Supplemental Figures and Table legends

Figure S1 Time-course array data of the expression of selected pluripotency genes.
PD SB NOG treatment generally led to a more rapid downregulation of gene expression compared with FGF2 SB NOG treatment. Note that at the 6 hr time point, *NANOG* expression was already downregulated, consistent with "direct" activation by the TGFβ/SMAD2 and FGF/ERK pathways. *OCT4* downregulation appeared to be delayed, suggesting that indirect regulatory mechanisms were acting on this gene.
Figure S2 Roles of FGF and BMP signaling in neuroectoderm induction.

(A) Gene expression analyzed by RT-qPCR at d 4 (HuES6, n = 2). Note that PAX6 induction specifically depends on inactivation of the FGF/ERK signaling axis.

(B) BMP stimulation analyzed by RT-qPCR (HuES6, n = 2). Note that SOX2 downregulation occurs before that of OCT4.

(C) Dorsomorphin can substitute for Noggin in the neuroectodermal induction of hESCs. RT-qPCR analysis with HuES6 hESCs (n = 1). SB treatment in the absence of FGF2 led to the induction of both PAX6 and HAND1. Addition of either Noggin or dorsomorphin abolished HAND1 upregulation in favour of PAX6 induction.

(D) PAX6 immunostain after 4 days with the indicated treatments (HuES6). Note the enhanced percentage of PAX6+ cells when autocrine BMP signaling was suppressed.
Figure S3 OTX2 is involved in neuroectoderm formation of hESCs.

(A) Time-course comparison investigating the necessity of TGFβ inhibition ("SB") for neuroectoderm induction. Note that without SB, NANOGL and OCT4 downregulation was somewhat delayed, whereas PAX6 levels were similar by d 6. Induction of the forebrain marker FOXL1 appeared to be repressed by SB (RT-qPCR data, line HuES6).

(B) Representative immunostains of samples treated as in A.

(C) Illustration of known OTX2 isoforms. FGF/ERK inhibition tended to preferentially induce the a isoform that differs from the b isoform by its transcription start site as well as by an in-frame insertion in the coding sequence.
(D) RNA-seq-based quantification of OTX2 isoform levels in hESCs.

(E) FGF/ERK inhibition induced OTX2 but not PAX6 within hours (RT-qPCR data, line HuES6).

(F) OTX2 immunostains of hESCs under FGF/TGFβ activating or FGF/TGFβ inactivating conditions, as indicated (d 3). Note that OTX2 upregulation was attributable to FGF inhibition rather than to inactivation of TGFβ signaling. All samples in presence of DM to suppress autocrine BMP signaling.

(G) NANOG downregulation and OTX2 overexpression cooperate in inducing PAX6. Cells were infected with corresponding lentiviruses and analyzed after 3 d in FGF2 medium that otherwise prevents PAX6 induction. Bars reflect qPCR normalization error, line HuES6, n = 1.
Figure S4 Addressing parameters in the two-step neuronal differentiation procedure and characterization of hiPSCs.

(A) Top: Control experiment ($n = 2$ per cell line) showing that 1-2 days of adaptation of hESCs to FGF2-based chemically-defined growth medium did not compromise the expression of hESC markers, prior to
inducing differentiation (left). Rather, pre-adaptation to N2B27 medium served to erase the primitive streak–like expression bias found in MEF-conditioned medium\(^4\) (middle-right), without significantly inducing neuroectodermal gene expression (PAX6, right). Bottom: Immunostain for hESC markers 1 d after replating N2B27-adapted hESCs further confirming that cells were fully undifferentiated at the beginning of experiments.

(B) Use of matrigel as a substrate for plating PD SB NOG–treated EBs yielded higher numbers and percentages of neurons staining positive for beta-III Tubulin on day 8 (HuES6).

(C) RT-qPCR analysis for neuronal markers on day 8 (NCL3). Larger EBs (diameter > 300 µm) plated on day 4 tended to give rise to neurons at somewhat higher efficiency (compare scheme in Figure 3B).

(D) Characterization of two foreskin fibroblast–derived hiPSC clones using standard assays.

Supplemental Table Legends

**Table S1 Microarray time-course data of HuES6 hESCs treated as indicated in Figure 1B.**
Probes that did not reveal gene expression are not indicated. Abbreviations (top row): S, array intensity signal (a.u.); D, detection \(P\) value (D < 0.01 denotes detectable expression); P, \(P\) value for differential gene expression against day 0; R, expression ratio against day 0.

**Table S2 Global gene expression profiles of HuES6 and NCL3 hESCs treated as indicated in Figure 3B.**
(A) Probes that did not reveal gene expression are not indicated. Abbreviations (top row): S, array intensity signal (a.u.); D, detection \(P\) value (D < 0.01 denotes detectable expression); P, \(P\) value for differential gene expression against day-0 EBs; R, expression ratio against day-0 EBs. 
(B) Gene ontology analysis of >5-fold upregulated genes. Highlighted terms support the interpretation that the obtained neurons present neural crest derivatives.

**Table S3 List of oligonucleotides used in this study.**