCs are derived under culture conditions similar to those recently reported for the derivation of murine EpiSCs (Brons et al., 2007; Tesar et al., 2007), but FAB-SCs and EpiSCs originate from different developmental stages of the embryo. To chart similarities and differences among FAB-SCs, ES cells, and EpiSCs, we performed global gene and microRNA (miRNA) expression analysis on these cells.

Using a Luminex bead platform (Lu et al., 2005), we analyzed the expression levels of >430 miRNAs in independent ES, EpiSC, and FAB-SC clones. Heatmap analysis of these samples revealed clear differences in the global miRNA profiles of these three cell lines (Figure 1D). Interestingly, FAB-SCs express miRNAs recently shown to be ES cell specific, such as the miR-290 cluster (Houbaviy et al., 2003), or enriched in self-renewing ES cells, such as miR-18a, miR-19a, and miR- 20a (Hayashi et al., 2008). In contrast, EpiSCs express low levels of these miRNAs. These data demonstrate that FAB-SCs express miRNAs typical of ES cells, reflecting the blastocyst origin of these cells. EpiSCs, on the other hand, express several miRNAs associated with postimplantation development including miR-1 and miR-206 (associated with muscle development) and

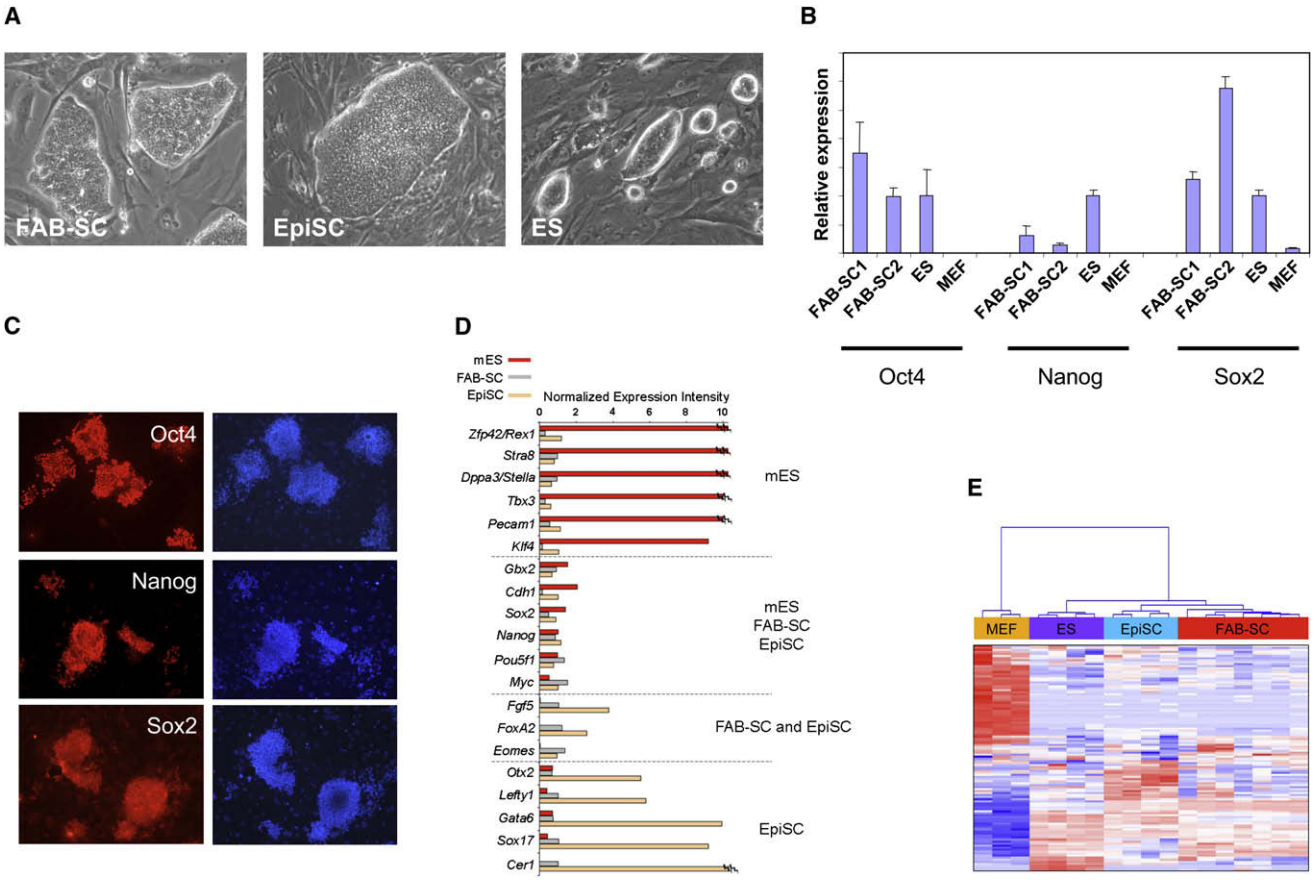

Figure 1. Novel Blastocyst-Derived Stem Cells

FAB-SCs were derived as described in the Results section.

(A) Brightfield image of FAB-SCs (top panel) and mES cells (bottom panel).

(B) Q-PCR expression analysis of Oct4, Sox2, and Nanog expression on FAB-SC and ES cell as indicated. Error bars, ± standard deviation (SD).

(C) Left panels: Immunofluorescence staining of FAB-SCs for Oct4, Sox2, and Nanog as indicated. Right panels: DAPI nuclear staining.

(D) Normalized expression intensity values (scaled median ratio) were obtained from Agilent whole-genome microarrays. Three biological replicates were used for all three cell types.

(E) Hierarchy clustering of MicroRNA profiles of MEF, mES, EpiSC, and FAB-SC cell lines.

miR-150 and miR-142 (hematopoietic differentiation) (Chen et al., 2004; Xiao et al., 2007). The microRNA expression profiling of these pluripotent stem cell lines underscores the unique character of FAB-SCs and reflects their early developmental origin. EpiSCs on the other hand demonstrate the expression of miRNAs associated with early lineage commitment, in line with the postimplantation epiblast origin of these cells.

To further interrogate the similarities and differences between FAB-SCs, mES cells, and EpiSCs, we performed microarray analysis of the transcriptional profile of these different cell lines. FAB-SCs express several known pluripotency factors, including Oct4, Sox2, and Nanog, at levels similar to those in mES and EpiSCs (Figure 1E). Yet, the expression of epiblast markers is absent or low in FAB-SCs compared to EpiSCs, demonstrating that while these cells are propagated under similar growth factor conditions, they are not the same (Figure 1E). FAB-SCs distinguish themselves from ES cells as well, as they do not express many of the genes associated with germ cell differentiation, such as Stella, Blimp1, or Dazl, which are commonly expressed in mES cells (Figure 1E). The mRNA expression analysis further demonstrates that FAB-SCs represent an alternative stable stem cell state that is distinct from both mES cells and EpiSCs.

FAB-SCs Fail to Demonstrate Pluripotency in Assays of Development

We next tested the ability of FAB-SCs to generate derivatives of all three germ layers in in vitro and in vivo assays of development. Embryoid body (EB) formation is a simple and widely used method in which aggregates of pluripotent cells initiate a differentiation program that is reminiscent of early embryonic development (Doetschman et al., 1985; Leahy et al., 1999). In the context of the EB, molecular interactions that drive early embryonic development are recapitulated and cells differentiate to form ectoderm, endoderm, and mesoderm derivatives.

Surprisingly, and in stark contrast to ES cells, EBs made from FAB-SCs remained small and failed to expand. We next interrogated the ability of FAB-SCs to form teratomas when injected into immunodeficient mice. FAB-SCs failed to form

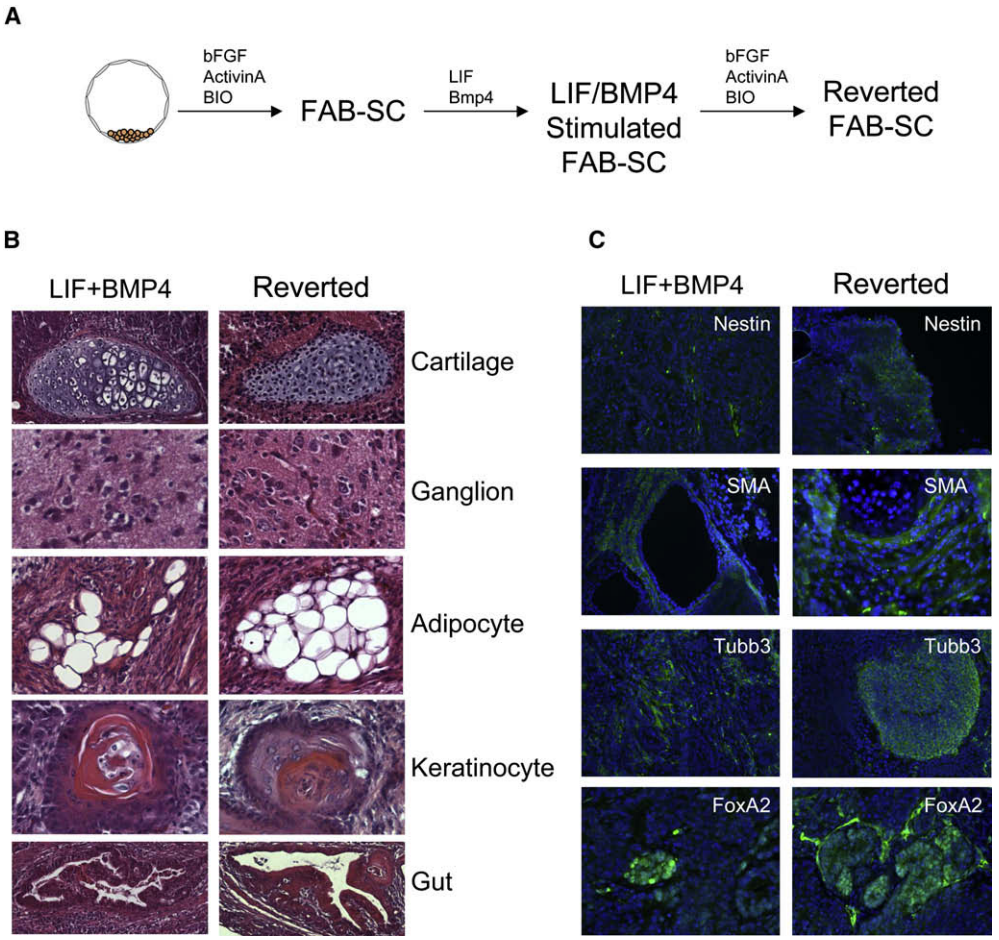

Figure 2. LIF and BMP4 Stimulate FAB-SC Teratoma Formation

(A) Schematic representation of FAB-SC derivation (with bFGF/ActivinA and BIO), LIF/BMP4-stimulated FAB-SCs cultured in the presence of LIF and BMP4, and reverted FAB-SCs, which are again maintained in bFGF/ActivinA and BIO.

(B) H&E staining of teratomas generated from a clonal FAB-SC line stimulated with LIF/BMP4 (left panels) or stimulated and subsequently cultured for 7 days in FAB-SC conditions (right panels). Derivatives of all three germ layers are observed as indicated.

(C) Immunohistochemistry analysis of markers of ectoderm (Nestin, Tubulin β3 [Tubb3]), mesoderm (Smooth Muscle Actin, [SMA]), or endoderm (FoxA2).

any teratomas at 3 months after injection (n = 20), whereas teratomas formed within 1 month in all mice injected with ES cells (n = 5).

Pluripotency is characterized by the ability of a stem cell to self-renew indefinitely while maintaining the capacity to differentiate into derivatives of all three germ layers. While FAB-SC cultures display sustained expression of hallmark molecular markers of pluripotency >30 passages (Figure 1C), the cells fail to pass standard in vitro and in vivo tests of pluripotency such as EB differentiation or teratoma formation. As such, FAB-SCs are also molecularly and functionally distinct from primitive epiblast-like cells (EPL cells) that are derived when ES cells are cultured in the presence of HEPG2 conditioned medium (Rathjen et al., 1999). Epiblast marker genes, which are expressed in EPL cells, are low or absent in FAB-SCs, and in contrast, FAB-SCs express the ICM marker Gbx2 and EPL cells do not. In addition, EPL cells are capable of forming teratomas, while FAB-SCs are not, excluding the possibility that FAB-SCs are EPL cells.

Growth Factor Stimulation Induces FAB-SC Pluripotency

To examine the influence of the growth factor milieu on FAB-SC pluripotency, we explored the effect of LIF and BMP4 stimulation on the ability of FAB-SCs to generate teratomas (Figure 2A).

To ensure a homogeneous FAB-SC population, we used flow cytometry to sort single-cell clones of FAB-SCs containing a GFP transgene into 96-well plates and visually confirmed the presence of a single cell in each well. Clonal FAB-SCs were stimulated with LIF (100 ng/ml) and BMP4 (50 ng/ml) for 1 week and injected subcutaneously into NOD-SCID mice (1×10^6 cells, n = 7) to assess their teratoma-forming potential. While none of the native FAB-SC clones gave rise to teratomas, injection of LIF/BMP4-stimulated FAB-SC clones resulted in the formation of teratomas in all recipients (3 independent clones, n = 7 for each clone). H&E staining and immunofluorescent detection of markers of germ layer differentiation demonstrated that the teratomas generated by the LIF/BMP4-stimulated

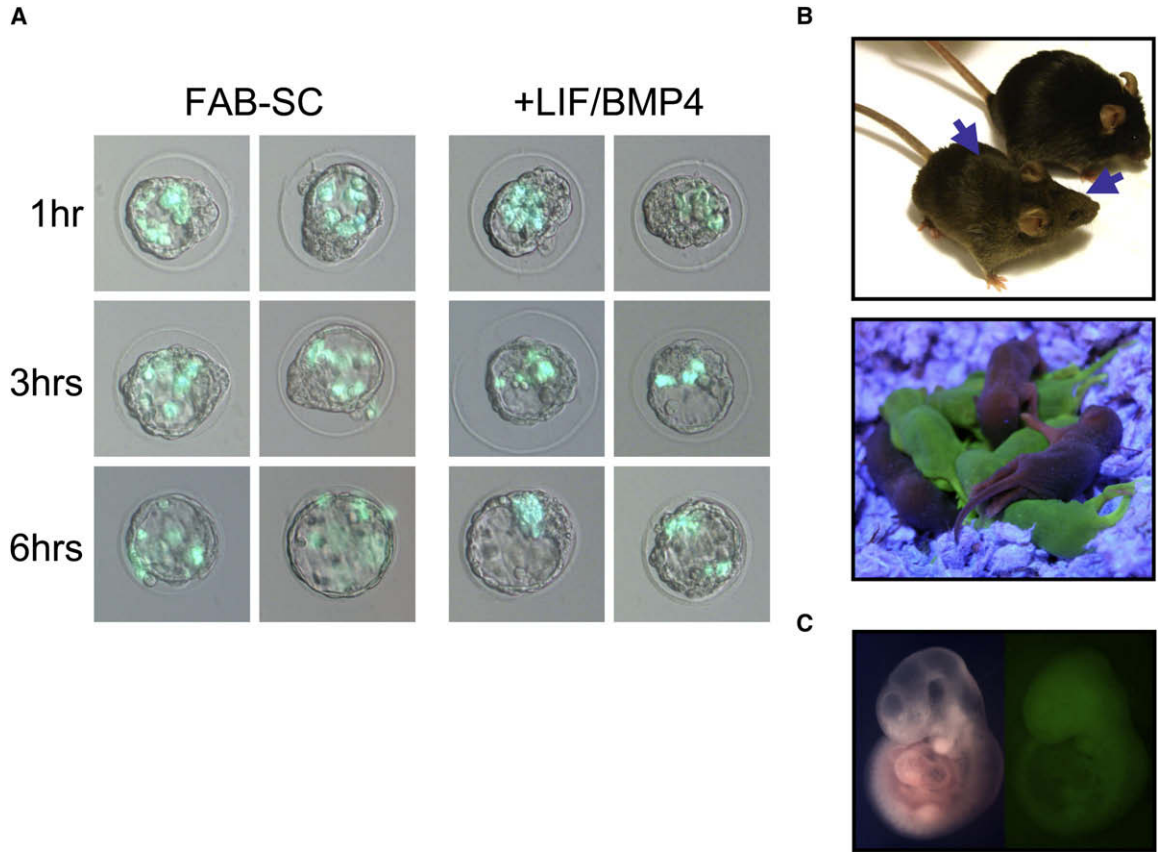

Figure 3. Growth Factor Stimulation Induces FAB-SC Chimera Formation and Germline Contribution

(A) Temporal analysis of the integration of GFP-transgenic FAB-SC before and after LIF/BMP4 stimulation into recipient blastocysts. Integration of FAB-SCs and LIF/BMP4-stimulated FAB-SCs was monitored at 1, 3, and 6 hr after injection as indicated.

(B) Top panel: High contribution chimera derived from LIF/BMP4-stimulated FAB-SC (48 hr stimulation); arrows indicate agouti coat color chimerism. Bottom panel: GFP+ offspring of the FAB-SC chimera, demonstrating germline transmission.

(C) Chimera from clonal FAB-SCs stimulated for 7 days with LIF/BMP4.

FAB-SCs displayed derivatives of all three embryonic germ-layers (Figures 2B and 2C, left panels), demonstrating that brief culture in LIF/BMP4 induces FAB-SC developmental potential. Interestingly, when the LIF/BMP4-stimulated FAB-SCs were returned back to the original FAB-SC growth factor conditions and cultured for another week, these "reverted" FAB-SCs retained the ability to generate teratomas (Figures 2B and 2C, right panels). Thus, the FAB-SC pluripotent state, induced by transient LIF/BMP4 stimulation, is retained even when the LIF/BMP4 stimulus is subsequently removed.

Blastocyst Contribution by FAB-SCs

To further explore our observation that LIF/BMP4 stimulation induced FAB-SC pluripotency, we examined the effect of growth factor stimulation on the ability of FAB-SCs to form chimeras upon transfer into recipient blastocysts. Control and LIF/BMP4-stimulated FAB-SCs, expressing the GFP transgene described above, were injected into blastocyst embryos and their integration into the recipient blastocyst was monitored over time. While the LIF/BMP4-exposed FAB-SCs integrated with the cells of the ICM within 6 hr after injection, unstimulated FAB-SCs remained dispersed in the blastocyst cavity, revealing that integration of FAB-SCs into the recipient embryos was impaired (Figure 3A).

To further analyze the developmental potential of FAB-SCs before and after growth factor stimulation, we transferred the embryos into pseudopregnant females and analyzed chimerism by the expression of the GFP transgene and/or coat color. No chimerism was observed in any of the >320 pups from blastocysts injected with 10–12 FAB-SCs each. Sectioning and immunohistochemistry staining of the embryos using an anti-GFP antibody revealed no GFP contribution to the recipient embryos at mid-gestation (E9.5–E11.5). In contrast, even brief 48 hr stimulation of FAB-SCs with LIF and BMP4 induced the ability of these cells to form chimeras. Although the chimeric frequency was low (7 out of 254 pups), the chimeras demonstrated high (40%–90%) FAB-SC contribution (Figure 3B, top panel). Mating with a wild-type female showed transmission of the FAB-SC-derived GFP transgene to the offspring, demonstrating that LIF/BMP4-stimulated FAB-SCs are capable of germline contribution as well (Figure 3B, middle panel). These embryo chimerism experiments were repeated with single-cell-derived clonal

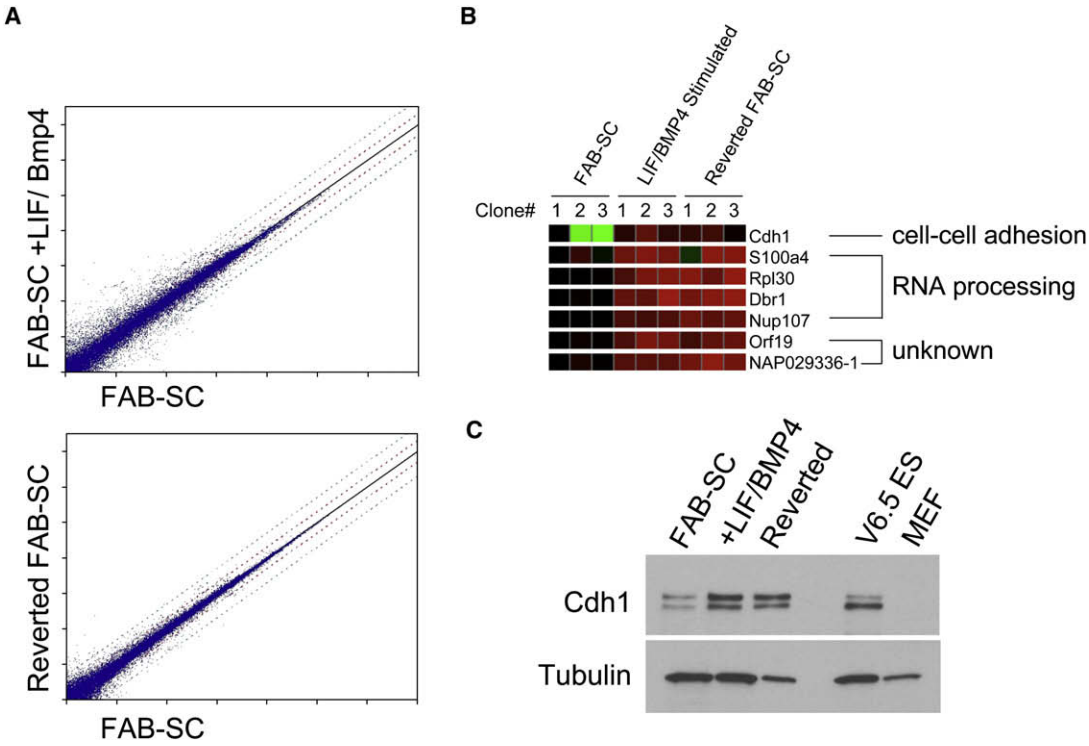

Figure 4. E-Cadherin Is Induced by LIF/BMP4 Stimulation of FAB-SC

(A) Microarray comparison of gene expression of FAB-SC and LIF/BMP4-stimulated FAB-SC (upper panel) or FAB-SC and growth-factor-reversed FAB-SC (lower panel).

(B) Heatmap of genes permanently upregulated by LIF/BMP4 stimulation of FAB-SC.

(C) Western blot analysis of E-cadherin expression in FAB-SC, LIF/BMP4-stimulated FAB-SC, and growth-factor-reverted FAB-SC.

FAB-SC lines. Again, no chimerism was observed upon blastocyst transfer of clonal FAB-SCs (n = 160). However, LIF/BMP4 stimulation of clonal FAB-SCs for 7 days induced the ability of these cells to contribute to recipient embryos with a frequency similar to the parental cell line (not shown).

Finally, we analyzed whether FAB-SCs exposed to LIF/BMP4 for 1 week would contribute to chimera formation after further culture in bFGF, ActivinA, and BIO. Clonal LIF/BMP4-stimulated FAB-SCs were cultured for an additional 7 days in FAB-SC media containing bFGF, ActivinA, BIO, and anti-LIF antibody and subsequently injected into recipient blastocyst embryos. GFP contribution by the injected reverted FAB-SCs was detected in 4 out of 123 embryos analyzed, with chimerism ranging from 20%–80% (Figure 3C), demonstrating that pluripotency is retained even after removal of the LIF/BMP4 signal.

The above data demonstrate that LIF/BMP4 stimulation of FAB-SCs induces their ability to contribute to chimera formation. The relatively low number of chimeras obtained suggests that only a fraction of the FAB-SCs undergoes full growth-factor-mediated conversion to the pluripotent state. Robust contribution by cells that do successfully undergo pluripotent conversion, including contribution to the germline, indicates however that the induced cells are truly pluripotent. Importantly, the ability of FAB-SCs to display this effect at the clonal level demonstrates that the LIF/BMP4-stimulated chimera formation is the result of an induction of FAB-SC pluripotency rather than clonal selection of "competent" cells from a heterogeneous starter population.

E-Cadherin Is Induced by Transient LIF/BMP4 Stimulation of FAB-SCs

The unique properties of FAB-SCs allowed us to further probe the molecular mechanism behind the induction of FAB-SC pluripotency by growth factor stimulation. Microarray comparison of the FAB-SC gene expression profile of three independent clonal FAB-SC lines with the expression profile of LIF/BMP4-stimulated FAB-SCs demonstrated profound changes in gene expression (Figure 4A, upper panel). Comparison of FAB-SCs to cells that were transiently stimulated with LIF/BMP4 and subsequently reverted to the original growth conditions of bFGF, ActivinA, and BIO enabled us to focus on gene expression changes linked to the induction of pluripotency (Figure 4A, lower panel). We compared the gene expression profiles of FAB-SCs, the LIF/BMP4-stimulated FAB-SC, and the growth-factor-reversed FAB-SCs and searched for genes that were up- or downregulated at least 3-fold by LIF/BMP4 stimulation and remained altered in the reverted FAB-SCs. Only a handful of genes displayed this expression profile consistently in three independent FAB-SC clones (Figure 4B). Four "hits" were genes involved in RNA translation and two were genes with unknown function. Cdh1 (E-cadherin) displayed the most profound induction, as it demonstrated a 4- to 6-fold upregulation on all seven features

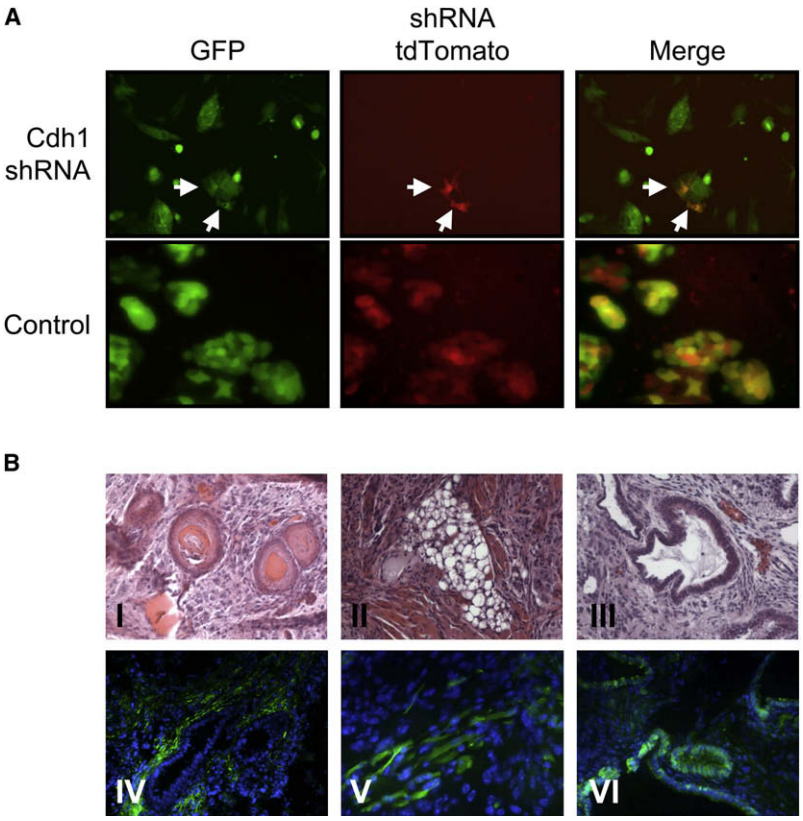

Figure 5. E-Cadherin Regulates FAB-SC Pluripotency

(A) FAB-SCs constitutively expressing a GFP transgene were transduced with control vector or shRNA to knock down E-cadherin. A tdTomato reporter gene was coexpressed from the lentiviral shRNA vector to allow identification of knockdown cells. Cdh1 knockdown results in FAB-SC differentiation (middle top panel, arrowhead).

(B) FAB-SCs were transduced with either control vector or Cdh1 expression vector and 1 × 10^6 cells were injected subcutaneously into NOD-SCID mice. Tumors were analyzed for germlayer differentiation 1 month after injection of the cells. Top panels: H&E staining of teratomas generated from Cdh1-FAB-SCs. (I) Keratinocyte, (II) adipocyte, (III) gut. Lower panels: Immunohistochemistry analysis of markers of (IV) Nestin, ectoderm, (V) Smooth Muscle Actin, mesoderm, and (V) FoxA2, endoderm.

of the microarray. Western analysis of E-cadherin expression in FAB-SCs, LIF/BMP4-stimulated FAB-SCs, and reverted FAB-SCs demonstrated that while FAB-SCs express low levels of E-cadherin, LIF/BMP4 stimulation results in upregulation of E-cadherin expression to levels comparable to those observed in mES cells; this upregulation was sustained in the reverted FAB-SCs (Figure 4C).

E-Cadherin Is a Critical Regulator of FAB-SC Pluripotency

To further analyze the function of E-cadherin in pluripotent stem cells, we generated lentiviral shRNA hairpins to examine the effect of downregulation of E-cadherin expression on the ability of FAB-SCs to generate teratomas. shRNA hairpins were tested to functionally downregulate E-cadherin expression in mES cells (Figure S1A available online). While E-cadherin expression was unaffected in the control hairpin, two E-cadherin hairpins (Cdh1-SH3 and Cdh1-SH4) demonstrated >90% downregulation of E-cadherin expression and were selected for further experiments. Functional knockdown of E-cadherin expression was tested in a cell aggregation assay. Cadherins mediate cell-cell adhesion through homotypic interactions. Two cell populations expressing similar levels of the same cadherin will form homogeneous aggregates, while dissimilarities in the nature or level of cadherin expression will result in segregation of the two cell types (Gibralter and Turner, 1985; Takeichi et al., 1981). While ES cells transduced with the control shRNA hairpin formed homogeneous aggregates when mixed with wild-type ES cells, ES cells expressing the E-cadherin hairpins did not mix with the wild-type cells but instead aggregated in spatially separate domains, demonstrating functional consequences of E-cadherin knockdown in these cell lines (Figure S1B). Next we explored the effect of knockdown of E-cadherin expression on LIF/BMP4-stimulated FAB-SCs, but we were unable to establish stable clones using the E-cadherin knockdown hairpins. Transduction of LIF/BMP4-stimulated FAB-SCs with the control vector yielded stable clones expressing the tdTomato reporter gene present in the lentiviral shRNA vector (Figure 5A). Loss of E-cadherin has been reported to induce anoikis-mediated apoptosis in certain cancer cell lines, yet no difference in apoptosis was observed between the control and E-cadherin knockdown samples (not shown). When we analyzed the transduced cells by fluorescent microscopy, however, we observed a striking difference in the morphology of the E-cadherin knockdown cells compared to control. In the control sample, tdTomato-positive (lentiviral-transduced) cells proliferated as undifferentiated colonies (Figure 5A, lower panels). In contrast, stimulated FAB-SC cells transduced with the E-cadherin hairpins had a fibroblast-like morphology, demonstrating that downregulation of E-cadherin resulted in rapid FAB-SC differentiation (Figure 5A, upper panels, arrows).

Induction of FAB-SC Pluripotency by Ectopic Expression of E-Cadherin

The upregulation of E-cadherin expression in FAB-SCs following LIF/BMP4 stimulation correlates with the induction of the ability of these cells to form teratomas containing derivatives of all three germ layers. We next examined whether this upregulation of E-cadherin expression is sufficient to induce the teratoma-forming potential of FAB-SCs. FAB-SCs transduced with a lentiviral vector expressing E-cadherin formed teratomas in immunodeficient mice after 1 month (5 out of 7), whereas none of 7 mice injected with control FAB-SCs developed teratomas. H&E staining and immunofluorescent detection of markers of germ layer differentiation demonstrated that the teratomas generated by the E-cadherin FAB-SCs displayed derivatives of all three embryonic germ layers (Figure 5B). These data demonstrate that overexpression

of E-cadherin alone is sufficient to induce robust teratoma-forming potential in FAB-SCs and suggest that a key target of LIF/BMP4 stimulation is upregulation of E-cadherin expression.

Accelerated ES Cell Differentiation in the Absence of E-Cadherin Expression

Above data demonstrate that E-cadherin plays an important role in regulating the FAB-SC pluripotent state. To test whether abrogation of E-cadherin expression would compromise the pluripotency of mES cells, we generated stable mES cell lines expressing the control or Cdh1 knockdown hairpins. Downregulation of E-cadherin expression in mES cells changes the morphology of the cells from the tight, three-dimensional colony shape to more flattened colonies of loosely connected cells, very much resembling FAB-SCs (Figure S2A). ES cells stably expressing the E-cadherin hairpins could be propagated for over 20 passages, and the proliferation and apoptotic rates between the control and E-cadherin knockdown ES cell lines were comparable (see Figure 6D below), suggesting that knockdown of E-cadherin did not impair ES self-renewal, proliferation, or apoptosis. Using immunohistochemistry we analyzed the expression of Oct4 in the control and E-cadherin knockdown ES cell lines (Figure S2B). No differences in the level, localization, or percentage of Oct4 expression were observed between control and E-cadherin knockdown ES cells. Furthermore, even cells that demonstrated a rounded-up clustered colony morphology were expressing Oct4 (arrowheads, Figure S2B). Thus, while knockdown of E-cadherin expression in traditional mES cells results in a change in morphology and loss of tight adhesion of the ES cells, loss of E-cadherin does not impair ES cell self-renewal. Like FAB-SCs, the E-cadherin knockdown ES cells formed small EBs (Figures 6A and 6B). Moreover, whereas E-cadherin knockdown ES cells form teratomas upon subcutaneous injection into NOD-SCID mice, loss of E-cadherin expression results in a profound reduction in teratoma size, as measured by the weight of the teratomas (Figure 6C). Analysis of the teratomas revealed multilineage differentiation, however, indicating that differentiation of the Cdh1 knockdown ES cells per se was not impaired (not shown). The reduced size of the E-cadherin knockdown EBs was not due to decreased proliferation or increased apoptosis of these cells (Figure 6D). Finally we analyzed the expression of pluripotency markers Oct4, Sox2, and Nanog before and during differentiation of control ES cells or the E-cadherin knockdown cell lines. While we observed no difference in the downregulation of Oct4 and Sox2 expression, western blot analysis revealed an accelerated loss of Nanog protein expression in the absence of E-cadherin expression as compared to control cells (Figure 6E). The rapid loss of Nanog expression in the E-cadherin knockdown cells was also observed when we monitored Nanog RNA levels by Q-PCR (Figure 6F). Oct4, Sox2, and Nanog have been shown to bind to promoter elements of genes involved in early cell fate decisions (Boyer et al., 2005, 2006). The concerted binding of these transcription factors mediates the recruitment of the polycomb silencing complex, thereby suppressing gene expression. Thus part of the role Oct4, Sox2 and Nanog play in ES cell self-renewal is to prevent the expression of genes associated with differentiation, such as genes in the Hox-cluster. Since loss of E-cadherin results in early downregulation of Nanog upon cell differentiation, we analyzed the expression of HoxA1 and HoxB1 in these cells. Upregulation of HoxA1 and HoxB1 is a hallmark sign of cell differentiation toward the somatic lineages and distinguishes pluripotent cells from early somatic cells in the primitive epiblast (Saitou et al., 2002; Yabuta et al., 2006). While we observed no upregulation of HoxA1 expression during early differentiation of control or E-cadherin knockdown ES cells, HoxB1 expression was upregulated prematurely in both E-cadherin knockdown cell lines compared to control (Figure 6F). Together our data demonstrate that loss of E-cadherin expression results in rapid differentiation of FAB-SCs, and while mES cells can maintain their pluripotent state in the absence of E-cadherin expression, they demonstrate an accelerated loss of Nanog expression and premature upregulation of HoxB1 expression upon induction of differentiation.

DISCUSSION

We have derived a novel stem cell line from murine blastocyst embryos using growth factor conditions previously reported for murine EpiSCs and human ES cells (Brons et al., 2007; Ginis et al., 2004; Tesar et al., 2007). Transcriptome and miRNA expression analyses demonstrate that EpiSCs, mES cells, and now FAB-SCs represent distinctive stem cell lines derived from early murine embryos. Our data demonstrate that stem cells can exist in a number of distinct metastable epigenetic states, which each display unique phenotypic properties that are determined by the growth factor environment and the developmental stage of the embryo from which the cell line is derived (Table 1). FAB-SCs may be trapped in a state of "partial pluripotency," akin to the partially reprogrammed states identified in cultures of induced pluripotent stem cells (iPS cells). In a seminal paper Takahashi and Yamanaka demonstrated that introducing four transcription factors, Oct4, Sox2, c-Myc, and Klf4, is sufficient to reprogram fibroblasts into pluripotent stem cells (Takahashi and Yamanaka, 2006). The authors selected successfully reprogrammed iPS cells by their re-expression of an ES cell-specific Fbx15 reporter. While these initial iPS cells displayed the ability to generate teratomas with derivatives of all three germ layers and contributed to somatic lineages in chimera experiments, they failed to contribute to the germline, indicating that full pluripotency was not achieved. Subsequent application of alternative reporter genes demonstrated that the pluripotent potential of the derived iPS cell lines is determined by the method of iPS colony selection, and germline-competent iPS cells can be obtained if cells are selected for the re-expression of Nanog or Oct4 transcription factors (Maherali et al., 2007; Meissner et al., 2007; Okita et al., 2007) or when selection is omitted entirely (Stadtfeld et al., 2008). These data suggested that iPS cells can exist in a stable, partially reprogrammed state. Indeed, a recent report demonstrates that incomplete reprogramming of chromatin marks associated with somatic lineages in some iPS lines results in failure of these iPS cells to repress lineage-specifying transcription factors and hampers their pluripotent potential (Mikkelsen et al., 2008). Similarly, our FAB-SCs may be in a stable ground-state of "near pluripotency" that can be reprogrammed to full pluripotency by altering the growth factor milieu. The role and mechanism of growth factor signaling in the

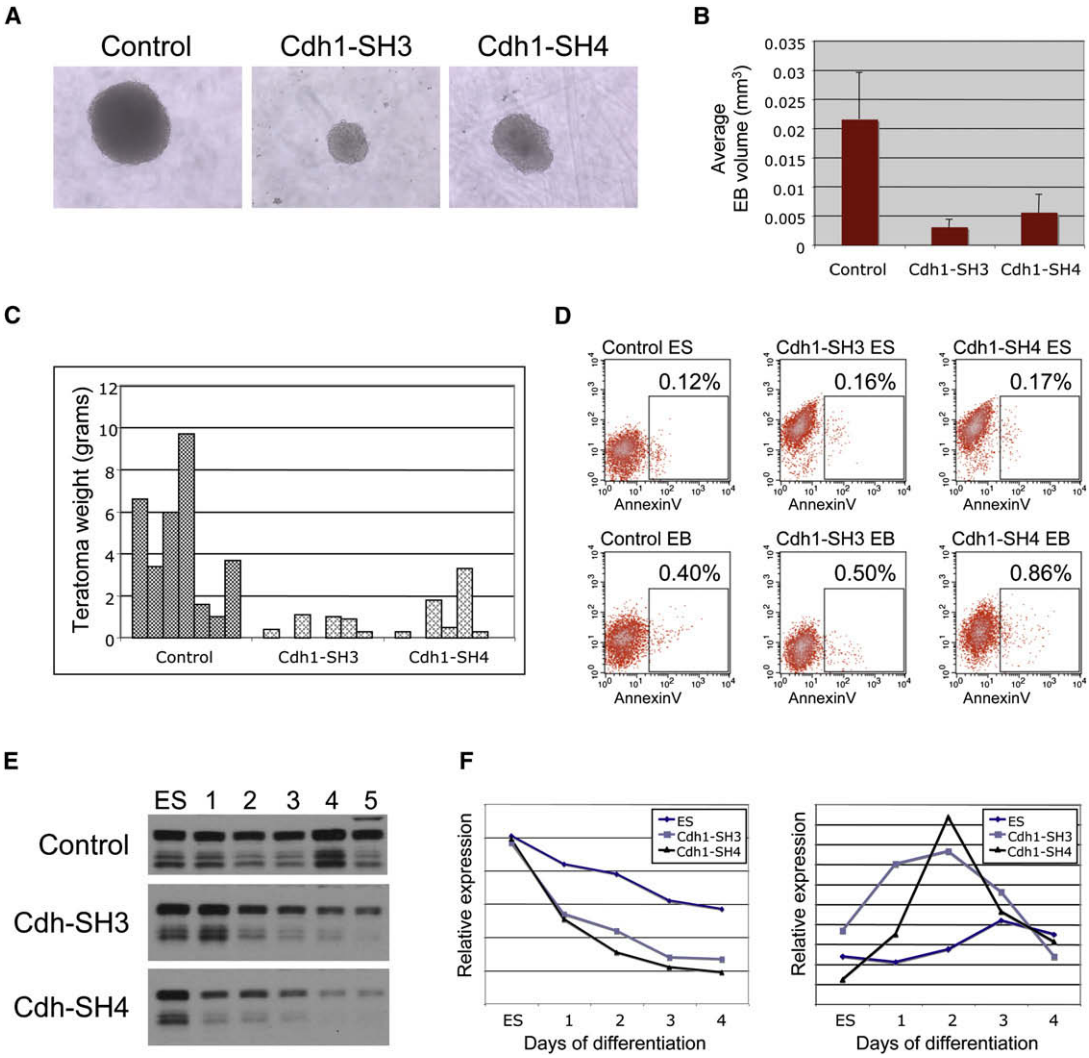

Figure 6. Loss of E-Cadherin Expression Compromises EB and Teratoma Formation by Accelerating ES Cell Differentiation

(A) Representative images demonstrating EB differentiation of wild-type and E-cadherin knockdown ES cells. Loss of cadherin expression results in small EB size compared to the wild-type EBs.

(B) Bar-graph analysis of the sizes of wild-type or E-cadherin knockdown EBs.

(C) The effect of loss of E-cadherin expression on teratoma formation. Plotted is the weight (in grams) of seven teratomas of wild-type and seven each of two independent E-cadherin knockdown ES cell lines. Loss of E-cadherin results in a profound reduction in teratoma size.

(D) Analysis of apoptotic frequency in control and E-cadherin knockdown cells. Stable ES cell lines transduced with control vector or two independent E-cadherin knockdown hairpins were analyzed by flow cytometry for AnnexinV staining. The ES cells expressed a tdTomato fluorescent reporter gene to distinguish them from the MEF feeder cells. Top panels: AnnexinV-FITC staining of control and two E-cadherin knockdown ES cell lines. The percentage of AnnexinV-positive cells is indicated. Bottom panels: AnnexinV staining of the same cell lines after 7 days of monolayer differentiation of the cells.

(E) E-cadherin downregulation results in early loss of Nanog expression and premature upregulation of HoxB1. Western blot analysis of Nanog protein expression during differentiation of control ES cells (top) and two independent E-cadherin knockdown ES cell lines (middle and lower panels). Numbers indicate days of differentiation.

(F) Left: Q-PCR analysis of the Nanog RNA levels in the same samples. Right: Q-PCR analysis of HoxB1 expression in the same samples.

reprogramming process has thus far not been recognized, and our FAB-SC system uniquely allows the dissection of this process at the molecular level.

We identified E-cadherin as a candidate gene that is induced upon transient LIF/BMP4 stimulation of FAB-SCs. E-Cadherin plays an essential role during preimplantation embryonic development. In its absence, proper cell polarization is absent and trophectoderm formation is impaired (Larue et al., 1994; Riethmacher et al., 1995). Remarkably, embryo compaction is unaffected in E-cadherin null embryos, but the cells fail to sustain the compacted state and do not form a blastocyst. E-Cadherin mutant embryos fail to hatch and do not demonstrate the typical compacted ICM morphology but instead form clusters of rounded cells, much like bunches of grapes (Larue et al., 1994), and shRNA knockdown of E-cadherin expression in mES cells results in similar colony morphology. In addition,

Table 1. Origin, Culture Conditions, and Functional Properties of Different Pluripotent Stem Cell Lines

Cell Line	Origin	Growth Factor Conditions	Teratoma Formation	Chimera Formation	Reference
Murine ES	Blastocyst	LIF, BMP4	All germ layers	Somatic and germline contribution	(Evans and Kaufman, 1981; Martin and Evans, 1975)
Murine EpiSC	Epiblast	bFGF, Activin	All germ layers	No	(Brons et al., 2007; Tesar et al., 2007)
Human ES	Blastocyst	bFGF, Activin, (BIO), MEF conditioned media	All germ layers	Not tested	(Thomson et al., 1998)
FAB-SC	Blastocyst	bFGF, Activin, BIO	No	No	This report
Reverted FAB-SC	Blastocyst	bFGF, Activin, BIO	All germ layers	Somatic contribution, germline contribution not tested	This report
Murine IPS cells	Somatic cells	LIF, BMP4	All germ layers	Somatic and germline contribution	(Maherali et al., 2007; Meissner et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006)
Human IPS cells	Somatic cells	bFGF, serum, MEF conditioned media	All germ layers	Not tested	(Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007)
Murine EG cells	Embryonic gonad	Derivation: bFGF, LIF, and SCF Maintenance: LIF and fetal bovine serum	All germ layers	Somatic and germline contribution	(Matsui et al., 1992; Resnick et al., 1992)
Human EG cells	Embryonic gonad	LIF, bFGF, and Forskolin	All germ layers	Not tested	(Shamblott et al., 1998)
Murine mGS cells	Postnatal testis	Derivation: GDNF, LIF Maintenance: LIF and fetal bovine serum	All germ layers	Somatic contribution	(Guan et al., 2006; Kanatsu-Shinohara et al., 2004; Seandel et al., 2007)

E-cadherin mutant ES cells fail to generate organized tissue structures in teratoma assays (Larue et al., 1996). Ectopic expression of E-cadherin or N-cadherin in E-cadherin mutant ES cells restored their ability to generate defined teratomas and revealed an unexpected differential effect on the type of tissue formed by the expression of these two cadherins, demonstrating that cell-cell adhesion plays an important role in directing tissue differentiation. Our data extend these observations by demonstrating that E-cadherin plays an active role in regulating the stem cell pluripotent state itself.

The differential need for E-cadherin expression for the maintenance of pluripotency in FAB-SCs and mES cells may be due to redundant effects of N-cadherin expression in mES cells. Indeed, a knockin ES cell line in which N-cadherin cDNA was inserted into the E-cadherin locus demonstrated that while N-cadherin failed to correct the trophectoderm phenotype of the E-cadherin mutant embryos, it did rescue ES cell colony morphology (Kan et al., 2007). Since FAB-SCs only express E-cadherin, downregulation of E-cadherin expression results in rapid differentiation. In contrast, mES cells express low levels of N-cadherin that may act redundantly to rescue stem cell self-renewal. Upon knockdown of E-cadherin expression, differentiating mES cells display premature downregulation of Nanog expression concomitant with an early upregulation of HoxB1 expression. We conclude that E-cadherin plays a critical role in regulating the stem cell pluripotent state and prevents premature differentiation by regulating Nanog expression.

The molecular link between E-cadherin and Nanog expression is unknown and may be founded in the close cell-cell contact mediated by E-cadherin. For example, DE-cadherin is an important component of the *Drosophila* germ cell niche, where it anchors germline stem cells to the somatic component of the niche (Song and Xie, 2002). Close contact between the stem cells and the soma assures that the stem cells receive high levels of growth factor signals that are required for their self-renewal (Song and Xie, 2002; Tulina and Matunis, 2001; Yamashita et al., 2003). It is interesting to hypothesize that pluripotent stem cells, such as FAB-SCs and ES cells, in a similar fashion require cadherin-mediated cell-cell contacts for optimal self-renewal, possibly by an unknown paracrine mechanism. Alternatively, E-cadherin itself could provide essential downstream signals that mediate the regulation of Nanog expression and maintenance of pluripotency. E-Cadherin associates with β-catenin, which serves dual roles as mediator of cell adhesion and transcriptional regulator. β-catenin activation through the Wnt signaling pathway has been implicated in ES cell self-renewal (Sato et al., 2004). In addition, TCF binding sites were recently found to colocalize with many Oct4 and Nanog binding sites in the genome, indicating that the Wnt-β-catenin signaling pathway is integral to the pluripotency circuitry mediated by these transcription factors (Cole et al., 2008). E-Cadherin may fine-tune this pathway by modulating intracellular β-catenin levels. Additional signaling pathways are known to be activated by cadherin stimulation and dissection of the molecular associations between the growth factor environment, The expression of E-cadherin by epithelial cells has been postulated as a reason for why transcription factor-induced reprogramming of epithelia is more efficient than the generation of iPS cells from fibroblasts, which lack

E-cadherin expression (Aoi et al., 2008). Thus, unraveling the role of the growth factor environment and cell-cell interactions in the induction and maintenance of FAB-SC pluripotency may serve as a paradigm for other systems of epigenetic reprogramming.

EXPERIMENTAL PROCEDURES

Mouse Strains and ES Cells

C57BL/6-TGN(ACTbEGFP) were from Jackson Laboratories, Bar Harbor, ME, USA (Okabe et al., 1997). 129SvEv were from Taconic, Germantown, NY, USA. ES and FAB-SC cell lines were derived from F1 cross between C57BL/6-TGN(ACTbEGFP)1Osb and 129SvEv. Antibodies used Oct4 (Santa Cruz), Sox2 (Chemicon), Nanog (Abcam), and E-cadherin 1:100 (Cell Signaling Technology).

Derivation and Maintenance of FAB-SC Cell Lines

E3.5 blastocysts were obtained from C57/BL6 female × 129SvEv-EGFP male mice. Briefly, blastocysts were flushed from uterus with M2 media (Chemicon) and placed on MEFs in media that consisted of DMEM, 15% KOSR, 2 mM L-glutamine, 1% nonessential amino acids, 100 U of penicillin, 100 μg of streptomycin, 1 mM Sodium Pyruvate (all from Invitrogen), 0.1 mM β-mercaptoethanol, 50 μg/ml ascorbic acid, 100 μg/ml FE-saturated transferring (all from Sigma). For the maintenance of FAB-SCs and reverted FAB-SCs, growth factors were added: 1 ng/ml bFGF (R&D systems), 50 ng/ml human ActivinA (Peprotech), 0.5 μM BIO (Sigma), and 100 ng/ml LIF-blocking antibody (R&D systems). Seven days after initiation of explant culture, blastocyst outgrowths were dissociated with Trypsin-EDTA (Invitrogen) and transferred to new wells containing MEFs. The established FAB-SC lines were passaged every other day at a subculture ratio of 1:10. The LIF/BMP4-stimulated FAB-SCs were cultured in the same basal media containing 100 ng/ml rmLIF and 50 ng/ml rhBMP4 (R&D systems).

miRNA Expression Profiling

MicroRNA expression profiling was performed as described (Lu et al., 2008; Mi et al., 2007). One microgram total RNA was used for profiling using the plate-capture method of labeling and using the bead-based platform for detection. MicroRNAs were captured using plates coupled with oligonucleotides antisense to microRNAs, ligated with adaptors, reverse-transcribed and amplified through PCR, incorporating biotin as a label. Labeled microRNAs were hybridized to detecting oligonucleotides on colored beads before detection using flow cytometry on a Luminex 100IS machine. Median fluorescence intensity was used for each microRNA as a measure of expression. Data were preprocessed as described (Lu et al., 2005). Briefly, samples were normalized assuming the same total fluorescence intensity. Data were then log2-transformed and thresholded at 5. Hierarchical clustering was performed in matlab after filtering out microRNAs that were detected in the noise range (with maximum expression in any sample of less than 7.25).

Culture of ES-EpiSC Cells

Cells were maintained on GFR-matrigel (BD Biosciences) or Laminin (Sigma) as indicated in FAB-SC medium (DME, 15% KOSR [Invitrogen], 0.1 mM nonessential amino acids [Invitrogen], 2 mM glutamine, penicillin/streptomycin [Invitrogen], 0.1 mM β-mercaptoethanol, 200 μg/ml iron-saturated transferrin (Sigma), and 50 μg/ml ascorbic acid [Sigma]), supplemented with growth factors: for ES cells 100 ng/ml LIF and 50 ng/ml BMP4 (R&D Systems), for ES-EpiSC and FAB-SC cells 50 ng/ml recombinant human ActivinA (Peprotech), 1 ng/ml bFGF (R&D Systems), 0.5 μM BIO (Sigma), and 500 ng/ml noggin (R&D Systems). For EB differentiation, cells were trypsinized, collected in EB medium (IMDM/15% IFS, 200 μg/ml iron-saturated transferrin [Sigma], 4.5 mM monothiolglycerol [Sigma], 50 μg/ml ascorbic acid [Sigma], and 2 mM glutamine). Cells were collected and plated in hanging drops at 300 cells per 25 μl droplet in an inverted bacterial petri dish. EBs were collected from the hanging drops at day 3 and transferred into 10 ml EB medium in slowly rotating 10 cm Petri dishes. At day 3, EBs were fed by exchanging half of their spent medium. Cells were harvested by lysis in Trizol (Invitrogen), followed by RNA extraction and cDNA synthesis for Q-PCR analysis of gene expression.

Blastocyst Injection

Blastocysts were collected from the uterine horns of superovulated C57BL/6 females that were mated with C57BL/6 males. Ten to twelve cells were injected per recipient blastocyst. Upon injection, blastocysts were transferred into the uterus of pseudopregnant recipient CD-1 females who were plugged 2.5 days earlier by vasectomized CD-1 males.

Microarray Analysis

For genome-wide expression analysis we compared the expression profile of three independent clonal FAB-SC lines as well as the LIF/BMP4-stimulated FAB-SCs and reverted FAB-SCs from these lines. Total RNA was extracted using Trizol reagent (Invitrogen) and labeled and hybridized to Agilent Whole Mouse Genome Oligo 4X44K Microarrays (one-color platform) according to the manufacturer's protocols. mES and EpiSC expression data were from the geo omnibus (Tesar et al., 2007). The gene expression results were analyzed using GeneSifter and/or Genespring microarray analysis software.

Single-Cell Cloning of FAB-SCs

FAB-SCs were dissociated by 0.05% Trypsin-EDTA at 37°C. Cell suspension was filtered through 40 μm cell strainer (BD bioscience) and single cells were FACS (FACS Aria, BD Bioscience) sorted individually into 96 wells with feeders. Presence of single cell in each well was confirmed visually. The subclones were subsequently propagated using standard FAB-SC culture conditions.

FACS Analysis

Cells were collected by trypsinization and resuspended in ice-cold RPMI + 0.5% FBS. Cells were incubated with the antibodies against the indicated surface antigens for 30 min at 4°C. Anti-SSEA1 and anti-SSEA2 were from the Hybridoma bank at the University of Iowa; TRA-1-60 and TRA-1-81 anti-human ES cell antibodies were from Chemicon. The cells were washed twice with RPMI/0.5% FBS and incubated with PE-conjugated rat anti-mouse IgM for 30 min at 4°C. Cells were washed twice with RPMI/0.5% FBS, resuspended in RPMI/0.5% FBS, and analyzed on a Becton Dickinson FACSCalibur analyzer or sorted using a Becton Dickinson FACSAria cell sorter.

Lentiviral shRNA Knockdown

The target sequences for murine E-cadherin RNAi knockdown were selected and the DNA oligos were designed using the pSicoligomaker 1.5 program (http://web.mit.edu/jacks-lab/protocols/pSico.html). The target sequences were GGAGATGCAGAATAATTAT (Cdh1 sh3) and GAAGAGAACATTCTA (Cdh1 sh4). For the lentiviral vector, we used a modified version of pLentiLox 3.7, in which the GFP is replaced by a tdtomato selection marker (gift from Dr. L. Daheron). The DNA oligos for the small hairpins were cloned into the XhoI and HpaI sites in the pLentiLox-tdtomato vector as described in http://web.mit.edu/jacks-lab/protocols/pll37cloning.htm. Lentivirus was produced in 293-FT cells (Invitrogen) according to the manufacturer's protocol and concentrated by centrifugation at 18000 rpm for 2 hr.

ACCESSION NUMBERS

Microarray data have been deposited in the GEO Database with the following accession numbers: GSM314554, GSM314555, and GSM314556.

SUPPLEMENTAL DATA

Supplemental Data include two figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01116-1.

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