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REGULAR ARTICLE

Role of Sonic hedgehog signaling and the expression of its components in human embryonic stem cells

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Abstract Human embryonic stem cells (hESC) are characterized by their ability to self-renew and differentiate into all cell types of the body, making them a valuable resource for regenerative medicine. Yet, the molecular mechanisms by which hESC retain their capacity for self-renewal and differentiation remain unclear. The Hedgehog signaling pathway plays a pivotal role in organogenesis and differentiation during development, and is also involved in the proliferation and cell-fate specification of neural stem cells and neural crest stem cells. As there has been no detailed study of the Sonic hedgehog (SHH) signaling pathway in hESC, this study examines the expression and functional role of SHH during hESC self-renewal and differentiation. Here, we show the gene and protein expression of key components of the SHH signaling pathway in hESC and differentiated embryoid bodies. Despite the presence of functioning pathway components, SHH plays a minimal role in maintaining pluripotency and regulating proliferation of undifferentiated hESC. However, during differentiation with retinoic acid, a GLI-responsive luciferase assay and target genes *PTCH1* and *GLI1* expression reveal that the SHH signaling pathway is highly activated. Besides, addition of exogenous SHH to hESC differentiated as embryoid bodies increases the expression of neuroectodermal markers Nestin, *SOX1*, *MAP2*, *MSI1*, and *MSX1*, suggesting that SHH signaling is important during hESC differentiation toward the neuroectodermal lineage. Our findings provide a new insight in understanding the SHH signaling in hESC and the further development of hESC differentiation for regenerative medicine.

Introduction

Human embryonic stem cells (hESC) are pluripotent cells capable of self-renewal and differentiation into lineages representative of all three primary germ layers (Thomson et al., 1998). These unique properties impart hESC the potential to be used in cell replacement therapy. It is essential that the mechanisms governing self-renewal and pluripotency be understood before they can be used in regenerative medicine. The developmentally important Fibroblast Growth Factor (FGF) and Activin/Nodal signaling pathways have been identified as fundamental pathways governing hESC self-renewal and pluripotency (Vallier et al., 2005). However, another important signaling pathway in early embryology is the Hedgehog (HH) pathway whose function and mechanism in hESC is less well understood.

HH signaling is a highly conserved pathway that plays a pivotal role in cell proliferation and differentiation during development. It mediates myriad embryonic processes such as cell fate specification in the central nervous system, and limb patterning and development of the bone, cartilage,

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lung, gut, and left right axis (Hooper and Scott, 2005; Ingham and McMahon, 2001). HH proteins are potent morphogens capable of acting through long distances. In mammals, there are three HH family ligands: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) which are synthesized as approximately 45-kDa precursors that undergo proteolytic autoprocessing and cholesterol modification to produce the active N-terminal fragments (Porter et al., 1996). The HH pathway signals via two transmembrane proteins, Smoothened (SMO) and its negative regulator, Patched1 (PTCH1). Other components of the pathway include the GLI family of transcriptional effectors GLI1, GLI2, and GLI3, whereby GLI1 acts as a transcriptional activator while GLI2 and GLI3 are able to act as both activators and repressors (Sasaki et al., 1999; Pan and Wang, 2007; Wang et al., 2000). In the absence of the HH ligand, PTCH1 inhibits SMO resulting in the cleavage of GLI to the N-terminal repressor form lacking an activation domain. On HH binding to PTCH1, the inhibitory effect on SMO is relieved, GLI repressor formation is inhibited, and full-length transcriptional activators are transported to the nucleus. This results in the transcription of HH target genes which include *PTCH1* and *GLI1* (reviewed in (Hooper and Scott, 2005; Ruiz i Altaba et al., 2002)). HH activity is regulated by the inhibitory Hedgehog-interacting protein (HIP) that binds directly with SHH (Chuang and McMahon, 1999). Another negative regulator of the pathway is Suppressor of Fused (SUFU) that interacts directly with the GLI proteins and sequestering it in the cytoplasm, thus reducing the ability of GLI to initiate transcription (Kogerman et al., 1999; Dunaeva et al., 2003).

One of the most extensively studied functions of SHH is its role in the dorsal ventral patterning of the neural tube during the development of the central nervous system in vertebrates. SHH derived from the notochord forms a concentration gradient of SHH along the dorsal ventral axis in the overlying neural tube, resulting in the specification of diverse and distinct neuronal cell fates (Briscoe et al., 1999). Its importance in embryonic development is also reflected in its *in vitro* counterpart, embryonic stem (ES) cells. During mouse embryonic stem cell (mESC) differentiation, the pathway antagonist forskolin inhibited the formation of the ectoderm layer in embryoid bodies (EBs) (Maye et al., 2000). SHH is also used as a patterning factor in various ES cell differentiation protocols. When applied in conjunction with other molecules like FGF8 or retinoic acid (RA) at different stages of differentiation, SHH promotes the differentiation to neuronal subtypes like ventral spinal progenitors (Li et al., 2008), dopaminergic neurons (Yan et al., 2005; Perrier et al., 2004; Roy et al., 2006), motor neurons (Wichterle et al., 2002), and dorsal interneurons (Murashov et al., 2005). Apart from its role in differentiation, it has been suggested that SHH signaling could play a role in hESC self-renewal. In genome-wide studies of transcriptional networks in mESC and hESC, the key regulators of pluripotency OCT4, SOX2, and NANOG were found to occupy sites upstream of *PTCH1* and *GLI3* (Boyer et al., 2005; Loh et al., 2006). Moreover, downregulation of OCT4 in hESC resulted in reduced levels of PTCH1 and GLI3 (Babaie et al., 2007), suggesting that they are downstream targets involved in maintaining hESC pluripotency.

Therefore, this study aims to elucidate the function of SHH signaling pathway in hESC and their differentiated progenies. Our results indicate that components of SHH signaling are expressed in undifferentiated hESC and differentiated EBs as shown by immuofluorescent staining and RT-PCR. We found that SHH signaling is minimal in hESC in the undifferentiated state but is upregulated during differentiation. SHH does not play a role in maintaining the undifferentiated hESC phenotype or regulating the proliferation of hESC. However, results showed that SHH influences the lineage determination of hESC during differentiation as confirmed by the increased expression of neuroectodermal markers. Thus, our results presented here extend the understanding of extrinsic factors regulating hESC pluripotency and self-renewal.

Results

Expression of SHH signaling pathway components

To determine whether SHH signaling is present in hESC, components of the SHH signaling pathway were analyzed by immunocytochemistry. PTCH1 and SMO receptors were localized to the plasma membrane while GLI1 and GLI3 colocalized with the nuclear dye, DAPI (Figs. 1A–D). The expression of pathway components was further confirmed by RT-PCR where low expression of *SHH* and *IHH* was detected whereas DHH was undetectable in undifferentiated hESC. hESC also express the *PTCH1* and *SMO* but not *PTCH2* receptors and transcription factors *GLI1* and *GLI3*, along with the pluripotent marker *OCT4* (Fig. 1E). This confirms that hESC express SHH signaling components necessary for signal transduction. During spontaneous differentiation, gene expression of SHH, DHH, and IHH in embryoid bodies was significantly higher compared to hESC over the entire 14-day differentiation period. Concomitantly, there was also an upregulation of *GLI3*, Hedgehog interacting protein (*HIP*), *PTCH1*, and *PTCH2* and a downregulation of *SMO* and *GLI1* (Fig. 1E). These results are consistent with previous data (Sasaki et al., 1997), that reported similar mRNA expression of pathway components in hESC and EBs. The presence of these SHH pathway components suggests that SHH signaling cascades may have functional importance in undifferentiated hESC and EBs.

Activation of SHH signaling in undifferentiated hESC and role of GLI mediators

The presence of the SHH pathway components in hESC led us to investigate if SHH signaling is active in undifferentiated hESC. This was achieved using the GLI-mediated transcriptional activation assay with GLI-responsive luciferase reporter plasmid (8XGli-BS) (Rho et al., 2006). Transfection of the 8XGli-BS luciferase reporter plasmid into hESC showed 7-fold induction of luciferase activity, as compared to the background luciferase levels of the 8XmutGli-BS luciferase reporter plasmid, indicating that there is endogenous activation of the SHH pathway in undifferentiated hESC (Fig. 2A).

To identify the role of GLI mediators in undifferentiated hESC, three GLI mediators, GLI1, GLI2, and GLI3 expression vectors, were also cotransfected with the wildtype 8XGli-BS or 8XmutGli-BS luciferase reporter plasmid into hESC.

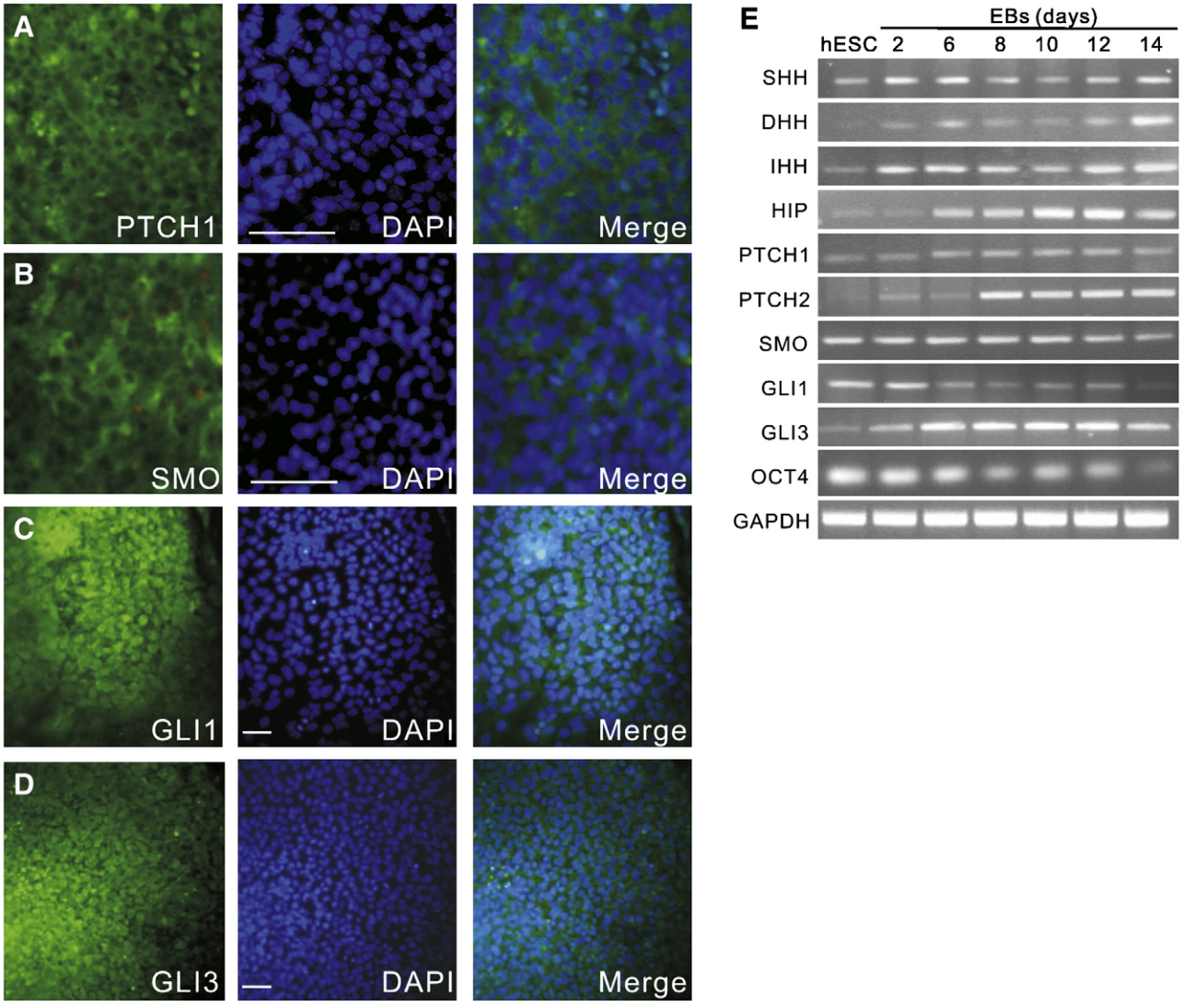

Figure 1 hESC express SHH pathway components. (A–D) Representative images showing immunofluorescent staining of (A) PTCH1, (B) SMO, (C) GLI1, (D) GLI3 in hESC. Middle panel shows corresponding DAPI nuclear staining in blue and right panel shows corresponding merged images. Scale bars represent 100 μm. (E) RT-PCR analysis of SHH signaling components in undifferentiated hESC and differentiating EBs over 14 days.

Expression of GLI1 and GLI2 in hESC induced a 4-fold and 3-fold increase in wildtype 8XGli-BS luciferase activity, respectively, while expression of GLI3 in hESC inhibited 8XGli-BS luciferase activity (Fig. 2A). Coexpression of GLI3 and GLI1 in hESC showed that GLI3 suppressed luciferase reporter activation by GLI1 in a dose-dependent manner (Fig. 2B). Another component of the SHH pathway, SUFU, a negative regulator of GLI mediators, was also found to significantly downregulate luciferase activity when it was coexpressed with GLI1 in hESC (Fig. 2C). These results are consistent with those previously reported (Stone et al., 1999) and confirm the role of the GLI mediators in SHH signal transduction in hESC.

Effect of SHH on hESC pluripotency and proliferation

Since the SHH pathway was shown to be active in hESC, we examined whether addition of exogenous SHH affects hESC pluripotency. hESC were cultured in conditioned medium (CM) for two passages with or without 1 μg/ml recombinant SHH and pluripotent surface marker Tra-1-60 expression was evaluated by flow cytometry analysis. Results showed that cells cultured over two passages in CM + SHH maintained high levels of Tra-1-60 expression which are comparable to cells cultured in CM (Fig. 3A). Similarly, quantitative real-time PCR analysis of pluripotent markers *OCT4* and *NANOG* showed that cells cultured in CM or CM + SHH had similar gene expression levels (Fig. 3B). Therefore, hESC remained undifferentiated in the presence of SHH, suggesting that SHH does not induce differentiation.

We were then interested to know if SHH maintains pluripotency when hESC are grown under spontaneous differentiation conditions. Cells were cultured in CM without FGF2 (CM − FGF2) to induce spontaneous differentiation, as FGF2 in CM has been shown to be necessary in the maintenance of pluripotency (Greber et al., 2007). After the second passage, there was a drop in expression of Tra-1-60,

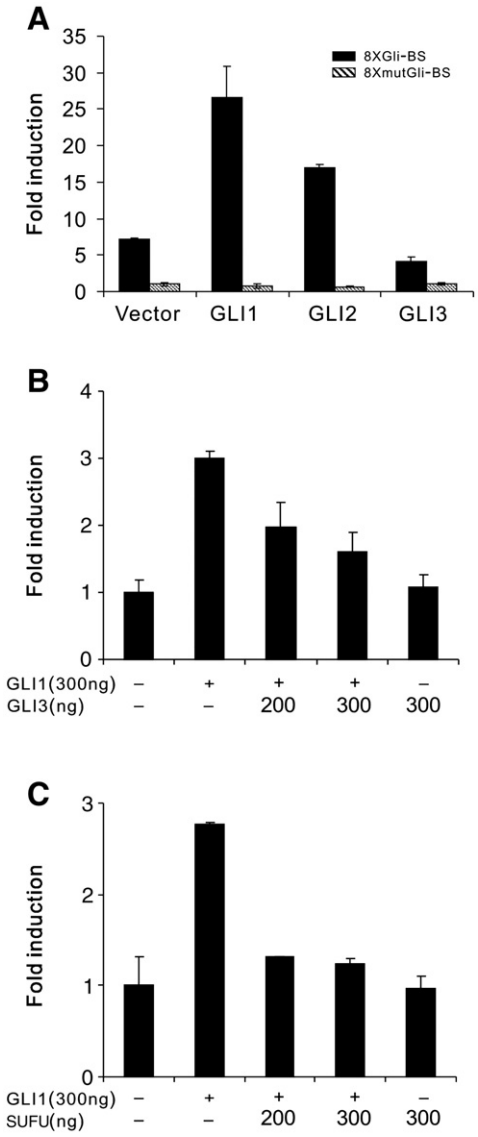

Figure 2 GLI mediators are functioning in undifferentiated hESC. Luciferase activity of 8XxGli-BS luciferase reporter plasmid. (A) hESC were transiently transfected with 8XGli-BS or 8XmutGli-BS luciferase reporter plasmid together with the indicated expression vectors encoding GLI1, GLI2, and GLI3. (B, C) The 8XGli-BS luciferase reporter plasmid and GLI1 expression vector were cotransfected with increasing concentrations of (B) GLI3 and (C) SUFU expression vector as indicated. Luciferase activities were calculated as a ratio of *Firefly* luciferase activity over *Renilla* luciferase activity and expressed as fold induction relative to vector control. Values shown are mean ± SD of a representative experiment carried out in triplicate and repeated at least three times.

OCT4, and *NANOG* in CM – FGF2 cells compared to cells cultured in CM (Figs. 3A and B), indicating that cells were undergoing spontaneous differentiation. When 1 μg/ml recombinant SHH was added daily to CM – FGF2 cells over two passages (CM – FGF2+SHH), there was a similar decrease in Tra-1-60, *OCT4*, and *NANOG* expression levels when compared to CM – FGF2 cells (Figs. 3A and B). These results suggest that SHH treatment does not maintain pluripotency of hESC and does not speed up the differentiation of hESC.

In order to study if SHH affects the proliferation of hESC, an EdU incorporation assay was used to investigate the cell proliferation of hESC after SHH treatment. hESC were treated with 1 μg/ml recombinant SHH for 24 h and cells were labeled with EdU during the last 2 h. Cells were costained with the pluripotent marker OCT4 and analyzed by flow cytometry. Results showed that despite treatment with 1 μg/ml SHH, the percentages of pluripotent OCT4 and EdU-positive proliferating cells were similar to those of the control (without SHH) (Fig. 3C). The effect of SHH on apoptosis was also evaluated using the annexin V apoptosis assay and the apoptotic cell population (annexin V positive and PI negative) was similar in cells treated with or without SHH (Fig. 3D). To further confirm our observations, an alternative method of introducing exogenous recombinant SHH was achieved by transfection of a CMV expression vector encoding the full-length SHH into hESC as it has been previously reported that recombinant HH protein and overexpression of Ihh had similar biological effects (Deckelbaum et al., 2002). Despite the differential expression of regulatory proteins both for cell cycle progression and for inhibition on overexpression of SHH, there was no change in the percentage of EdU-positive proliferating cells in the SHH overexpression compared to vector control (Supplemental Figs. 1A and B). Taken together, these results suggest that SHH does not stimulate the proliferation of undifferentiated hESC.

Activation of SHH signaling in hESC during differentiation

Having established the activation of the SHH pathway in undifferentiated hESC, we next studied the pathway activation during early differentiation by interrogating SHH target gene expression. hESC were either maintained in an undifferentiated state with C or induced to differentiate by culturing in differentiation medium (DM) or DM supplemented with 5 μM retinoic acid (DM + RA) for 96 h. Gene expression of *OCT4*, *NANOG*, and the SHH target genes *PTCH1* and *GLI1* was then analyzed by real-time PCR. When cells were differentiated with DM, there was a 50% reduction in *OCT4* and *NANOG* gene expression compared to undifferentiated hESC, and addition of RA (DM + RA) abolished *OCT4* and *NANOG* expression, showing loss of pluripotency (Fig. 4A). The drop in pluripotency marker expressions was accompanied by 1.5-fold upregulation of *PTCH1* expression in DM-treated cells and a 4-fold upregulation in DM + RA-treated cells. *GLI1* gene expression also increased by 2.5-fold in DM + RA-treated cells, implying that the SHH pathway is activated during differentiation.

In a different approach to the study of pathway activation, the 8XGli-BS luciferase reporter plasmid was transfected into hESC and treated under similar culture conditions as above. No increase in luciferase activity was observed for hESC cultured in DM. However, a significant induction of luciferase activity (2-fold) was observed in differentiating hESC treated with RA (DM + RA) (Fig. 4B). This induction in luciferase activity was inhibited by cyclopamine, a specific antagonist of the pathway that inhibits at the level of the SMO receptor (Taipale et al., 2000). Addition of 10 μM

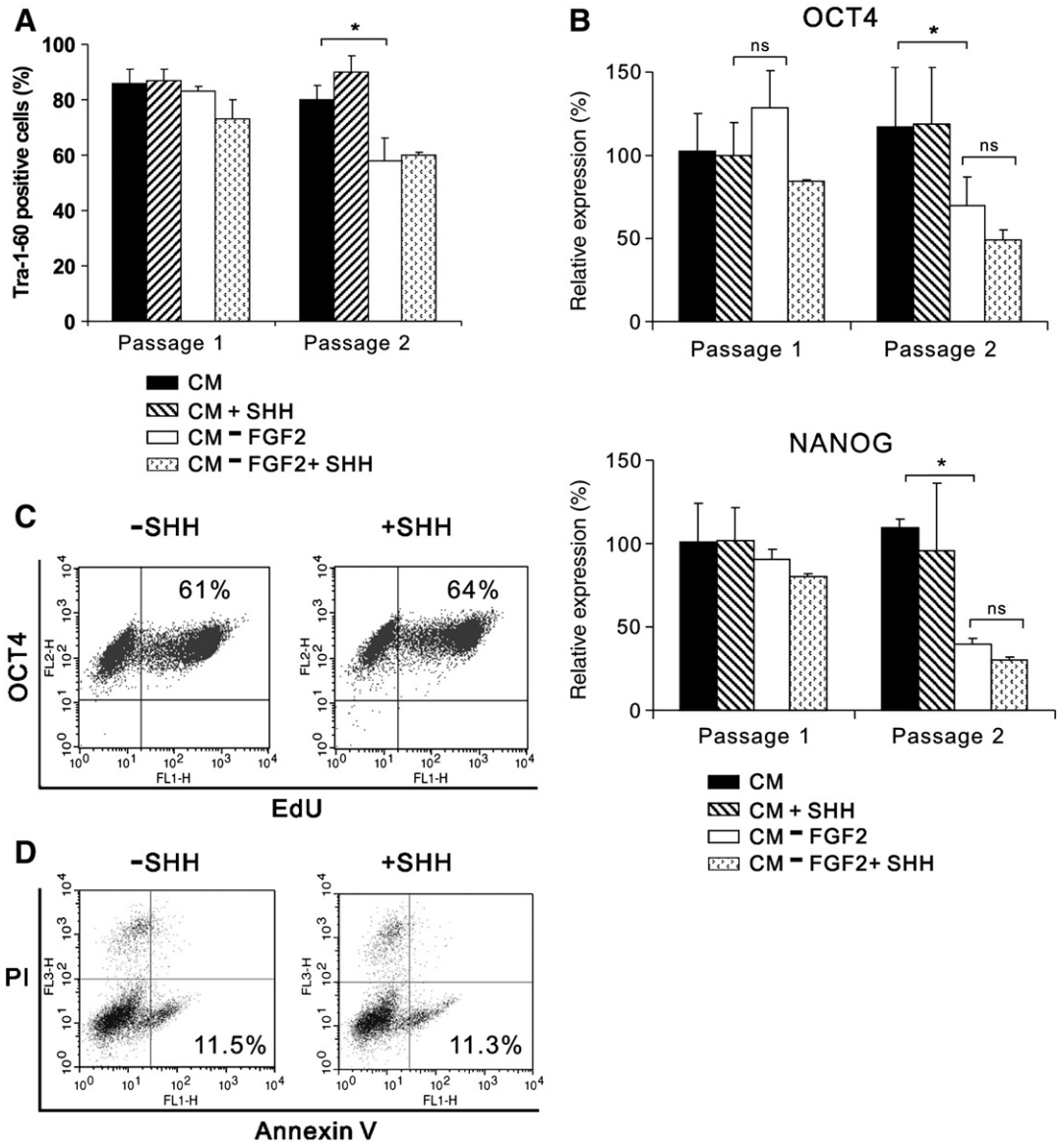

Figure 3 Exogenous SHH does not affect pluripotency and proliferation in undifferentiated hESC. (A) FACS analysis of Tra-1-60-positive cells and (B) quantitative real-time PCR analysis of pluripotent markers *OCT4* and *NANOG* expression in hESC maintained in conditioned media (CM), CM supplemented with 1 μg/ml SHH (CM + SHH), CM without FGF2 (CM − FGF2), or CM without FGF2 supplemented with 1 μg/ml SHH (CM − FGF2 + SHH) over two passages. Gene expression was normalized to that of GAPDH and expressed as percentages relative to undifferentiated hESC maintained in CM. The values shown are mean ± SD of a representative experiment performed in triplicate and repeated three times. *$P<0.05$; ns, nonsignificant. (C) FACS analysis of EdU incorporation assay in undifferentiated hESC. Cells were synchronized with nocodazole for 16 h and then treated with or without 1 μg/ml SHH for 24 h. Representative dot plots showing EdU incorporation in hESC costained for OCT4. (D) FACS analysis of annexin V apoptosis assay in undifferentiated hESC whereby cells were treated with or without 1 μg/ml SHH for 24 h prior to assay. Representative dot plots showing apoptotic cells (annexin V positive and PI negative).

cyclopamine reduced the luciferase activity in CM and DM to a lower basal level as compared to the vehicle control in CM and DM. There was also approximately 75% reduction in luciferase activity in DM + RA cells as compared to vehicle control under the same conditions (Fig. 4B). This indicates that endogenous SHH ligands activated the SHH pathway during differentiation as it is SMO dependent. Similarly, the luciferase activity induction was also inhibited by forskolin, an inhibitor of the pathway which activates protein kinase A (PKA) which in turn phosphylates the GLI mediator proteins to the repressor form (Wang et al., 2000; Sheng et al., 2006). Here, the luciferase activity in all culture conditions was significantly reduced by more than 50% with the addition of 50 μM forskolin as compared to vehicle control (Fig. 4B). Together with the target gene expression studies, the results provide evidence that endogenous SHH signaling in hESC is present and can be further highly activated by the endogenous SHH during RA-mediated differentiation.

As exogenous SHH is commonly added as a patterning factor in hESC neuronal differentiation protocols, we wanted

to confirm that exogenous SHH activates the pathway during differentiation. Hence, SHH was overexpressed along with the 8XGli-BS luciferase reporter plasmid. Subsequently, there was a 2-fold increase in luciferase activity in hESC cultured in CM and in DM on overexpression of SHH compared to the vector control (Fig. 4C). On top of that, overexpression of SHH elicited a greater increase in luciferase activity of around 3-fold in hESC cultured in DM + RA compared to the vector control (Fig. 4C). This result indicates that there is greater pathway activation by exogenous SHH during hESC differentiation with RA. Specificity of SHH-induced luciferase activity was demonstrated by the addition of 10 μM cyclopamine which resulted in a 50% reduction in luciferase activity in cells cultured in CM, DM, or DM + RA when compared to vehicle control. Overexpression of GLI1 in this assay served as a positive control for pathway activation (Fig. 4C). Exogenous SHH was also introduced in the form of recombinant SHH to confirm the pathway activation effect of overexpressing SHH ligand and similar results were obtained (Supplemental Fig. 2). Therefore, the results indicate that both endogenous and exogenous SHH can activate the pathway in hESC and higher activation occurs during RA differentiation.

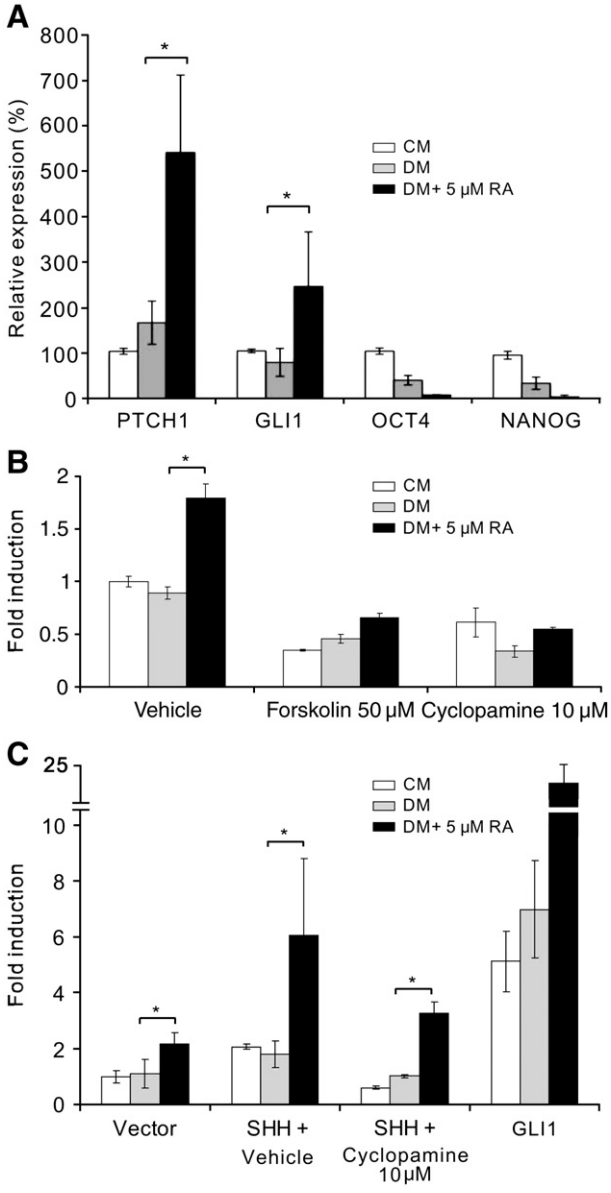

Figure 4 Activation of SHH signaling by endogenous and exogenous SHH. (A) Quantitative real-time PCR analysis of target gene *PTCH1* and pluripotent markers *OCT4* and *NANOG* expression in hESC maintained in conditioned media (CM), or induced to differentiate with differentiation media (DM) or DM supplemented 5 μM RA (DM + 5 μM RA) for 48 h. Gene expression was normalized to that of *GAPDH* and expressed as percentages relative to hESC in CM. The values shown are mean ± SD of a representative experiment performed in triplicate and repeated twice. (B, C) Luciferase activity of the 8XGli-BS luciferase reporter plasmid. hESC were transfected with the 8XGli-BS luciferase reporter plasmid and cultured under similar conditions as above for 48 h and then assayed for luciferase activity. (B) Cells were treated with the vehicle control (DMSO/ethanol) or pathway inhibitors 10 μM cyclopamine and 50 μM forskolin. (C) SHH expression vector was cotransfected with the 8XGli-BS luciferase reporter plasmid in the absence (vehicle-DMSO) or presence of 10 μM cyclopamine. GLI1 was overexpressed as a positive control. Luciferase activities were calculated as a ratio of *Firefly* luciferase activity over *Renilla* luciferase activity and expressed as fold induction relative to vehicle or vector control. Values shown are mean ± SD of a representative experiment carried out in triplicate and repeated at least three times. * $P<0.05$.

SHH signaling influences lineage determination during spontaneous differentiation

Expression of key components of the SHH pathway in EBs and its activation during differentiation suggest that SHH may play a functional role during spontaneous differentiation. To examine the effect of SHH signaling in lineage determination during hESC differentiation, exogenous SHH secreted in the conditioned medium (SHH-CM) of an inducible SHH overexpressing cell line (293-EcR Shh) (Cooper et al., 1998) with DM was used to culture differentiated hESC grown as EBs for 14 days. The levels of secreted SHH in SHH-CM were confirmed using ELISA (data not shown). Conditioned media from the normal HEK293 cells (Control-CM) was used as control. Production of active N-terminal SHH from 293-EcR Shh cells was used as a more convenient and economical source of SHH than commercial recombinant SHH. Also its usefulness in differentiation studies has been demonstrated whereby the SHH producing cells were cocultured with mESC to promote motor neuron differentiation (Soundararajan et al., 2007).

After 14 days in culture with SHH, expressions of the pluripotent marker *OCT4*, SHH target genes *GLI1* and *PTCH1*, and differentiation markers were analyzed using quantitative real-time PCR. Long-term treatment of SHH-CM enabled sustained activation of the SHH pathway as shown by the upregulation of *GLI1* and *PTCH1* expression (Fig. 5A). *OCT4* expression was downregulated under all conditions as compared to undifferentiated hESC, indicating that the media used did not inhibit the ability of cells to differentiate (Fig. 5A). An increased expression of neuroectodermal

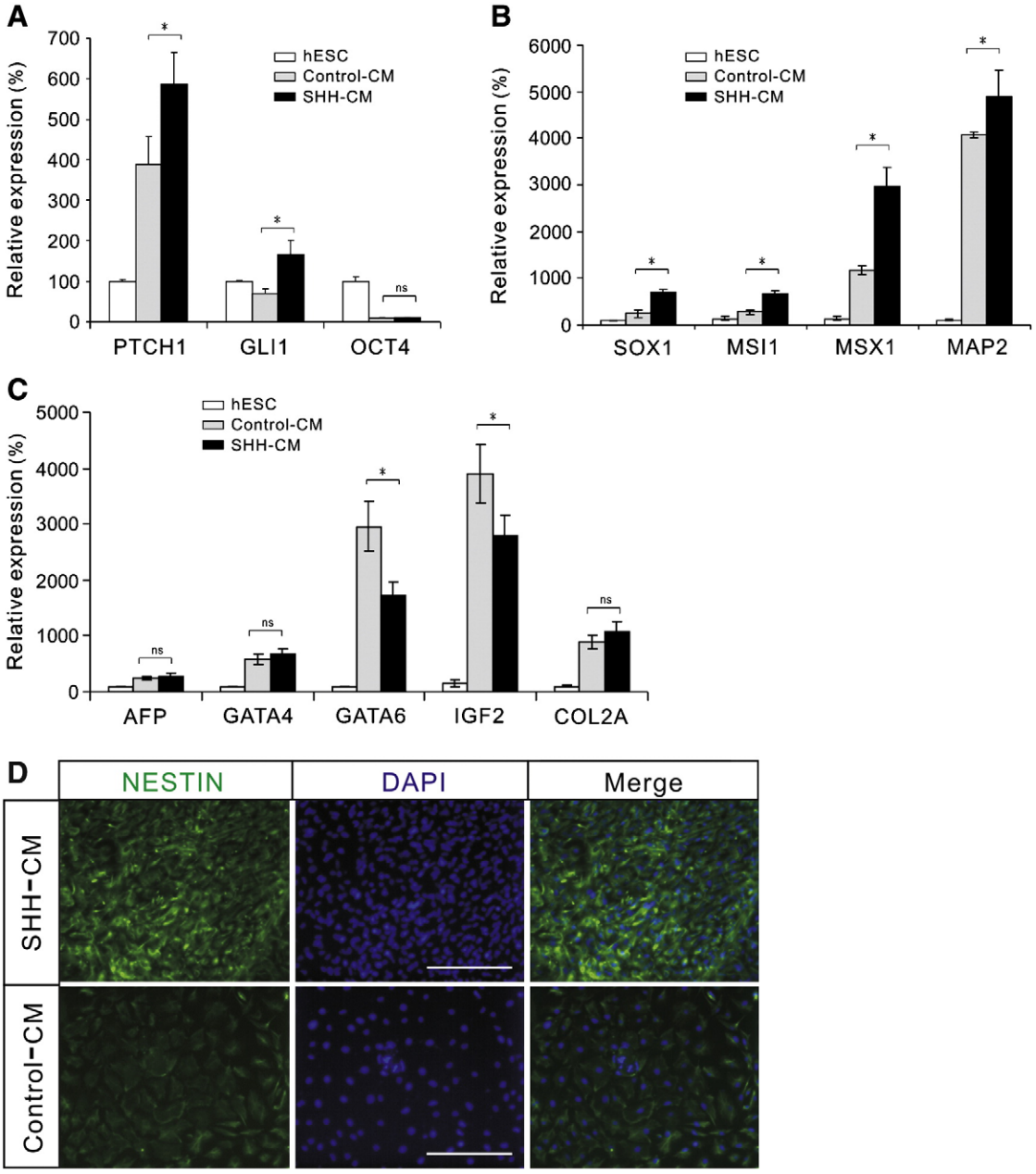

Figure 5 Neuroectoderm marker expressions are upregulated in EBs after 14 days of exposure to SHH. (A–C) EBs were grown in SHH-CM or Control-CM suspension culture for 14 days and mRNA expression was analyzed by quantitative real-time RT-PCR to determine the expression of (A) SHH target genes, (B) neuroectoderm, and (C) mesoderm and endoderm markers. Gene expression was normalized to that of GAPDH and expressed as percentages relative to undifferentiated hESC. Values shown are mean ± SD of a representative experiment carried out in triplicate and repeated at least three times. * $P<0.05$ (significant difference compared to Control-CM-treated EBs). (D) Immunofluorescent staining of neural stem cell marker Nestin in SHH-CM and Control-CM-treated EBs. Middle panel shows corresponding DAPI nuclear staining in blue and right panel shows corresponding merged images. Scale bars represent 50 μm.

markers *SOX1, Musashi 1(MSI1), MSX1*, and *MAP2* was observed in SHH-CM-treated EBs compared to the control group (Fig. 5B). The expressions of endodermal markers *AFP* and *GATA4* were not significantly altered, although a 30% decrease in *GATA6* mRNA was observed. *IGF2*, a mesoderm marker, was downregulated, whereas there was no effect on the *COL2A* expression level (Fig. 5C).

Following the 14-day differentiation in suspension, the EBs were replated onto gelatin-coated dishes to further differentiate for an additional 7 days in SHH-CM or Control-CM. Immunofluorescent staining results showed that in SHH-CM-treated differentiated cells, the neural stem cell marker, Nestin, is more highly expressed compared with Control-CM-treated cells (Fig. 5D). Quantitative analysis also confirmed that there was about 15 % increase in Nestin-positive cells when treated with SHH (Supplemental Fig. 3). Therefore, our results indicate that long-term exposure of EBs to exogenous SHH promotes differentiation toward the

neuroectoderm lineage and increases Nestin-positive neural derivatives, but with no significant influences on mesodermal or endodermal lineages.

Discussion

In this study, we investigated the expression of SHH and its pathway components in undifferentiated hESC and hESC during EBs differentiation. We found that hESC express the key components of the SHH signaling pathway, including the receptors PTCH1, SMO, and transcription factors GLI1 and GLI3 (Fig. 1). It was recently shown that the H1 and H9 hESC lines possess primary cilia containing the SMO and PTCH1 receptors and the authors also found that low levels of SHH are located at the base of the cilia (Kiprilov et al., 2008). Primary cilia have been shown to be required for SHH signaling to occur (Han et al., 2008) and preliminary data indicate that primary cilia are present in our HES-3 hESC line (unpublished data). It suggests that primary cilia and SHH signaling are important in hESC and work is underway to investigate the presence of SMO and PTCH1 receptors or other important signaling receptors on the primary cilia of undifferentiated and differentiated hESC.

Using a GLI-mediated transcriptional activation assay, we detected that the SHH signaling pathway was active in undifferentiated hESC (Fig. 2A). Results also established that GLI1 and GLI2 were able to activate the pathway (Fig. 2A), while GLI3 repressed GLI1-mediated activation (Fig. 2B), as reported elsewhere (Sasaki et al., 1999; Taipale et al., 2000). Another pathway component, SUFU that controls the processing and activity of the GLI proteins (Lum et al., 2003); (Ruel et al., 2003) was also shown expectedly to act as a negative regulator of GLI1 in hESC (Fig. 2C). The results show that the SHH pathway is present in hESC and the signaling cascade downstream of SMO is functioning, supporting the previous findings that PTCH1 and SMO receptors on the cilia were responsive to SHH stimulation (Kiprilov et al., 2008).

Having established the presence of the SHH pathway in hESC, we examined its function in hESC. Our results showed that exogenous SHH did not induce differentiation of hESC cultured in CM as there was no difference in levels of pluripotent markers Tra-1-60, *OCT4*, and *NANOG* expression in a 2-week culture with 1 μg/ml recombinant SHH. Removal of FGF2 from CM resulted in spontaneous differentiation with the loss of pluripotent markers and addition of recombinant SHH did not increase the loss of pluripotent marker expression nor did it increase the expression of pluripotent markers (Figs. 3A and B). These observations are in line with data reported by Heo et al. (Heo et al., 2007), whereby mESC maintained their undifferentiated status with long-term treatment of 0.5 μg/ml SHH over 5 passages. SHH was also shown to stimulate undifferentiated mESC proliferation via the canonical Gli pathway and noncanonical Ca^{2+}/PKC and EGF receptor activation. The authors observed an increase in cell proliferation and cell cycle component expression on stimulation with 50 ng/ml SHH (Heo et al., 2007). However, in our study, we found that neither the addition of recombinant SHH nor overexpression of SHH result in any significant differences in cell proliferation or cell death despite changes in the level of cyclin D1, a known SHH target (Wechsler-Reya and Scott, 1999) (Figs. 3C and D; Supplemental Fig. 1A). This discrepancy could be due to the inherent difference in self-renewal properties between mESC and hESC or that the stimulatory effect of SHH is tissue specific as SHH has been shown, depending on the cell type, to be a mitogen as well as a negative regulator of the cell cycle (Neumann, 2005); (Roy and Ingham, 2002). Therefore, although the SHH pathway is functional and present in undifferentiated hESC, it does not appear to play a role in self-renewal or pluripotency. Rather, we suggest that the pathway is minimally activated in hESC and is poised for activation on differentiation.

This hypothesis is supported by real-time PCR data that show when cells were differentiated in DM with RA, there was significant upregulation of the SHH target genes *PTCH1* and *GLI1* (Fig. 4A), indicating pathway activation. The GLI-mediated transcriptional activation assay corroborated with the target gene expression data, showing that the SHH pathway was activated in cells in DM with RA, and that the activation was SMO/GLI dependent (Fig. 4B). The SHH pathway does not appear to be activated in DM-treated cells because they were partially differentiated as demonstrated by the presence of small amount of *OCT4* and *NANOG* transcripts (Fig. 4A). And on treatment with RA, a known potent inducer of differentiation, there was rapid differentiation as *OCT4* and *NANOG* expression was downregulated, and this was accompanied by high activation of the SHH pathway (Fig. 4A). RA treatment has been shown to induce the differentiating cells to express the SHH ligand (unpublished data) (Okada et al., 2004), thereby highly activating the SHH pathway. The GLI-mediated transcriptional activation assay also showed that there was the greatest pathway activation by exogenous overexpressing SHH or recombinant SHH when hESC were differentiated with RA (Fig. 4C; Supplemental Fig. 2). This implies that in the undifferentiated state, activation of the pathway by exogenous SHH is minimal but when the cells begin to differentiate, they become more responsive to SHH stimulation. RA is commonly used in several ES cell differentiation protocols as a caudalizing factor to generate differentiated cells of the neural linage (Li et al., 2008; Wichterle et al., 2002; Schuldiner et al., 2001; Bibel et al., 2004) and its function could be partly attributed to its activation of the SHH signaling pathway (Okada et al., 2004). Coupled with the expression of SHH pathway components in EBs (Fig. 1E), SHH could play a more important role during differentiation and led us to study the effect of SHH signaling on the lineage determination during early EBs differentiation.

During hESC differentiation as EBs, the aggregates consist of various types of cells representing the three embryonic germ layers, ectoderm, mesoderm and endoderm (Xu et al., 2001), whose lineage commitment is governed by growth factors (Schuldiner et al., 2000). Since SHH is a known developmental patterning cue that regionalizes neural progenitor cells, we investigated the effect of SHH on hESC EBs differentiation by using SHH-CM. Here, we demonstrated that CM containing SHH was able to influence hESC lineage commitment to the neuroectoderm lineage as shown by the increase in gene expression of neuroectoderm markers *SOX1*, *MSI1*, *MSX1*, and *MAP2*. Differentiated hESC treated with SHH also showed increase expression of Nestin, a neural stem cell marker (Fig. 5; Supplemental Fig. 3). Our findings are consistent with the report showing the requirement of HH signaling in establishing the neuroectoderm in mESC EBs

whereby EBs derived from Smo or Ihh mESC mutant lines were not be able to generate neuroectoderm and their neural derivatives (Maye et al., 2004). The increase in Nestin-positive differentiated cells from the SHH-treated population could be due to SHH supporting the proliferation of cells expressing Nestin. A recent study also showed that SHH promoted the survival of Sox1-positive mESC-derived neuroprogenitors (Cai et al., 2008). Similarly, current gain-of-function studies have also found that SHH increases the expression of Sox1 and Nestin in hESC-derived neuroprogenitors using a defined neural differentiation protocol (unpublished data).

In conclusion, this is the first study to show that the SHH pathway is minimally active in undifferentiated hESC and activation of SHH signaling does not maintain hESC pluripotency and proliferation. Instead, SHH signaling is highly activated during differentiation and influences the determination of early differentiated hESC toward the neuroectodermal lineage. Our study significantly increases the knowledge of SHH signaling in hESC and expands the understanding of extrinsic factors regulating hESC pluripotency. This understanding can be applied to control the developmental fate of hESC during differentiation to achieve a more efficient and homogenous population of the required cell type for future regenerative medicine.

Materials and methods

Cell culture and reagents

Human embryonic stem cell lines HES-2 and HES-3 from ES Cell International were cultured in medium conditioned by mitomycin-C-inactivated immortalized mouse embryonic fibroblast feeder (ΔE-MEF) supplemented with 10 ng/ml of fibroblast growth factor-2 (FGF2) (Invitrogen) on Matrigel (BD Bioscience)-coated plates as described (Chan et al., 2008). ΔE-MEF conditioned medium contained Knockout-DMEM (KO-DMEM) supplemented with 15% Knockout serum replacer (KO-SR), 1% nonessential amino acids, 4 ng/ml FGF2, 1 mM L-glutamine, and 1% penicillin-streptomycin (all from Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma). Medium was changed daily. Cells were kept in a 5% CO_2 incubator at 37 °C and passaged by mechanical dissociation of colonies following collagenase IV treatment every 7 days. HEK293 cells (CRL-1573) and 293-EcR Shh cells (CRL-2782) were obtained from the American Type Culture Collection (ATCC) and cultured at 37 °C/ 5% CO_2 in complete medium containing DMEM (high glucose) and 10% fetal bovine serum (FBS) according to the manufacturer's protocol. Reagents used for treatment of cells were nocodazole (Sigma), cyclopamine (10 μM, Calbiochem), recombinant SHH N-terminal protein (0.5–1 μg/ml, R&D Systems), forskolin (50 μM, Sigma), and retinoic acid (5 μM, Sigma).

Embryoid body formation and differentiation

hESC were dissociated into small clumps by collagenase IV and cultured in suspension as embryoid bodies in differentiation medium (KO-DMEM supplemented with 20% fetal bovine serum, 1% nonessential amino acids, 1 mM L-glutamine, and 1% penicillin-streptomycin [all from Invitrogen] and 0.1 mM β-mercaptoethanol [Sigma]) on ultralow attachment plates (Corning). The culture was maintained in suspension for 14 days. The EBs were then plated for 1 week on gelatin-coated dishes for further differentiation.

Immunocytochemistry

hESC were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 30 min and washed in PBS containing 1% BSA (PBS/BSA). Cells were incubated overnight at 4 °C with primary antibodies: rabbit anti-GLI1 and anti-PTCH1 (Abcam), rabbit anti-GLI3, anti-SMO (Santa Cruz), and mouse anti-Nestin (Neuromics) at 1:100 dilutions. Cells were then incubated with secondary antibody Alexa Fluor 488 (Invitrogen) for 2 h at room temperature. Mounting medium containing DAPI (Vector Lab) was used for nuclei counterstaining. Images were acquired using a Zeiss Axiovert 200 M inverted fluorescent microscope (Carl Zeiss). After immunostaining and images capturing, the clusters were randomly picked up and the population of Nestin-positive cells among total differentiated cells (DAPI-positive labeled nuclei) was counted and scored within random at 400× magnification. Three to five clusters in each group were counted and data were expressed as mean ± SEM.

RNA extraction, RT-PCR, and real-time PCR analysis

RNA was extracted with the RNeasy kit (Qiagen) and treated with DNase according to the manufacturer's protocol. One microgram of RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen) and amplification of genes was performed using Platinum *Taq* (Invitrogen) with a program of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 54–64 °C for 1 min, and 72 °C for 1 min and an extension step at 72 °C for 10 min. For real-time PCR analysis, all samples were run in triplicates at a reaction volume of 25 μl containing Power SYBR Green PCR Master Mix (Applied Biosystems), and 200 nM primers. The reaction was run on the ABI Prism7000 Sequence Detection System (Applied Biosystems) using the following amplification parameters: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analyzed using the $\Delta\Delta C_T$ method to obtain expression levels relative to endogenous GAPDH control in each sample as previously described (Chan et al., 2008). The primers used for these analyses are listed in Supplemental Tables 1 and 2.

Flow cytometry analysis

For detection of pluripotent markers OCT-4 and Tra-1-60, cells were dissociated with trypsin and harvested as single cells before fixation and permeabilization using Fix and Perm Cell Permeabilization reagents (Invitrogen). Cells were incubated with mouse monoclonal antibodies against OCT-4 (Santa Cruz) and Tra-1-60 (Chemicon), washed with PBS/BSA, and then incubated with FITC-conjugated goat anti-mouse secondary antibody (DAKO). Cells were washed and resuspended in PBS/BSA for flow cytometry analysis. As a negative control, cells were stained with the appropriate isotype control. Cells were acquired using a FACS Calibur

(Becton Dickinson) and results were analyzed with the CellQuest Software (Becton Dickinson).

Cell proliferation assay

To detect DNA synthesis in proliferating cells, incorporation of a thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) was used (Invitrogen). hESC were transfected with the CMV expression vector containing SHH (Open Biosystems) using Lipofectamine 2000 (Invitrogen) and harvested 24, 48, and 96 h posttransfection, where EdU was added in 2 h prior to harvest for assay. In a different approach, hESC were treated with 10 mM nocodazole for 16 h for cell cycle synchronization. Cells were then treated with 1 μg/ml recombinant SHH for 24 h. EdU incorporation in cells was detected according to the manufacturer's instructions by using a FACS Calibur (Becton Dickinson) and results were analyzed with the CellQuest Software (Becton Dickinson).

Apoptosis assay

hESC were treated with 1 μg/ml recombinant SHH for 24 h and apoptosis assay was carried out using the annexin V-FITC apoptosis detection kit (Bender MedSystems) according to the manufacturer's instructions. Cells were counterstained with propidium iodide (PI) to identify apoptotic cells. Samples were analyzed on the FACS Calibur and results were analyzed with the CellQuest Software (Becton Dickinson).

Western blot analysis

hESC lysate prepared using 1% Igepal lysis buffer were resolved on 4–12% NuPAGE gels (Invitrogen) and transferred onto polyvinylidene fluoride (PVDF) membranes (BioRad). Membranes were blocked in PBS with 5% low-fat milk and probed overnight with primary antibodies; mouse anti-cyclin A, anti-cyclin D1, anti-pRb, anti-p21, rabbit anti-p15 and anti-SHH (Cell Signaling Technologies), mouse anti-OCT4, and anti-actin (Santa Cruz). Anti-actin was used as a loading control. This was followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and signals were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Transfection and luciferase reporter assay

CMV expression vectors containing mouse Gli1, mouse Gli2, and human GLI3 were kind gifts from Chi-Chung Hui (Hospital for Sick Children, Toronto, Canada). The 8XGli-BS luciferase reporter plasmid containing 8 copies of Gli-binding sites and 8XmutGli-BS luciferase reporter plasmid containing 8 copies of the mutated binding site were obtained as a gift from Hiroshi Sasaki (Centre for Developmental Biology, Riken, Japan) (Rho et al., 2006). The entire open-reading frame of full-length human cDNA encoding *Suppressor of Fused* (*SUFU*) (Open Biosystems) was subcloned into expression vector pcDNA3.1(+) (Invitrogen) and sequence-verified. hESC were plated in 24-well plates 4 days before transfection and transfections were performed using Lipofectamine 2000 (Invitrogen). The 8XGli-BS or 8XmutGli-BS luciferase reporter plasmid was cotransfected with 20 ng TK-Renilla (pRL-TK) (Promega) internal control plasmid and DNA expression vectors as indicated. Wherever required, the total amount of plasmid DNA transfected was adjusted to 1 μg by adding the control plasmid pCDNA3.1(+) (Invitrogen). To induce differentiation, hESC growth medium was changed 16 h after transfection to DM or DM supplemented with 5 μM retinoic acid (Sigma). Cells were harvested after 48 h and assayed for luciferase activity using the Dual-Luciferase system (Promega) according to manufacturer's protocol. Luciferase activities were measured for luminescence by the Infinite M200 microplate reader (TECAN). Luciferase activities were calculated as a ratio of *Firefly* luciferase activity over *Renilla*luciferase activity and expressed relative to an empty vector control. All transfection experiments were performed at least with three biological replicates using different batches of cells and preparations of plasmid DNAs and similar results were obtained.

SHH-conditioned media production

To produce conditioned medium with SHH, 4×10^6 of 293-EcR Shh cells and HEK293 cells as control were plated on T-175 flasks (Nalgene). Once the cells reached 50% confluence, growth media were changed to DM and Ponasterone A (Invitrogen) was added to293-EcR Shh cells to induce expression of SHH. Culture medium was collected after 24 h and filtered through a 0.22-μm filter unit (Nalgene) and used as growth media for hESC-derived EBs. Bulk quantities of CM were produced and pooled to ensure consistency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2009.09.002.

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