FGF signalling inhibits neural induction in human embryonic stem cells

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Human embryonic stem cells (hESCs) can exit the self-renewal programme, through the action of signalling molecules, at any given time and differentiate along the three germ layer lineages. We have systematically investigated the specific roles of three signalling pathways, TGF β /SMAD2, BMP/SMAD1, and FGF/ERK, in promoting the transition of hESCs into the neuroectoderm lineage. In this context, inhibition of SMAD2 and ERK signalling served to cooperatively promote exit from hESC self-renewal through the rapid downregulation of *NANOG* and *OCT4*. In contrast, inhibition of SMAD1 signalling acted to maintain *SOX2* expression and prevent non-neural differentiation via *HAND1*. Inhibition of FGF/ERK upregulated *OTX2* that subsequently induced the neuroectodermal fate determinant *PAX6*, revealing a novel role for FGF2 in indirectly repressing *PAX6* in hESCs. Combined inhibition of the three pathways hence resulted in highly efficient neuroectoderm formation within 4 days, and subsequently, FGF/ERK inhibition promoted rapid differentiation into peripheral neurons. Our study assigns a novel, biphasic role to FGF/ERK signalling in the neural induction of hESCs, which may also have utility for applications requiring the rapid and efficient generation of peripheral neurons.

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Introduction

The TGF β and BMP pathways, two evolutionary conserved signalling cascades, activate the set of transcription factors SMAD2/3 and SMAD1/5/8, respectively, which in turn either activate or repress the expression of target genes (Schmierer and Hill, 2007; Figure 1A; ‘SMAD1’ and ‘SMAD2’ hereafter). The balance between these pathways has a major impact on cell fate specification in human embryonic stem cells (hESCs; Yao *et al*, 2006; Greber *et al*, 2008; Vallier *et al*, 2009b). hESCs resemble epiblast stem cells, which are derived from pregastrulation mouse embryos, in their responsiveness to extracellular signals (Brons *et al*, 2007; Tesar *et al*, 2007). Consistent with the absence of inductive signals in the anterior mouse epiblast, neuroectoderm formation from hESCs is facilitated by the inhibition of either TGF β signalling (Smith *et al*, 2008), BMP signalling (Pera *et al*, 2004), or both (Chambers *et al*, 2009).

FGF2 is a major factor involved in the self-renewal of hESCs and has been shown to activate the ERK cascade (Kang *et al*, 2005; Figure 1A). The effects of FGF2 signalling in hESCs appear to be complex and relatively subtle. In addition to positive effects on cell survival and/or proliferation, FGF2 signalling sustains expression of the pluripotency controlling gene *NANOG* (Greber *et al*, 2010; Yu *et al*, 2011), in collaboration with TGF β /SMAD2 signalling (Greber *et al*, 2008; Xu *et al*, 2008; Vallier *et al*, 2009a). On the other hand, FGF2 signalling antagonizes BMP signalling, a major (non-neural) differentiation-inducing pathway (Xu *et al*, 2005, 2008; Greber *et al*, 2007; Peerani *et al*, 2007). FGF2 signalling has also been implicated in neuroectoderm formation from hESCs, and from the related mouse epiblast stem cells, but whether it bears a positive or negative effect remains controversial: on the one hand, some studies reported negative effects on neural commitment when inactivating FGF signalling using pharmacological inhibitors (LaVaute *et al*, 2009; Vallier *et al*, 2009b; Cohen *et al*, 2010; Na *et al*, 2010). On the other hand, data by others suggest that neuroectodermal gene expression in hESCs can be induced by withdrawing FGF2 from the culture medium (Greber *et al*, 2008; Chambers *et al*, 2009; Rosa and Brivanlou, 2011). Here, we seek to clarify these seeming discrepancies by systematically investigating the interplay of the three pathways in neural induction of hESCs, with an emphasis on the role played by FGF signalling in this context.

Results

Combined TGF β /SMAD2 and FGF/ERK inhibition promotes rapid exit from self-renewal

We sought to systematically investigate the specific roles of the three key pathways by using recombinant proteins and

Figure 1 Inhibition of TGFβ/SMAD2 and FGF/ERK pathways cooperatively reduces hESC-specific gene expression. **(A)** Simplified scheme of signalling pathways. Red colour denotes pathway inhibition. Small molecule inhibitors are PD0325901 (PD), SB431542 (SB), and dorsomorphin (DM). **(B)** Design of microarray time-course experiment. Four time series of hESCs treated in the indicated ways were monitored after 0 h, 6 h, 1, 2, 3, 4, and 6 days. **(C)** Array expression data for selected pluripotency genes in hESC line HuES6. **(D)** Immunostains for NANOG and OCT4 in hESC line NCL3. Note that FGF2 SB NOG treatment did largely but not fully diminish the expression of NANOG and OCT4 in all cells but additional FGF/ERK inhibition did. **(E)** RT-qPCR analysis in two hESC lines. Note the cooperation between SB and PD treatment in downregulating *NANOG* and *OCT4* expression.

pharmacological inhibitors to selectively activate or repress their activity (Figure 1A). Four types of treatment conditions were designed to monitor the effects of the individual cascades on global gene expression over a period of 6 days (Figure 1B). As a baseline, hESCs were treated with FGF2 and the BMP antagonist Noggin (FGF2 NOG) in chemically defined medium, a condition that has been reported to promote self-renewal (Xu *et al*, 2005). A comparison between FGF2 NOG and FGF2 SB NOG-treated cells is expected to reveal the transcriptional response of SMAD2 inactivation, whereas a comparison of FGF2 SB NOG with PD SB NOG treatment would reveal the effects of the FGF/ERK cascade. Finally, a comparison of PD SB NOG with PD SB treatment should help to clarify the role of BMP inactivation (Figure 1A and B).

A prerequisite for hESC differentiation is the shutdown of the self-renewal machinery. Analysis of the array data confirmed that FGF2 NOG treatment largely sustained the expression of pluripotency markers. A decline in the expression of certain markers was observed towards the end of the time course, which could have been due to the near confluency of the cultures by day 6. In contrast, additional supplementation with SB led to a strong decline in the expression of self-renewal markers over the course of 6 days, indicating loss of hESC identity (Figure 1C). *NANOG* downregulation occurred very rapidly, consistent with the finding that it is a direct target of SMAD2. It is therefore likely that the expression of other members of the hESC self-renewal network, such as *OCT4*, were affected by the downregulation of *NANOG*. Interestingly, expression of *SOX2*, which is

required not only by undifferentiated hESCs but also by neuroectoderm, was maintained with FGF2 SB NOG treatment (Figure 1C). This configuration—low *NANOG* and *OCT4* but high *SOX2* levels—is compatible with neuroectoderm formation. However, a time point-by-time point comparison of the two conditions revealed that the expression of only a few genes was induced by SB treatment (Supplementary Table S1). Based on this observation, we conclude that inactivation of TGF β /SMAD2 signalling is effective in downregulating the expression of hESC-specific genes, but that in the presence of FGF2 and NOG, inhibition of TGF β /SMAD2 signalling is not sufficient for activating the expression of prominent differentiation-associated markers.

Consistent with these negative effects of SB treatment on hESC self-renewal, immunocytochemistry analysis revealed that most—albeit not all—cells stained negative for *NANOG* and *OCT4* after 4 days in culture (Figure 1D, top). Like TGF β /SMAD2 signalling, FGF/ERK signalling has been suggested to also sustain *NANOG* expression in hESCs (Greber *et al*, 2010; Yu *et al*, 2011). We therefore compared the downregulation kinetics of hESC markers in two treatment conditions: FGF2 SB NOG and PD SB NOG. More effective downregulation of prominent hESC markers was found with PD SB NOG compared with FGF2 SB NOG. An 80% downregulation in *NANOG* mRNA levels was seen within 6 h of treatment with PD SB NOG; *NANOG* and *OCT4* mRNA levels were virtually undetectable by day 4 (Supplementary Figure S1; Figure 1D, bottom). The cooperativity between inactivation of SMAD2 and ERK signalling was confirmed by RT-qPCR in two hESC lines—HuES6 and NCL3—on day 4 (Figure 1E). We therefore conclude that inhibition of both TGF β /SMAD2 and FGF/ERK signalling produces the most prominent downregulation of hESC-specific gene expression (Supplementary Figure S1).

FGF/ERK inhibition specifically induces *PAX6*

Next, we investigated the induction of neuroectodermal markers with an emphasis on *PAX6* expression (Zhang *et al*, 2010). A time-course comparison revealed a pronounced upregulation of *PAX6* in the PD SB and PD SB NOG-treated samples, but not in the FGF2 NOG or FGF2 SB NOG-treated samples, suggesting the presence of a strong association between FGF/ERK inhibition and *PAX6* induction (Figure 2A). This result was confirmed by RT-qPCR of samples that had been exposed to different degrees of FGF/ERK signalling activity (as well as using alternative pharmacological inhibitors or PD in the presence of FGF2; Supplementary Figure S2A). These data also revealed that inhibition of the FGF/ERK cascade was superior in inducing *PAX6* expression over simply withdrawing FGF2 from the culture medium (Figure 2B). Immunocytochemistry analysis confirmed the suppression of *PAX6* induction by FGF2, whereas many—albeit, by far, not all—cells grown in the absence of FGF2 or with FGF/ERK inhibition showed pronounced *PAX6* staining (Figure 2C). These data strongly suggest that FGF2/ERK signalling specifically represses *PAX6* expression in hESCs. This effect of FGF2/ERK signalling must be independent of its *NANOG*-maintaining function, as SB treatment alone was effective in reducing *NANOG* expression, but in the presence of FGF2, SMAD2 inhibition was not sufficient in causing strong *PAX6* upregulation.

BMP/SMAD1 inhibition enhances the specificity of neuroectodermal induction

We next sought to assess the effect of BMP/SMAD1 suppression. Global time-course analysis suggested a high overall similarity in gene expression changes between the PD SB and PD SB NOG-treated samples. 3D principal component analysis, however, suggested differences in a subset of genes (Figure 2D). Strikingly, *SOX2* expression was not well maintained with PD SB treatment, whereas *HAND1*, encoding a basic helix-loop-helix transcription factor involved in extra-embryonic as well as mesodermal development, was strongly induced in the absence of NOG (Figure 2E). These effects are reminiscent of the activation of BMP signalling in hESCs (Greber *et al*, 2008). The rapid suppression of *SOX2* expression concomitant with the induction of *HAND1* expression by BMP4 stimulation was confirmed using an independent hESC line (Supplementary Figure S2B). We hypothesized that FGF/ERK suppression has ambivalent effects on neuroectodermal induction: on the one hand, it leads to *NANOG* downregulation as well *PAX6* induction (productive), while on the other hand, it activates autocrine BMP/SMAD1 signalling (Greber *et al*, 2007; Peerani *et al*, 2007), which may induce non-neural differentiation (Li *et al*, 2007) (counterproductive). Consistent with this, *HAND1*, a BMP/SMAD1 target, became induced within only 6 h of FGF/ERK inactivation; additional NOG supplementation was able to rescue this effect (Figure 2F). Hence, FGF/ERK inhibition may cause differentiation into different types of lineages—neuroectodermal or extraembryonic/mesodermal. Not surprisingly, hESCs treated with PD or PD SB stained heterogeneously positive for both *PAX6* and *HAND1*. However, addition of NOG suppressed the induction of *HAND1* in favour of *PAX6* expression (Figure 2G). Based on these data, we conclude that the addition of NOG is required to antagonize the BMP/SMAD1 activating effect of FGF/ERK inhibition. Hence, in this context, NOG acts as a specificity enhancer for neuroectodermal commitment, consistent with its role in model organisms. This interpretation was confirmed by substituting NOG with a small molecule inhibitor of BMP/SMAD1 signalling, dorsomorphin (Yu *et al*, 2008), yielding pronounced *PAX6* expression in 80–90% of all cells by day 4 (Figure 2H; Supplementary Figure S2C and D).

FGF/ERK inactivation upregulates *OTX2* that acts as a positive regulator of *PAX6*

The above data suggest that FGF/ERK inactivation is the key trigger for *PAX6* induction in hESCs, which could imply that concomitant inactivation of TGF β /SMAD2 is dispensable. Indeed, by day 6, FGF plus BMP inactivation was almost as effective in inducing *PAX6* as triple pathway inhibition (Supplementary Figure S3A and B). Notably, however, *PAX6* induction started only once *NANOG* and *OCT4* were almost completely downregulated. *NANOG* and *OCT4* have been found to bind to the *PAX6* promoter in hESCs, and we confirmed these data using ChIP-qPCR (Figure 3A; Boyer *et al*, 2005). Next, we sought to functionally assess whether the two factors indeed repress *PAX6* induction. Following stable overexpression and 4 days of triple pathway inhibition, immunocytochemical analysis revealed that *NANOG*-overexpressing cells were largely—and *OCT4*-overexpressing cells completely—non-overlapping with transgene-negative/*PAX6*-positive cells (Figure 3B). These observations strongly

Figure 2 FGF/ERK inhibition induces *PAX6* and *HAND1* expression. (A) Time-course array data. Note that *PAX6* expression became induced only upon inhibition of the FGF/ERK cascade. (B) RT-qPCR analysis of *PAX6* expression in two hESC lines (HuES6 and NCL3; $n = 2$). Note the strong negative correlation between *PAX6* induction and FGF/ERK activity (* $P < 0.05$; ** $P < 0.01$). (Right) Western blot confirming inactivation of ERK in the presence of PD. (C) Immunostain confirming the negative correlation between *PAX6* induction and FGF/ERK signalling. Additional blockage of TGF β signalling did not have any obvious effect (line NCL3, similar results were obtained with line HuES6). (D) 3D-PCA plot of time-course data revealed differences between PD SB and PD SB NOG-treated samples. (E) Array expression data. PD SB treatment in the absence of Noggin leads to *SOX2* repression and *HAND1* induction. (F) FGF/ERK inhibition activates autocrine BMP signalling (RT-qPCR, HuES6; $n = 2$). (G) *PAX6*/*HAND1* immunostain. (H) Quantification of *PAX6*-positive cells ($n = 6$ representative images analysed per cell line).

suggest that *NANOG* and, especially, *OCT4* do indeed act as repressors of neural fate in hESCs and of *PAX6* in particular. Since PD treatment cooperates with SB in diminishing *NANOG* and *OCT4* expression, this must constitute one mechanism by which FGF inactivation promotes *PAX6* induction.

When treating hESCs with SB plus FGF2 for up to 8 days, *NANOG* and *OCT4* got eventually diminished to essentially

undetectable levels. Still, however, *PAX6* was not strongly induced under these conditions (Figures 1C and 4A, below). We concluded that downregulation of *NANOG*/*OCT4* is necessary for *PAX6* induction—but not sufficient. We therefore filtered the array data set of Supplementary Table SI for potential positive inducers of *PAX6*, requiring that such a factor shall become immediately upregulated following FGF/ERK inactivation. One of the very few candidates passing our

Figure 3 PAX6 induction requires NANOG/OCT4 downregulation and OTX2 upregulation. (A) ChIP-qPCR using antibodies against NANOG ($n = 1$) and OCT4 ($n = 3$) confirms binding to PAX6 promoter. (B) NANOG and OCT4 overexpression interferes with PAX6 induction. Note that transgene-expressing cells in red do not (OCT4 OE) or only partially (NANOG OE) overlap with PAX6-positive cells. Control vector-infected samples were NANOG/OCT4-negative and ~90% PAX6-positive (not shown). (C) RT-qPCR time course showing OTX2 upregulation precedes that of PAX6 and NR2F2. The induction of total OTX2 transcript appears to be driven by isoform a (see Supplementary Figure S3C). (D) Western blot of time-course samples treated as in (C). Note that both downregulation of NANOG/OCT4 and upregulation of OTX2 precedes PAX6 induction. (E) Immunocytochemistry: OTX2 is already weakly expressed in hESCs (left) but becomes markedly upregulated following FGF inactivation (middle), and is essentially colocalized with PAX6 in PD SB DM-induced neuroectoderm (right). (F) OTX2 knockdown interferes with PAX6 induction. Gene expression of lentivirus-infected cells was analysed following 4 days of PD SB DM treatment. Note that the degree of OTX2 knockdown was similar to the reduction in PAX6 and NR2F2 upregulation ($n = 2$). (G) Colonies showing successful OTX2 silencing as judged by immunocytochemistry also showed reduced PAX6 expression. (H) OTX2 overexpression experiment in FGF2-containing media. The addition of SB in the right panel served to selectively compromise NANOG and OCT4 levels in the cells. Note the cooperation between SB treatment—i.e., NANOG/OCT4 downregulation—and OTX2 overexpression in inducing PAX6 ($n = 2$). (I) Immunocytochemistry revealing PAX6 induction in patches of OTX2-overexpressing cells, despite the presence of FGF2 in the culture medium. (J) OTX2 ChIP-qPCR in putative enhancer regions of PAX6. The qPCR amplicons used (see Supplementary Table SIII) included the sequences shown at the bottom. The canonical OTX binding motif is highlighted in bold. All experiments in this figure: HuES6 hESCs.

Figure 4 Prolonged FGF/TGF β /BMP inactivation gives rise to functional neurons within 8 days. (A) Immunostain for β -III Tubulin and PAX6 (NCL3). (B) Outline of two-step neuronal induction procedure involving 4 days of pathway inhibitor treatment as EBs, followed by 4 days on matrigel. The media switch before EB formation served to erase a primitive streak-like gene expression footprint caused by MEF-CM (see Supplementary Figure S4A). ROCK inhibitor Y-27632 was additionally supplemented for the first 3 days to enhance cell survival (see Materials and methods). (C) β -III Tubulin stain on day 8 according to (B) (HuES6). (D) FACS analysis for β -III Tubulin (NCL3 hESCs). Negative control: 293T cells. (E) Immunocytochemical characterization of day 8 neurons (NCL3 hESCs). (F) Electrophysiological characterization of NCL3-derived neurons on day 12 using whole-cell patch clamping. (Top left) Voltage responses to current injections causing fast, large-amplitude action potentials. (Top right and bottom) Generation of successive action potentials was reversibly inhibited by TTX. Similar data were obtained with HuES6 at earlier time points (day 8). (G) Neuron formation procedure from (B) applied to two hiPSC clones.

filtering criteria was the homeobox transcription factor OTX2. Although it is already moderately expressed in undifferentiated hESCs, RT-qPCR and western blot analyses of independent samples confirmed that OTX2 became rapidly and markedly upregulated by day 2—just before the main induction of PAX6 and other neuroectodermal genes like NR2F2 (Figure 3C and D; Rosa and Brivanlou, 2011). Interestingly, the overall OTX2 upregulation appeared to be driven by isoform 'a', which is only expressed at low levels in the undifferentiated state (Figure 3C; Supplementary Figure S3C and D). The upregulation of OTX2 was specific to inactivation of the FGF/ERK signalling cascade (Figure 3E, left; Supplementary Figure S3F) and detectable within just 2 h of PD treatment (Supplementary Figure S3E). In line with a

putative role in neuroectoderm formation, OTX2 colocalized with PAX6 in neuroectoderm cells (Figure 3E, right).

Given the rapid and pronounced induction kinetics upon neuroectoderm formation, we next tested in functional assays whether OTX2—in collaboration with downregulation of NANOG/OCT4—may act as a positive inducer of PAX6. Indeed, counteracting the upregulation of OTX2 during neuroectoderm formation by RNA interference clearly compromised the induction of PAX6 and also of NR2F2 (Figure 3F and G). Conversely, overexpressing OTX2 'a' under FGF2 culture conditions clearly induced PAX6 to some degree (Figure 3H, left panel). Not surprisingly, this effect on PAX6 expression was rather moderate given that lentiviral infection rates were imperfect and that NANOG/OCT4 were fully

expressed under these conditions. However, addition of SB (to selectively diminish *NANOG/OCT4*) lead to a pronounced increase of *PAX6* (and *NR2F2*) levels, as expected—despite the presence of FGF2 in the culture medium (Figure 3H and I). Similarly, we observed synergistic effects on *PAX6* induction between *OTX2* overexpression and *NANOG* RNA interference (Supplementary Figure S3G). Finally, we scanned conserved upstream regions of the *PAX6* locus for the presence of canonical *OTX* motifs and tested these for *OTX2* occupancy using ChIP-qPCR. Two out of three upstream regions tested displayed clear *OTX2* binding in hESCs undergoing neuroectoderm induction (Figure 3J). Taken together, these data strongly suggest that besides negative effects on *NANOG/OCT4*, inactivation of FGF/ERK signalling upregulates *OTX2*, which then acts as a positive inducer of *PAX6*.

FGF/ERK inhibition promotes peripheral neuron differentiation following neuroectoderm formation

We next asked whether prolonged triple pathway inhibition of hESCs would give rise to neuron-like cells, as was the case for mouse epiblast stem cells (Greber *et al*, 2010). Cells staining positive for β -III Tubulin were readily observed within 8 days in adherent culture. However, these cultures still contained many *PAX6*⁺ cells, indicating asynchronous differentiation (Figure 4A). We therefore switched to a modified procedure involving neuroectoderm formation in embryoid bodies (EBs) for 4 days, followed by plating these EBs on matrigel-coated dishes for another 4 days (Figure 4B). Strikingly, a large number of neuron-like cells were seen to grow out of the plated EBs, in a radial manner, which stained strongly positive for β -III Tubulin (Figure 4C). About two-thirds (line HuES6) or up to 100% (line NCL3) of the plated EBs displayed this pattern. Matrigel appeared to be a preferred substrate for the outgrowth of these cells (Supplementary Figure S4B). Fluorescence-activated cell sorting (FACS) of bulk cultures revealed that most cells were positive for β -III Tubulin, with an apparent correlation between the size of the plated EBs and the efficiency of neuronal marker induction (Figure 4D; Supplementary Figure S4C).

Cells at the periphery of the outgrowths stained positive for doublecortin, a marker for immature neurons. MAP2 expression was rather confined to the intersection between the centre and the periphery of the outgrowths. Only rarely did cells stain positive for specific markers such as GABA or tyrosine hydroxylase (TH). However, many cells appeared positive for vesicular glutamate transporter 2, indicative of glutamatergic identity, which was also confirmed by pronounced *VGLUT2* mRNA levels (Figure 4E; Supplementary Table SIIA). Electrophysiological cell characterization on days 8 and 12 using the whole-cell patch clamp technique revealed the ability of the cells to fire one or more action potentials upon injecting depolarizing currents, with an average amplitude of 69 ± 3 mV ($n=9$, resting membrane potential: -44 ± 1.3 mV). Addition of tetrodotoxin (TTX) reversibly erased the action potentials, demonstrating the functionality of fast voltage-gated sodium channels (Figure 4F). Finally, when induced pluripotent stem cells (iPSCs) were subjected to the differentiation procedure, results were similar to those obtained with hESCs (Figure 4G; Supplementary Figure S4D). Collectively, these data demonstrate that extended inhibition of the three pathways in human pluripotent cells rapidly gives rise to functional neurons at high efficiency.

To delve deeper into the dispensability of FGF/ERK signalling in this entire differentiation process, we took a closer look at marker gene expression in these neuronal cultures (Supplementary Table SIIA). Mapping panels of known markers of diverse neuronal fates to these data as well as Gene Ontology analysis revealed that the neurons generated upon continuous pathway inhibition were likely to be neural crest-derived neurons of the peripheral nervous system (PNS), not neurons of the central nervous system (CNS) (green dots in Figure 5A and Supplementary Table SIIIB). The strong upregulation of neural crest and peripheral neuron markers was confirmed in independent samples (Figure 5B). Confirming this interpretation, between 60 and 80% of all cells in the EB outgrowths stained positive for *BRN3A* (also known as *POU4F1*), a prominent marker for sensory neurons (Howard, 2005; Figure 5C).

To determine the signalling requirements for the formation of these neurons from neuroectoderm, we varied the exogenous factors added during the adherent phase of the procedure outlined in Figure 4B. The addition of FGF2 between days 4 and 8 almost completely suppressed terminal differentiation into peripheral neurons, whereas withdrawal of inhibitors of the other two pathways did not appear to have any effect (Figure 5D and E). The suppression of peripheral neuron fate by FGF2 may fit well with the common use of FGF2 to stabilize an accepted precursor stage in CNS development (Elkabetz *et al*, 2008). Consistent with this, we found that when PD SB NOG-treated EBs were plated out on day 3 (but not later) into FGF2-containing medium, we reproducibly observed formation of neural rosettes that displayed sustained *PAX6* and *Nestin* expression (Figure 5F). Taken together, these data suggest that in addition to repressing the initial induction of *PAX6* in hESCs, FGF2 later acts as a repressor of peripheral neuron fate to arrest differentiation and stabilize neural precursor stages.

Discussion

Overall, our data set assigns a novel role to FGF2 signalling in hESCs, namely prevention of neuroectoderm induction by the specific repression of *PAX6*, the key fate determinant of that lineage in human (Zhang *et al*, 2010; Figure 5G). As FGF2 supplementation inhibited *PAX6* induction throughout all our experiments and as sheer FGF2 withdrawal or FGF/ERK inhibition alone was sufficient in activating *PAX6* expression, we conclude that inactivation of FGF2 signalling—by FGF2 withdrawal from the culture medium or by pharmacological inhibition—may be the key trigger underlying neuroectoderm conversion from hESCs. According to our time-course data, inhibition of TGF β /SMAD2 signalling mainly serves to promote downregulation of hESC-specific genes (Figure 5G; Brown *et al*, 2011). FGF inactivation cooperates with TGF β /SMAD2 inhibition to downregulate *NANOG* and *OCT4* levels, and overexpression of these factors strongly interfered with *PAX6* induction. Hence, as a necessary requirement, *NANOG* and *OCT4* need to be turned off to enable derepression of *PAX6*—in principle.

In the presence of FGF2, however, SMAD2 inhibition was not sufficient for inducing neuroectoderm development—even after prolonged time periods of treatment when *NANOG* and *OCT4* were essentially shut down. We therefore

Figure 5 FGF2 blocks peripheral neuron formation from PAX6-positive neuroectoderm. (A) Mapping of known neural marker genes to global expression data of cells differentiated according to Figure 4B. (B) Neural crest-associated and peripheral neuron markers are strongly upregulated after 4 + 4 days of PD SB DM treatment (combined array and RT-qPCR data). (C) Immunostain and quantification of BRN3A-positive cells following 4 + 4 days of PD SB DM treatment. (D) Variation of culture conditions between days 4 and 8. Note that if FGF2 was added between days 4 and 8, only very few neurons were formed. PAX6 was downregulated in all conditions. (E) RT-qPCR for TUBB3 of samples treated in a similar manner as in (D) ($n = 2$ per cell line; $*P < 0.05$). Note that TGFβ/BMP inhibition was dispensable for TUBB3 induction between days 4 and 8 (compare PD SB DM with PD only). (F) Formation of neural rosettes after plating PD SB NOG-treated EBs into FGF2-containing medium on day 3 (top). Putative Nestin⁺/PAX6[−] neural crest cells were predominantly formed when exposing cells to FGF2 after day 4 (bottom, line HuES6). (G) Model summarizing transcriptional effects of three signalling pathways on gene expression in the context of neuroectoderm induction in hESCs. (H) Model illustrating the biphasic role of FGF signalling in controlling neural cell fate in hESCs.

conclude that NANOG and OCT4 downregulation alone is not sufficient for promoting substantial induction of PAX6. Rather, we hypothesized that FGF inactivation additionally promotes the upregulation of a positively acting inducer of PAX6. Rosa and Brivanlou (2011) recently implicated the transcription factor NR2F2 in promoting neuroectoderm differentiation of hESCs, in part acting by repressing OCT4. Notably, in their study, differentiation was induced by switching to FGF2-free culture media. Consistent with the data by Rosa and Brivanlou, NR2F2 was strictly coregulated with PAX6 throughout all our experiments. This was, however, also true for the initial time points in which PAX6 and NR2F2 were only very modestly upregulated. Here, we identified

OTX2 to be one of the earliest upregulated genes following FGF inactivation. OTX2 silencing in differentiating cells interfered with the induction of both PAX6 and NR2F2, and OTX2 overexpression—in cooperation with NANOG/OCT4 downregulation—promoted induction of both these key genes. OTX2 therefore appears to be an early driver of neuroectoderm commitment, being immediately linked to inactivation of FGF signalling (Figure 5G). Our hESC data are consistent with the *in-vivo* expression pattern of mouse *Otx2* in the Fgf-expressing pregastrulation epiblast and in anterior neuroectoderm (Kurokawa *et al*, 2004). In future studies, it will be interesting to investigate the link between FGF/ERK and OTX2 in more detail.

The model of Figure 5G initially appears to be at odds with published work, as FGF signalling has frequently been implicated in promoting neural induction. However, most of these data have been generated in model organisms, such as chick and *Xenopus*, and little is known about pregastrulation-stage mammalian embryos or hESCs. Moreover, inactivation of FGF/ERK signalling has been reported to disable neural induction from mouse ESCs (Ying *et al*, 2003; Kunath *et al*, 2007; Stavridis *et al*, 2007), but these data may better be interpreted as a failure of mouse ESCs to enter an epiblast-like intermediate stage under these conditions (Lanner and Rossant, 2010). Furthermore, models suggesting FGF2 as a driver of neuroectodermal induction in hESCs were based partly on the observation that inhibition of FGF signalling alone or in combination with TGF β /SMAD2 inactivation may give rise to non-neural fates (LaVaute *et al*, 2009; Vallier *et al*, 2009b; Na *et al*, 2010). We argue, however, that these observations rather highlight yet another role of FGF signalling in hESCs, namely to antagonize endogenous BMP signalling (Xu *et al*, 2005, 2008; Greber *et al*, 2007; Peerani *et al*, 2007). When FGF2 is withdrawn from the media or when the pathway is inhibited by pharmacologic agents, autocrine BMP signalling becomes activated enough to interfere with neuroectodermal commitment. Addition of NOG or DM was therefore necessary to prevent BMP-mediated effects, such as activation of *HAND1* and repression of *SOX2*, to enable the most pronounced induction of neuroectoderm (Chambers *et al*, 2009; Figure 5G).

After neuroectoderm formation, FGF signalling appears to play another critical role, as prolonged inhibition of the FGF/ERK cascade immediately gave rise to PNS derivatives—mostly sensory neurons—at high efficiency. The occasional emergence of TH-positive cells is consistent with this finding, as TH is also a marker for sympathetic neurons (Howard, 2005; Figure 4E). The procedure may also present a valuable platform for modelling PNS-associated diseases or for compound screens requiring the rapid generation of neurons from human pluripotent cells. FGF2 almost completely blocked peripheral neuron formation to redirect cell fate into neural rosettes—an accepted CNS precursor state—or putative Nestin⁺/PAX6⁺ neural crest cells. Indeed, FGF2 is frequently used to promote the self-renewal of neural stem and precursor cells, and protocols describing their derivation from ESCs incorporate the addition of FGF2 at some point (Zhang *et al*, 2001; Conti *et al*, 2005; Koch *et al*, 2009). However, this is not inconsistent with our finding that FGF2 represses the onset of *PAX6* induction in hESCs (Figure 5G). Rather, it appears that FGF signalling plays a biphasic role in the neural induction of hESCs (Figure 5H).

Materials and methods

Cell culture

Fully characterized hESC lines HuES6 (Cowan *et al*, 2004) and NCL3 (Zhang *et al*, 2006) were received from the Harvard Office of Technology Development and the UK Stem Cell Bank, respectively, and used at a passage number below 50. Both lines were confirmed to give rise to derivatives of all three germ layers by spontaneous *in vitro* differentiation. hESCs tested negative for mycoplasma. All experimental conditions were carried out under adherence using matrigel-coated 6- or 12-well dishes. Proper matrigel coating was crucial for both maintenance of self-renewal and efficient neuronal differentiation: matrigel HC (growth factor-reduced, BD) was thawed on ice overnight and then diluted 1:3 with ice-cold

knockout DMEM (Invitrogen). In all, 1-ml stocks were then kept frozen at -20°C . For coating of plates, each 1-ml stock was resuspended in 24 ml of conventional hESC medium (Amit *et al*, 2004) (without growth factors) on ice (1:75 total dilution, final protein concentration: ~ 0.25 mg/ml). Precooled (at 4°C) 6- or 12-wells were covered with 1 ml or 0.4 ml of diluted matrigel, respectively. Plates were then wrapped in parafilm and kept at room temperature overnight. The next day, plates were transferred to 4°C for at least 1 day. Before use, coating quality was verified under a phase contrast cell culture microscope (a network of polymerized matrigel, such as e.g., in Figure 4C, must readily be visible).

hESCs were routinely maintained in MEF-conditioned medium (Xu *et al*, 2001) and split using colony precutting with sterile injection needles, followed by treatment with dispase (2 mg/ml), repeated washing in PBS with $\text{Mg}^{2+}/\text{Ca}^{2+}$, lifting off the cell clumps using a sharp plastic scraper (PAA), and several seconds of centrifugation to collect the aggregates without single cells. Experiments were exclusively conducted in chemically defined medium. hESCs were always preadapted to defined medium for 1–2 days (see Supplementary Figure S4A). Defined medium—which was modified from Yao *et al* (2006)—consisted of DMEM/F12 (Hyclone), supplemented with $1 \times$ N2 supplement, $1 \times$ B27 supplement, 0.1 mM β -mercaptoethanol (Invitrogen), $1 \times$ non-essential amino acids, $1 \times$ L-glutamine, $1 \times$ penicillin/streptomycin (PAA), and 0.2% human serum albumin (Biological Industries). For maintenance of self-renewal, N2NB27 medium was supplemented with 20 ng/ml FGF2 or FGF2 plus dorsomorphin/Noggin. Upon splitting, 10 μM of ROCK inhibitor Y-27632 (Watanabe *et al*, 2007) (Ascent) was added to the medium as well. Growth factor or inhibitor treatments were typically initiated 1–2 days after splitting: FGF2 (PeproTech; 20 ng/ml), Noggin ('NOG'; PeproTech; 250 ng/ml), BMP4 (PeproTech; 20 ng/ml), TGF β 1 (PeproTech; 1 ng/ml), SB431542 ('SB'; Sigma; 15 μM), PD0325901 ('PD'; Axon Medchem; 0.5–1 μM), dorsomorphin ('DM'; Merck; 0.5 μM). For some of the time-course experiments, for genetic manipulation, and for quantifying PAX6-positive cells by immunocytochemistry, cells were treated with inhibitors 2 days after splitting with Accutase (PAA), which resulted in colonies of more homogeneous sizes. For EB formation, cell aggregates were collected as for routine splitting and then transferred to bacteriological 6-cm dishes containing defined medium with FGF2 and ROCK inhibitor. For media changes, dishes were slowly rotated under a stereo microscope and EBs were collected from the centre and then transferred to new dishes with minimal liquid. ROCK inhibitor Y-27632 was added to all EB cultures for the first 3 days of treatment to support cell survival. Medium was changed daily in all experiments.

RT-qPCR, RNA profiling, and computational analysis

Cells were lysed directly in culture dishes and RNA was isolated using RNeasy kits with on-column DNA digestion (Qiagen). RT-qPCR and microarray analyses were carried out as described (Greber *et al*, 2010), using fully validated primers with normalization to two housekeeping genes (Supplementary Table SIII) and HumanRef-8 v3 bead chips (Illumina), respectively. Processed array data are in Supplementary Tables SI and SII. Principal component analysis was performed using an in-house developed algorithm in Matlab. Gene Ontology analysis was carried out using DAVID. RNA sequencing was performed on Illumina HiScan SQ instrumentation. hESC library preparation, cluster generation, and 2×50 bp paired-end sequencing was carried out using kits by the manufacturer. Data were analysed using freely available software packages. For the identification of putative OTX2 binding sites in the *PAX6* locus, we used an in-house database of putative regulatory elements in upstream regions of known genes. As a general procedure, the binding energy m for the affinity of a given motif with a position weight matrix (PWM) σ to a sequence $X = (x_1, \dots, x_w)$ of length w was calculated by:

$$m(X, \sigma) = \frac{\sum_{i=1}^w \log \left(\frac{P(x_i | \sigma_i)}{P(x_i | \sigma_i^*)} \right)}{w}$$

where σ_i is the background frequency of the four nucleotides. $P(x_i | \sigma_i)$ denotes the normalized frequency of a nucleotide x_i in the PWM column σ_i . The multiple alignment format data set was obtained from the UCSC genome browser website. Binding energies were obtained for each sequence in 40 000 conserved upstream regions of known genes, followed by calculating the binding energy

z-score for each motif. Only hits with an energy z-score ≥ 2.0 were accepted. OTX binding matrix information was obtained from Transfac.

Immunocytochemistry, western blotting, ChIP-qPCR, and FACS

For immunocytochemistry, cells in 12-well dishes were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2–1% Triton X-100 for 10 min, and blocked with a solution comprising 2% BSA, 5% FCS, and 0.1% Tween 20 in PBS for 45 min. Primary and secondary antibodies were applied overnight at 4°C and for 1 h at room temperature, respectively, in 0.5% BSA in PBS-T. Hoechst stain was applied in the second-to-last washing step at 1 µg/ml. Samples were mounted with Aqua-Poly/Mount (Polysciences) and round glass coverslips. SDS-PAGE and western blotting was performed according to standard procedures with loading 40 µg of protein per gel lane. ChIP-qPCR was performed as described, using about 5×10^6 formaldehyde-fixed cells as starting material (Greber *et al*, 2010). FACS analysis was performed using the Fix and Perm intracellular staining kit (Invitrogen GAS-003) and BD FACSAria instrumentation. Primary antibodies were α AFP (Sigma A8452, IF 1:500); α Doublecortin (Santa Cruz sc-8067, IF 1:100); α BRN3A (Santa Cruz sc-8429, IF 1:500); α GABA (Sigma A2052, IF 1:1000); α GAPDH (Ambion 4300, WB 1:5000); α HAND1 (Santa Cruz sc-9413, IF 1:25); α MAP2 (Millipore MAB3418, IF 1:200); α MAPK (Cell Signaling Technology 9102, WB 1:1000); α NANOG (R&D AF1997, IF 1:50, WB 1:250, ChIP 2 µg); α Nestin (Chemicon MAB5326, IF 1:200); α OCT4 (Santa Cruz sc-5279, IF 1:100; Santa Cruz sc-8629, IF 1:100, WB 1:5000; Santa Cruz sc-8628 X, ChIP 2 µg); α OTX2 (R&D AF1979, IF 1:100; Abcam ab21990, IF 1:500, WB 1:5000, ChIP 2 µg); α PAX6 (Covance PRB-278P, IF 1:500, WB 1:5000); α Phospho-MAPK (Cell Signaling Technology 9106, WB 1:2000); α SMA (DakoCytomation M0851, IF 1:100); α β -III Tubulin (Sigma T8660, IF 1:1000; Covance PRB-435P IF 1:2000; BD Pharmingen 560381, FACS 20 µl per sample); α TH (Millipore AB152, IF 1:250); α VGLUT2 (Millipore MAB5504, IF 1:50). Secondary Alexa 488, 568, and 594 labelled antibodies were diluted 1:1000 (catalogue numbers A21468, A21206, A11078, A11008, A11001, A11057, A11079, A11061, A11011—all from Invitrogen). Unless otherwise stated, representative pictures are shown.

Gene silencing and overexpression

For overexpression, open reading frames were PCR amplified from hESC or neuroectoderm cDNA using primers in Supplementary Table SIII, cloned into pCRII-TOPO (Invitrogen), sequence-validated, and subcloned into the *PmeI* and *SpeI* sites of pLVTHM (Addgene plasmid 12247). The OTX2 ORF contained the insert sequence shown in Supplementary Figure S3C. Lentiviral particles were produced using 293T cells grown in MEF-conditioned medium for 24 h, following cotransfection of the pLVTHM construct and Addgene plasmids 12260 and 12259 at a mass ratio of 4:3:1 using Lipofectamine 2000 (Invitrogen). hESCs were infected in MEF-conditioned medium 1 day after splitting with Accutase or upon replating. Virus supernatants were adjusted to result in around >50% infection efficiency, as judged based on GFP controls. Defined medium was used from the day after infection onwards. OTX2 RNAi sequences were designed using the Invitrogen Stealth RNAi design tool. By following the accompanying guidelines, silencing constructs were prepared by annealing and cloning short-hairpin oligonucleotides shown in Supplementary Table SIII into pLKO.1 puro (Addgene plasmid 8453). Plasmid inserts were

sequence-validated and lentiviruses were produced as above. At 48 h after infection, cells were treated as described in text and concomitantly selected using 1 µg/ml puromycin. Virus supernatants with three different shRNA constructs were pooled as this tended to yield slightly higher knockdown efficiencies. Addgene plasmid 1864 expressing scrambled shRNA served as control. The NANOG RNAi vector used was LL-NANOGi (Zaehres *et al*, 2005).

Electrophysiology

Recordings were performed in culture dishes at room temperature and at 33°C. Cell cultures were superfused with a solution containing 120 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 30 mM HEPES, 2 mM MgSO₄, 2 mM CaCl₂, 10 mM dextrose, 25 µM Alexa 594 hydrazide; pH: 7.25 with HCl; osmolality: 305 mOsmol/kg. Electrodes for whole-cell recordings with a resistance of 6–8 MΩ were pulled from borosilicate glass capillaries. Electrodes were filled with a saline solution containing 95 mM κ -gluconate, 20 mM K₃-citrate, 10 mM NaCl, 10 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, 3 mM BAPTA, 3 mM Mg-ATP, 0.5 mM Na-GTP; pH: 7.25 with KOH; osmolality: 295 mOsm/kg. Electrodes were connected to an EPC-10 amplifier (HEKA Elektronik, Lamprecht, Germany). After gaining access to the cell, dyes were allowed to equilibrate by diffusion for at least 5 min. Access resistances were in the range of 5–20 MΩ. Experiments were controlled by PatchMaster software (HEKA Elektronik). Liquid junction potential was not corrected for. TTX (0.5–1 µM) was applied via bath perfusion to block Na⁺ channels and abolish action potentials.

hiPSC generation

Human iPSCs were produced from foreskin fibroblasts (ATCC #CRL-2097) by following Melton's protocol (Huangfu *et al*, 2008) with three factors (OCT4, SOX2, and KLF4). Retroviruses were produced in 293T cells using Fugene 6 (Roche) and Addgene plasmids 8454, 8449, 17217, 17218, and 17219 (Takahashi *et al*, 2007). Two lines showing typical hESC morphology and growth characteristics were further characterized according to the standard assays (Supplementary Figure S4D). Bisulphite sequencing was carried out using an EpiTect Bisulfite Kit (Qiagen), followed by PCR cloning using primers listed in Supplementary Table SIII and sequencing of inserts.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: BG, PC, H-CP, and HRS designed the experiments. BG, PC, MZ, and DWH performed the experiments. BG, PC, AJMM, MJA-B, MZ, SM, and SF analysed the data. BG and HRS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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