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ScienceDirect
Developmental Biology 313 (2008) 107–117
DEVELOPMENTAL BIOLOGY
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Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm

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Received for publication 28 November 2006; revised 11 September 2007; accepted 2 October 2007
Available online 11 October 2007

Abstract

Nodal, a member of the TGF-β family of signaling molecules, has been implicated in pluripotency in human embryonic stem cells (hESCs) [Vallier, L., Reynolds, D., Pedersen, R.A., 2004a. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol. 275, 403–421], a finding that seems paradoxical given Nodal's central role in mesoderm/endoderm specification during gastrulation. In this study, we sought to clarify the role of Nodal signaling during hESC differentiation by constitutive overexpression of the endogenous Nodal inhibitors Lefty2 (Lefty) and truncated Cerberus (Cerb-S) and by pharmacological interference using the Nodal receptor antagonist SB431542. Compared to wildtype (WT) controls, embryoid bodies (EBs) derived from either Lefty or Cerb-S overexpressing hESCs showed increased expression of neuroectoderm markers Sox1, Sox3, and Nestin. Conversely, they were negative for a definitive endoderm marker (Sox17) and did not generate beating cardiomyocyte structures in conditions that allowed mesendoderm differentiation from WT hESCs. EBs derived from either Lefty or Cerb-S expressing hESCs also contained a greater abundance of neural rosette structures as compared to controls. Differentiating EBs derived from Lefty expressing hESCs generated a dense network of β-tubulin III positive neurites, and when Lefty expressing hESCs were grown as a monolayer and allowed to differentiate, they generated significantly higher numbers of β-tubulin positive neurons as compared to wildtype hESCs. SB431542 treatments reproduced the neuralising effects of Lefty overexpression in hESCs. These results show that inhibition of Nodal signaling promotes neuronal specification, indicating a role for this pathway in controlling early neural development of pluripotent cells.

Keywords: Human embryonic stem cells; Lefty; Nodal; Activin; Neuroectoderm; Neurons

Introduction

Embryonic stem cells (ESCs) derive their pluripotency from their embryonic origin in the inner cell mass of blastocysts (Evans and Kaufman, 1981; Thomson et al., 1998). They are unique among mammalian cells in being able to grow indefinitely *in vitro* while maintaining a capacity to differentiate into all adult cell types. Interestingly, recent studies have shown that mouse ESCs (mESCs) and human ESCs (hESCs) maintain their pluripotent status using different signaling pathways. LIF signaling is essential for mESC self-renewal (Nichols et al., 1990; Yoshida et al., 1994), but it is not active in hESCs (Daheron et al., 2004; Humphrey et al., 2004). Instead, Activin/Nodal signaling is essential for the maintenance of hESC pluripotency and self-renewal, with FGF-2 acting as a competence factor (Vallier et al., 2004a, 2005; James et al., 2005; Beattie et al., 2005; Xiao et al., 2006).

Activin and Nodal, members of the TGF-β superfamily, are also responsible for inducing mesendoderm, the precursor of the definitive mesodermal and endodermal lineages during gastrulation (reviewed in Schier, 2003). Nodal signaling is regulated by Cripto, an extracellular GPI-linked protein that

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doi:10.1016/j.ydbio.2007.10.003

acts as a co-factor. Endogenous inhibitors of Nodal signaling including Lefty1, Lefty2 and Cerberus, are capable of modulating early vertebrate development (Perea-Gomez et al., 2002; Meno et al., 1999; Belo et al., 1997). Recent evidence suggests that Lefty2 binds to Cripto and prevents the formation of the Nodal/Cripto/receptor complex, thereby inhibiting Nodal signaling (Chen and Shen, 2004; Cheng et al., 2004). A truncated form of Cerberus, Cerb-S, has been found to block Nodal specifically by direct binding to the ligand (Piccolo et al., 1999). Lefty expression at peri-implantation stages of mouse development further suggests an early role for it in anterior–posterior patterning in the mouse embryo (Takaoka et al., 2006). In each of these cases the probable mechanism of inhibitor action is the disruption of signaling through the Activin/Nodal pathway.

Although numerous studies have shown the importance of diminished BMP signaling for neural induction during vertebrate development (reviewed in Munoz-Sanjuan and Brivanlou, 2002), less is known about the role of other members of the TGFβ superfamily. Nodal$^{-/-}$ embryos show enhanced neuroectoderm differentiation (Camus et al., 2006), suggesting that Nodal acts as an inhibitor of neuroectoderm specification *in vivo*. In addition, Cripto$^{-/-}$ mESCs show a propensity for neuronal differentiation (Liguori et al., 2003; Sonntag et al., 2005). Furthermore, recent studies suggest a role of Nodal inhibition in neural induction in mouse and human ESCs *in vitro* (Vallier et al., 2004a; Watanabe et al., 2005). Lefty expression has also been characterised in hESCs during their differentiation and has been implicated in ectoderm specification (Dvash et al., 2007). Thus, Nodal signaling not only controls mesendoderm differentiation during gastrulation, but may also have a reciprocal developmental role in preventing cells from differentiating into ectodermal or neural fates both *in vivo* and *in vitro*.

In this study, we investigated whether the inhibition of Nodal signaling in hESCs would promote their specification into neural progenitors *in vitro*. Nodal inhibition was initially accomplished by overexpressing full-length Lefty2 (Lefty) or a truncated form of Cerberus (Cerb-S) in hESCs grown in Chemically Defined Medium (CDM), where BMP signaling is quiescent (Vallier et al., 2005). We found that overexpression of either Lefty or Cerb-S resulted in a pronounced increase in neuroectoderm development. Furthermore, similar effects could be achieved with the specific pharmacological inhibitor of type I Activin/Nodal receptor signaling, SB431542. These data strongly support the hypothesis that Nodal signaling inhibits neuroectoderm specification during early hESC differentiation (Vallier et al., 2004a; Sonntag et al., 2005; Camus et al., 2006), and provide a robust means of achieving neural differentiation from hESCs.

Materials and methods

Human embryonic stem cell culture

H9 (WiCell Inc., Madison, Wisconsin, USA) and hSF-6 (UCSF, San Francisco CA, USA) hESCs were routinely cultured as described (Schatten et al., 2005) in KSR medium containing KO-DMEM supplemented with Serum Replacement (Invitrogen), glutamate (1 mM) and β-mercaptoethanol (0.1 mM). Every 4 days, cells were harvested using 1 mg/ml collagenase IV (Gibco) and then plated into 60 mm plates (Costar) pre-coated with 0.1% porcine gelatin (Sigma) and containing irradiated mouse embryonic fibroblasts. For serum replacer-free culture, hESCs were grown in Chemically Defined Medium (CDM) (Johansson and Wiles, 1995), supplemented with Activin (10 ng/ml, R&D systems) and FGF2 (12 ng/ml, R&D systems). The composition of CDM was 50% IMDM (Gibco) plus 50% F12 NUT-MIX (Gibco), supplemented with 7 μg/ml of insulin (Roche), 15 μg/ml of transferrin (Roche), 450 μM of monothioglycerol (Sigma) and 5 mg/ml bovine serum albumin fraction V (Sigma). To enable hESCs to adhere in CDM, plates were pre-coated with foetal bovine serum (FBS)(Hyclone) or Fibronectin 10 μg/ml (Chemicon) for 24 h at 37 °C and then washed twice in PBS.

Karyotypic analyses were performed on H9 and hSF-6 cells at various passages. Abnormalities involving chromosomes 9, 5, and 19 were occasionally observed at late passages (p80–p115) confirming that hESCs can incur genetic alterations under certain conditions (Draper et al., 2004). Consequently only hESCs from earlier passages (p50–p70) were used for these experiments.

Generation of expression constructs and stable transfection

Plasmid pTP6 (Pratt et al., 2000) containing the CAGG (hCMV/Chicken β-Actin chimeric promoter) driving the expression of GFP-IRES-Puromycin, was used as the basis for constructing Lefty and Cerb-S expression vectors. pTP6 was digested with EcoR1, removing the GFP open reading frame (ORF), and either mouse Lefty2 or Xenopus Cerb-S ORFs were cloned into this site to generate pTP6-Lefty2 and pTP6-Cerb-S respectively. For stable expression with vectors encoding Lefty2 or Cerb-S, 3 confluent 60 mm plates containing around 2000 hESC colonies each were plated onto one 6 well gelatin-coated plate containing mouse feeders. After 48 h the cells were transfected using Lipofectamine 2000 (Invitrogen) as described (Vallier et al., 2004b). Three days after transfection, the cells were passaged onto 60 mm gelatin-coated tissue-culture plates containing puromycin-resistant mouse feeders. After 3 additional days, puromycin (1 μg/ml final concentration) was added. Puromycin-resistant colonies that appeared by 12 days of selection were picked, dissociated and plated onto 24-well gelatin-coated, feeder containing plates and expanded for further analysis as described above.

Differentiation of hESCs as EBs

Lefty or Cerb-S expressing hESCs (as well as wildtype H9 and hSF-6 hESCs) were grown in 6 cm dishes (Corning), on mouse feeders. When confluent, hESCs were passaged using collagenase 1 mg/ml, as previously described (Schatten et al., 2005) and cultured in non-adherent conditions in CDM supplemented with either SU5402 (Calbiochem; 10 μM) or SB431542 (Tocris; 20 μM). Alternatively, hESCs were incubated in DMEM supplemented with 10% FCS and 1 mM glutamine (MEF medium) to promote mesoderm formation. EBs were then grown for 12–16 days at 37 °C at 5% CO_2 before being harvested for histological or molecular marker analysis. For some experiments, EBs grown in CDM were plated directly on fibronectin.

Differentiation of hESCs as a monolayer

Wildtype and Lefty expressing hESCs were grown on fibronectin in FGF(12 ng/ml)/Activin (20 ng/ml) as previously described as a monolayer in the absence of a feeder layer (Vallier et al., 2005). When cells were 70–80% confluent medium was changed to either CDM only, CDM/FGF (12 ng/ml), CDM/Noggin 200 ng/ml, CDM/SU5402 and differentiated for 24 days.

RT-PCR and real-time PCR

Total RNA was extracted from EBs using the Qiagen RNeasy Micro kit according to manufacturer instructions. For RT-PCR, 50–100 ng of RNA were

used for each RT-PCR reaction, using the OneStep RT-PCR kit according to manufacturer instructions. For real-time PCR, 1 μg RNA was reverse-transcribed using the Qiagen QuantiTect Reverse Transcription kit. 1/20–1/40 of each cDNA reaction was used for each real-time PCR reaction using the QuantiTect SYBR Green PCR Kit (Qiagen) according to manufacturer instructions. Relative quantification of gene expression was done by normalising the detected levels of gene of interest mRNA to those of the housekeeping GAPDH gene. All primers were purchased from Qiagen, with the exception of Xenopus Cerb-S: Forward 5′-CTGTGACAGGATGGTGATAC-3′; Reverse 5′-ATGGTGCAGGGTAG TAGATG-3′. Mouse Lefty2: Forward 5′-GGAGATGTACCTGGACCTGC-3′; Reverse 5′-CATCTGAGGCGCAGC-TACAG-3′.

In situ hybridisation

EBs were fixed in 4%PFA in PBS at 4 °C o/n, and stored in methanol at −20 °C. *In situ* hybridisation was performed as described previously (Harland, 1991), with slight modifications.

Immunocytochemistry

Immunocytochemistry for pluripotency markers Oct4 and Tra-1-60 has been described previously (Vallier et al., 2004b). Immunocytochemistry was performed for the pluripotency marker Oct4 (Santa-Cruz) and the differentiation markers Sox17 (R&D systems), Sox1 (kind gift of Dr. Robin Lovell-Badge), Nestin (Abcam), β-tubulinIII (Chemicon), Neurofilament (Sigma), GABA (Sigma), glutamate (Sigma). Cells were fixed with 4% paraformalyhyde for 15 min and blocked with 10% fetal calf serum/0.01% Triton X-100 for 1 h. Primary antibodies were incubated at 4 °C for 16 h in 1% fetal calf serum/0.01% Triton X-100. Cells were washed repeatedly and incubated with appropriate fluorescent secondary antibodies (Jackson laboratories) for 4 h. For cell counting studies, random fields were observed and the number of positive cells averaged over 10 fields.

Southern blot analysis

Genomic DNA was isolated from hESCs using QIAGEN Genomic-Tip 100/G according to the manufacturers' instructions. 10 mg of genomic DNA was digested with Xba1 (60 units) for 16 h Southern blot and hybridisation and using [P^{32}]dCTP labelled probe has been described previously (Sambrook et al., 2001). Probe used corresponds to the first 600 bp of the CAG promoter.

Results

Characterisation of Lefty and Cerb-S expressing hESCs

Human ESCs grown in non-adherent conditions aggregate to form embryoid bodies (EBs) in which differentiation is initiated along both extra-embryonic and primary embryonic germ layer pathways (Vallier et al., 2004a). We used this model to study mechanisms controlling neuroectoderm specification of hESCs, as EBs reproduce three-dimensional cellular interactions. To explore the function of Nodal signaling in these EBs, we stably over-expressed Lefty2 and Cerb-S in the hESCs from which the EBs were derived, using the robust expression vector pTP6 (Pratt et al., 2000).

Southern blot analysis using the CMV promoter as a probe (first 600 bp of CMV/chicken β-Actin chimeric promoter) showed single copy integration for both Lefty and Cerb-S overexpressing hESC H9 lines. As expected, wildtype H9 genomic DNA did not hybridise with this probe (Fig. 1A). Only single copy integrants were used for further study. Stable clones generated using the Lefty-pTP6 vector ($N=18$ for H9 cell line and $N=10$ for hSF-6 cell line) and the Cerb-S-pTP6 vector ($N=26$ for H9 cell line and $N=8$ for hSF-6 cell line) were screened for Lefty or Cerb-S expression using RT-PCR. Fig. 1B shows representative RT-PCR analyses of Lefty and Cerb-S overexpressing clones. In addition, an hrGFP overexpressing hESC cell line (Vallier et al., 2004a) was included as a negative control in RT-PCR analysis along with wild type hESCs to control for any effects induced by the genetic manipulation procedure itself (data not shown). The numbers of stably transfected colonies generated using the Lefty-pTP6 and Cerb-S-pTP6 vectors were similar to those generated using the hrGFP-pTP6 vector (data not shown). Three independent clones for both Lefty and Cerb-S expressing H9 and hSF-6 hESCs were further characterised and used for subsequent experiments.

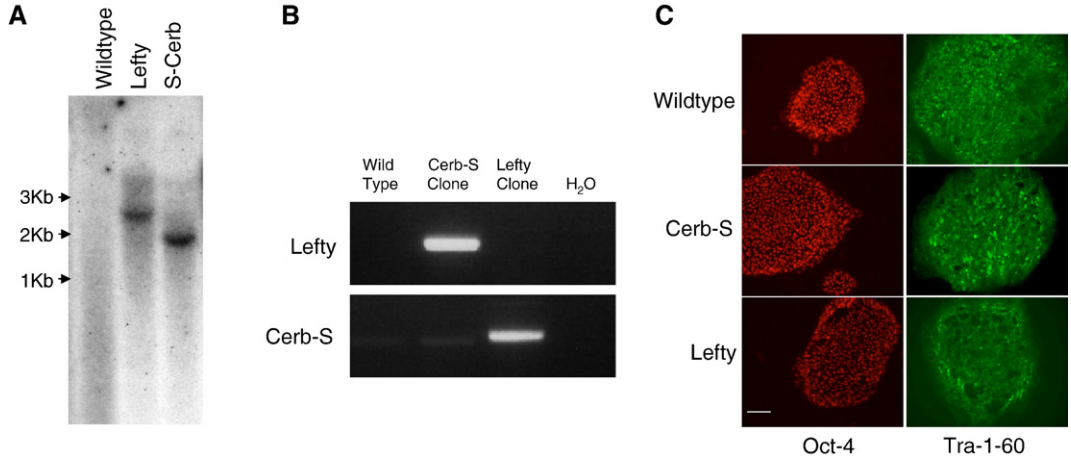


Fig. 1. (A) Southern blot analysis using a 600 bp fragment of the CAG promoter as a probe reveals single-copy integration of expression vectors for Lefty and Cerb-S expressing hESC clones. (B) RT-PCR analysis of Lefty and Cerb-S expressing human embryonic stem cells, shows robust, specific expression of either mouse Lefty or xenopus Cerb-S expression from stable single copy integration clones. (C) Immunocytochemistry of Lefty and Cerb-S expressing hESCs shows homogenous expression of pluripotency markers Oct-4 (Red) and TRA-1-60 (Green) (Scale bar=200 μM).

Lefty and Cerb-S protein were secreted from EBs of either Lefty or Ceberus expressing hESCs. This was clear from the ability of supernatants of such cultures to induce differentiation of EBs derived from Nodal overexpressing hESCs (data not shown). However, pluripotent Lefty and Cerb-S expressing hESC lines, like wildtype hESCs, expressed the pluripotency markers Oct4 and Tra-1-60 (Fig. 1C) during a prolonged period of culture with feeder cells (30 passages/5 months). These results demonstrate that inhibition of the endogenously produced Nodal by either Lefty or Cerb-S expressing clones did not lead to differentiation of hESCs grown in conditions supportive of pluripotency. This could reflect the ability of mouse feeders to secrete sufficient Activin to maintain pluripotency under these conditions (Vallier et al., 2005). Furthermore, Lefty expressing hESCs grown in feeder-free conditions in CDM medium supplemented with Activin/FGF maintained their expression of pluripotent markers Oct4 and Tra-1-60 (data not shown) also indicating that Activin can circumvent the inhibiting effects of Lefty on Nodal Activity.

Lefty or Cerb-S overexpression drives differentiation of hESCs toward the neuroectodermal pathway at the expense of other lineages

To investigate the development of EBs derived from Lefty or Cerb-S overexpressing hESCs, we grew the cells as EBs in suspension culture using CDM medium. As previously observed (Vallier et al., 2004a), wildtype control EBs grown in CDM developed almost exclusively as simple spheres with no apparent tissue organisation (Fig. 2A). By contrast, the majority of EBs derived from Lefty overexpressing hESCs showed a unique phenotype, consisting of epithelial layers organised into regular folded structures and bearing a striking resemblance to the neural folds formed during early vertebrate development (Fig. 2A). Cerb-S expressing EBs showed an epithelial phenotype similar to Lefty EBs, but with fewer folded structures (Fig. 2A). Hematoxylin/Eosin staining of sectioned EBs confirmed that these folded structures found in Lefty overexpressing EBs were neuroectoderm-like, with internal epithelial rosettes (Fig. 2B). Lefty and Cerb-S expressing EBs contained respectively 5 times ($P<0.05$) and 2 times (not significant, N.S.) more epithelial rosettes than control EBs (Fig. 2C). These results indicate that inhibition of the Activin/Nodal signaling pathway by Lefty produces a striking alteration of developmental fate of hESCs.

To test whether the EBs derived from either Lefty or Cerb-S overexpressing hESCs were neural in character, EBs were fixed, sectioned and immuno-stained for markers of definitive neuroectoderm (Nestin and Sox1), endoderm (Sox17) and mesoderm (Brachyury). Lefty and Cerb-S expressing EBs grown in CDM showed an overall increase in expression of the neuroectoderm markers Nestin and Sox1 compared to wildtype EBs, while there was a concomitant reduction in expression of the endoderm marker Sox17 in Lefty and Cerb-S expressing cells when EBs were grown in MEF medium (Fig. 3A), a condition known to promote mesendoderm specification. Brachyury expression was observed neither in control EBs nor in Lefty or Cerb-S expressing EBs (data not shown). *In situ* hybridisation analysis supported these results, showing that at 14 days EBs derived from Lefty overexpressing hESCs showed up-regulation of the neuroectoderm markers Nestin and Sox3 and the neuronal differentiation marker Neurogenin2 (NGN2) (Fig. 3B). In addition, Lefty expressing EBs showed a more pronounced down-regulation of the pluripotency marker Oct4 as compared to controls (Fig. 3B). Real time qPCR analysis was employed to quantify these effects. Comparison of the expression of Nestin, Sox3, Sox1 and NGN2 in four independent batches of WT and Lefty EBs showed a clear increase in the mean expression levels of most markers ($P<0.05$ for Nestin, Sox3 and Sox1), though the level of NGN2 was not significantly greater (Student's *T*-test, $P=0.094$) (Fig. 3C).

To evaluate further the differentiation potential of Lefty and Cerb-S overexpressing hESCs, EBs were cultured in MEF medium containing fetal bovine serum (FBS), which promotes the formation of mesoderm lineages including cardiomyocytes, as recognised by rhythmically beating structures. Lefty overexpressing EBs grown in these conditions displayed a decreased incidence of rhythmically contracting structures as compared to wildtype EBs, [~ 79% decrease for Lefty ($P<0.01$), ~ 53% for Cerb-S (NS)], suggesting a reduced ability to generate mesoderm (Fig. 3D). Nodal expressing EBs grown in these conditions were not seen to generate such beating structures, and instead maintained expression of the pluripotency marker Oct-4 (Fig. 3D and data not shown).

To observe the ability of Lefty and Cerb-S expressing EBs to differentiate into specific neuronal cell types, WT, Lefty or Cerb-S EBs grown for 12 days as suspension cultures in CDM were re-plated on fibronectin coated plates in CDM and cultured in these conditions for 12 more days. EBs derived from Lefty expressing hESCs (both H9 and hSF-6) showed substantial neurite outgrowths as compared to Cerb-S and wildtype cells. This was particularly evident when the cultures were stained with an antibody to the pan neuronal marker β-tubulin III (Fig. 4A). Cultures from plated Lefty and Cerb-S EBs were positive for the neurotransmitters Glutamate and GABA and for the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 4B), confirming that Activin/Nodal inhibition promotes specification of neural progenitors that are able, under appropriate conditions, to generate both differentiated neurons and astrocytes.

To quantify the neurogenic potential of Lefty expressing hESCs, we cultured Lefty expressing hESCs in feeder-free conditions as a monolayer in CDM supplemented with Activin and FGF as described previously (Vallier et al., 2005). When cells reached 70–80% confluency, medium was changed to CDM only, CDM plus FGF (12 ng/ml) (CDM/FGF), CDM plus the BMP inhibitor Noggin (200 ng/ml) (CDM/Noggin) or CDM plus the FGF receptor inhibitor SU5402 (10 μM) (CDM/SU5402), and cells were cultured in these conditions for 24 days. Immunostaining for β-tubulin III, a neuronal marker, revealed abundant neurons generated by Lefty expressing hESCs, although wildtype hESCs also showed a background level of neuronal differentiation in these conditions (Fig. 4C). The incidence of neural differentiation was determined by

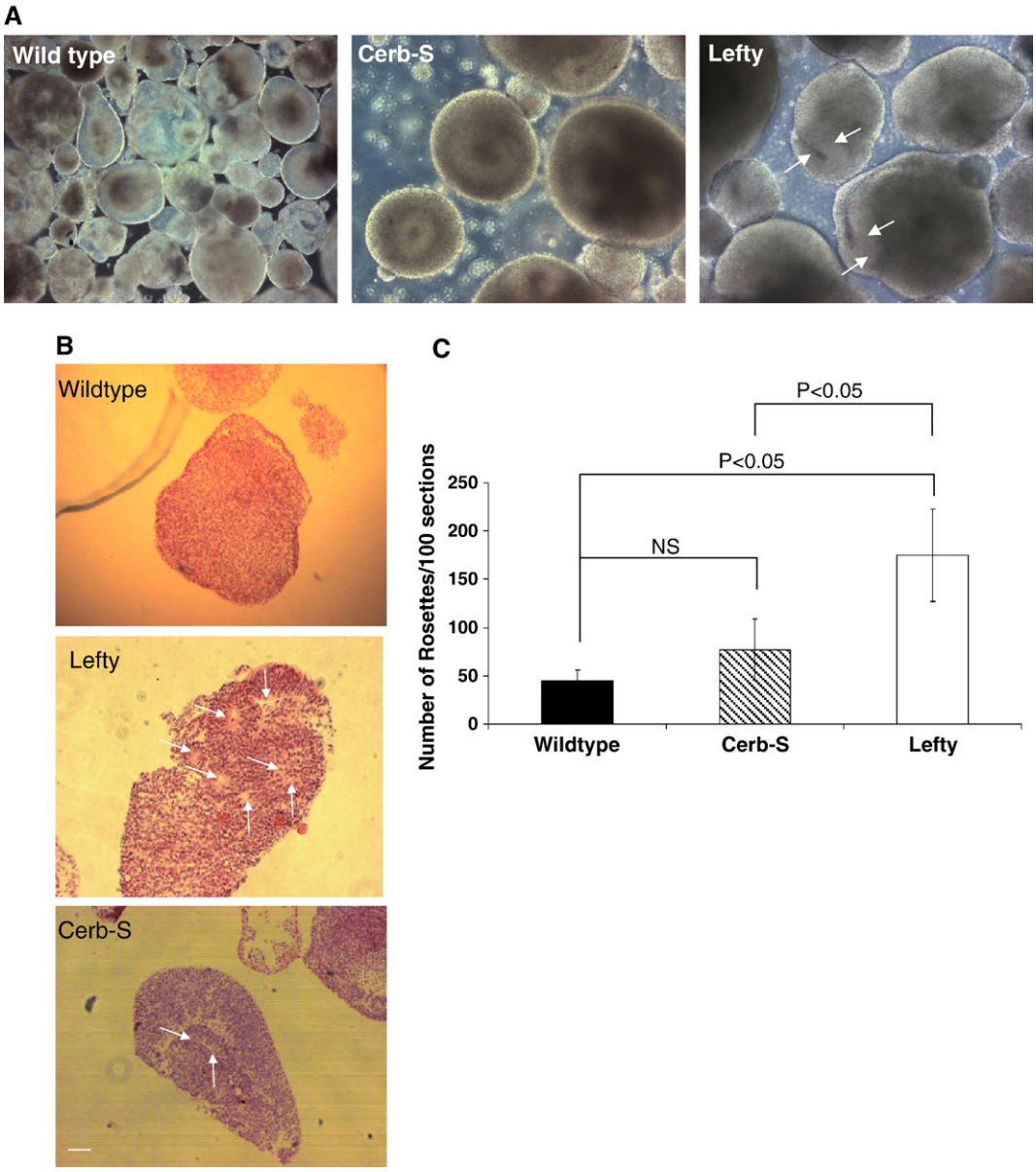


Fig. 2. (A) EBs derived from Lefty expressing hESCs form folded epithelial rosette structures (white arrows). (B) Hematoxylin and Eosin stained sections of Wildtype, Lefty and Cerb-S expressing EBs showing epithelial rosettes (white arrows). (C) Quantitation of rosette structures in EBs of either Lefty or Cerb-S expressing and wildtype EBs. Lefty expressing EBs had significantly greater numbers of rosette structures as compared to Cerb-S and wildtype (NS=not significant) (Scale bar=200 μM).

counting the number of β-tubulin III positive cells in random fields. This showed that when differentiated in CDM, Lefty expressing hESCs generated more β-tubulin III positive cells/field (69±13) than wildtype cells, (25±9) ($P<0.01$) (Fig. 4D). FGF signaling was essential for neuronal differentiation in these conditions, as shown by the effect of the pharmacological inhibitor SU5402, which induced a significant loss of β-tubulin III positive cells (Fig. 4E) ($P<0.001$). In addition, inhibition of BMP signaling significantly increased ($P<0.05$) the number of β-tubulin III positive cells, suggesting that BMPs may be released by differentiating cells in the cultures and restrain neural cell development. Interestingly, SU5402 treatment of Lefty expressing hESCs differentiating as EBs generated small neurospheres-like structures, which strongly expressed β-tubulin III (Fig. 4F). These structures were not observed when wildtype hESCs were differentiated as EBs in the presence of SU5402.

Pharmacological inhibition of Nodal signaling in hESCs mimics Lefty activity in vitro

In view of the complexity of generating transgenic hESC lines expressing Nodal inhibitors, we also investigated whether a specific pharmacological inhibitor of Nodal signaling, SB431542, would also lead to increased neuroectoderm specification in hESC derived EBs. Comparison of gene expression by real time qPCR in DMSO- and SB431542-treated EBs grown in CDM for 14–16 days in the presence of

SB431542 showed a similar increase in the mean expression levels of Nestin, Sox3, and NGN2 to that observed in Lefty expressing EBs (Fig. 5A), though there was not a significant increase in Sox1 expression levels (Student's *T*-test; $P=0.103$). No substantial changes were observed when comparing expression of these markers in untreated and DMSO-treated cells (data not shown). The addition of Activin to cultures had the opposite effects of SB431542, namely decreasing expression of the neuroectoderm markers, Sox1, Sox3, Nestin and NGN2 (data not shown). We also compared the time course of hESC differentiation toward neuroectoderm in the presence or in the absence of SB431542 by harvesting EBs every 4 days

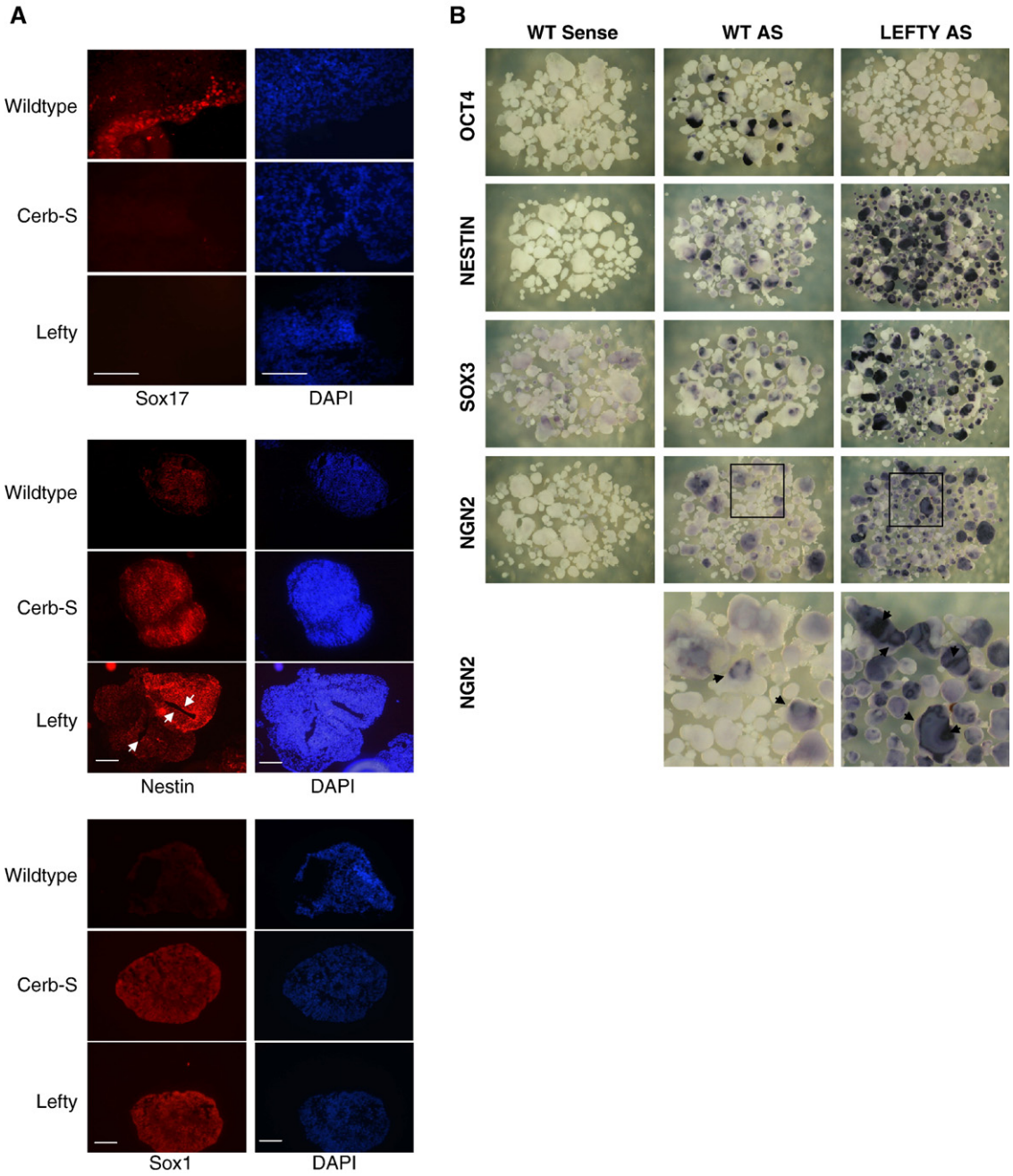


Fig. 3. (A) Immunocytochemistry of Lefty expressing, Cerb-S expressing and wildtype EBs. Immunostaining of EB sections revealed a loss of mesoderm marker Sox17 and an increase in neuroectoderm markers Nestin and Sox1 in Lefty and Cerb-S expressing EBs as compared to wildtype EBs. Intensified Nestin staining was seen in Lefty EBs was localised to folded epithelial rosette structures (white arrows). (B) *In situ* hybridisation on EBs derived from WT or Lefty expressing hESCs. Lefty expressing EBs showed an increase in the expression of neuroectoderm markers Nestin and Sox3 and the neural differentiation marker NGN2. The boxed area for NGN2 is shown in higher magnification, below. NGN2-positive patches of tissue are indicated (black arrowheads). Lefty expressing EBs also showed a more robust down-regulation of the pluripotency marker Oct4 (AS, anti-sense). (C) Real time qPCR analysis showed significantly increased average levels of Nestin, Sox3 and Sox1 expression in Lefty expressing EBs as compared to wildtype. Four independent batches of WT and Lefty EBs were used for this analysis. (D) Incidence of beating structures in Lefty expressing EBs. Lefty, Cerb-S expressing and wildtype EBs were plated in MEF medium, and beating structures were counted. Lefty expressing EBs generated fewer beating structures as compared to wildtype EBs (NS=not significant) (Scale bar=200 μM).

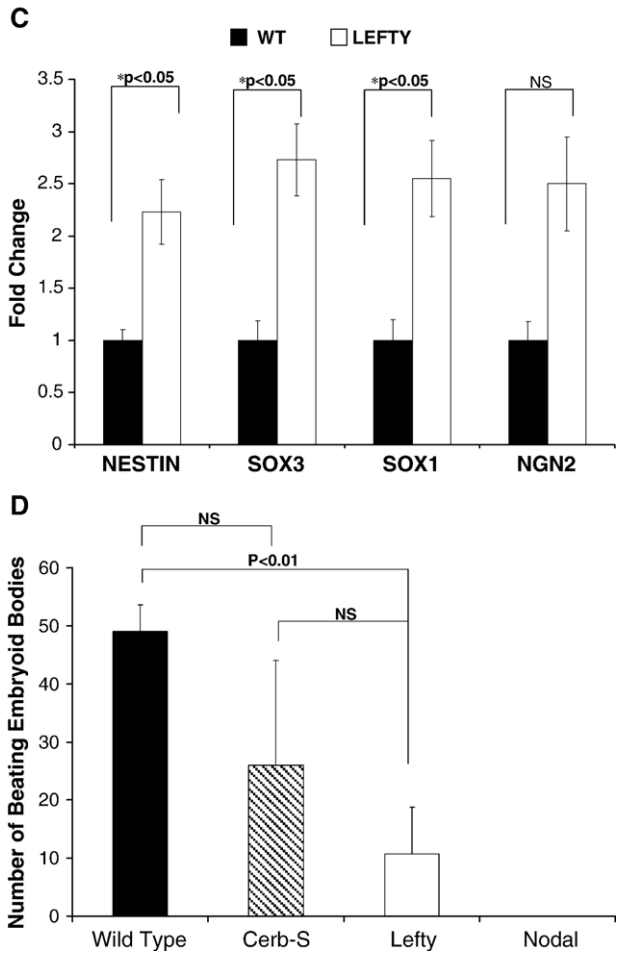


Fig. 3 (*continued*).

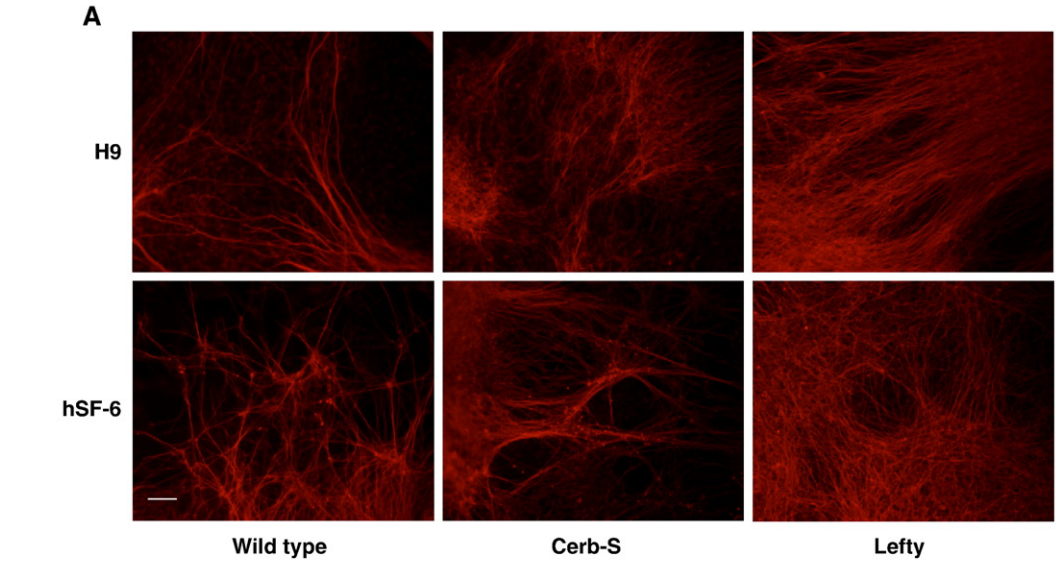
over a 16 day interval and analysing gene expression by RT-PCR. Inhibition of Activin/Nodal signaling by SB431542 caused a rapid down-regulation of the pluripotency marker Oct4 as compared to controls, thus apparently priming the cells for differentiation (Fig. 5B). By contrast the expression of the neuroectoderm markers Nestin, Sox3, Sox1 and NGN2 appeared to be upregulated earlier in SB431542-treated EBs than in control EBs (Fig. 5B). These findings indicate that forced inhibition of Nodal/Activin signaling results in more rapid loss of pluripotency and acquisition of neural character by hESCs compared to spontaneous differentiation, and strongly support the conclusion that inhibition of Activin/Nodal signaling, whether by natural or pharmacological inhibitors, results in highly efficient neuroectoderm specification *in vitro* under defined culturing conditions in which BMP signaling is quiescent.

Discussion

The majority of previous studies attempting to drive either mouse or human embryonic stem cells along the neuroectoderm pathway have focused on exposing pluripotent embryonic stem cells to soluble factors known to induce neurogenesis in the developing mammalian neural tube. Successful protocols promoting neurogenesis from embryonic stem cells have utilised sonic hedgehog, FGFs, Noggin, WNTs and retinoic acid, depending upon the regional specification sought (Reubinoff et al., 2001; Wichterle et al., 2002; Li et al., 2005; Yan et al., 2005; Irioka et al., 2005). Other studies have co-cultured hESCs with specific types of stromal cells to induce neuronal differentiation (Kawasaki et al., 2002; Perrier et al., 2004). Although effective, these studies have focused on a developmental time point that is significantly later than when neuroectoderm is initially specified. By contrast, our findings address cell fate decisions made early in the process of neuroectoderm development, which has been studied extensively in experimental embryonic systems. By expressing the Nodal inhibitors Lefty and Cerb-S in hESCs or using the pharmacological inhibitor of Activin/Nodal signaling SB431542, we found that neuroectoderm specification during early development of hESCs as EBs depends on the repression of Nodal signaling.

The default model of neuroectoderm specification is based on animal cap experiments in Xenopus showing that in the absence of exogenous growth factors, particularly BMPs, pluripotent animal cap cells differentiate into neuroectoderm (reviewed in Munoz-Sanjuan and Brivanlou, 2002). However, results obtained in the chick embryo demonstrate that neuroectoderm specification is dependent on FGF signaling. Accordingly, it was recently proposed that FGF acts first as a competence factor and then cooperates to inhibit BMP signaling, thereby blocking the negative effect of BMPs on neuroectoderm differentiation (Stern, 2005). Moreover, other findings in Xenopus (Delaune et al., 2005) and in mouse ESCs are consistent with this recent view (Ying et al., 2003), suggesting that a neural inductive function of FGF signaling is conserved through evolution.

While the current models for neuroectoderm specification are mainly based on the function of FGF and BMP signaling pathways, much less is known about the involvement of Activin/Nodal signaling. This pathway is a potent inducer of mesendoderm differentiation. Since mesendoderm is specified early in the progression from pluripotency to gastrulation, active inhibition of this signaling pathway would seem crucial to allow neuroectoderm specification (Schier, 2003). The absence of Nodal expression in regions of the mouse embryo giving rise to neuroectoderm descendants is consistent with this proposed negative role of Nodal in neural induction (Lawson et al., 1991; Collignon et al., 1996). Moreover, in chimeric embryos containing mESCs mutant for Nodal, the ESC progeny preferentially colonise the anterior region of the embryo, which gives rise to brain (Lu and Robertson, 2004). Nodal$^{-/-}$, Cripto$^{-/-}$ or Smad4$^{-/-}$ embryos all show an increase in neuroectoderm differentiation, with an absence of mesendoderm differentiation (Camus et al., 2006; Sonntag et al., 2005). These results strongly suggest that loss of Nodal activity is conducive to normal neuroectoderm specification during early embryogenesis. We have shown that active inhibition of Nodal by Lefty, Cerb-S, or the pharmacological Activin/Nodal inhibitor SB431542 in hESCs all lead to an increase in neuronal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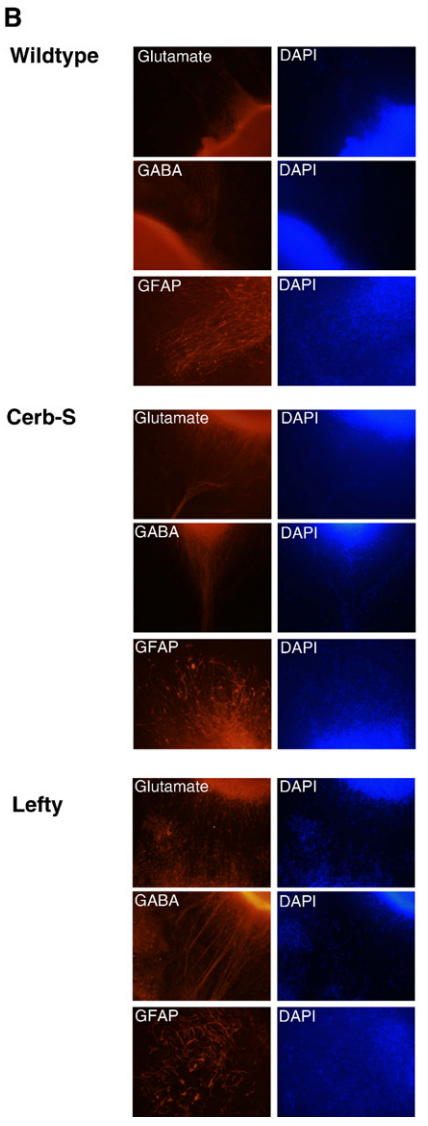
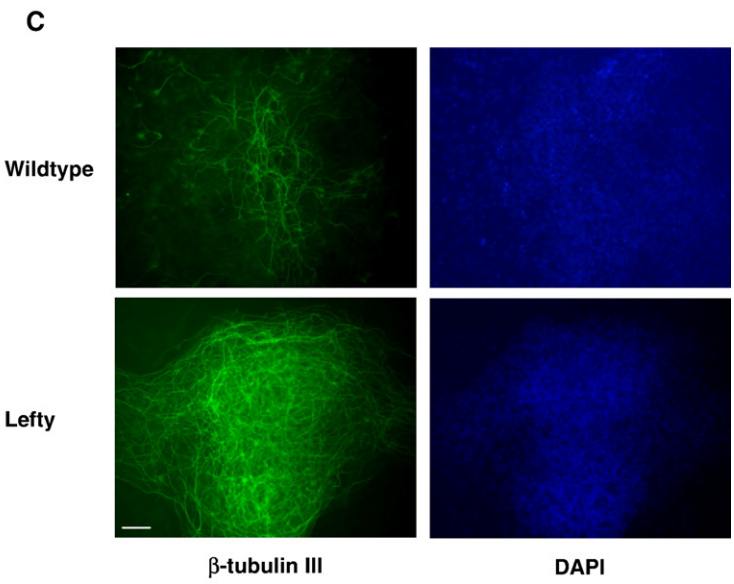
hSF-6
Wild type
Cerb-S
Lefty


B
Wildtype
Glutamate
DAPI
GABA
DAPI
GFAP
DAPI
Cerb-S
Glutamate
DAPI
GABA
DAPI
GFAP
DAPI
Lefty
Glutamate
DAPI
GABA
DAPI
GFAP
DAPI


C
Wildtype
Lefty
β-tubulin III
DAPI

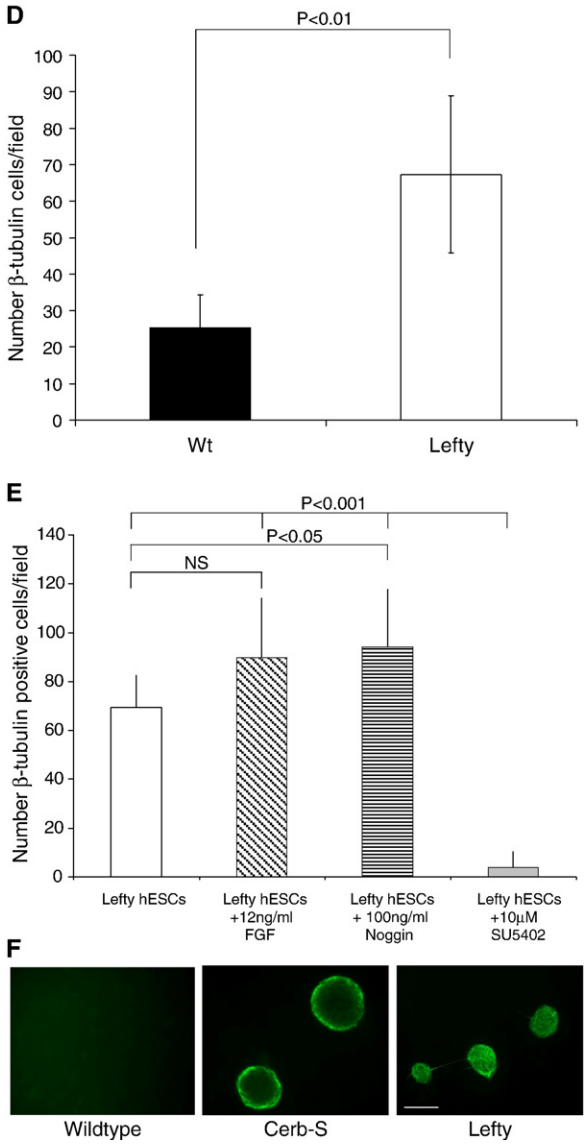


Fig. 4. (A) Immunocytochemistry for the neuronal marker β-tubulin III on re-plated wildtype Cerb-S expressing and Lefty expressing EBs. Lefty expressing EBs appeared to generate denser networks of neurite outgrowths as compared to Cerb-S expressing and wildtype EBs. (B) Immunocytochemistry for the neuronal phenotype markers, Glutamate and GABA, and the astrocyte marker GFAP shows abundant formation of each cell type. (C) Immunocytochemistry of β-tubulin III positive cells in monolayer culture of either Lefty expressing or wildtype hESCs. (D) Quantitation of β-tubulin III positive cells in 6 independent experiments from Lefty expressing and wildtype cells grown as a monolayer revealed a Lefty induced increase in the number of β-tubulin III positive cells. (E) Quantitation of β-tubulin III positive cells in 6 independent experiments from Lefty expressing cells grown in feeder free conditions as a monolayer and then differentiated in CDM medium containing either FGF (12 ng/ml), the BMP inhibitor Noggin (200/ml) or FGF receptor inhibitor SU5402 (20 μM). Lefty expressing hESCs exposed to FGF did not show a significant increase in the number of β-tubulin III positive cells as compared to controls, whereas Noggin produced a significant increase in β-tubulin III positive cells. Interestingly, inhibition of FGF signalling by SU5402 resulted in a highly significant reduction in neuron formation as shown by β-tubulin III immunoreactivity. (F) Immunocytochemistry for β-tubulin III on Lefty or Cerb-S expressing EBs exposed to SU5402 showed that compact neurospheres were formed when FGF signalling was inhibited. (NS=not significant) (Scale bar 400 μM).

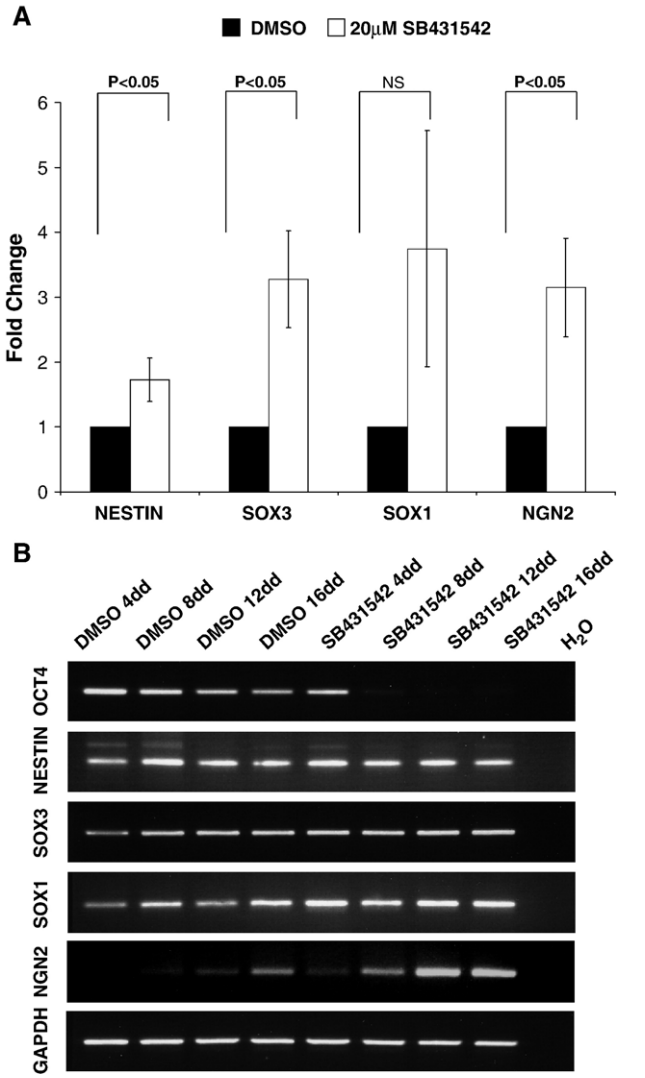


Fig. 5. (A) Real time qPCR analysis on DMSO- and SB431542 (20 μM)-treated EBs cultured for 14–16 days. Activin/Nodal inhibition led to a significant average increase in Nestin, Sox3, and NGN2 expression. Five independent batches of DMSO- and SB431542-treated EBs were used for this analysis. (B) RT-PCR on the time course of SB431542 incubation of hESCs reveals a more rapid downregulation of Oct4 and upregulation of neuroectoderm markers compared to controls. EBs were treated with DMSO or SB431542 (20 μM) and analysed by RT-PCR after 4, 8, 12 and 16 days of culture.

differentiation and a concomitant decrease in mesendodermal differentiation. Therefore, these results confirm that inhibition of the Activin/Nodal signaling pathway is sufficient for neuroectoderm differentiation in hESCs when they are grown in conditions of combined BMP quiescence and FGF activity.

As Nodal is expressed at the late epiblast stage, before the mesendoderm *versus* neuroectoderm decision, it is likely that Nodal plays a key role that is relevant to neural induction. Collignon and co-workers showed that the epiblast cells of Nodal$^{-/-}$ embryos lack expression of the pluripotent markers Nanog and Oct-4, suggesting that Nodal is required to maintain the pluripotent status of the epiblast layer before gastrulation and thus before Nodal's role in mesendoderm induction (Camus et al., 2006). Their observation that the expression of

pluripotency markers is replaced by the expression of neuroectoderm markers shows importantly that Nodal is not simply influencing the decision between mesendoderm and neuroectoderm, but that it is acting earlier to block neuralisation in the epiblast. This is consistent with results in hESCs where Nodal overexpression is sufficient to maintain their pluripotent status (Vallier et al., 2004a). It is also consistent with our finding that inhibition of Nodal leads to more rapid and more efficient neuroectoderm differentiation of hESCs, together with rapid loss of pluripotency markers (Vallier et al., 2005). Compared to wild-type hESCs, differentiating cultures of Lefty overexpressing hESCs or hESCs treated with the Activin/Nodal receptor inhibitor SB431542 express significantly higher levels of early neuroectoderm markers, including Nestin and Sox3. These observations suggest that higher numbers of neural precursors are generated in these conditions. Taken together, these results demonstrate that Activin/Nodal signaling blocks neuroectoderm differentiation by maintaining pluripotency of the epiblast layer *in vivo* and of hESCs *in vitro*. We therefore concur with Camus et al. (2006) that the current model of neuroectoderm differentiation can be expanded to include inhibition of Activin/Nodal signaling as a prerequisite for the induction of neuroectoderm specification during early mammalian development.

We also determined whether decreased levels of Activin/Nodal signaling are permissive for further differentiation of such neuroepithelial progenitors by assaying the expression of molecular markers known to be up-regulated at later steps along the neural differentiation pathway. During vertebrate neurogenesis, progression of Sox1-positive neural progenitors towards terminal differentiation involves up-regulation of bHLH proteins, such as Neurogenin (Kan et al., 2004). Neurogenin2 (NGN2) plays an important role in the progression of neural precursors (Ma et al., 1996; Nieto et al., 2001; Scardigli et al., 2001). Our time-course assays using SB431542 indicated that NGN2 expression increases between 4 and 16 days of EB differentiation, thus suggesting that neural progenitors generated through Activin/Nodal inhibition are able to progress along the neurogenic pathway. As expected, Activin treatment of differentiating EBs caused opposite effects, maintaining Oct4 expression and repressing the expression of Nestin, Sox1 and NGN2.

The development of chemically defined and animal product-free media is a major focus of human stem cell research. The characterisation of neuronal cells generated by growing EBs in CDM supplemented with SB431542 is an important step toward the generation of fully functional cells for therapeutic use. Indeed, the use of chemically defined medium avoids the use of other, more complex additives including animal-derived stroma cells and extracellular matrix. However, as originally formulated (Johansson and Wiles, 1995) CDM contains Bovine Serum Albumin (BSA) whose substitution by human albumin is needed to achieve culture conditions fully compatible with clinical application. In sum, Activin/Nodal signaling inhibition provides a unique *in vitro* approach to study the mechanisms controlling the first steps of neuroectoderm development of hESCs. Accordingly, it will permit a better understanding of the molecular mechanisms controlling cell fate specification in early mammalian development.

Note added in proof

Recent work by Chang et al. (2007) suggests that Neural induction requires continued suppression of both Smad1 and Smad2 signals during gastrulation.

Acknowledgments

This work was supported by Christopher Reeve Foundation (JS), Diabetes UK (LV) the BBSRC (BH, GL), and the Medical Research Council (RAP). We thank Ingrid Pradel and Kamran Hussain for their technical contributions, H Hamada for providing the mouse Lefty cDNA, and E M Robertis for providing the Xenopus Cerb-S cDNA. We would also like to thank Paula Mcphee for her assistance in typing and proofreading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.003.

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