SIP1 Mediates Cell-Fate Decisions between Neuroectoderm and Mesendoderm in Human Pluripotent Stem Cells

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SUMMARY

Human embryonic stem cells (hESCs) rely on fibroblast growth factor and Activin-Nodal signaling to maintain their pluripotency. However, Activin-Nodal signaling is also known to induce mesendoderm differentiation. The mechanisms by which Activin-Nodal signaling can achieve these contradictory functions remain unknown. Here, we demonstrate that Smad-interacting protein 1 (SIP1) limits the mesendoderm-inducing effects of Activin-Nodal signaling without inhibiting the pluripotency-maintaining effects exerted by SMAD2/3. In turn, Activin-Nodal signaling cooperates with NANOG, OCT4, and SOX2 to control the expression of SIP1 in hESCs, thereby limiting the neuroectoderm-promoting effects of SIP1. Similar results were obtained with mouse epiblast stem cells, implying that these mechanisms are evolutionarily conserved and may operate in vivo during mammalian development. Overall, our results reveal the mechanisms by which Activin-Nodal signaling acts through SIP1 to regulate the cell-fate decision between neuroectoderm and mesendoderm in the progression from pluripotency to primary germ layer differentiation.

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells derived from embryos at the blastocyst stage. Despite an apparent common origin, hESCs and mouse embryonic stem cells (mESCs) rely on different signaling pathways to self-renew and to maintain their pluripotent status. Mouse ESCs rely on leukemia inhibitory factor and bone morphogenetic protein (BMP) signaling (Chambers, 2004), whereas hESCs rely on fibroblast growth factor (FGF) and Activin-Nodal signaling (Beattie et al., 2005; Vallier et al., 2005). Indeed, hESCs resemble pluripotent stem cells derived from postimplantation mouse embryos (mouse epiblast stem cells [mEpiSCs]), suggesting that hESCs and mEpiSCs share a common embryonic identity and pluripotent state (Brons et al., 2007; Tesar et al., 2007). The similarity between hESCs and mEpiSCs has been reinforced at the molecular level by studies showing that in both pluripotent cell types, Activin-Nodal signaling directly regulates the expression of the pluripotency factor NANOG through its intracellular effectors SMAD2/3 and that NANOG in turn blocks neuroectoderm differentiation induced by FGF signaling (Vallier et al., 2009a; Xu et al., 2008). These results obtained from in vitro studies complement in vivo observations showing that NODAL is necessary to maintain NANOG expression and to prevent precocious neuroectoderm differentiation in the mouse epiblast (Camus et al., 2006; Mesnard et al., 2006). Taken together, these results clarify the mechanisms by which Activin-Nodal signaling blocks neuroectoderm differentiation while maintaining pluripotency of hESCs, mEpiSCs, and the late epiblast during early mammalian development. However, Activin-Nodal signaling is also known to be necessary for driving differentiation of pluripotent stem cells toward mesendoderm, the common progenitor of the definitive mesoderm and endoderm lineages, in vitro (D’Amour et al., 2005) and during gastrulation in vivo (Arnold and Robertson, 2009). The mechanisms by which Activin-Nodal signaling maintains pluripotency without inducing differentiation into mesendoderm remain unknown. We determined the gene expression profile of hESCs grown in the presence or absence of Activin-Nodal signaling, observing that Smad-interacting protein 1 (SIP1) was one of the most significantly upregulated genes upon pharmacological inhibition of Activin-Nodal signaling. SIP1 is a member of the ZFHX1 family of two-handed zinc finger/homeodomain proteins and was initially discovered with the yeast two-hybrid system as a binding partner of SMAD1 and SMAD2/3 (Verschueren et al., 1999). The SIP1 protein contains two zinc finger clusters flanking a homeodomain-like segment, a C-terminal-binding-protein binding site, and a Smad-binding domain (Postigo et al., 2003). Each zinc finger cluster binds to one CACCT(G) site (Verschueren et al., 1999) to repress target genes such as E-CADHERIN (Comijn et al., 2001) and BRACHYURY (Lerchner et al., 2000). In addition, SIP1 has been implicated in neuroectoderm development in Xenopus (van Grunsven et al., 2000; van Grunsven et al., 2007), mice (Van de Putte et al., 2003), chick (Sheng et al., 2003), and humans, in which heterozygous mutations or deletions of SIP1 cause Mowat-Wilson syndrome (Dastot-Le Moal et al., 2007). Finally, SIP1 has been shown to antagonize the transforming growth factor β (TGFβ) family signaling pathway, which includes Activin-Nodal and BMP signaling, by direct interaction with the MH2 domain of activated SMAD proteins (SMAD1, 2, and 3). These findings led us to hypothesize that...
SIP1 regulates the effects of Activin-Nodal and BMP signaling in hESCs and in mEpiSCs.

To test this hypothesis, we studied the function of SIP1 in pluripotency and early cell-fate decisions of hESCs and mEpiSCs. Gain- and loss-of-function experiments confirmed that SIP1 has an essential role in both processes, revealing that SIP1 overexpression enhances neuroectoderm differentiation whereas its knockdown allows for the expression of mesendodermal genes induced by Activin-Nodal signaling. We show that SIP1 establishes equilibrium between neuroectoderm and mesendoderm differentiation, thus conferring on these hESCs and mEpiSCs their pluripotent status.

RESULTS

SIP1 Is Upregulated upon Neuroectoderm Differentiation

We have previously shown that inhibition of Activin-Nodal signaling in the presence of FGF2 drives differentiation of hESCs into neuroectoderm (Smith et al., 2008). To explore the underlying mechanisms, we performed microarray analyses on hESCs grown for 48 h in a chemically defined medium (CDM) in the presence of FGF2 (12 ng/ml) and SB431542 (SB, 10 μM), an Activin receptor inhibitor. As expected, SB-treatment resulted in downregulation of known SMAD2/3 target genes such as LEFTYA, LEFTYB, NODAL, and NANOG, and upregulation of neural genes such as OLIG3, HOXA1, GBX2, and SIX1 (Figure 1A). We also observed that the expression of SIP1 was strongly upregulated upon inhibition of Activin-Nodal signaling, suggesting that SMAD2/3 could repress the transcription of SIP1 in hESCs. To confirm these observations, we performed quantitative reverse transcription polymerase chain reaction (QPCR). Neuroectoderm specification (defined as the unambiguous onset of lineage-specific gene expression) occurred after 2 days of treatment with SB. This was marked by the downregulation of pluripotency genes NANOG and OCT4 accompanied by the upregulation of early neuroectoderm genes such as GBX2 and HOXA1 (Figure 1B). Expression of genes marking neural tube development (OLIG3, SOX1, and SIX1) started later, at day 4 (Figure 1B), suggesting that neuroectoderm generated under these conditions followed a normal sequence of development. SIP1 expression was detected in pluripotent hESCs (data not shown), but increased by an order of magnitude after 2 days of neural induction. The marked increase of SIP1 upon differentiation thus paralleled the expression of early neuroectoderm markers GBX2 and HOXA1 (Figure 1B). These observations showed that SIP1 expression is limited by Activin-Nodal signaling in hESCs and suggested that SIP1 might have a role in the specification and progression of hESCs along the neural pathway.

SIP1 Overexpression Enhances Neuroectoderm Differentiation of hESCs

To determine how SIP1 functions in pluripotency and differentiation, we stably overexpressed SIP1 in hESCs and in mEpiSCs. Fewer SIP1-overexpressing clones were generated as compared to the green fluorescent protein (GFP) control (Figure S1A available online), suggesting that SIP1 overexpression was antagonistic to hESC and mEpiSC survival or proliferation. Nevertheless, three SIP1-overexpressing H9 hESC lines (SIP1-hESCs) and a control GFP-expressing H9 hESC line (GFP-hESCs) were expanded for further analyses. Growth curves showed that SIP1-hESCs grew slower than control GFP-hESCs (Figure S1B), confirming that high levels of SIP1 expression diminished hESC self-renewal.

We analyzed the levels of pluripotency and differentiation markers in SIP1-hESCs by using QPCR analysis. SIP1-hESCs expressed 20- to 30-fold higher levels of SIP1 transcripts than control GFP-labeled hESCs (Figure 1C, Day 0), a level comparable to SIP1 expression in wild-type hESCs after 2 days of neural induction (see Figure 1B). SIP1 overexpression decreased NANOG, but not OCT4 or SOX2 expression. Conversely, SIP1 overexpression increased both early (GBX2 and HOXA1) and later (OLIG3, SOX1, and SIX1) neuroectoderm marker expression (Figure 1C, day 0). QPCR analysis of SIP1-hESCs grown on feeders showed similar results (Figure S1C). Together, these observations suggest that SIP1 overexpression inhibits pluripotency and instead favors neuroectoderm development.

To explore the function of SIP1 in neuroectoderm development, we grew SIP1-hESCs in CDM supplemented with SB + FGF2. Whereas control GFP-hESCs adopted a stellar morphology typical of early neural precursors after SB-treatment, SIP1-hESCs displayed a more pronounced neural morphology with NEUROFILAMENT-positive neurite projections (Figure 1D). Importantly, SIP1 overexpression also increased neuroectoderm differentiation of hESCs grown as embryoid bodies in CDM, showing that SIP1 promotes neural development independent of the culture conditions used for inducing differentiation (Figure S1E). These results were confirmed in an independent hESC line by overexpressing SIP1 in hSF-6 cells (Figure S1B and data not shown). Finally, SIP1 overexpression in mEpiSCs similarly enhanced neuroectoderm differentiation (Figures S1B and S1F), suggesting that SIP1 function is evolutionarily conserved between human and mouse. Taken together, these results lead to the hypothesis that SIP1 plays a key role in neuroectoderm development in vitro as it does in vivo.

SIP1 Is Required for Neuroectoderm Differentiation of hESCs

Accordingly, we carried out loss-of-function experiments, knocking down SIP1 in hESCs with shRNA. More colonies were generated by transfecting hESCs with SIP1 shRNA vectors than with a control nontargeting scrambled shRNA vector in both H9 and hSF-6 lines (Figure S2A), suggesting that SIP1 knockdown has a positive effect on hESC pluripotency or self-renewal. Three H9 lines expressing shRNA against SIP1 (shSIP1-hESCs) and one control line (shScrambled-hESCs) were analyzed. QPCR showed that expression of SIP1 was knocked down by 90% in shSIP1-hESCs. Expression of the pluripotency marker NANOG increased by 1.5-fold in SIP1 knockdowns, whereas expression of OCT4 and SOX2 remained at control levels (Figure 2A, day 0). In addition, knockdown of SIP1 also decreased the basal levels of early (GBX2 and HOXA1) and later (OLIG3 and SOX1) neuroectoderm genes in hESCs (Figure 2A, day 0). Together, these results corroborate the effects of SIP1 overexpression, showing its proneural effects.
ShSIP1-hESCs induced to differentiate into neuroectoderm (CDM + SB + FGF2) showed decreased expression of NANOG and OCT4, as did control shScrambled-hESCs and wild-type hESCs grown under these conditions (Figure 2A and Smith et al., 2008). Expression of early neuroectoderm markers (GBX2 and HOXA1) was slightly decreased by SIP1 knockdown, suggesting that it was involved but not essential for neuroectoderm specification. However, expression of later markers OLIG3, SOX1, and SIX1 was dramatically decreased in shSIP1-hESCs, suggesting that SIP1 has a more important role in the progression of neuroectoderm differentiation than on its initial specification (Figure 2A). These observations were confirmed by flow cytometry, showing that during neuroectoderm induction, fewer cells expressed neural cell adhesion molecule (NCAM) in the absence of SIP1 (Figure 2B). Moreover, when differentiated as embryoid bodies in CDM, shSIP1-hESCs displayed lower levels of neuroectoderm markers (SIP1, OLIG3, and SOX1) and late neural markers (NGN2 and NEUROD1) than controls (Figure S2B). Importantly, similar results were obtained when control shScrambled-hESCs and shSIP1-hESCs were grown on feeders and differentiated for 3 days with SB (Figure S2C), confirming that the effects of SIP1 knockdown were independent of the culture conditions. Taken together, these results demonstrate that while SIP1 is not necessary for initial neuroectoderm specification, it is required for the normal progression of in vitro neural development.

**SIP1 Knockdown Bypasses the Role of Exogenous Activin**

The results described above led us to hypothesize that SIP1 knockdown could bypass the requirement for exogenous Activin to block neuroectoderm differentiation. Accordingly, shSIP1-hESCs and control shScrambled-hESCs were grown in CDM without Activin for 6 days and analyzed for the expression of pluripotency and differentiation markers by QPCR. In the absence of exogenous growth factors, control hESCs differentiated into neuroectoderm as shown by the downregulation of NANOG and OCT4 and upregulation of SIP1, SIX1, and SOX1.
In contrast, shSIP1-hESCs retained the expression of *NANOG* and *OCT4*, whereas expression of neural markers *SIX1*, *SOX1*, and endogenous *SIP1* itself was almost undetectable (Figure 2C). Similar results were obtained by knocking down SIP1 in hSF-6 hESCs, confirming that these effects are independent of the cell line used (Figure S2D). Together, these results show that absence of SIP1 blocks neuroectoderm differentiation induced by decreasing Activin-Nodal pathway activity.

**SIP1 Blocks BMP-Induced Mesendoderm Fates during Neuroectoderm Differentiation**

In amphibians, SIP1 protein is known to interact with SMAD1 and promote neuroectoderm specification by inhibiting BMP signaling (Nitta et al., 2004). Consequently, the neuroectoderm-enhancing effects of SIP1 in hESCs could be due to blockade of the inhibitory role of BMP signaling on neuroectoderm differentiation. Because BMP signaling is not active in hESCs grown in CDM + Activin + FGF (Vallier et al., 2005), we analyzed the effect of SIP1 overexpression on extra-embryonic differentiation induced by BMP4 (Figure 3A and Figure S3A) (Vallier et al., 2009b; Xu, 2006). QPCR showed that SIP1-hESCs grown in CDM + BMP4 expressed higher levels of extraembryonic tissues markers such as *CDX2* and *HAND1* (for trophectoderm) and *SOX7* (for primitive endoderm) than control GFP-hESCs (Figure 3A), suggesting that SIP1 promotes extraembryonic differentiation induced by BMP4 rather than inhibiting it. In addition to inducing this apparent extraembryonic phenotype, BMP4 increases the basal expression of definitive mesendoderm and endoderm markers in hESCs (*BRACHYURY*, *GOOSECOID*, and *SOX17*). SIP1-hESCs expressed lower levels of mesendoderm and endoderm markers, suggesting that although SIP1 enhances the effect of BMP signaling on the expression of extra-embryonic genes, it reduces the expression of mesendodermal and definitive endodermal genes.

We further investigated the importance of SIP1 on the effect of BMP signaling in the context of neuroectoderm differentiation. ShSIP1-hESCs and control shScrambled-hESCs were grown in neuroectoderm-inducing conditions (CDM + SB + FGF2) for 3 days, followed by treatment with BMP4 for three additional days. QPCR analyses showed that the addition of BMP4 to control shScrambled-hESCs decreased the levels of neuroectoderm markers *SOX2*, *GBX2*, *OLIG3*, and *SOX1* (Figure 3B), confirming the inhibitory effect of exogenous BMP on neuroectoderm differentiation. However, BMP4 treatment did not restore the expression of pluripotency genes *NANOG* and *OCT4*, suggesting that BMP4 addition in such cultures did not revert neuroectodermal cells to the pluripotent state. When BMP4 was
Statistical analyses were as in Figure 1. Gene expression levels in shScrambled-hESCs (SCR) and shSIP1-hESCs (SIP1.1–SIP1.3) of genes expressed in the primitive streak (mesendoderm markers) (Figure S3B) in shScrambled-hESC. Interestingly, exogenous BMP4 did not increase the expression of the definitive endoderm marker SOX17 (Figure S3B) in shScrambled-hESCs or in shSIP1-hESCs, confirming that BMP signaling is not sufficient for inducing endoderm differentiation in the absence of Activin-Nodal signaling. Taken together, these results demonstrate that SIP1 knockdown enables the mesendoderm-inducing effect of BMP to predominate even after initiation of neuroectoderm differentiation, thereby revealing a role of SIP1 in protecting neural differentiation from the effects of BMP.

**SIP1 Prevents Activin-Nodal Signaling from Driving Pluripotent hESCs into the Mesendoderm Lineage**

SIP1 protein has been shown to inhibit the transcriptional activity associated with the Activin-Nodal signaling cascade through its interaction with SMAD2/3 proteins (Verschueren et al., 1999). Consequently, we investigated whether such a mechanism could potentially account for the observed effects of SIP1 overexpression and knockdown described here. We first analyzed the effects of SIP1 expression on the activity of SBE4-luc, a luciferase reporter gene specific for Activin-Nodal signaling. Overexpression of SIP1 decreased SBE4-luc reporter activity, while knockdown of SIP1 using shRNA increased it. In addition, SIP1 overexpression limited the potent transcriptional activation associated with transient SMAD3 overexpression, whereas SIP1 knockdown further stimulated it (Figure 4A). Finally, SIP1 overexpression in hESCs decreased the expression of known SMAD2/3 target genes, such as NANOG, NODAL, LEFTY, A, and LEFTYB, whereas SIP1 knockdown increased their expression (Figure S4). Together, these data confirm that SIP1 inhibits the transcriptional activity of Activin-Nodal signaling in hESCs.

Because SIP1 limits Activin-Nodal signaling, we hypothesized that SIP1 prevents this signaling cascade from driving pluripotent stem cells into the mesendoderm (precursor of mesoderm and endoderm) and definitive endoderm lineages. We thus investigated the effects of SIP1 knockdown in hESCs cultured in the presence of a high dose of Activin. ShScrambled-hESCs and shSIP1-hESCs were grown for 4 days in CDM with different Activin doses (10 ng/ml and 100 ng/ml). QPCR analyses showed that shSIP1-hESCs expressed moderately higher levels of the pluripotency marker NANOG and dramatically higher levels of mesendoderm and endoderm markers (BRA, MIXL1, EOMES, GSC, and SOX17) as compared to control shScrambled-hESCs when they were cultured in 10 ng/ml of Activin (the dose capable of maintaining pluripotency) (Figure 4B, blue bars). Increasing Activin concentration to 100 ng/ml upregulated the expression of NANOG, MIXL1, EOMES, GSC, and SOX17 in shScrambled-hESCs to levels similar to those for shSIP1-hESCs grown in 10 ng/ml of Activin (Figure 4B, maroon bars). High Activin further upregulated the expression of mesendoderm and endoderm markers in shSIP1-hESCs above those shown at 10 ng/ml of Activin. These results suggest that SIP1 expression in pluripotent hESCs is necessary to block the expression of mesendoderm and endoderm markers induced by Activin-Nodal signaling. These results were further confirmed by analyzing the expression of mesendoderm and endoderm markers (BRACHYURY, GOOSECOID, and SOX17) in shSIP1-hESCs differentiated into neuroectoderm (Figure 4C). QPCR analysis revealed that shSIP1-hESCs expressed higher levels of BRACHYURY and SOX17 as compared to shScrambled hESCs even during the early stages of neuroectoderm differentiation (Figure 4C, day 2), further supporting the hypothesis that endogenous SIP1 is required in hESCs to block the expression of
mesendoderm and endoderm markers and thereby to preserve the pluripotent state.

These results were further supported by analysis of the expression of mesendoderm, endoderm, and extraembryonic lineage markers in SIP1-hESCs. Indeed, SIP1 overexpression decreased the background expression of mesendoderm and endoderm genes (e.g., BRACHURY, GOOSECOID, and SOX17) in hESCs, but did not affect the expression of extraembryonic lineage genes (e.g., HAND1 and SOX7) (Figure S1D, day 0), suggesting that SIP1 specifically limits mesendoderm differentiation of hESCs. Taken together, these observations confirm that SIP1 plays a crucial role in diminishing the expression of mesendoderm markers induced by Activin-Nodal signaling in pluripotent stem cells.

Figure 4. SIP1 Inhibits Activin-Nodal Signaling and Activin-Nodal-Dependent Expression of Mesendodermal Genes in hESCs
(A) Effect of SIP1 on SMAD transcriptional activity was determined by measuring luciferase activity of SBE4-luc reporter. Firefly luciferase activity (normalized to control renilla activity) is expressed as mean ± standard deviation from three independent experiments.
(B) Gene expression levels in control shScrambled-hESCs and shSIP1-hESCs (shSIP1_A – shSIP1_C) grown for 4 days in CDM + FGF2 + Activin (either 10 ng/ml or 100 ng/ml) were determined by QPCR.
(C) Gene expression levels in control shScrambled-hESCs and shSIP1-hESCs (shSIP1_A – shSIP1_C) during neuroectoderm differentiation (CDM + SB + FGF2) were determined by QPCR. Statistical analyses were as in Figure 1. *p ≤ 0.05 for all shSIP1 lines compared to control shScrambled line. See also Figure S4.

SIP1 Overexpression Blocks Activin-Dependent Endoderm Differentiation and Developmental Progression
To explore further the inhibitory effect of SIP1 on the Activin-Nodal signaling cascade, we determined whether SIP1 would block endoderm differentiation, which relies on high levels of Activin-Nodal signaling (D’Amour et al., 2005; Ninomiya et al., 1999). SIP1-hESCs were induced to differentiate into definitive endoderm with a chemically defined protocol that mimics in vivo development (Vallier et al., 2009b). This protocol first induces differentiation of hESCs into mesendodermal cells expressing primitive streak markers BRACHURY, MIXL1, EOMESODERMIN, PDGFRα, and TBX6 and then drives the differentiation of these cells into definitive endoderm cells expressing CXCR4, GOOSECOID, and SOX17. QPCR analysis showed that expression of BRACHURY, a primitive streak and pan-mesoderm marker, was lower in SIP1-hESCs than control GFP-hESCs (Figure 5A). This is consistent with studies of amphibian development showing that BRACHURY is directly repressed by SIP1 (Papin et al., 2002). However, some mesendoderm markers (MIXL1, PDGFRα, and EOMESODERMIN) were expressed at similar levels in SIP1-hESCs as in control GFP-hESCs. In contrast, expression of definitive endoderm markers GOOSECOID, CXCR4, and SOX17 was strongly reduced in SIP1-hESCs (Figures 5A and 5B). This shows that during mesendoderm induction with this protocol, SIP1 overexpression did not block the initial differentiation into mesendodermal cells, but blocked the differentiation of mesendoderm cells into definitive endoderm. In addition, we observed that
SIP1-overexpressing mEpiSC lines induced to differentiate with the same protocol failed to express SOX17 (Figure S5B), showing that the role of SIP1 in blocking endoderm development is evolutionarily conserved between mouse and human. Finally, the expression of neuroectoderm (SOX2, SOX1, HOXA1, and CDX2) and extraembryonic markers (CDX2 and SOX7) was not increased in SIP1-hESCs grown in culture conditions inductive for endoderm differentiation (Figure S5A), demonstrating that SIP1 overexpression did not block endoderm specification by promoting neuroectoderm or extraembryonic differentiation in these conditions.

To extend these observations to later stages of endoderm development, we analyzed the effect of SIP1 overexpression on liver differentiation, taking advantage of a protocol recently established in our laboratory (see Supplemental Experimental Procedures). With this protocol, control GFP-hESCs showed upregulation of hepatic nuclear factors (HNF4α, HNF1β, and HNF6), regulators of liver-specific gene expression. They also showed upregulation of definitive liver markers such as ALBUMIN and α-feto protein (AFP). However, SIP1-hESCs cultured in these conditions expressed lower levels of the HNFs as compared to control GFP-hESCs and showed no increase in expression of ALBUMIN and AFP (Figure 5C). Evidently, SIP1 overexpression blocks liver differentiation, confirming our observations that SIP1 inhibits endoderm differentiation.

We then defined the effect of SIP1 in later mesoderm development, specifically the capacity to differentiate into cardiac cells during embryoid body (EB) formation. After 10 days of EB formation, 24% ± 9% of GFP-EBs displayed beating structures (Figure S5C and Movie S1), whereas none of the EBs from SIP1-hESCs were beating. QPCR analyses showed that expression of cardiac specific homeobox (NKX2.5), cardiac myosin heavy chain 6 (MYH6), and ventricular myosin light chain (MLC2V) was considerably upregulated in the GFP-EBs (Figure S5D) but not in SIP1-overexpressing EBs. Interestingly, these observations show that overexpression of SIP1 impedes differentiation to later stages of mesoderm, even though it did not affect the expression of some mesoderm markers during early stages of differentiation (Figure 5A).

Activin-Nodal Signaling Cooperates with NANOG, OCT4, and SOX2 to Regulate SIP1 Expression in hESCs

SIP1 expression appears to be tightly controlled in hESCs, with higher levels favoring neuroectoderm and lower levels favoring
pluripotency. Therefore, we sought to identify the transcriptional network controlling SIP1 expression in hESCs. Our observations that Activin-Nodal inhibition increased SIP1 expression, whereas a high dose of Activin strongly downregulated SIP1 expression, suggest that SIP1 could be a direct target gene of Activin-Nodal signaling in hESCs. Furthermore, a previous genome-wide study showed that NANOG, OCT4, and SOX2 co-occupy the SIP1 promoter (Boyer et al., 2005), suggesting that SIP1 expression could also be controlled by the core transcriptional circuitry in hESCs. Accordingly, we performed chromatin immunoprecipitation (ChIP) analyses to determine whether SMAD2/3, NANOG, OCT4, and SOX2 were present on the SIP1 promoter. We observed that SMAD2/3 binds the SIP1 promoter at a region located 5.7 to 6.8 kb upstream of the SIP1 ATG, this being the same region where NANOG, OCT4, and SOX2 binding occurs (Figure 6A). SIP1 expression in hESCs could thus be directly controlled by these transcription factors. To define precisely the function of each transcription factor in the regulation of SIP1 expression, we individually knocked them down or overexpressed them in hESCs and then analyzed the expression of SIP1 by using QPCR. Decrease in SMAD2, NANOG, or OCT4 expression each increased SIP1 expression, whereas knockdown of SOX2 decreased SIP1 expression (Figure 6B, top row). Conversely, overexpression of SMAD2, NANOG, or OCT4 each increased SIP1 expression, whereas overexpression of SOX2 increased SIP1 expression (Figure 6B, bottom row). These results indicate that SMAD2, NANOG, and OCT4 repress SIP1 expression, whereas SOX2 activates its expression. Importantly, only SOX2 binds the SIP1 promoter during differentiation of hESCs toward the neuroectoderm lineage (Figure 6C), suggesting that SOX2 could favor neuroectoderm differentiation through the activation of SIP1 expression. Taken together, these results suggest that in hESCs, SMAD2/3 cooperate with the core transcriptional circuitry to regulate the expression of SIP1, an important factor required to block the expression of mesendoderm markers in pluripotent stem cells and to protect neuroectoderm differentiation.
differentiation against inhibition by Activin-Nodal and BMP signaling cascades.

**DISCUSSION**

The results presented here demonstrate that SIP1 plays an important role in pluripotency and differentiation of hESCs and mEpiSCs. Our findings reinforce previous in vivo studies showing that SIP1 plays a key role in neuroectoderm specification (zebrafish, Delalande et al., 2008; Xenopus, van Grunsven et al., 2000; chick, Sheng et al., 2003; and mouse, Van de Putte et al., 2003). Importantly, our data show that SIP1 overexpression enhances neuroectoderm differentiation of hESCs, but its role in this process is important for progression of neuroectoderm development rather than initial specification. This is supported by our SIP1 knockdown data showing that neuroectoderm can be initially specified but is unable to progress to maturity. These data are in agreement with previous genetic studies carried out in the mouse, which show that SIP1 is neither necessary nor sufficient for initial differentiation of neuroectoderm, but affects later stages of neural development (Miyoshi et al., 2006).

Several studies have revealed an evolutionarily conserved role of SIP1 in the inhibition of BMP signaling (Nitta et al., 2004; Van de Putte et al., 2003; van Grunsven et al., 2007). Interestingly, in hESCs grown in chemically defined culture conditions (in which BMP signaling is quiescent), SIP1 mainly functions to inhibit mesendoderm differentiation induced by Activin-Nodal signaling. Paradoxically, SIP1 enhances, rather than blocks, extraembryonic differentiation of hESCs induced by exogenous BMP4. This could be accounted for by the inhibitory effect of SIP1 on Activin-Nodal signaling, which in turn may relieve Activin/Nodal’s inhibitory effect on BMP signaling, reported by Xu et al. (2008). It has also been shown that pharmacological inhibition of Activin-Nodal signaling augments the extraembryonic-lineage-inducing effect of BMP (Sumi et al., 2008; Vallier et al., 2009b). Therefore, a SIP1-induced reduction in Activin-Nodal signaling activity as observed here may actually potentiate the impact of BMP signaling. Moreover, the differential effects of SIP1 on mesendodermal (BRA and TBX6 affected, but not PDGFRα, MIXL1, or EOMES) and definitive endoderm marker genes (GSC, SOX17, and CXCR4 all strongly impeded by SIP1) could be explained by the differential requirement of Activin-Nodal and BMP signaling in this cell-fate decision. Accordingly, the more pronounced effect of SIP1 on endodermal genes could mainly be due to the greater dependency of this cell-fate decision on Activin-Nodal signaling, as demonstrated in previous studies (D’Amour et al., 2005; Nostro et al., 2008; Sumi et al., 2008; Vallier et al., 2009b). Nonetheless, a role of SIP1 in inhibiting BMP signaling is observed in the context of neuroectoderm differentiation (in conditions where Activin-Nodal signaling is blocked or diminished). In this case, SIP1 protects neuroectoderm differentiation from the mesendoderm-inducing effects of BMP. This supports previous genetic studies demonstrating that SIP1 favors neural induction by blocking mesendoderm differentiation (Nitta et al., 2007; Papin et al., 2002; Sheng et al., 2003; van Grunsven et al., 2001; Verschueren et al., 1999). Evidently, SIP1 acts to sustain neuroectoderm differentiation by inhibiting signaling pathways that induce the formation of other germ layers, particularly mesendoderm and definitive endoderm.

In addition to a role in enhancing neural development, SIP1 plays a key role in maintaining stem cell pluripotency by inhibiting Activin/Nodal-dependent mesendoderm differentiation. This explains how Activin-Nodal signaling can maintain pluripotency rather than induce differentiation. We have previously shown that NANOG interacts directly with SMAD2/3 to limit Activin/Nodal-induced endoderm differentiation (Vallier et al., 2009a). Importantly, knockdown of NANOG expression increases neuroectoderm markers but not mesendoderm markers, suggesting that another factor (identified here as SIP1) is required to limit the inductive effect of Activin-Nodal signaling leading to mesendoderm differentiation. The subsequent developmental defects of SIP1 in hESCs were demonstrated by their failure to differentiate into beating cardiomyocytes and by failure of SIP1-overexpressing hESCs and mEpiSCs to upregulate SOX17 or differentiate into liver cells. Taken together, our findings underscore the importance of SIP1 in limiting the endoderm-inducing effect of Activin-Nodal signaling, whereby SIP1 balances opposing tendencies toward neuroectodermal and mesendodermal differentiation, thus preserving the pluripotent state.

Importantly, our demonstration here of the repression of SIP1 transcription by NANOG provides a missing link between Activin-Nodal signaling and its inhibition of neuroectoderm differentiation. Our previous demonstration that Activin-Nodal signaling inhibits neuroectoderm differentiation in hESCs and mEpiSCs (Vallier et al., 2004a) led to the further mechanistic insight that this effect is mediated through the activation of NANOG transcription (Vallier et al., 2009a). Specifically, SMAD2/3 bind to the NANOG promoter and induce NANOG transcription in hESCs (Vallier et al., 2009a; Xu et al., 2008). Our finding shows that SMAD2/3, NANOG, and OCT4 repress SIP1 expression in hESCs, thus revealing the mechanisms by which Activin-Nodal signaling acts through the pluripotency transcriptional circuit to prevent differentiation into neuroectodermal fates.

These mechanisms can be summarized in the form of a model by which Activin-Nodal signaling maintains pluripotency, preventing neuroectoderm induction without inducing mesendoderm differentiation (Figure 7). In pluripotent hESCs, the transcriptional regulation of SIP1 by Activin-Nodal signaling is implemented through repressive effects (NANOG and OCT4) and activating effects (SOX2) that act in opposition to each other, thereby resulting in a tight regulation of SIP1 expression. This limits the capacity of SMAD2/3 to activate mesendoderm markers, but does not block the pluripotency-maintaining effects of Activin-Nodal signaling. Neuroectoderm specification is triggered by a decrease in Activin-Nodal signaling (which is driven in embryos by the natural inhibitors of Nodal, Lefty and Cerberus [Menno et al., 1997; Smith et al., 2008]). The consequent loss of NANOG and OCT4 expression enables SOX2 to fully activate the SIP1 gene. Increased SIP1 expression in turn inhibits residual Activin-Nodal signaling and diminishes the mesendoderm-inducing effects of BMP signaling. This allows the neuroectodermal cell fate to prevail over mesendodermal fates. This model reveals how extracellular signals cooperate with the core pluripotency transcriptional network to maintain a stasis between neuroectodermal and mesendodermal differentiation, which is resolved in favor of neuroectoderm when SIP1 expression climbs in response to decreased Activin-Nodal activity.
thereby enabling SOX2 to fully activate the transcription of inhibition of SIP1 expression imposed by SMAD2/3, NANOG and OCT4, differentiation. SIP1 inhibits mesendoderm differentiation by diminishing inhibition of hESCs. NANOG inhibition of neuroectoderm development is achieved through gene expression described were reproducible from one experiment to the next. See Supplemental Information for cardiac and liver cell differentiation.

**Gene expression profiling with microarray has been described in Vallier et al. (2004b).**

**Figure 7. SIP1 Mediates Differentiation of hESCs, Favoring Neuroectoderm and Inhibiting Mesendoderm**

During pluripotency, Activin-Nodal signaling drives the expression of NANOG, which acts together with OCT4 and SMAD2/3 to inhibit expression of SIP1 in hESCs. NANOG inhibition of neuroectoderm development is achieved through inhibition of SIP1, whose function is necessary for progression of neural differentiation. SIP1 inhibits mesendoderm differentiation by diminishing Activin-Nodal signaling. Inhibition of Activin-Nodal signaling releases the inhibition of SIP1 expression imposed by SMAD2/3, NANOG and OCT4, thereby enabling SOX2 to fully activate the transcription of SIP1, which favors neuroectoderm differentiation, inhibiting mesendoderm differentiation.

In conclusion, our insight into the function of SIP1 shows how Activin-Nodal signaling can act both to maintain pluripotency and to promote mesendoderm differentiation at close developmental intervals. It also offers a mechanism by which the cell fate decision to form neuroectoderm is maintained in the face of stimuli to form other germ layers. Future studies will permit a thorough understanding of the full molecular cascade by which SIP1 controls these cell-fate choices, providing knowledge-based approaches for controlling differentiation of human pluripotent cells into clinically useful cell types.

**EXPERIMENTAL PROCEDURES**

**Human ESC and Mouse EpiSC Culture in Chemically Defined Conditions**

Human ESCs (H9 [WiCell, Madison, WI] and hSF-6 [UCSF, San Francisco, CA]) and mouse EpiSCs (129S2-EpiSCs) were grown in a chemically defined medium (CDM) as previously described (Brons et al., 2007). For neuroectoderm differentiation, cells were grown in CDM + SB431542 10 μM (Tocris) + FGF2 20 ng/ml + BMP4 10 ng/ml (R&D Systems) + FGF2 1 ng/ml. For differentiation into extraembryonic lineages, cells were grown in CDM + BMP4 10 ng/ml. For embryoid body (EB) formation, hESC colonies were grown in CDM in low attachment plates (Costar) on a rotating shaker. All differentiation experiments were repeated at least twice on different passages of cells to ensure that the patterns of gene expression described were reproducible from one experiment to the next. See Supplemental Information for cardiac and liver cell differentiation.

**Microarray Analysis**

Gene expression profiling with microarray has been described in Vallier et al. (2009a).

**Vectors for Overexpression**

The pTP6 vector backbone (Pratt et al., 2000) containing the CAGG (hCMV/Chicken β-Actin chimeric promoter) promoter and an IRES-Puromycin, was used as the basis for constructing all expression vectors for stable overexpression of transgene in hESCs. Flag-SIP1 and YFP-SIP1 (Long et al., 2005) were subcloned into pTP6 for generating pTP6-Flag-SIP1 and pTP6-YFP-SIP1. Expression vectors were transfected into hESCs using Lipofectamine 2000 according to Vallier et al. (2004b), pTP6-hrGFP (human recombinant green fluorescence protein) was used as a transfection control.

**Quantitative Polymerase Chain Reaction**

Total RNA was extracted with RNeasy Mini kit with a DNase digestion step per the manufacturer’s instructions (QIAGEN). One microgram of RNA was reverse-transcribed with Moloney Murine Leukaemia Virus Reverse Transcriptase ( Gibco, 28025-013). Quantitative polymerase chain reaction (QPCR) mixtures were prepared as described (Quantace Sensimix dT, QT6 T3), then denatured at 94°C for 5 min and cycled at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; this was followed by final extension at 72°C for 10 min after completion of 40 cycles. QPCR reactions were performed with Stratagene Mx3005P in duplicate and normalized to Porphobiligen Deaminase (PBGD) in the same run. Primer sequences can be found in Tables S2 and S3. Error bars on all QPCR graphs represent standard deviation from three independent replicates. Where student’s t tests (two-tailed assuming nonequal variance) were performed, asterisks indicate that all three experimental lines showed significantly different gene expression (p < 0.05) compared to control line.

**Immunostaining and Flow Cytometry**

Detailed immunostaining methods are described in Vallier et al. (2005). Antibodies used for immunostaining were as follows: Neurofilament, 1:100 (Sigma N2912); SOX17, 1:100 (R&D Systems, AF1924); Cy3-donkey-anti-mouse IgG, 1:400 (Chemicon, AF192C); and Texas red-donkey-anti-goat IgG, 1:400 (Jackson Lab, 705-75-147). Antibodies used for flow cytometry were as follows: NCAM, 1:200 (BD Pharmingen, 557699); and mouse IgG isotype control (BD Pharmingen, 557549).

**Luciferase Assays**

DNA plasmids containing firefly luciferase reporter constructs (SBE4-luc), CMV-Renilla (Promega), and expression vectors were cotransfected into hESCs with Lipofectamine 2000 (Invitrogen). The ratio between luciferase reporters and CMV-Renilla was 10:1. Medium was changed after 18 hr and cells were harvested 48 hr later for luciferase assay. Luciferase activity was measured with the dual luciferase assay kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for cell density and transfection efficiency.

**Knockdown and Cell Sorting**

Transient knockdown of OCT4 and SOX2 were carried out with pSilencer-eGFP, Successfully transfected cells, coexpressing eGFP, were selected by fluorescence-activated cell sorting (FACS) after 48 hr of growth posttransfection. Stable knockdown of SIP1, SMAD2, and NANOG were carried out with pLKO.1-shRNA vector (Sigma) by Lipofectamine transfection. See Table S4 for details.

**Chromatin Immunoprecipitation**

Detailed chromatin immunoprecipitation methods are described in Vallier et al. (2009a). Sixteen micrograms of the following antibodies were used for immunoprecipitation: SMAD2/3 (Santa Cruz, sc-8332x), NANOG (R&D, AF1997), SOX2 (R&D, AF2018), and OCT4 (R&D, AF1759). Purified DNA was used as template for QPCR to amplify the proximal promoter of SIP1. Primer sequences can be found in Table S5.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, five tables, and one movie and can be found with this article online at doi:10.1016/j.stem.2009.11.015.
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REFERENCES


