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Regulation of developmental competence and commitment towards the definitive endoderm lineage in human embryonic stem cells

Kate Lynn Jaremko, Yusuke Marikawa*

Institute for Biogenesis Research, University of Hawaii John A. Burns School of Medicine, Hawaii, USA

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Abstract Human embryonic stem cells (hESCs) can self-renew and become all three germ layers. Nodal/Activin signaling specifies developmental status in hESCs: moderate Nodal/Activin signaling maintains pluripotency, while enhancement and inhibition promote definitive endoderm (DE) and neuroectoderm (NE) development, respectively. However, how modulation of Nodal/Activin signaling influences developmental competence and commitment toward specific lineages is still unclear. Here, we showed that enhancement of Nodal/Activin signaling for 4 days was necessary and sufficient to upregulate DE markers, while it diminished the upregulation of NE markers by inhibition of Nodal/Activin signaling. This suggests that after 4 days of enhanced Nodal/Activin signaling, hESCs are committed to the DE lineage and have lost competence toward the NE lineage. In contrast, inhibition of Nodal/Activin signaling using LY364947 for 2 days was sufficient to impair competence toward the DE lineage, although cells were still able to activate *LEFTY1* and *NODAL*, direct targets of Nodal/Activin signaling. Expression analyses indicated that the levels of pluripotency regulators *NANOG* and *POU5F1* were significantly diminished by 2 days of LY364947 treatment, although the expression of *NANOG*, but not *POU5F1*, was restored immediately upon Activin A treatment. Thus, downregulation of *POU5F1* coincided with the abrogation of DE competence caused by inhibition of Nodal/Activin signaling.
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Introduction

Pluripotency is defined as the ability to give rise to derivatives of all three germ layers, namely ectoderm, mesoderm, and endoderm. Several types of pluripotent stem cell lines have been established, such as embryonic stem cells (ESCs), epiblast stem cells (EpiSCs), and embryonal germ cells (EGCs). These pluripotent stem cell lines are derived from specific groups of pluripotent embryonic tissues. ESCs, EpiSCs, and EGCs originate from the inner cell mass (ICM) of the blastocyst, the epiblast of the post-implantation embryo, and the primordial germ cell, respectively (Pera et al., 2000; Brons et al., 2007; Yu and Thomson, 2008). Pluripotent stem cells can be maintained and propagated in vitro for an extended period while retaining various developmental properties of the original pluripotent embryonic tissues. In addition, non-pluripotent somatic cells can also be reprogrammed into pluripotent stem cells, or induced pluripotent stem cells (iPSCs), by introduction of defined factors, which exhibit developmental ability similar to

* Corresponding author at: 651 Ilalo Street, Biomedical Sciences Building 163A, Honolulu, HI 96813, USA. Fax: +1 808 692 1962.
E-mail address: marikawa@hawaii.edu (Y. Marikawa).

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ESCs or EpiSCs (Takahashi and Yamanaka, 2006; Lowry and Plath, 2008; Park et al., 2008; Yu et al., 2007).

Pluripotent stem cells can differentiate into various types of cells in response to distinct environments. Differentiation can take place in semi-in vivo conditions, such as chimerization with a developing embryo or transplantation into an adult body, the latter of which typically gives rise to teratoma, containing differentiated tissues of all three germ layers. Differentiation of pluripotent stem cells can also be induced in vitro by culturing in specific media. Directed differentiation of pluripotent stem cells, particularly of human ESCs (hESCs), into desired cell types in vitro is of medical importance, because differentiated cells can be used for cell replacement therapy, drug screening, and investigations into disease pathogenesis (Zhu et al., 2011; Gearhart, 1998). Thus, many studies have been focusing on establishing culture conditions and protocols that enable differentiation of specific cell types from hESCs.

During the course of directed differentiation, pluripotent stem cells become committed to adopting specific developmental pathways and lose the competence to take on other pathways. Acquisition of commitment and loss of competence are the fundamental aspects of embryogenesis. Namely, the fertilized egg is a single cell that is competent to give rise to all cell types in the body, but after a series of cell divisions, individual cells receive distinct developmental instructions, such as inductive signals, and gradually become committed towards specific developmental lineages while the competence to undertake the other lineages is lost. The concept of developmental commitment and competence has served as the foundation of classic as well as modern Developmental Biology to describe the process of animal development (Waddington, 1940; Wolpert and Tickle, 2010; Gilbert, 2010). Studies utilizing pluripotent stem cells offer invaluable opportunities to elucidate in depth the molecular mechanisms of developmental commitment and competence. This is especially important for studying mechanisms of human development, as the manipulation of early human tissues is not as accessible as other animal models. An understanding of how these events are regulated is also significant from the viewpoint of medical applications of pluripotent stem cells, especially for cell replacement therapy. One of the concerns associated with transplantation of cells that are derived from pluripotent stem cells into patients is that transplants may contain cells that are not fully committed to desired cell types and are still competent to give rise to other unwanted cell types. Any uncommitted pluripotent stem cells that are included in the transplants are likely to give rise to teratoma in the patients (Blum and Benvenisty, 2008; Fong et al., 2010; Ben-David and Benvenisty, 2011).

Nodal/Activin signaling is an evolutionarily conserved signal transduction pathway, and plays critical roles in the maintenance and differentiation of hESCs. Nodal and Activin (Activin A and Activin B [Thompson et al., 2004]) are members of the transforming growth factor beta (TGFβ) superfamily, and bind to heteromeric complexes between type I (Alk4, Alk5, and Alk7) and type II (ActRIIB) activin receptors, which in turn activate Smad2/3 to regulate the expressions of various target genes (Schier, 2003; Valdimarsdottir and Mummery, 2005; Payne et al., 2011). Moderate activation of Nodal/Activin signaling is essential for the maintenance of pluripotency in hESCs (Beattie et al., 2005; James et al., 2005; Vallier et al., 2005). A small amount (e.g.,10 ng/mL) of Activin A or TGFβ1 is an essential ingredient in the chemically defined culture media that are formulated for routine maintenance of hESCs under feeder-free conditions (Amit et al., 2004; Brons et al., 2007; Ludwig et al., 2006). Inhibition of Nodal/Activin signaling with small molecule inhibitors of Alk4, Alk5, and Alk7, such as SB431542 (Callahan et al., 2002; Inman et al., 2002) or LY364947 (Sawyer et al., 2003; Peng et al., 2005), downregulates the expressions of pluripotency regulators NANOG and OCT4 (POU5F1) in hESCs (James et al., 2005). Concomitantly, inhibition of Nodal/Activin signaling directs hESCs toward the neuroectoderm (NE) lineage, and upregulates the expression of NE marker genes, such as *PAX6*, *GBX2*, and *NFH* (Wassarman et al., 1997; Camus et al., 2006; Smith et al., 2008; Patani et al., 2009; Chng et al., 2010; Hu et al., 2010; Zhou et al., 2010). NE differentiation can be promoted by a pharmacological inhibitor of Nodal/Activin signaling, such as SB431542, or by ectopic expression of Nodal antagonists, such as Lefty2 and Cer-S (Smith et al., 2008; Patani et al., 2009), whereas it is inhibited by overexpression of NODAL (Vallier et al., 2004). On the other hand, a high level (e.g., 50 ng/mL) of Activin A promotes the differentiation of hESCs into the definitive endoderm (DE) lineage, which is characterized by the expression of *SOX17*, *CXCR4*, and *FOXA2* (D'Amour et al., 2005; Borowiak et al., 2009; Mayhew and Wells, 2010; Sulzbacher et al., 2009; Wang et al., 2011). Thus, the level of Nodal/Activin signaling imposes critical impacts on hESCs, and regulates three distinct developmental states: a low level or absence of signaling for the NE lineage, an intermediate level for the maintenance of pluripotency, and a high level for the DE lineage. However, the mechanisms of how and when each developmental state is determined by a specific level of Nodal/Activin signaling are still not well-understood. For example, questions like how long hESCs need to be exposed to a high level of Activin A to become committed toward the DE lineage, and at what point hESCs lose competence to undertake the DE lineage when Nodal/Activin signaling is inhibited, have not been addressed.

In the present study, we conducted a series of experiments to gain insight into the regulation of competence and commitment towards the DE lineage in hESCs. We found that the duration of exposure to a high level of Activin A had a cumulative effect on the activation of the DE marker genes up to 6 days of culture. When hESCs were exposed to Activin A for 4 days, the inhibition of Nodal/Activin signaling was no longer able to downregulate the expression of DE markers, suggesting that hESCs had already become committed to the DE lineage. We also showed that the commitment toward DE was associated with the loss of competence toward NE. To investigate the nature of the competence toward DE, we tested 5 different pharmacological inhibitors of Nodal/Activin signaling. While all of them strongly inhibited Nodal/Activin signaling, only LY364947 was fully reversible, with the property to be washed way without impairing activation of Nodal/Activin signaling in response to Activin A treatment. By taking advantage of the reversible nature of LY364947, we determined that 2 days, but not 1 day, of Nodal/Activin signaling inhibition was detrimental to the DE competence. Finally, we showed that the downregulation of *POU5F1* coincided with the loss of DE competence in response to Nodal/Activin signaling inhibition, raising the possibility that this key pluripotency regulator plays a critical role in maintaining the competence toward DE in hESCs.

Results

Enhancement and inhibition of Activin signaling in hESCs promotes DE and NE, respectively

Since the first establishment of hESC lines from human blastocysts (Thomson et al., 1998), methods have been created for the induction of differentiation toward all three germ layers (ectoderm, mesoderm, and endoderm). Specifically, a high dosage of recombinant Activin A in a low-serum culture medium has been used to induce definitive endoderm (DE) (D'Amour et al., 2005; Borowiak et al., 2009), whereas the inhibition of Nodal/Activin signaling using a chemical inhibitor, such as SB431542, has been used to promote neuroectoderm (NE) formation in hESCs (Smith et al., 2008; Patani et al., 2009). In the present study, we first examined whether activation or inhibition of Nodal/Activin signaling is sufficient to promote the DE or NE lineage in the same basal medium. Undifferentiated hESCs were treated for 6 days with a high dose of Activin A (50 ng/mL) or SB431542 (10 μM) added to the basal medium. When cultured with Activin A for 6 days, the expression of the DE markers, *SOX17*, *FOXA2*, and *CXCR4*, were significantly activated, as compared to culture in the basal medium only. On the other hand, when treated with SB431542 in the same basal medium for 6 days, there was a significant increase in the expression of the NE markers, *PAX6*, *GBX2*, and *NFH* (Figure 1A). In mouse embryos, *Gbx2* is expressed in the primitive streak, from which DE emerges (Wassarman et al., 1997; Waters et al., 2003). In hESC differentiation, however, *GBX2* was expressed only in SB431542-treated but not in Activin A-treated cells (FIG. S1). This suggests that *GBX2* is mainly activated in the NE lineage but not in the DE lineage, consistent with previous reports (Chng et al., 2010; Camus et al., 2006; Hu et al., 2010; Patani et al., 2009; Wassarman et al., 1997). The basal medium used in the present study included 1% of fetal calf serum (Materials and methods). Fetal calf serum has been shown to contain BMP4 activity, which may impact the course of hESC differentiation (Xu et al., 2002; Ying et al., 2003; Kodaira et al., 2006). However, 1% fetal calf serum in the basal medium did not activate *ID1*, *ID2*, or *ID3* (FIG. S2), all of which are known target genes of BMP4 signaling in hESCs (Miyazono and Miyazawa, 2002; López-Rovira et al., 2002). This suggests that BMP4 activity in this serum-containing basal medium is negligible. Thus, manipulation of Nodal/Activin signaling level alone in the same basal medium was sufficient to drive the differentiation toward the DE or NE lineage.

To further investigate the mode of cell differentiation toward DE or NE, time courses of DE and NE marker expression during 8 days of culture were examined. Transcript levels of the DE markers *SOX17* and *CXCR4* peaked at day 6, while *FOXA2* peaked at day 4 of culture with high Activin A (Figure 1B). We further examined expression of SOX17 protein by immunocytochemistry and FACS analysis to assess the extent of DE induction under this culture condition. Distinct nuclear staining was detected in approximately 70–80% of cells at day 6 of high Activin A treatment (Figure 1C). The number of SOX17-positive cells gradually increased during 6 days of culture with high Activin A in a manner similar to the profile of *SOX17* transcript level (FIG. S3). These results suggest that the majority of the cell population was induced toward DE within 6 days of Activin A treatment. Expression time course for NE induction by SB431542 treatment was more variable among the marker genes examined. *PAX6* continued to rise throughout 8 days of culture in the presence of SB431542 (Figure 1B). In contrast, *GBX2* expression peaked at day 6, which is similar to the expression time course of the DE genes in response to Activin A treatment. The transcript levels of *NFH* rose through days 2 and 4, slightly dropped on day 6, and rose again by day 8 (Figure 1B). PAX6 protein was detected in approximately 70–80% of cells at day 6 of SB431542 treatment, indicating that NE induction was efficient under this culture condition (Figure 1D). Thus, treatment of hESCs with high Activin A and SB431542 were able to induce the expressions of DE and NE markers, respectively, with distinct temporal profiles.

A total of 4 days of enhanced Nodal/Activin signaling is required for induction of DE

While the expression of DE genes was robustly elevated after 6 days of culture with high Activin A, it is not clear whether continuous presence of Activin A throughout the culture period is required to upregulate the DE genes. Also unclear is whether cells need to be exposed to high Activin A at particular time points during 6 days of culture. To address these questions, we designed 6 day-culture schemes with a total of 2 days or 4 days of high Activin A treatment at different time points, as outlined in the diagram (Figure 2A). hESCs were cultured in the basal medium alone (as denoted in white) or in the basal medium containing high Activin A (as denoted in red). Significant upregulation of *SOX17* and *FOXA2* ($p<0.05$) was observed when cells were treated with high Activin A for a total of 4 days, regardless of at which time points Activin A was present in the medium during 6 days of culture (Figure 2A). Namely, similar expression levels of the DE genes were achieved by either initial 4 days of Activin A followed by 2 days of no Activin A (4dAA-2dBM), or initial 2 days of no Activin A followed by 4 days of Activin A (2dBM-4dAA), or two rounds of 2 day Activin A treatment separated by 2 days of no Activin A (2dAA-2dBM-2dAA). A total of 4 days of Activin A treatment also elevated the expression level of *CXCR4* relative to that in the basal medium (6dBM), although it was not statistically significant ($p=0.057$) for 2dAA-2dBM-2dAA. In contrast, there was essentially no significant upregulation of all DE genes by 2 days of Activin A treatment (2dAA-4dBM, 4dBM-2dAA, or 2dBM-2dAA-2dBM) as compared to 6 days of basal medium (6dBM), except for a slight but significant ($p<0.01$) upregulation of *CXCR4* by 2 days of Activin A treatment after day 4 (4dBM-2dAA) (Figure 2A). These results overall suggest that a total of 4 days of high Activin A treatment was sufficient and necessary for upregulation of DE markers.

Enhancement of Nodal/Activin signaling for 4 days promotes commitment toward DE and diminishes competence toward NE

As shown above, 4 days of high Activin A treatment was sufficient to upregulate the DE genes even when Activin A was not added in the culture medium for 2 days. However, it is possible that endogenous Nodal/Activin signaling is still active at a low level even in the absence of exogenous

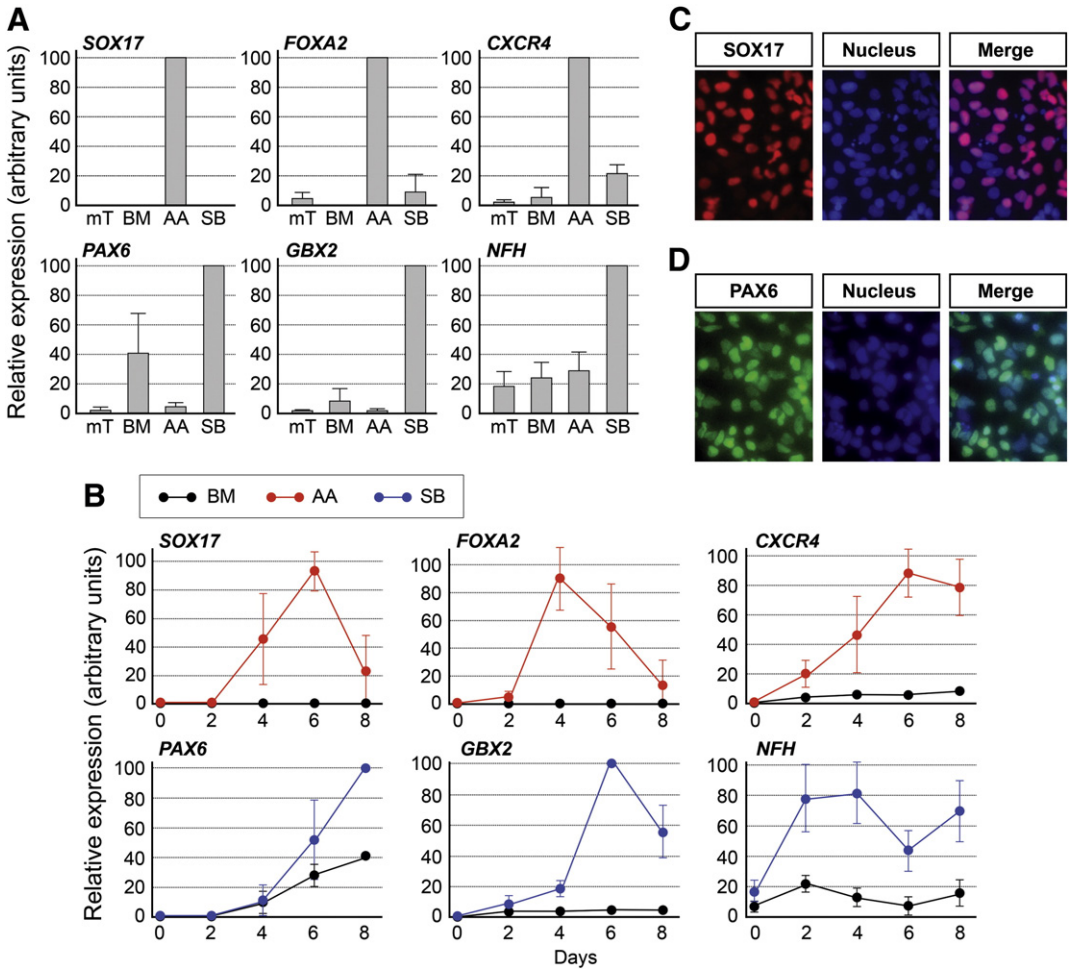


Figure 1 Enhancement and inhibition of Activin signaling in hESCs promotes DE and NE, respectively. (A) qRT-PCR analysis showing the expression levels of the DE markers (*SOX17*, *FOXA2* and *CXCR4*) and NE markers (*PAX6*, *GBX2* and *NFH*) after 6 days of culture in 4 different media: mT = mTeSR, BM = the basal medium, AA = Activin A (50 ng/mL) in the basal medium, and SB = SB431542 (10 μM) in the basal medium. For the DE and NE markers, the expression levels in AA and SB are used for standardization, respectively (i.e., relative expression = 100). (B) qRT-PCR analysis showing temporal expression profiles of the DE and NE markers over 8 days of culture in different media. In each experiment (AA compared with BM for the DE markers, and SB compared with BM for the NE markers), the highest expression level is used for standardization. (C) Immunocytochemistry showing expression of DE marker SOX17 in hESCs cultured for 6 days in the basal medium containing high Activin A. (D) Immunocytochemistry showing expression of NE marker PAX6 in hESCs cultured for 6 days in the basal medium containing SB431542. All nuclei are visualized by staining with DAPI in (C) and (D).

Activin A. Thus, to further delineate the role of Nodal/Activin signaling in developmental commitment and competence toward the DE lineage, another set of experiments were designed, in which the endogenous Nodal/Activin signaling was forcefully inhibited with SB431542 (as denoted in blue) before or after Activin A treatment (Figure 2B). Basically, we conducted the same 2 or 4 days total of high Activin A treatment during 6 days of culture, with the addition of SB431542 on days treated with the basal medium alone in the previous scheme (compare Figs. 2A and B). As demonstrated by robust upregulation of *SOX17*, *FOXA2* and *CXCR4*, the initial 4 days of enhanced Nodal/Activin signaling was sufficient to promote the DE lineage even when Nodal/Activin signaling was inhibited afterward for 2 days (4dAA-2dSB). This suggests that hESCs are already committed to the DE lineage after 4 days of Nodal/Activin signaling enhancement. However, when cells were treated with SB431542 prior to a total of 4 days of high Activin A treatment (i.e., 2dSB-4dAA or 2dAA-2dSB-2dAA), the activation of *SOX17* and *FOXA2* expression was essentially abrogated. The response of *CXCR4* expression to SB431542 treatment was not as distinct as those of *SOX17* and *FOXA2*, implicating that the regulation of *CXCR4* by Nodal/Activin signaling may be different from the other two DE markers (see Discussion). Nonetheless, these results suggest that inhibition of Nodal/Activin signaling for 2 days with SB431542 compromises competence of hESCs to DE. When Nodal/Activin signaling was enhanced for 4 days initially, however, cells did not downregulate DE markers and were thus committed toward DE.

We then inquired how the competence of hESCs to give rise to NE would be affected with respect to their commitment toward the DE lineage. To address this question, we first

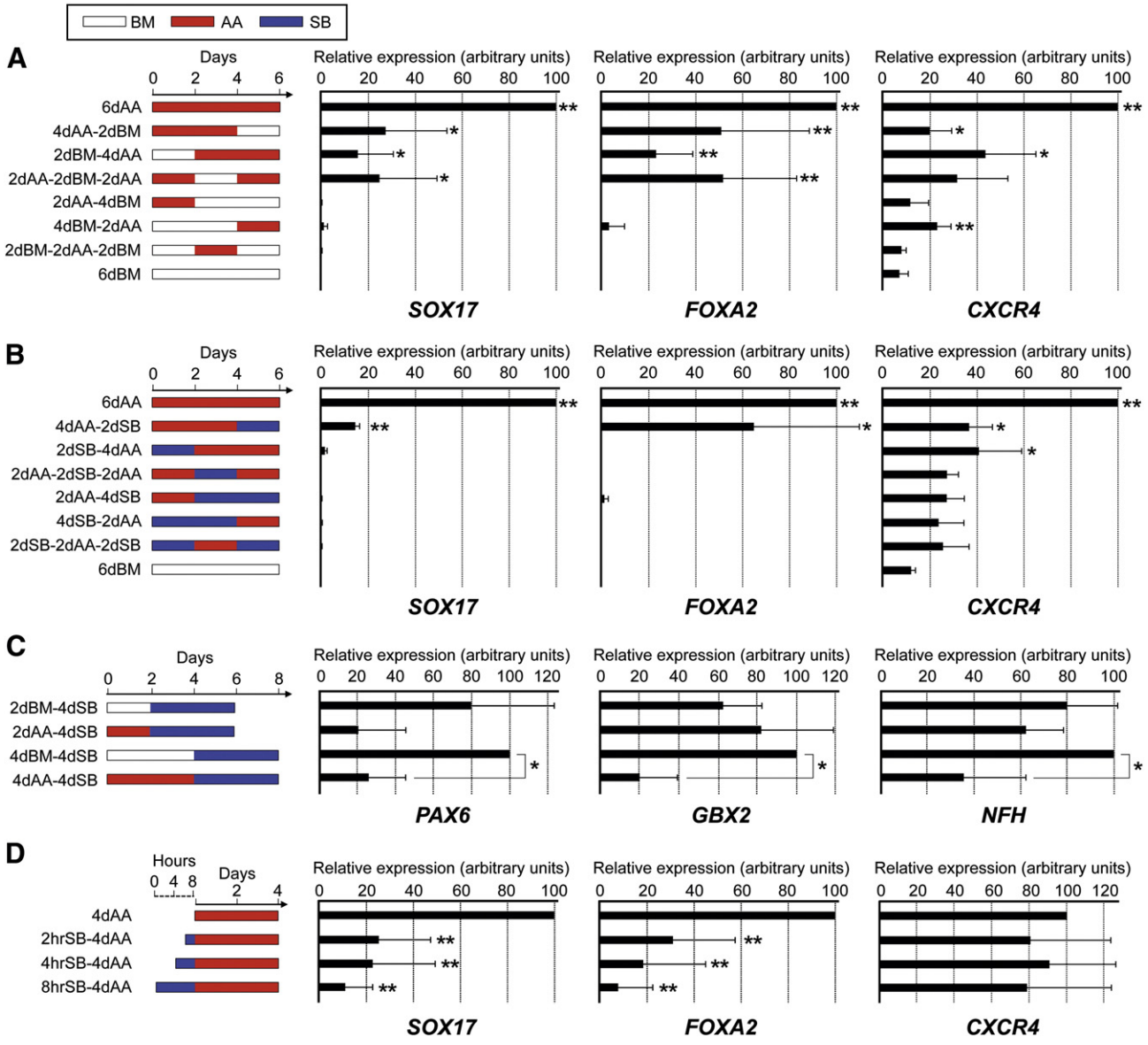


Figure 2 Four days of enhanced Nodal/Activin signaling promotes commitment toward the DE lineage and impairs competence toward the NE lineage. (A) Left: Schematic representation of the hESC culture scheme, in which cells are cultured for a total of 6 days in the basal medium containing high Activin A at different time points and durations. Right: qRT-PCR analysis showing expression levels of the DE markers, *SOX17*, *FOXA2*, and *CXCR4*. In each experiment, the level in 6dAA (6 days of culture in the presence of high Activin A) is used for standardization (i.e., relative expression = 100). The expressions that are significantly different from that in 6dBM (6 days of culture in the basal medium alone) are marked with asterisks (* for $p<0.05$ and ** for $p<0.01$). (B) Left: Schematic representation of the hESC culture scheme, in which cells are cultured for a total of 6 days in the basal medium containing high Activin A or SB431542 at different time points and durations. Right: qRT-PCR analysis showing expression levels of the DE markers. In each experiment, the level in 6dAA is used for standardization. The expressions that are significantly different from that in 6dBM are marked with asterisks (* for $p<0.05$ and ** for $p<0.01$). (C) Left: Schematic representation of the hESC culture scheme, in which cells are initially cultured for 2 days or 4 days in the presence or absence of high Activin A, and then cultured for 4 days with SB431542. Right: qRT-PCR analysis showing expression levels of the NE markers, *PAX6*, *GBX2*, and *NFH*. In each experiment, the level in 4dBM-4dSB is used for standardization. The expression levels are compared for statistical significance between the two treatments with the same total duration of culture, that is, 2dBM-4dSB versus 2dAA-4dSB, and 4dBM-4dSB versus 4dAA-4dSB. Significant differences ($p<0.05$) are marked with an asterisk. (D) Left: Schematic representation of the hESC culture scheme, in which cells are cultured with SB431542 for different durations (0, 2, 4, or 8 h), followed by 4 days of Activin A treatment. Right: qRT-PCR analysis showing expression levels of the DE markers. In each experiment, the level in 4dAA is used for standardization. The expressions that are significantly different from that in 4dAA are marked with asterisks ($p<0.01$). BM = the basal medium, AA = Activin A (50 ng/mL) in the basal medium, and SB = SB431542 (10 μM) in the basal medium.

treated hESCs with high Activin A for 2 days or 4 days, followed by 4 days of SB431542 treatment, and then examined the expression levels of the three NE markers, *PAX6*, *GBX2*, and *NFH* (Figure 2C). When hESCs were pre-treated with Activin A for 2 days (2dAA-4dSB), the expressions of the NE markers were not significantly reduced compared to the control (2dBM-4dSB), although the *PAX6* level appeared to be slightly lower (not statistically significant). This indicates that hESCs were still able to give rise to NE in response to the inhibition of Nodal/Activin signaling even when signaling was enhanced for 2 days prior to inhibition. In contrast, when pre-treated with Activin A for 4 days (4dAA-4dSB), the expression levels of all three NE markers were significantly reduced compared to the control (4dBM-4dSB) (Figure 2C). These results suggest that hESCs lose the competence toward NE when they become committed toward the DE lineage by 4 days of enhanced Nodal/Activin signaling.

The competence to give rise to DE is impaired after 2 days, but not 1 day, of Nodal/Activin signaling inhibition

The loss of DE competence by the inhibition of Nodal/Activin signaling occurred more rapidly than the loss of NE competence by the activation of Nodal/Activin signaling (i.e., the former took 2 days while the latter took 4 days). This implicates that DE competence is more fragile than NE competence. To assess how quickly DE competence is impaired, we inhibited Nodal/Activin signaling for much shorter durations, namely 2, 4, and 8 h and tested whether DE competence would be affected. After the short duration of SB431542 treatment, hESCs were cultured with high Activin A for 4 days and the expression levels of DE markers *SOX17* and *FOXA2* were examined. Surprisingly, the upregulation of these DE marker genes was significantly diminished even with just 2 h of SB431542 treatment (Figure 2D). This result implicates either that the DE competence is extremely fragile to the inhibition of Nodal/Activin signaling or that treatment with SB431542 irreversibly impaired its targets, namely ALK4, 5 and 7, and thus compromised activation of Nodal/Activin signaling even after removal of the inhibitor from the culture medium.

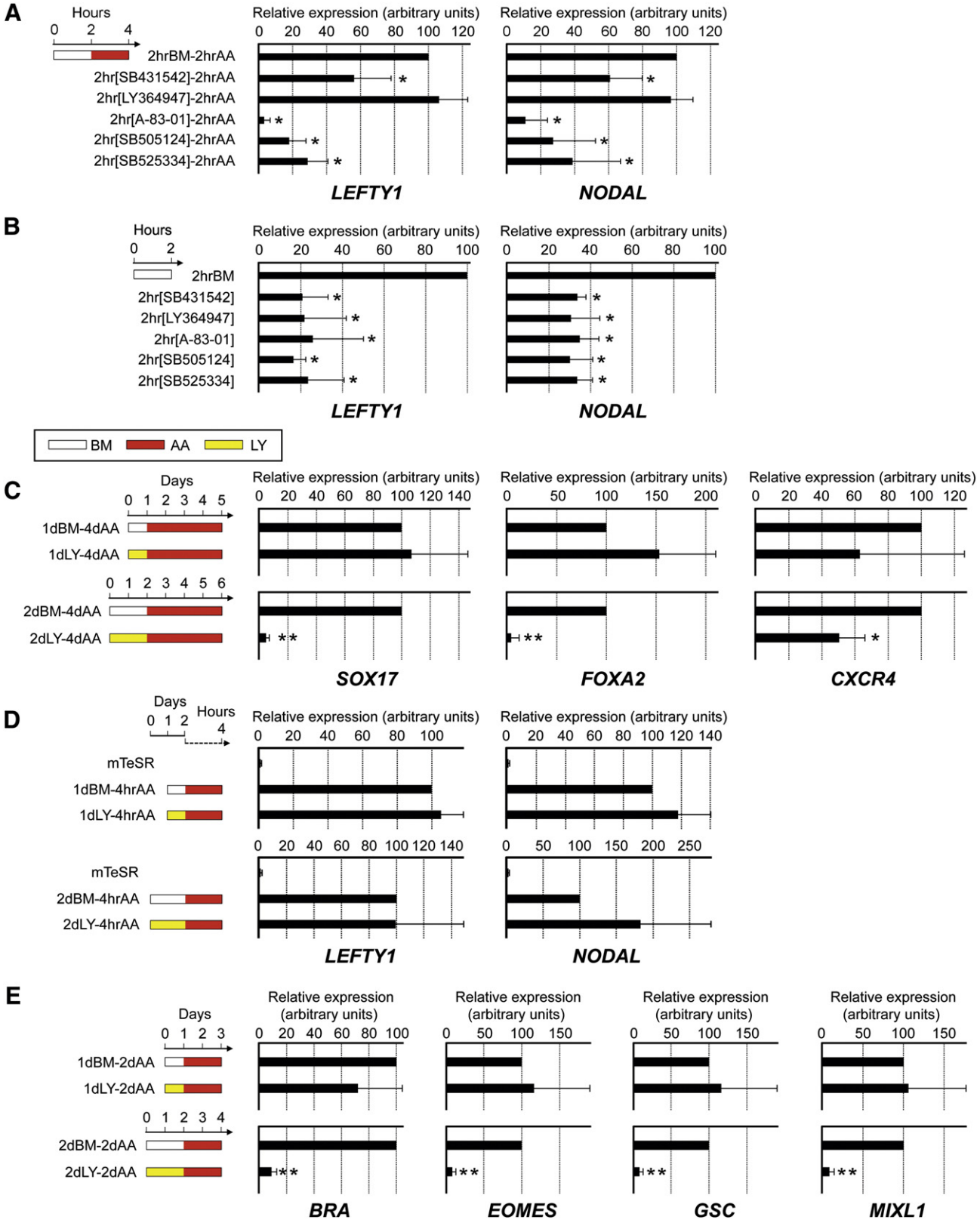
To test the latter possibility, we examined whether Activin A treatment after SB431542 can still activate the expression of *LEFTY1* and *NODAL*, known target genes of Nodal/Activin signaling in hESCs (Besser, 2004). hESCs were first cultured for 2 h in the absence or presence of SB431542 (i.e., 2hrBM or 2 hr[SB431542]), and then treated with Activin A for 2 h (i.e., 2hrAA). The levels of *LEFTY1* and *NODAL* were significantly lower in 2 hr[SB431542]-2hrAA than in 2hrBM-2hrAA (Figure 3A), indicating that Nodal/Activin signaling was not effectively activated by Activin A after SB431542 removal. The reduction in Nodal/Activin signaling was unlikely due to residual SB431542 in the culture medium, because additional washing before Activin A treatment did not fully restore the activation of *LEFTY1* and *NODAL* (FIG. S4). These results suggest that inhibition of Nodal/Activin signaling by SB431542 is not immediately reversible after its removal. This finding obscures the interpretation of the previous experiments (Figs. 2B,D), because SB431542 might have irreversibly damaged the machinery for Nodal/Activin signaling, rather than impacting the developmental competence to give rise to DE.

To address this issue, we tested several other pharmacological inhibitors of Nodal/Activin signaling that are commonly used, with a hope to identify a reversible inhibitor, of which impact on Nodal/Activin signaling can be abated after removal from the culture medium. Treatment with 10 μM of LY364947, A-83-01, SB505124, or SB525334 (Vogt et al., 2011) robustly and equally downregulated the expression of *LEFTY1* and *NODAL* within 2 h (Figure 3B), confirming that all of these compounds are potent inhibitors of Nodal/Activin signaling in hESCs. After 2 h of inhibitor treatment, hESCs were cultured with Activin A for 2 h, and examined for the activation of *LEFTY1* and *NODAL*. The expression levels of these target genes in response to Activin A treatment were significantly lower in A-83-01-treated, SB505124-treated, and SB525334-treated cells, as compared to the control (2hrBM-2hrAA) (Figure 3A). This indicates that these inhibitors also compromised the Nodal/Activin signaling machinery even after removal, similar to SB431542. In striking contrast, however, the activation of *LEFTY1* and *NODAL* in LY364947-treated cells was as robust as in the

Figure 3 Two days of Nodal/Activin signaling inhibition compromises the competence toward DE. (A) qRT-PCR analysis showing the expression levels of Nodal/Activin signaling target genes, *LEFTY1* and *NODAL*, in cells that are initially treated for 2 h with one of the five pharmacological inhibitors (SB431542, LY364947, A-83-01, SB505124, SB525334; all at 10 μM), followed by treatment with Activin A (50 ng/mL) for 2 h. The expression level in the no-inhibitor control (2hrBM-2hrAA) is used for standardization (i.e., relative expression = 100). The expressions that are significantly lower than that in 2hrBM-2hrAA are marked with asterisks ($p<0.05$). (B) qRT-PCR analysis showing the expression levels of Nodal/Activin signaling target genes in cells that are treated with one of the five pharmacological inhibitors for 2 h. The expression level in the no-inhibitor control (2hrBM) is used for standardization. The expressions that are significantly lower than that in 2hrBM are marked with asterisks ($p<0.05$). (C) Left: Schematic representation of the hESC culture scheme, in which cells are cultured with LY364947 for 1 day or 2 days, followed by 4 days of Activin A treatment. Right: qRT-PCR analysis showing expression levels of the DE markers, *SOX17*, *FOXA2*, and *CXCR4*. The level in 1dBM-4dAA or 2dBM-4dAA is used for standardization. The expressions that are significantly lower than the matching control (1dBM-4dAA or 2dBM-4dAA) are marked with asterisks (* for $p<0.05$ and ** for $p<0.01$). (D) Left: Schematic representation of the hESC culture scheme, in which cells are cultured with LY364947 for 1 day or 2 days, followed by 4 h of Activin A treatment. Right: qRT-PCR analysis showing the expression levels of Nodal/Activin signaling target genes. The level in 1dBM-4hrAA or 2dBM-4hrAA is used for standardization. (E) Left: Schematic representation of the hESC culture scheme, in which cells are cultured with LY364947 for 1 day or 2 days, followed by 2 days of Activin A treatment. Right: qRT-PCR analysis showing expression levels of the mesendoderm markers, *BRACHYURY* (*BRA*), *EOMES*, *GSC*, and *MIXL1*. The level in 1dBM-2dAA or 2dBM-2dAA is used for standardization. The expressions that are significantly lower than the matching control (1dBM-2dAA or 2dBM-2dAA) are marked with asterisks ($p<0.01$).

control (Figure 3A). This result demonstrates that LY364947 is an effective and reversible inhibitor, and does not impair the transduction of Nodal/Activin signaling after its removal. Thus, LY364947 serves as an ideal tool to address our original question about how long hESCs retain DE competence when Nodal/Activin signaling is inhibited.

In order to address this question, hESCs were treated with LY364947 for 1 day or 2 days (i.e., 1dLY or 2dLY), followed



by Activin A treatment for 4 days (i.e., 4dAA), and examined for the activation of the DE markers. The expression levels of *SOX17*, *CXCR4*, and *FOXA2* in 1dLY-4dAA were similar to those of the control, 1dBM-4dAA (Figure 3C). Thus, the DE competence was not impaired after 1 day of inhibition of Nodal/Activin signaling. In contrast, the levels of all the DE markers in 2dLY-4dAA were significantly lower than those in 2dBM-4dAA (Figure 3C). Importantly, this loss of DE competence was not due to permanent impairment of the machinery for Nodal/Activin signaling, as it was still intact even after 2 days of LY364947 treatment, as demonstrated by robust activation of *LEFTY1* and *NODAL* in response to Activin A treatment for 4 h (Figure 3D). These results suggest that hESCs lose the DE competence after 2 days, but not 1 day, of Nodal/Activin signaling inhibition and that this competence loss is not due to irreversible impairment of Nodal/Activin signaling.

To gain further insight into how hESCs lose DE competence, we examined the expression of mesendoderm genes in response to Activin A after LY364947 treatment. Mesendoderm is the common precursor of definitive endoderm and mesoderm, which arises in the primitive streak during embryogenesis (Rodaway and Patient, 2001; Tada et al., 2005). Key mesendoderm genes, such as *BRACHYURY* (*BRA*), *GSC*, and *MIXL1*, are activated in hESCs by Activin A treatment, which temporarily precedes the activation of DE genes (Chen et al., 1997; Labbé et al., 1998; D'Amour et al., 2005; Yu et al., 2011). When hESCs were cultured with LY364947 for 1 day prior to Activin A treatment for 2 days (1dLY-2dAA), the expression levels of the mesendoderm genes were as high as those of the control (1dBM-2dAA) (Figure 3E). In contrast, their expression levels were substantially diminished by 2 days of LY364947 treatment (2dLY-2dAA) compared to the control (2dBM-2dAA), indicating that hESCs were no longer capable of activating the mesendoderm genes in response to Activin A after 2 days of Nodal/Activin signaling inhibition (Figure 3E). This result raises the possibility that the loss of DE competence may be due to the lack of mesendoderm formation.

Downregulation of *POU5F1* coincides with the loss of the DE competence caused by Nodal/Activin signaling inhibition

The above studies implicate that properties of hESCs are dramatically altered with respect to the DE competence between 1 day and 2 days of Nodal/Activin signaling inhibition. To gain further insight into the molecular nature of this alteration, we examined whether the expression levels of key pluripotency regulators, namely *POU5F1*, *NANOG*, *SOX2*, *KLF4*, *PBX1* and *ZFP42* (also known as *REX1*) (Ben-Shushan et al., 1998; Loh et al., 2006; Boyer et al., 2005; Chan et al., 2009), are affected by 1 day and/or 2 days of Nodal/Activin signaling inhibition. The transcript levels of *PBX1* and *ZFP42* were largely unchanged during 2 days of LY364947 treatment, although the level of *ZPF42* was only slightly lowered by LY364947 (Figure 4A). The transcript levels of *KLF4* and *SOX2* were significantly elevated after 2 days of LY364947 treatment (Figure 4A). In contrast, LY364947 treatment downregulated the expression of *POU5F1* and *NANOG*. The transcript level of *POU5F1* was essentially unchanged after 1 day of LY364947 treatment, while it was significantly reduced after 2 days of treatment down to about 40% of the level in the untreated control (Figure 4A). The transcript level of *NANOG* was already decreased within 1 day of LY364947 treatment down to about 10% of the control (Figure 4A). Downregulation of POU5F1 and NANOG by LY364947 was also confirmed at the protein level by FACS analysis and immunocytochemistry (FIG. S5).

Reduction in the *POU5F1* and *NANOG* expression levels in response to Nodal/Activin signaling inhibition is particularly intriguing in light of several studies, which have suggested potential involvement of these two genes in mesendoderm formation in mouse ES cells, EC cells, and hESCs (Niwa et al., 2000; Chambers et al., 2007; Stefanovic et al., 2009; Yu et al., 2011; Marikawa et al., 2011; Thomson et al., 2011). Consistent with these notions, the levels of *POU5F1* and *NANOG* transcripts were not decreased during the first 6 days of DE differentiation culture, while they were distinctly downregulated during NE differentiation culture (Figure 4B). Thus, the expression of these two pluripotency regulators at a level similar to undifferentiated cells appears to be compatible with the differentiation toward mesendoderm and DE.

As shown above, the levels of *POU5F1* and *NANOG* were significantly reduced in 2 days of Nodal/Activin signaling inhibition. However, it is possible that their expression levels may be immediately restored upon activation of Nodal/Activin signaling by Activin A, which would discredit the potential link between their downregulation and the loss of DE competence. Thus, we examined the expression levels of *POU5F1* and *NANOG* in those cells that were cultured with Activin A (for 4 h) after the treatment with LY364947 (for 1 day or 2 days). The level of *NANOG* transcript was markedly elevated in response to Activin A treatment (Figure 4C), even after 2 days of pre-treatment with LY364947. In contrast, the level of *POU5F1* remained significantly lower than the basal level (in mTeSR) after 4 h of Activin A treatment (Figure 4C). Thus, reduction in the expression of *POU5F1* appears to correlate well with the loss of DE competence that is caused by 2 days of Nodal/Activin signaling inhibition.

Discussion

In this study, we investigated the mode of developmental commitment and competence toward the DE and NE lineages in hESCs in response to changes in Nodal/Activin signaling activity. Four days of enhanced Nodal/Activin signaling was sufficient to commit hESCs toward the DE lineage, as the inhibition of signaling afterward could no longer suppress the expression of DE genes. Concomitantly, competence toward the NE lineage was significantly impaired by prior treatment with high Activin A for 4 days. Thus, the commitment to DE was associated with the loss of competence to NE, suggesting that the developmental potential of hESCs is critically altered after 4 days of enhanced Nodal/Activin signaling. Interestingly, the key regulators of developmental pluripotency, *POU5F1* and *NANOG*, were still robustly expressed after 4 days of Activin A treatment, indicating that the loss of NE competence was not due to lack of expression of these pluripotency regulators. However, it is possible that DE-specific genes that

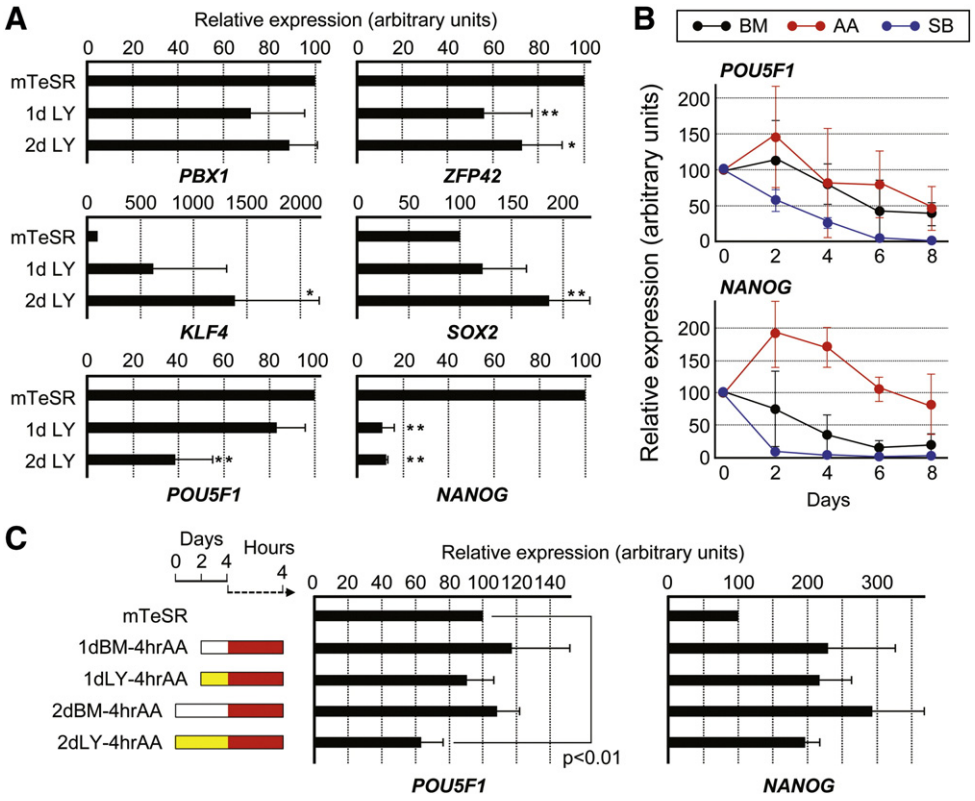


Figure 4 Downregulation of *POU5F1* is associated with the impairment in DE competence. (A) qRT-PCR analysis showing the expression levels of the pluripotency regulators, *PBX1*, *ZFP42*, *KLF4*, *SOX2*, *POU5F1*, and *NANOG*, in cells that are treated with LY364947 (10 μM) for 1 day (1dLY) or 2 days (2dLY). The level in the mTeSR control is used for standardization (i.e., relative expression = 100). The expressions that are significantly different from that in mTeSR are marked with asterisks (* for $p<0.05$ and ** for $p<0.01$). (B) qRT-PCR analysis showing temporal expression profiles of *POU5F1* and *NANOG* over 8 days of culture in different media. In each experiment, the expression level at day 0 is used for standardization. BM = the basal medium, AA = Activin A in the basal medium, and SB = SB431542 in the basal medium. (C) Left: Schematic representation of the hESC culture scheme, in which cells are cultured with LY364947 for 1 day or 2 days, followed by 4 h of Activin A treatment. Right: qRT-PCR analysis showing the expression levels of *POU5F1* and *NANOG*. The level in mTeSR is used for standardization.

were activated by 4 days of Activin A treatment, namely *SOX17*, could impose inhibitory effects on the pluripotency regulators and the expression of NE-specific genes. A study showed that POU5F1 has a dual function by working with both SOX2 and SOX17 to maintain pluripotency and to promote the mesendoderm lineage, respectively (Stefanovic et al., 2009). POU5F1 binds to SOX17, and both together occupy the promoter of endoderm genes, such as *HEX* and *SOX17* itself. Elevation of SOX17 by enhanced Nodal/Activin signaling may deprive POU5F1 from operating with SOX2, which is involved in both pluripotency maintenance as well as NE development (Avilion et al., 2003; Li et al., 2005; Zappone et al., 2000; Papanayotou et al., 2008; Archer et al., 2011; Thomson et al., 2011). Another possibility is that the expression levels or epigenetic status of genes important for NE differentiation may be altered after 4 days of enhanced Nodal/Activin signaling. For example, Nodal/Activin signaling, along with NANOG and POU5F1, repress the expression of SIP1 (Smad-interacting protein 1; also known as ZEB2), which is essential for NE differentiation (Chng et al., 2010). It is possible that the extended enhancement of Nodal/Activin signaling may result in permanent interference with the expression of NE regulators, such as SIP1, thereby inhibiting competence toward NE.

A moderate level of Nodal/Activin signaling is essential for the maintenance of pluripotency in hESCs (Beattie et al., 2005; James et al., 2005; Vallier et al., 2005). In the present study, hESCs were maintained in a chemically defined medium, mTeSR1, which contains TGFβ1 to activate Nodal/Activin signaling (Ludwig et al., 2006). Our basal medium itself was not supplemented with any growth factors to activate Nodal/Activin signaling. Nonetheless, a low level of Nodal/Activin signaling was likely to be active in the basal medium, owing to the endogenous expression of NODAL in hESCs (Vallier et al., 2004). This endogenous low level of Nodal/Activin signaling may be sufficient to sustain the expression of pluripotency regulators *POU5F1* for up to 4 days and *NANOG* for up to 2 days (Figure 4B). This is in striking contrast to the basal medium supplemented with SB431542, which downregulated *POU5F1* and *NANOG* much more rapidly (Figure 4B). However, the level of Nodal/Activin signaling in the basal medium was possibly too low to prevent the appearance of NE features, specifically the expression of *PAX6*, as it was gradually upregulated in the basal medium alone (Figure 1B). *PAX6* has been shown to act as the NE fate determinant in hESCs, as its expression is both necessary and sufficient to induce NE differentiation (Zhang et al., 2010). Regardless, the other NE markers, *GBX2* and *NFH*,

were not upregulated in the basal medium even after 8 days of culture, raising the possibility that expression of *PAX6* alone is not enough to activate these NE markers under this culture condition.

In the present study, we tested five different pharmacological agents that are commonly used as selective inhibitors of Nodal/Activin signaling, namely SB431542, LY364947, A-83-01, SB505124, and SB525334 (Inman et al., 2002; Callahan et al., 2002; Laping et al., 2002; DaCosta Byfield et al., 2004; Laping et al., 2007; Sawyer et al., 2003; Peng et al., 2005; Tojo et al., 2005; Vogt et al., 2011), and found that only LY364947 was fully reversible such that Nodal/Activin signaling was activated efficiently after its removal from the culture medium. To our knowledge, this is the first study to demonstrate this unique characteristic of LY364947. It is unclear which chemical and/or physical characteristics of LY364947 enable effective and swift reversal of its negative impact on Nodal/Activin signaling. It may simply be due to its smaller molecular weight (MW = 272.30) compared to the other inhibitors, namely SB431542 (MW = 384.39), A-83-01 (MW = 421.52), SB505124 (MW = 335.40), and SB525334 (MW = 343.42), that is, LY364947 may diffuse out of the cells much more easily than the others. Regardless, this unique property of LY364947 allowed us in the present study to investigate critically the duration of Nodal/Activin signaling inhibition that impairs the developmental competence toward DE, without sustained impairment on Nodal/Activin signaling.

Compared to *SOX17* and *FOXA2*, the response of *CXCR4* to Nodal/Activin signaling was not substantially impaired by the inhibition of Nodal/Activin signaling (Figs. 2B,D). This is interesting in light of a recent study that identified conserved triple SOX17-binding sites within the *CXCR4* proximal enhancer, which implicates that the expression of *CXCR4* is under the control of SOX17 (Katoh and Katoh, 2010). Nonetheless, *CXCR4* was still expressed when the expression of *SOX17* was significantly downregulated after the inhibition of Nodal/Activin signaling. It is possible that even with reduced *SOX17* expression, *CXCR4* expression was maintained by POU5F1 as well as Activin A, because the *CXCR4* enhancer also contains a POU-binding site and SMAD-binding sites (Katoh and Katoh, 2010).

We only used inhibition of Nodal/Activin signaling to induce NE in this study, because the main purpose of the study was to investigate specifically how Nodal/Activin signaling controls developmental commitment and competence toward DE and NE. Several studies have shown that the inhibition of Nodal/Activin signaling alone is indeed sufficient to induce NE lineages in hESCs (Smith et al., 2008; Patani et al., 2009). However, regulation of other signaling pathways is also involved in the promotion of NE development in hESCs, namely the activation of FGF signaling and the inhibition of BMP signaling (Chambers et al., 2009; Chng et al., 2010). Additionally, enhancement of other pathways, such as Wnt signaling can promote differentiation toward the DE lineage (Payne et al., 2011; Mayhew and Wells, 2010; Bone et al., 2011). Future studies are essential to determine how these other signaling pathways can impact the commitment and competence toward DE and NE. For example, does the activation of FGF signaling or inhibition of BMP signaling impair the competence toward DE? Also, does the activation of Wnt signaling impair competence toward NE? These studies should shed light on the molecular nature of the machinery that is responsible for the maintenance of DE and NE competence.

A recent study shows that SIP1 controls cell-fate decisions between the NE and mesendoderm lineages in hESCs (Chng et al., 2010). SIP1 antagonizes TGFβ family signaling via direct interaction with MH2 domains of SMAD1/2/3. The expression of SIP1 is inhibited by the pluripotency regulators, POU5F1 and NANOG, which suppresses NE differentiation. On the other hand, SIP1 expression is activated by SOX2, which limits the mesendoderm-inducing effects of Nodal/Activin signaling. Inhibition of Nodal/Activin signaling results in the upregulation of SIP1, which further interferes with this signaling through positive-feedback to promote NE lineage development (Chng et al., 2010). In addition, the repression of *SIP1* expression by POU5F1 and NANOG is relieved when these pluripotency regulators are downregulated by the inhibition of Nodal/Activin signaling. Is the loss of DE competence by LY364947 treatment, as shown in the present study, due to the inhibition of Nodal/Activin signaling by SIP1? This is unlikely the case, because even after LY364947 treatment, hESCs were still able to activate *LEFTY1* and *NODAL*, the downstream target genes of Nodal/Activin signaling, in response to Activin A (Figure 3D). Therefore, the cause of DE competence loss cannot be attributed to impaired transduction of Nodal/Activin signaling.

Among six pluripotency regulators examined, only *NANOG* and *POU5F1* were downregulated by 2 days of LY364947 treatment, which impaired DE competence. *NANOG* is a direct target of Nodal/Activin signaling, and the maintenance of its expression requires active Nodal/Activin signaling (Xu et al., 2008; Chambers et al., 2007; Vallier et al., 2009; Yu et al., 2011). Furthermore, *NANOG* is essential to maintain DE competence in hESCs, because the knockdown of *NANOG* expression by RNAi compromises the ability of hESCs to become DE in response to enhanced Nodal/Activin signaling (Vallier et al., 2009). Nonetheless, the loss of the DE competence by LY364947 treatment is unlikely caused by downregulation of *NANOG* for the following two reasons. First, either 1 day or 2 days of LY364947 treatment caused substantial downregulation of *NANOG*, while only 2 days of treatment abrogated DE competence. Second, the expression of *NANOG* was almost immediately restored by Activin A treatment even after 2 days of LY364947 treatment. Thus, downregulation of *NANOG* does not always correlate with the loss of DE competence. On the other hand, however, the downregulation of *POU5F1* correlates with the abrogation of DE competence. Specifically, both the level of *POU5F1* and the DE competence were significantly reduced only after 2 days, but not 1 day, of LY364947 treatment. In addition, the expression of *POU5F1* remained low after 2 days of LY364947 treatment even when Nodal/Activin signaling was activated afterward with Activin A. Repression of *POU5F1* in human and mouse ESCs results in differentiation to trophectoderm (Nichols et al., 1998; Matin et al., 2004; Zaehres et al., 2005; Babaie et al., 2007), while overexpression of POU5F1 causes expression of mesendoderm genes, including *Sox17*, *Hex*, and *T* (Niwa et al., 2000; Stefanovic et al., 2009). Recent studies with mouse ESCs and P19 embryonal carcinoma cells suggested that *Pou5f1* is also required for mesendoderm differentiation, as knockdown of *Pou5f1* impaired the upregulation of Brachyury in response to Wnt/β-catenin signaling activation (Thomson et al., 2011; Marikawa et al., 2011). Thus, downregulation of *POU5F1* may be contributing to the loss of DE competence, although other factors may also be involved. Future studies should be designed to identify molecular alterations at the transcriptional,

protein, and epigenetic levels that occur in response to 2 days, but not 1 day, of Nodal/Activin signaling inhibition, which lead to a loss of competence to the DE lineage.

Materials and methods

hESC culture

hESCs (H9; WiCell Research Institute, Madison, WI) were maintained on plates coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) in the serum-free, defined media, mTeSR1 (Stemcell Technologies, Vancouver, BC), which was changed daily. For routine passaging (every 3–4 days), cells were harvested by incubating with Dispase (1 mg/mL; Invitrogen, Carlsbad, CA) at 37 °C for approximately 5 min and 1:4 to 1:6 portion was plated into 6-well plates pre-coated with Matrigel. Because chromosome abnormalities have often been observed for late passages (p80–p115) in H9 cells (Smith et al., 2008), only earlier passages (p40–p60) were used for the present study.

hESC differentiation

H9 cells were plated in 4-well plates pre-coated with Matrigel, and cultured for 3–4 days in mTeSR1 until colonies grew to 50–70% confluency. For the differentiation protocols, medium was replaced with the basal medium (DMEM-F12, 1% fetal calf serum, 1× non-essential amino acids [Sigma-Aldrich, St. Louis, MO], and 1×2-mercaptoethanol [Millipore, Billerica, MA]). For differentiation to definitive endoderm (DE), medium was replaced with the basal medium supplemented with 50 ng/mL of recombinant Activin A (R&D Systems, Minneapolis, MN). For differentiation to neuroectoderm (NE), cells were placed in the basal medium with 10 μM SB431542 (EMD Biosciences, San Diego, CA). For both differentiation protocols, media were changed every 2 days unless otherwise noted.

RNA extraction and quantitative polymerase chain reaction

After maintenance or differentiation culture, cells were lysed with Trizol Reagent (Sigma-Aldrich), and total RNA was isolated according to the manufacturer's instructions. cDNA was synthesized from total RNA, using oligo dT(18) primer and M-MLV Reverse Transcriptase (Promega, Madison, WI). Real-time PCR was performed using iCycler Thermal Cycler with MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). cDNA was amplified using iQ SYBR Green Supermix (Bio-Rad) with the following conditions: the initial denaturation at 94 °C (5 min) followed by up to 45 cycles of 94 °C (15 s), 60 °C (20 s), and 72 °C (40 s). The primer sequences for reverse transcription-polymerase chain reaction (RT-PCR) are shown in Supporting Information. GAPDH levels were used to normalize the expression levels of all other genes. All experiments were conducted using at least 3 independent sets of samples (biological replicates), and data are presented as average ± standard deviation, and statistical comparisons were performed using Student's *t*-test.

Immunocytochemistry

Cells were fixed for 30 min in 4% paraformaldehyde (PFA) in PBS (phosphate-buffered saline) at room temperature and permeabilized with 0.5% Triton-X100 in PBS for 15 min at room temperature. After blocking in 5% bovine serum albumin (BSA) in PBS, cells were incubated overnight with anti-SOX17 (AF1924; R&D Systems), anti-PAX6 (PRB-278P; Covance Inc., Princeton, NJ), anti-OCT4 (C-10; Santa Cruz), or anti-NANOG (AF1997; R&D Systems) primary antibody in 0.1% Tween-20 in PBS (PBSw) at 4 °C. Cells were washed and incubated with Alexa488-conjugated or Alexa546-conjugated secondary antibodies (Invitrogen) in PBSw for 2 h at room temperature. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI) and examined with an Axiovert200 fluorescence microscope (Carl Zeiss).

Fluorescence activated cell sorting (FACS) analysis

Cells were dissociated to single cells in 0.25% Trypsin-EDTA for 10 min at 37 °C, fixed in 4% PFA in PBS for 20 min at room temperature, and permeabilized with 0.5% Triton-X100 in PBS for 20 min at room temperature. After blocking in 5% BSA in PBS, cells were incubated overnight with either anti-SOX17, anti-OCT4, or anti-NANOG primary antibody in PBSw at 4 °C. Cells were washed and incubated with Alexa488-conjugated secondary antibodies (Invitrogen) in PBSw for 3 h at room temperature. Cells were then washed, sorted with a FACSAria III, and analyzed using the FACSDiva software (BD Biosciences).

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Author contributions

K.L.J. and Y.M.: conception and design; Y.M.: financial support; K.L.J.: collection and assembly of data; K.L.J. and Y.M.: data analysis and interpretation; K.L.J. and Y.M.: manuscript writing; K.L.J. and Y.M.: final approval of manuscript.

Conflict of interest

The authors indicate no potential conflict of interest.

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