

Activin/Nodal signalling maintains pluripotency by controlling Nanog expression

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The pluripotent status of embryonic stem cells (ESCs) confers upon them the capacity to differentiate into the three primary germ layers, ectoderm, mesoderm and endoderm, from which all the cells of the adult body are derived. An understanding of the mechanisms controlling pluripotency is thus essential for driving the differentiation of human pluripotent cells into cell types useful for clinical applications. The Activin/Nodal signalling pathway is necessary to maintain pluripotency in human ESCs and in mouse epiblast stem cells (EpiSCs), but the molecular mechanisms by which it achieves this effect remain obscure. Here, we demonstrate that Activin/Nodal signalling controls expression of the key pluripotency factor Nanog in human ESCs and in mouse EpiSCs. Nanog in turn prevents neuroectoderm differentiation induced by FGF signalling and limits the transcriptional activity of the Smad2/3 cascade, blocking progression along the endoderm lineage. This negative-feedback loop imposes stasis in neuroectoderm and mesendoderm differentiation, thereby maintaining the pluripotent status of human ESCs and mouse EpiSCs.

KEY WORDS: Nanog, hESCs, Activin, Nodal, Smad2/3, Neuroectoderm, Endoderm, Mesendoderm, Extraembryonic, Mouse, Human

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells derived from embryos cultured from the blastocyst stage. Their embryonic origin confers upon hESCs the ability to proliferate indefinitely in vitro while maintaining their capacity to differentiate into the three primary germ layers, ectoderm, mesoderm and endoderm, from which all the cells of the adult body are derived. Despite their apparent common origin, recent studies have revealed that mouse and human ESCs use different signalling pathways to maintain their pluripotent status. Mouse embryonic stem cells depend on leukaemia inhibitory factor (Smith et al., 1992) and bone morphogenetic protein (Ying et al., 2003) to maintain pluripotency, whereas their human counterparts rely on Activin/Nodal (James et al., 2005; Vallier et al., 2004) and fibroblast growth factors (Xu et al., 2005). However, in vitro and in vivo studies have established that a core transcription factor circuit involving Oct4, Sox2 and Nanog is necessary for pluripotency in both species. Indeed, constitutive expression of Nanog is sufficient to prevent differentiation of mouse and human ESCs (Chambers et al., 2003; Darr et al., 2006), and loss of function confirms that Nanog is necessary to block primitive endoderm differentiation (Chambers et al., 2003; Hyslop et al., 2005; Mitsui et al., 2003). These apparent contradictory observations underline the lack of knowledge concerning the mechanisms linking extracellular signalling and the core transcriptional network (including Nanog), especially in hESCs.

Here, we show that Smad2/3, the downstream effectors of Activin/Nodal signalling, bind and directly control the activity of the Nanog gene in hESCs. Accordingly, inhibition of Activin/Nodal signalling resulted in a loss of Nanog expression while inducing differentiation toward neuroectoderm. Knockdown of Nanog expression mimicked this effect, which is strictly dependent on FGF signalling. Conversely, constitutive expression of Nanog was sufficient to maintain the pluripotent status of hESCs in the absence of Activin/Nodal signalling, by specifically blocking neuroectoderm differentiation. In addition, our biochemical analyses showed that Nanog interacts directly with Smad2/3 proteins, the direct effectors of Activin/Nodal signalling, to limit their transcriptional activity, which is crucial for the cell fate choice between pluripotency and differentiation in hESCs. Importantly, similar results were obtained using pluripotent stem cells derived from the epiblast layer of pre-gastrula stage mouse embryos (mEpiSCs) demonstrating that these mechanisms are evolutionarily conserved, consistent with the proposed homology between hESCs and EpiSCs (Brons et al., 2007; Tesar et al., 2007). Taken together, these results demonstrate that Activin/Nodal signalling blocks neuroectoderm differentiation of pluripotent cells by maintaining Nanog expression, and they also provide the basis for a model explaining for the first time how Activin/Nodal signalling can maintain the pluripotency of hESCs without inducing differentiation towards endoderm.

MATERIALS AND METHODS

mEpiSC and hESC culture in feeder free and serum free conditions
hESCs [H9 (WiCell, Madison, WI, USA) and hSF-6 (UCSF, San Francisco, CA, USA)] and mEpiSCs (NOD-EpiSCs and 129S2-EpiSCs) were grown in chemically defined culture conditions, as previously described (Brons et al., 2007). For embryoid body (EB) formation and differentiation, hESC colonies were grown in non-adherent conditions as described (Vallier et al., 2004). For differentiation into extraembryonic tissues, hESCs and EpiSCs were grown in CDM with 10 ng/ml BMP4 for 7 days. For neuroectoderm differentiation, hESCs and EpiSCs were grown in CDM with 10 μ M SB431542 and 12 ng/ml FGF2 for 7 days. For mesendoderm differentiation, hESCs were grown in CDM with 5 ng/ml Activin but

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without FGF2 for 3 days, and then for 4 additional days in the presence of 100 ng/ml Activin, 10 ng/ml BMP4 and 20 ng/ml FGF2 (our unpublished results).

Microarray analysis

Microarray analyses were performed as described by Brons et al. (Brons et al., 2007). All hybridisations employed are publicly available in MIAME format from the ArrayExpress microarray repository (European Bioinformatics Institute; <http://www.ebi.ac.uk/arrayexpress>) under Accession Number E-MEXP-1741.

RT-PCR and real-time PCR

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen). For each sample, 0.6 µg of total RNA was reverse transcribed using Superscript II (Invitrogen). RT-PCR was performed as described (Vallier et al., 2004). Real-time PCR reaction mixtures were prepared as described (SensiMix protocol Quantace), denatured at 94°C for 5 minutes, cycled at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds for 40 cycles, then subjected to a final extension step at 72°C for 10 minutes. Primer sequences have been described elsewhere (Vallier et al., 2004; Brons et al., 2007). RT-PCR reactions were performed using a Stratagene Mx3005P in triplicate and normalised to porphobilinogen deaminase (PBGD) in the same run. Data represent the mean of three independent experiments and error bars indicate standard deviation.

Nuclear extracts and Smad/Nanog co-immunoprecipitations

The nuclear extract was prepared as described (Dyer and Herzog, 1995). The final protein concentration was 2 mg/ml in HEMG110 buffer (Hepes pH 7.6, 0.5 mM EDTA, 5 mM MgCl₂, 110 mM KCl, 20% glycerol, 0.2 mM DTT, Roche Complete cocktail, 0.5 mM PMSF). In each experiment, 0.5–1.0 mg nuclear protein and 3 µl antibody [α Smad2/3 (Cell Signalling), rabbit IgG (Sigma)] were used and incubated for 3 hours at 4°C. Protein G beads (10 µl, Roche) were added and samples incubated for an additional hour. Beads were washed five times with HEMG110 and proteins eluted with LDS-loading buffer at 70°C. PAGE and western blotting was performed with the Invitrogen NuPAGE system. Primary antibodies [α Nanog (R&D systems), α Sox2 (Abcam)] were probed with secondary HRP antibodies (Sigma) and detected using the LumilitePlus kit (Roche). The HA and FLAG immunoprecipitations (IPs) were essentially performed as described above, using Roche and Sigma M2 affinity matrixes. The Flag-HA-hNanog construct is driven by the CAG promoter and was transfected by Lipofectamine 2000 (Invitrogen). Cells were harvested after 42 hours.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out as previously described by Forsberg and colleagues (Okabe et al., 1996), using antibody directed against Smad2 (Abcam) or Nanog (R&D Systems). Enrichment was measured by quantitative real-time PCR using SYBR green (SensiMix Quantace). Results were normalised against control region H located in the 3' untranslated region of the *Nanog* gene (Fig. 1D) and are expressed as \pm s.d. from three experiments. Previous studies have described the location of the Smad2/3-binding regions in the promoter of Lefty (Besser, 2004) and of Smad7 (Denissova et al., 2000).

Mutation of Smad2/3-binding sites

Potential Smad-binding sites in the *Nanog6* promoter construct were mutated using the Quick Change II Mutagenesis Kit (Stratagene) following the manufacturer's instructions. Constructs were sequenced to confirm the presence of the desired mutation and to check the integrity of the promoter sequence. The sequence of the Smad2/3-(1) binding site was mutated from AGAC to GGCC (–310 to –307) and the sequence of the Smad2/3-(2) binding site from AGAC to GGCC (–302 to –299).

Generation of hESCs with stable knockdown of Nanog

Five shRNA-Nanog expression vectors (Sigma, SHGLY-NM_024865) and one shRNA-non-targeting expression vector (Sigma, SC001) were stably transfected into H9 hESC lines in CDM supplemented with Activin and FGF2. After selection, 60 puro-resistant colonies were picked (10 colonies for each shRNA-Nanog and 10 colonies for the shRNA-Non-Targeting control) and each sub-line was screened for the expression of Nanog using

immunostaining analyses (Fig. 3B). Importantly, absence of Nanog protein was not observed in any of the 10 sub-lines expressing the shRNA-non-targeting control. Knockdown of Nanog expression was confirmed by real-time PCR in 12 shRNA-Nanog-hESCs sub-lines with two randomly chosen shRNA-non-targeting-hESCs used as controls (Fig. 3A).

RESULTS

Activin/Nodal signalling is necessary to maintain Nanog expression in hESCs and in mEpiSCs

To discover the identity of genes controlled by Activin/Nodal signalling in hESCs, we compared the gene expression profile of hESCs grown in chemically defined medium (CDM) containing Activin with that of hESCs grown for two days in CDM without Activin and instead containing the Activin receptor inhibitor SB431542. As expected, inhibition of Activin/Nodal signalling induced a strong downregulation of genes known to be downstream of this pathway, including *LEFTYA* (*LEFTY2* – Human Gene Nomenclature Database), *LEFTYB* (*LEFTY1* – Human Gene Nomenclature Database) and *NODAL* (Besser, 2004), and induced a marked increase in early markers of neuroectoderm differentiation, including *GBX2*, *HOXA1*, *OLIG3* and *SIX1* (Fig. 1A; see also Table S1 in the supplementary material). More surprisingly, the expression of *NANOG* was strongly downregulated in the absence of Activin/Nodal signalling, whereas the expression of *OCT4* (*POU5F1* – Human Gene Nomenclature Database) and *SOX2* did not change as substantially (Fig. 1A). These observations were confirmed by real-time PCR analyses showing that the expression of *NANOG* was downregulated by 75% after 24 hours of SB431542 treatment, similar to the decrease in expression of *NODAL* and *LEFTYA* (see Fig. S1A in the supplementary material). By contrast, the decrease in *OCT4* expression occurred more progressively, while the expression of *SOX2* remained relatively constant (Fig. S1A in the supplementary material). Interestingly, normal levels of *NANOG*, *NODAL* and Lefty transcripts could be re-established in hESCs by adding Activin after SB431542 treatment (see Fig. S1B in the supplementary material). Similar results were obtained either in the presence or absence of cycloheximide, indicating that reactivation of *NANOG* transcription by Activin/Nodal signalling did not require de novo protein synthesis. Consistent with the marked downregulation of *NANOG* expression resulting from the inhibition of Smad2/3-mediated TGF β signalling, overexpression of SMAD3 in hESCs resulted in a doubling of *NANOG* transcription. Conversely, expression of a dominant-negative form of SMAD3 resulted in a 10-fold decrease in *NANOG* transcription (see Fig. S1C in the supplementary material). Finally, similar results were obtained in mEpiSCs (Fig. S1D,E in the supplementary material). Taken together, these results show that Activin/Nodal signalling is necessary for the expression of *NANOG* in hESCs, and that this requirement is evolutionarily conserved in pluripotent cells derived from the epiblast of post-implantation mouse embryos.

Smad2/3 proteins directly control Nanog expression in hESCs

To distinguish between the hypotheses of direct transcriptional regulation of *Nanog* by Smad2/3 and action through an unknown intermediate, we looked for functional SMAD2/3-binding sites in the human *NANOG* promoter. We first determined that a 379-bp region located upstream of the *NANOG* ATG was sufficient to recapitulate the transcriptional activity induced by Activin/Nodal signalling (Fig. 1B). Interestingly, this region contains binding sites for *OCT4*, *SOX2* and Nanog (Boyer et al., 2005), and also

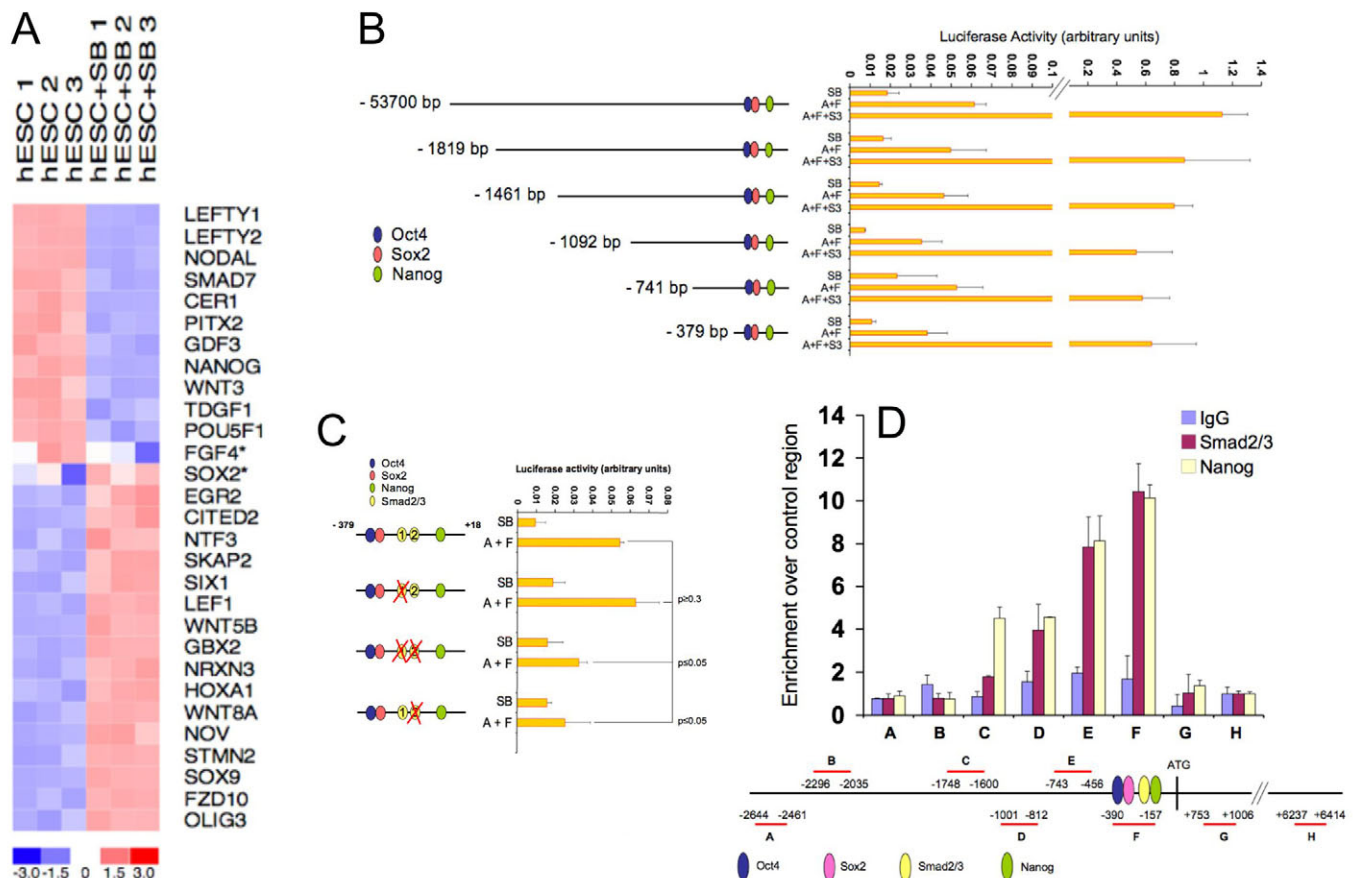


Fig. 1. Expression of NANOG in hESCs depends on Activin/Nodal signalling. (A) Microarray gene expression heat map comparing hESCs grown in CDM supplemented with Activin and FGF (hESC) with hESCs grown for 48 hours in CDM supplemented with FGF and SB431542 (hESCs+SB). For each gene (row), the heat map colours depict gene expression in units of standard deviation from the mean across all samples (columns). Upregulation is coloured in shades of red and downregulation in shades of blue. Gene names marked with an asterisk denote genes that did not pass a significant differential regulation threshold with a false discovery rate (FDR) of 1% when global sample group expression profiles were compared. (B) Promoter regions of the *NANOG* gene responsive to Activin/Nodal signalling. Luciferase reporter genes containing different sized fragments of the *NANOG* promoter were co-transfected into H9 cells along with renilla expression vector in the presence of Activin and FGF (A + F), or in the presence of Activin and FGF along with an expression vector for SMAD3 (A + F + S3), or in the presence of SB431542 (negative control, SB). Firefly luciferase activity (normalised to renilla luciferase activity) is expressed as mean±s.d. from three independent experiments. (C) Mutation of putative Smad2/3-binding sites in the *NANOG* promoter inhibits the transcriptional activation induced by Activin/Nodal signalling. Luciferase reporter genes containing the promoter of the human *NANOG* gene (−379 to +18) with or without mutated Smad2/3-binding sites were co-transfected into H9 cells along with the renilla expression vector in the presence of Activin and FGF (A + F), or in the presence of SB431542 (negative control, SB). Firefly luciferase activity (normalised to renilla luciferase activity) is expressed as mean±s.d. from three independent experiments. (D) Genomic regions of the *NANOG* gene bound by NANOG and SMAD2/3 proteins. ChIP assays were performed using antibodies directed against SMAD2/3 or NANOG. The immunoprecipitated DNA was then amplified using quantitative PCR and specific primers to detect enrichment in the denoted genomic regions. Results were normalised against control region H (−6237 to +6414) and are expressed as mean±s.d. from three experiments.

two consensus Smad2/3-binding sites [S2/3-(1) and S2/3-(2), see Fig. S1F in the supplementary material]. Mutation of these Smad2/3-binding sites revealed that the site nearest to the Nanog-binding site, S2/3-(2), was crucial for the transcriptional activity induced by Activin/Nodal signalling (Fig. 1C, see also Fig. S1G in the supplementary material), suggesting that this Smad2/3-binding site is functional. Interestingly, sequence alignment of the human *NANOG* promoter region to mouse, dog and cow equivalents revealed that the mouse promoter does not contain similar Smad2/3-binding sites (Fig. S1F in the supplementary material). This provides a further indication that the location of binding sites for highly evolutionarily conserved transcription factors can vary between humans and mice (Odom et al., 2007).

Finally, chromatin immunoprecipitation (ChIP) assays were performed to identify genomic regions bound by SMAD2/3 in the *NANOG* promoter (Fig. 1D). These analyses showed that Smad2/3 binds the same genomic region (containing the putative Smad2/3-binding sites) that was identified using luciferase assays. Taken together, these results reinforce the recent study by Thomson and colleagues (Xu et al., 2008), which showed that *NANOG* expression is directly controlled by SMAD2/3 in hESCs. The similar dependence of *Nanog* transcription on Smad2/3 in hESCs and mEpiSCs suggests that humans and mice share this direct link between extracellular growth factors and the core controlling transcriptional network despite their distinct genomic organisation.

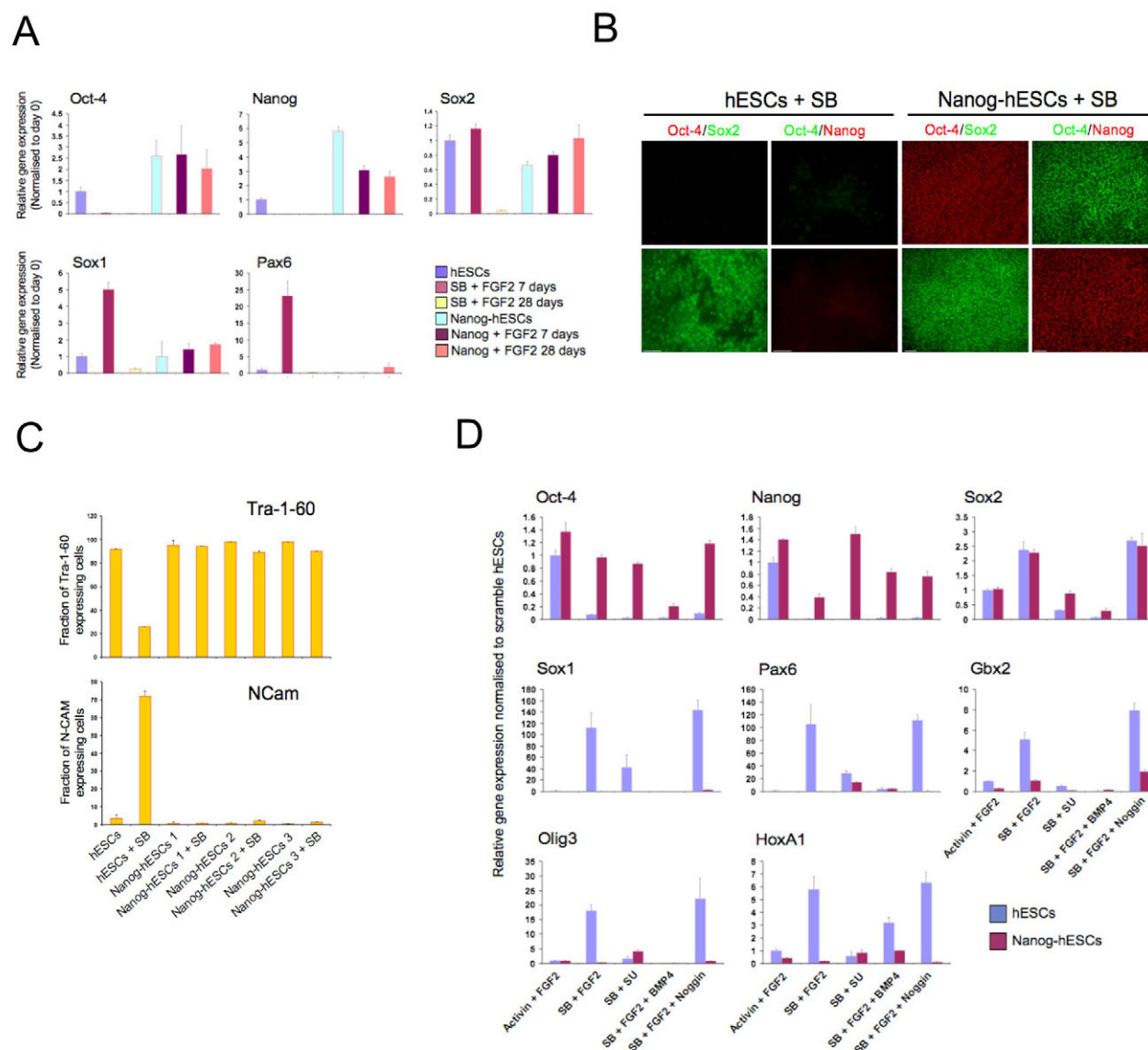


Fig. 2. Constitutive expression of Nanog is necessary and sufficient to prevent neuroectoderm differentiation of hESCs induced by inhibition of Activin/Nodal signalling. (A) Constitutive expression of NANOG blocks neuroectoderm differentiation. H9 cells (hESCs) and NANOG-hESC subline 2 (Nanog) were grown for 7 days or for 28 days in CDM supplemented with SB431542 and FGF2 and then real-time PCR was performed to detect the expression of the genes denoted. H9 cells grown in Activin and FGF2 were used as normalisation controls. (B) Immunofluorescence analysis for the co-expression of the pluripotency markers OCT4, NANOG and SOX2 in hESCs (left panels) and in NANOG-hESCs (right panels) grown for 7 days in CDM supplemented with SB431542 and FGF2. Scale bar: 50 μ m. (C) FACS analysis showing the percentage of cells expressing the pluripotency marker Tra-1-60 (upper panel) or the neuroectoderm marker NCAM (lower panel). The first column shows wild-type hESCs cultured in conditions maintaining pluripotency (CDM supplemented with Activin and FGF2), and the second column in neuroectoderm-inducing conditions (CDM supplemented with SB431542 and FGF2). The subsequent six columns show NANOG-hESCs (sublines 1, 2 and 3) grown either in pluripotency-maintaining or neuroectoderm-inducing (SB) conditions. (D) Neuroectoderm differentiation depends on FGF signalling, and the Nanog inhibitory effect is independent of BMP4 signalling. H9 cells (hESCs) and NANOG-hESC subline 2 (Nanog-hESCs) were grown for 7 days in CDM supplemented with SB431542 and FGF2; 10 μ M SB431542 and 10 μ M SU5402; 10 μ M SB431542, 12 ng/ml FGF2 and 10 ng/ml BMP4; or 10 μ M SB431542, 12 ng/ml FGF2 and 200 ng/ml Noggin. Real-time PCR was then performed to detect the expression of the genes denoted. H9 cells grown in CDM supplemented with Activin and FGF2 were used as normalisation controls.

Constitutive expression of Nanog blocks neuroectoderm differentiation induced by the inhibition of Activin/Nodal signalling in hESCs and in mEpiSCs

To further understand the relationship between Activin/Nodal signalling and *Nanog* transcription, we generated hESCs and mEpiSCs constitutively expressing NANOG (NANOG-hESCs and

Nanog-EpiSCs) (see Fig. S2A-C in the supplementary material). We then analysed the effect of Nanog overexpression when Activin/Nodal signalling was pharmacologically inhibited for 7 days. Under these conditions, wild-type cells differentiate into nearly homogenous populations of neuroectoderm progenitors (Smith et al., 2008) expressing *SOX1*, *PAX6*, *SOX2*, *HOXA1*, *GBX2* and *NCAM* (Fig. 2A,B; Fig. S2E in the supplementary material; our

unpublished results). By contrast, NANOG-hESCs grown under the same conditions did not undergo neuroectoderm differentiation, as shown by the expression of the pluripotency markers *OCT4* and *TRA-1-60*, and by the absence of *NCAM*, *SOX1*, *PAX6*, *HOXA1* and *GBX2* expression (Fig. 2A-C; see also Fig. S2E in the supplementary material). In addition, *FGF5* was not detected in NANOG-hESCs grown in the absence of Activin/Nodal signalling, thereby excluding the possibility that NANOG could block the differentiation of hESCs at a primitive ectoderm-like stage in these culture conditions (Fig. S2E in the supplementary material) (Darr et al., 2006). Interestingly, NANOG-overexpressing cells could be grown for prolonged periods in the presence of SB431542 while maintaining the expression of pluripotency markers and the absence of *SOX1* and *PAX6* (Fig. 2A). These findings show that NANOG overexpression was sufficient to substitute for Activin/Nodal signalling in the maintenance of hESC pluripotency. Similar results were obtained with Nanog-overexpressing mEpiSCs (see Fig. S3A,B in the supplementary material). These results were also confirmed by growing hESCs as embryoid bodies (EBs), showing that NANOG inhibited neural development regardless of the culture conditions used to induce differentiation (Fig. S4A-C in the supplementary material). Finally, overexpression of *OCT4* or *SOX2* in hESCs was unable to prevent the neuroectoderm differentiation of hESCs grown in the presence of SB431542 (data not shown), demonstrating that the ability to inhibit hESC differentiation was unique to Nanog and was not shared by other components of the core pluripotency transcriptional circuit. Taken together, these results indicate that among the effectors of pluripotency downstream of Activin/Nodal signalling, Nanog expression is sufficient to block neuroectoderm differentiation in both hESCs and mEpiSCs.

Knockdown of Nanog expression in hESCs results in an induction of neuroectoderm marker expression

The neural differentiation caused by inhibiting Activin/Nodal signalling in hESCs (Smith et al., 2008) led us to determine whether this effect was related to NANOG. Accordingly, we knocked down NANOG in hESCs by stably expressing a short hairpin RNA (shRNA) directed against *NANOG* transcripts (see Materials and methods). Immunostaining and real-time PCR analyses showed that NANOG expression was reduced by 90% (Fig. 3A-C), which represents a decrease in NANOG expression similar to that induced by the inhibition of Activin/Nodal signalling in hESCs (see Fig. 1B). *OCT4* expression was also decreased by 60%, whereas *SOX2* expression was maintained at normal hESC levels (Fig. 3C). Importantly, shNanog-hESCs were able to proliferate almost indefinitely in CDM supplemented with Activin and FGF2 while maintaining a low level of *OCT4* expression (Fig. 3D,E), confirming that Nanog is not necessary for self renewal (Chambers et al., 2007). The analysis of markers for extraembryonic tissues and for each of the three primary germ layers showed that the decrease in NANOG expression did not induce increases in primitive endoderm (*SOX7*, *GATA6*), trophoblast (*HAND1*, *omesodermin*) or mesoderm (brachyury, *omesodermin*, *MIXL1*, *SOX17*) markers (Fig. 3C). Notably, these experiments were performed in CDM containing Activin and FGF2, which does not possess any BMP-like activity (Vallier et al., 2005) that could interfere with the outcome of Nanog knockdown or could induce extraembryonic tissue differentiation. In striking contrast, NANOG knockdown increased the expression of neuroectoderm markers (*SOX1*, *SIX1*, *GBX2*, *OLIG3*, *HOXA1*; see Fig. 3C-E), showing that NANOG plays a key role in preventing neuroectoderm differentiation of hESCs. Importantly, NANOG

knockdown in hESCs resulted in increases in neuroectoderm marker expression (*GBX2*, *HOXA1* and *SOX1*) that were similar to those observed following pharmacological inhibition of Activin/Nodal signalling (Fig. 2A; Fig. S2E in the supplementary material). Taken together, these observations indicate that NANOG is responsible for inhibiting the neuroectoderm differentiation of hESCs, and that it mediates the effects of Activin/Nodal signalling in achieving this important element of pluripotency.

Nanog blocks the expression of neuroectoderm markers induced by FGF2 through mechanisms independent of BMP signalling

FGF signalling has been shown to be necessary for inducing neuroectoderm specification in amphibian and chick embryos (Stern, 2005), whereas BMP signalling is known to inhibit the same differentiation (Munoz-Sanjuan and Brivanlou, 2002). In principle, Nanog could block neuroectoderm differentiation by interfering with an FGF inductive effect or by enhancing a BMP inhibitory effect. To distinguish between these two possibilities, wild-type hESCs and Nanog-overexpressing hESCs were grown in CDM supplemented with SB431542 and FGF2, with SB431542 and SU5402 (a chemical inhibitor of FGF receptors), with SB431542, FGF2 and BMP4, or with SB431542, FGF2 and Noggin (an inhibitor of BMPs; see Fig. 2D). The inhibition of FGF signalling resulted in a decrease in *GBX2*, *SOX2*, *SOX1*, *PAX6*, *OLIG3* and *HOXA1* expression in wild-type cells grown in the presence of SB431542, confirming that FGF signalling is necessary for the neuroectoderm specification of hESCs (Fig. 2D). Importantly, addition of BMP4 completely inhibited the expression of neuroectoderm markers (Fig. 2D, data not shown), confirming the inhibitory effect of BMP signalling on neuroectoderm specification. However, the presence of BMP4 did not maintain pluripotency (as shown by the decrease in *OCT4* expression, see Fig. 2D), but instead drove the differentiation of wild-type hESCs and NANOG-hESCs into extraembryonic tissues (see below). These results demonstrate that BMP signalling is capable of blocking the neuroectoderm specification of hESCs but that this occurs only by promoting differentiation along the extraembryonic pathway. Moreover, the inhibition of BMPs using Noggin did not induce the expression of neuroectoderm markers in NANOG-hESCs (Fig. 2D) grown in the absence of Activin/Nodal signalling. These observations exclude the possibility that Nanog can block neuroectoderm differentiation through mechanisms involving BMP signalling. We then determined whether the induction of neuroectoderm markers induced by the knockdown of NANOG in hESCs was dependent on FGF signalling by growing shNanog-hESCs in CDM containing Activin and SU5402. The inhibition of FGF signalling strongly decreased the expression of *SOX2*, *GBX2*, *SOX1*, *PAX6*, *HOXA1* and *OLIG3* (Fig. 3E), confirming that FGF signalling is necessary for the expression of these neuroectoderm markers. However, the inhibition of FGF signalling did not restore pluripotency markers (Fig. 3E), confirming that FGF signalling is also involved in mechanisms controlling the pluripotency of hESCs. Taken together, these results demonstrate that Nanog is necessary to block the expression of neuroectoderm markers induced by FGF signalling, which is also necessary for hESC self renewal.

Constitutive expression of Nanog is unable to prevent extraembryonic differentiation

Genetic studies in the mouse have shown that the function of Nanog in mESCs and in the pre-implantation embryo is to block extraembryonic endoderm differentiation (Mitsui et al., 2003). To

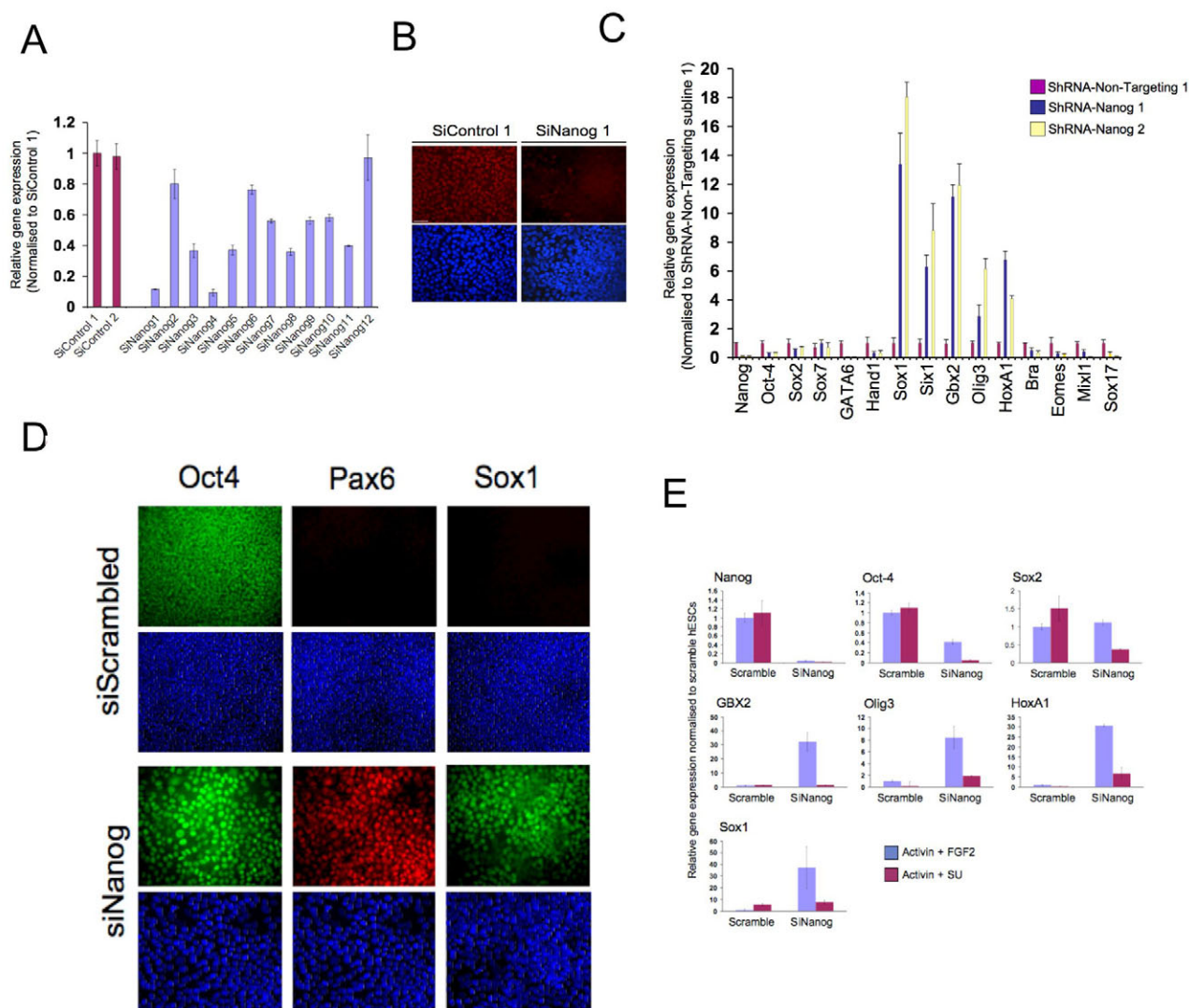


Fig. 3. Knockdown of NANOG expression in hESCs induces the expression of neuroectoderm markers. (A) Expression of NANOG in shRNA-NANOG-hESC sublines. shRNA-NANOG-hESCs (SiNanog) were grown for two passages in CDM supplemented with Activin and FGF and then real-time PCR analyses were performed to determine the level of Nanog transcripts. hESC lines expressing a scrambled shRNA were used as negative controls (SiControl). (B) Absence of NANOG protein in hESCs stably expressing shRNA directed against *NANOG* mRNA sequence (shRNA-treated, top; DAPI-stained nuclei, bottom). Scale bar: 100 μ m. (C) Knockdown of *NANOG* mRNA increased the expression of neuroectoderm markers. The expression of the genes denoted was analysed in shRNA-NANOG-hESCs using real-time PCR. (D) Expression of neuroectoderm markers in shRNA-NANOG-hESCs. shRNA-NANOG-hESCs (SiNanog) were grown for 10 passages in CDM supplemented with Activin and FGF and then immunostaining analyses were performed to detect the expression of OCT4, PAX6 and SOX1 proteins. Scale bar: 100 μ m. (E) The expression of neuroectoderm markers provoked by NANOG knockdown depends on FGF signalling. Control H9 shRNA-hESCs (Scramble) and shRNA-NANOG-hESC subline 1 (SiNanog) were grown for 7 days in CDM supplemented with Activin and FGF2, or with Activin and 10 μ M SU5402, and then real-time PCR was performed to detect the expression of the genes denoted.

examine whether this function was conserved in hESCs, we analysed the effect of NANOG overexpression on extraembryonic differentiation induced by BMP4 (Xu et al., 2002) (our unpublished results). After 7 days of culture in the presence of BMP4, NANOG-hESCs adopted a homogeneous, broad cellular morphology typical of extraembryonic differentiation induced in these conditions (data not shown). BMP4-treated NANOG-hESCs became OCT4 negative (Fig. 4A,B; see also Fig. S5A-C in the supplementary material), indicating that Nanog was not sufficient to maintain pluripotency in the presence of BMP4. These observations were confirmed by FACS analyses determining the proportion of undifferentiated hESCs in culture before and after BMP4 treatment. In control

conditions, 90% of both wild-type and NANOG-hESCs were positive for the pluripotency marker TRA-1-60 (see Fig. S5B in the supplementary material), whereas after 7 days of BMP4 treatment only 25-35% of either wild-type or NANOG-hESCs remained positive for TRA-1-60 (Fig. S5B in the supplementary material). In addition, expression of markers for primitive endoderm (*SOX7*, *GATA4*, *GATA6*, *AFP*, *H19*) and trophectoderm (*CDX2*, *EOMES*, *HAND1*, *ahCG*) could be detected in Nanog-expressing cells, as well as in wild-type cells (Fig. 4B, see also Fig. S5C in the supplementary material). Immunostaining analyses showed that NANOG and these markers (*CDX2*, *EOMES*, *GATA4*, *GATA6*) were co-expressed in the same cells (Fig. 4A), further demonstrating

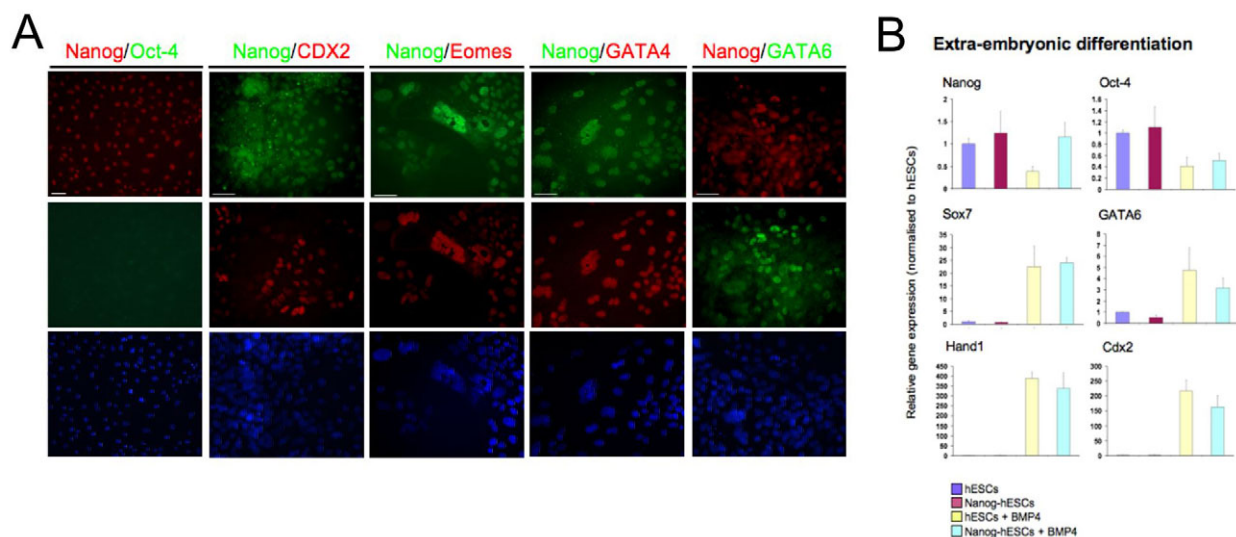


Fig. 4. Nanog is not sufficient to prevent BMP4-induced extraembryonic differentiation. (A) Immunofluorescence analysis for the co-expression of Nanog (top row) and OCT4 or extraembryonic markers (middle row) in NANOG-hESCs grown in the presence of BMP4. Bottom row shows DAPI-stained nuclei. Scale bar: 50 μ m. (B) Expression of extraembryonic markers in wild-type and NANOG-hESCs grown in the presence of BMP4. H9 cells (hESCs) and NANOG-hESCs (subline 11) were grown for 4 days in culture conditions maintaining pluripotency or for 8 days in the presence of BMP4 to induce extraembryonic differentiation. Then real-time PCR was performed to detect the expression of the genes denoted.

that Nanog did not block extraembryonic differentiation. Importantly, NANOG-hESCs also differentiated into cells expressing extraembryonic markers when induced to form EBs in a medium containing FBS (see Fig. S6A,B in the supplementary material). The extraembryonic outcome was thus independent of the culture system used to induce the differentiation of NANOG-hESCs. Finally, similar results were obtained with Nanog-mEpiSCs grown in CDM in the presence of BMP4 (see Fig. S5D,E in the supplementary material). Taken together, these results show that Nanog functions specifically to prevent neuroectoderm differentiation, rather than acting as a general blocker of differentiation.

Constitutive expression of Nanog allows mesendoderm specification but not further progression of endoderm differentiation

We then analysed the effect of NANOG overexpression on mesendoderm/endoderm differentiation by using an approach that mimics *in vivo* development (our unpublished results). The first step of this protocol induces the differentiation of hESCs into mesendoderm cells expressing brachyury, Mixl1 and eomesodermin, and the second step drives the differentiation of these progenitors into definitive endoderm cells that nearly homogeneously (~90% of cells) express SOX17, CXCR4, MIXL1 and GSC (Fig. 5A-C; data not shown). Gene expression profiling analysis of hESCs cultured under these conditions confirms that they express a large number of known definitive endoderm markers, including *GSC*, *LIM1*, *GATA4*, *GATA6* and *FOXA2*, whereas the expression of extraembryonic tissue markers (*SOX7*, *AFP*) and neuroectoderm markers (*SOX1*, *SOX2*) cannot be detected (data not shown). hESCs constitutively expressing NANOG that were grown in these conditions maintained the expression of OCT4 at levels similar to those in pluripotent cells while showing a limited induction of expression of endoderm markers (GSC, MIXL1, SOX17; see Fig. 5A,B). In addition, FACS analysis revealed that 90% of the wild-type cells expressed the definitive endoderm

marker CXCR4 after differentiation compared with only 30% of the NANOG-expressing cells, suggesting that constitutive expression of NANOG could deter endoderm differentiation (Fig. 5C). However, the expression of SOX2 was strongly diminished in NANOG-hESCs subject to this protocol, whereas expression of the mesendoderm markers brachyury and eomesodermin was induced (Fig. 5B). Immunostaining analyses confirmed that NANOG protein was systematically co-expressed in the same cells as brachyury (Fig. 5A), indicating that NANOG did not prevent the onset of expression of mesendoderm markers but suggesting that it was able to interfere with further progression to definitive endoderm. This interpretation is not contradicted by the maintenance of OCT4 expression in NANOG-hESCs, as Oct4 (like Nanog) is also expressed in the mesendoderm of gastrulating mouse embryos (Hart et al., 2004). Furthermore, permissiveness for mesendoderm but not endoderm differentiation in NANOG-hESCs is also reinforced by the induction of the mesendoderm marker PDGFR α and by the relatively low number of NANOG cells expressing CXCR4 (Fig. 5C). Finally, similar results were observed when Nanog-mEpiSCs were grown in endoderm-inducing culture conditions (see Fig. S7A,B). Taken together, these results indicate that rather than preventing mesendoderm specification in hESCs and mEpiSCs, Nanog limits the progression of mesendoderm progenitors towards definitive endoderm cells.

Nanog binds Smad2/3 in hESCs and modulates activity of the Activin/Nodal signalling pathway

Studies in amphibians and in mice have shown that high activity of Activin/Nodal signalling is necessary to specify the endoderm germ layer (Dunn et al., 2004). In addition, BMP signalling has been shown to be essential for mesendoderm specification (Davis et al., 2004; Fujiwara et al., 2002). Therefore, the effect of Nanog on mesendoderm progression could involve modulating the activity of these signalling pathways. To address this hypothesis, NANOG-overexpressing cells were grown in culture conditions inductive for endoderm differentiation in the presence of increasing doses of

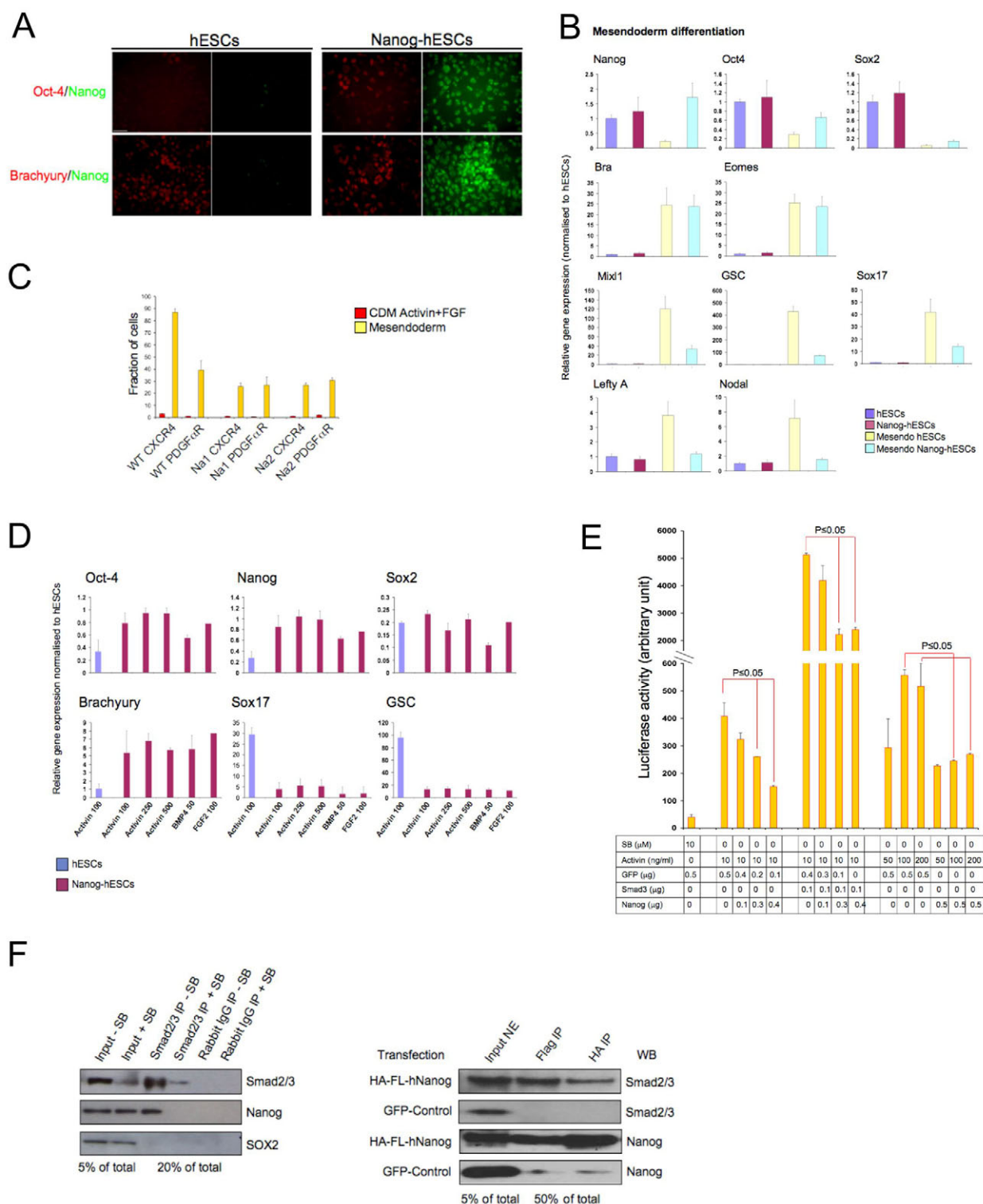


Fig. 5. See next page for legend.

Activin, BMP or FGF2 (Fig. 5D). A high dose of Activin, BMP or FGF was not sufficient to restore normal levels of endoderm markers (*SOX17*, *GSC*) in NANOG-hESCs, while expression of the mesendoderm marker brachyury was maintained in all these conditions (Fig. 5D). These observations show that an increase in extracellular factors cannot bypass the inhibitory effect of Nanog, and thus that Nanog could interfere directly with intracellular components of the Activin or BMP pathways. Interestingly, Nanog

has been shown to interact directly with Smad1 to modulate mouse ESC differentiation (Suzuki et al., 2006). However, we have been unable to detect any direct interaction between Smad1 and Nanog in hESCs (data not shown), and we have shown that Nanog overexpression does not block the inductive effects of BMP extraembryonic differentiation. Therefore Nanog is unlikely to effectively regulate hESC or mEpiSC differentiation through direct interaction with Smad1, as has been reported for mESCs (Suzuki et

Fig. 5. Nanog inhibits endoderm differentiation by limiting the transcriptional activity of the Activin/Nodal signalling pathway.

(A) Nanog expression is compatible with mesendoderm specification. Immunofluorescence analysis for the co-expression of NANOG and the mesendoderm marker brachyury in hESCs and in NANOG-hESCs grown in culture conditions inducing mesendoderm differentiation. Scale bar: 50 μm . (B) Expression of mesendoderm markers in wild-type and NANOG-hESCs differentiated into mesendoderm-like cells. H9 cells (hESCs) and NANOG-hESCs (subline 11) were grown for 4 days in culture conditions maintaining pluripotency or for 8 days in culture conditions inducing mesendoderm differentiation. Then, real-time PCR was performed to detect the expression of the genes denoted. H9 cells grown in CDM supplemented with Activin and FGF2 were used as normalisation controls. (C) FACS analysis showing the percentage of hESCs expressing the definitive endoderm marker CXCR4 and the mesendoderm/mesoderm marker PDGFR α . H9 cells and NANOG-hESCs (sublines 1, 2) were grown for 7 days in culture conditions driving the differentiation of hESCs into mesendoderm progenitors and then the expression of CXCR4 and PDGFR α was analysed using FACS. hESCs and NANOG-hESCs grown in CDM supplemented with Activin and FGF2 were used as negative controls. (D) A high dose of Activin, BMP or FGF is not sufficient to bypass the inhibitory effect of Nanog on endoderm differentiation. H9 cells (hESCs) and NANOG-hESCs (subline 11) were grown for 4 days in culture conditions maintaining pluripotency or for 8 days in culture conditions inducing mesendoderm differentiation with increasing doses of Activin (100 ng/ml, 250 ng/ml, 500 ng/ml), BMP (10 ng/ml, 50 ng/ml) and FGF2 (20 ng/ml, 100 ng/ml). Then, real-time PCR was performed to detect the expression of the genes denoted. H9 cells grown in CDM supplemented with Activin and FGF2 were used as normalisation controls. (E) Effect of Nanog on Smad transcriptional activity. A reporter gene for the transcriptional activity of Activin/Nodal signalling (containing four Smad-binding elements, SBE4) was co-transfected into H9 cells, in CDM supplemented with Activin or in CDM supplemented with SB431542, along with the renilla expression vector and with the expression vectors listed below the chart. Firefly luciferase activity (normalised to renilla luciferase activity) is expressed as mean \pm s.d. from three independent experiments. (F) Nanog protein interacts with Smad2/3. Co-immunoprecipitation of endogenous NANOG (hNanog) with SMAD2/3 (left panel) and of transfected HA-Flag-hNanog with Smad2/3 (right panels). Immunoprecipitations (IPs) were performed on nuclear extracts of hESCs grown in CDM supplemented with Activin and FGF2, or in the presence of SB431542. Input nuclear extracts (Input NE) and IP lanes were probed with the indicated antibodies. Loading percentage of the total material is indicated.

al., 2006). However, Nanog protein itself shares homology with the common (C-) Smad4 protein (Hart et al., 2004) and thus Nanog might interact with Smad2/3. To test this hypothesis, the SBE4 luciferase reporter (Jonk et al., 1998) for Activin/Nodal-induced, Smad2/3-mediated signalling was transfected into hESCs (with or without SMAD2/3 or/and NANOG expression vectors) in the presence of increasing doses of Activin. This analysis showed that overexpression of NANOG decreased (but did not abolish) the transcriptional activity associated with increasing doses of Activin and with increased levels of SMAD2 (data not shown) or SMAD3 (Fig. 5E). This result suggests that NANOG overexpression is able to modulate the Activin/Nodal signalling pathway in hESCs. Interestingly, a similar Nanog-mediated modulation of Smad3-induced transcriptional activity was observed in mEpiSCs (see Fig. S8A in the supplementary material). Together, these observations

suggest the existence of a negative-feedback loop in which Activin/Nodal-induced Nanog limits the transcriptional activity induced by Activin/Nodal signalling. To understand the mechanism of this interaction, we performed co-immunoprecipitation of NANOG or SMAD2/3, followed by western blot analyses. These revealed that the protein complexes containing NANOG also contained SMAD2/3 proteins (Fig. 3F). Importantly, these experiments were performed on nuclear extracts, and addition of SB431542 to the medium blocked the Smad2/3-Nanog interaction. This suggests that Nanog-complexed Smad2/3 must be phosphorylated and thus located in the nucleus, where it could affect the transcription of target genes. Similar Smad2/3-Nanog interactions were observed in mEpiSCs (see Fig. S8B in the supplementary material), suggesting that they are evolutionarily conserved. Finally, ChIP analyses showed that NANOG and SMAD2/3 proteins were capable of binding to the same genomic region in four different target genes of Activin/Nodal signalling, including *LEFTYA*, *SMAD7*, *SnoN* (*SKIL* – Human Gene Nomenclature Database) and *NANOG* itself (Fig. 1D, see also Fig. S1H in the supplementary material, data not shown), suggesting that the protein complexes containing NANOG and SMAD2/3 could regulate the promoters of target genes in hESCs. Interestingly, constitutive expression of NANOG during mesendoderm differentiation of hESCs limits the upregulation of known Smad2/3 target genes (*LEFTYA* and *NODAL*) to levels equivalent to those in hESCs (Fig. 5B). Consequently, the negative-feedback loop involving Activin/Nodal-mediated Nanog expression could act by limiting, but not entirely blocking, the transcriptional activity of Smad2/3.

DISCUSSION

The results presented here demonstrate that the Activin/Nodal signalling pathway directly controls the expression of Nanog in hESCs and in mEpiSCs, and thereby blocks their differentiation towards the neuroectoderm pathway. In turn, Nanog interacts directly with the Smad2/3 proteins to modulate their transcriptional activity. In this way, Nanog also blocks the progression of mesendoderm differentiation towards endoderm. This negative-feedback loop (Fig. 6) enforces stasis in both neuroectoderm and mesendoderm differentiation, resulting in the pluripotency of hESCs and mEpiSCs. This model represents a first step towards understanding the mechanisms by which Activin/Nodal signalling maintains the pluripotent status of hESCs and mEpiSCs, and also of pluripotent cells in the post-implantation mouse embryo. It also raises several questions concerning the mechanisms controlling early cell-fate specification. Indeed, although our results reveal the mechanisms by which Activin/Nodal signalling maintains pluripotency (i.e. by the direct control of Nanog expression), they do not explain how Activin/Nodal signalling can both maintain pluripotency and induce differentiation towards mesendoderm. However, our recent studies (our unpublished results) have demonstrated that BMP4 in combination with Activin is sufficient to drive the differentiation of hESCs into mesendoderm and then into endoderm in a chemically defined medium, suggesting that BMP4 is capable of converting Activin/Nodal signalling into an inductive signal for differentiation. Further studies will be necessary to understand the molecular interactions between these two signalling pathways during early cell-fate specification. In addition, a decrease in Nanog expression in the presence of Activin/Nodal signalling might be expected to result in mesendoderm differentiation. However, knockdown of Nanog

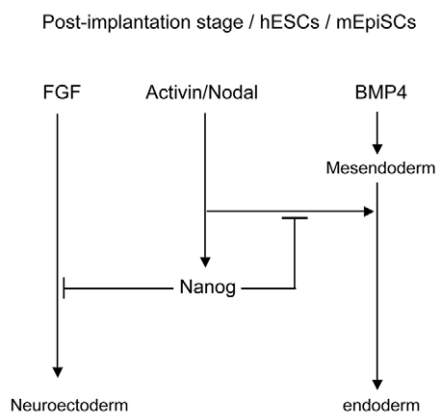


Fig. 6. Model explaining the regulation of Nanog in hESCs/mEpiSCs and its function in both cell types. Nanog expression in hESCs, mEpiSCs and post-implantation embryos is controlled by Activin/Nodal signalling and, in turn, Nanog prevents neuroectoderm differentiation induced by FGF signalling. However, Nanog also decreases the transcriptional activity of Smad2/3 proteins to limit the positive effect of the Activin/Nodal signalling on the progression of mesendoderm differentiation towards definitive endoderm. This feedback loop generates stasis of both neuroectoderm and mesendoderm differentiation, resulting in pluripotency of hESCs and mEpiSCs.

instead increases the expression of neuroectoderm markers, suggesting that other proteins besides Nanog block the positive effect of the Activin/Nodal signalling pathway on mesendoderm differentiation.

The effect of Nanog knock down on hESC pluripotency reveals a mechanism distinct to that described for mESCs by Chambers and colleagues (Chambers et al., 2007). Their work showed that suppression of Nanog expression was not sufficient to induce full differentiation of mESCs, and suggested that Nanog acts to limit the probability of differentiation into multiple lineages induced by extracellular signals. Our results showing that Nanog blocks neuroectoderm differentiation in hESCs and mEpiSCs indicate a more lineage-specific role for Nanog than that in mESCs. Indeed, Nanog overexpression blocked neither mesendoderm differentiation nor BMP-induced extraembryonic differentiation in hESCs and mEpiSCs. Interestingly, the neuroectoderm markers analysed in our study have been shown to be direct target genes of Nanog in hESCs (*SIX1*, *GBX2*, *PAX6*, *HOXA1*, *OLIG3*) (Boyer et al., 2005), suggesting that Nanog could block the activity of transcription factors downstream of FGF signalling (as it does with Smad2/3 for Activin/Nodal signalling). Together, these observations suggest that Nanog acts in hESCs by blocking differentiation induced by signalling pathways that are also necessary for hESC pluripotency and self renewal (i.e. Activin/Nodal and FGF).

Therefore, the generalised function of Nanog in pluripotent stem cells is to safeguard pluripotency against the differentiation-inducing effects of essential extracellular signals. Importantly, however, Nanog does not protect hESCs, mEpiSCs and mESCs against the same differentiative events. Indeed, gain- and loss-of-function studies have shown that Nanog primarily blocks primitive endoderm differentiation of mESCs in vitro (Hamazaki et al., 2004) and of mouse inner cell mass in vivo (Mitsui et al., 2003). Conversely, Nanog blocks neuroectoderm and definitive endoderm differentiation of hESCs and of mEpiSCs. In vivo studies reinforce

our results, as mouse embryos mutant for Nanog fail to develop beyond the late epiblast stage (Hamazaki et al., 2004), when Nanog expression in the epiblast becomes dependent on Nodal signalling (Mesnard et al., 2006; Mitsui et al., 2003). In addition, absence of Nodal expression and consequently of Nanog expression in post-implantation mouse embryos results in neuralisation of the epiblast, suggesting that Nanog acts in vivo to prevent neuroectoderm differentiation of the mouse epiblast before gastrulation (Camus et al., 2006), thus reinforcing our findings on its role in hESCs and mEpiSCs. The apparent similarity in the role of Nanog in hESCs, mEpiSCs and the pluripotent epiblast (and the dissimilarity of its role in mESCs) reinforces the hypothesis that hESCs and mESCs represent distinct stages of early mammalian development (Brons et al., 2007; Tesar et al., 2007). In this hypothesis, hESCs closely resemble pluripotent cells from post-implantation stages in vivo, in contrast to mESCs, which closely resemble pluripotent cells from the inner cell mass (Nichols et al., 2001). This hypothesis also implies that the functions of pluripotency factors change progressively during early mammalian development. Before implantation, the core pluripotency transcription factor circuit blocks formation of the extraembryonic lineages, whereas after implantation it blocks formation of the primary germ layers.

In this context, a recent study by Smith and colleagues (Ying et al., 2008) showed that general repression of differentiation signals by small molecules results in a 'ground state of pluripotency' in mouse ESCs. This model does not appear to apply to hESCs and mEpiSCs, which strictly depend on Activin/Nodal signalling to maintain the expression of Nanog and thereby to maintain their pluripotent state. Therefore, two or more distinguishable pluripotent states relying on different growth factors but on similar core transcriptional networks appear to exist in vitro and in vivo during embryonic development. Interestingly, we recently observed that human induced pluripotent stem cells rely, like hESCs, on Activin/Nodal signalling to maintain their pluripotent state (our unpublished results). Therefore, understanding the function of pluripotency factors in each of these pluripotent states will be crucial to achieving control over the differentiation of human pluripotent cells, whether derived from mammalian embryos or by inducing pluripotency in cells of somatic origin (Takahashi et al., 2007; Yu et al., 2007).

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/8/1339/DC1>

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