Antagonism between the transcription factors NANOG and OTX2 specifies rostral or caudal cell fate during neural patterning transition

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**ABSTRACT**

During neurogenesis, neural patterning is a critical step during which neural progenitor cells (NPCs) differentiate into neurons with distinct functions. However, the molecular determinants that regulate neural patterning remain poorly understood. Here, we optimized the “dual-SMAD inhibition” method to specifically promote differentiation of human pluripotent stem cells (hPSCs) into forebrain and hindbrain NPCs along the rostral-caudal (R-C) axes. We report that neural patterning determination occurs at the very early stage in this differentiation. Undifferentiated hPSCs expressed basal levels of the transcription factor orthodenticle homeobox 2 (OTX2) that dominantly drove hPSCs into the “default” rostral fate at the beginning of differentiation. Inhibition of glycogen synthase kinase 3β (GSK3β) through CHIR99021 (CHIR) application sustained transient expression of the transcription factor NANOG at early differentiation stages through Wnt signaling. Wnt signaling and NANOG antagonized OTX2 and in the later stages of differentiation, switched the default rostral cell fate to the caudal one. Our findings have uncovered a mutual antagonism between NANOG and OTX2 underlying cell fate decisions during neural patterning, critical for the regulation of early neural development in humans.

The embryonic neurodevelopment is a spatiotemporally regulated process, during which
distinct cell fates are progressively restricted based on spatial regions (1, 2). At early stage of neurogenesis, the specified neural ectoderm divides into functionally distinct cell fates along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes (3). This neural patterning process is a critical step to specify different neural precursors such as forebrain, midbrain, hindbrain and spinal cord. It has been known that the neural patterning is induced by the temporal and special morphogen gradients along the A-P and D-V axes (4, 5). These morphogens, including BMPs, WNTs, FGFs, RA and SHH (sonic hedgehog), coordinate and form gradients to specify regionally transcriptional program and distinct neural progenitors (6-10). However, the precise timing and mechanisms underlying morphogen-induced neuraxial patterning has not been fully elucidated in mammals, especially in human.

Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) could differentiate into neuroepithelial cells, regionally specified neural precursor cells (11-16), thus provides a valuable model to investigate molecular determinants on neural patterning in human background. The mostly used method to induce neural differentiation in hPSCs is through suppression of both TGFβ and BMP signaling (17, 18). Dual-inhibition of SMAD-dependent TGF-β and BMP signaling by their inhibitors (SB431542 and Noggin), or SB431542 and Dorsomorphin can efficiently trigger hPSCs differentiation into NPCs (19-21). Dual-SMAD inhibition triggered NPCs are believed to be more close to the anterior forebrain fate (22, 23). Interestingly, the anterior fate has also been considered as a “default” fate for hPSCs to initiate neural differentiation (24, 25). To initiate the caudal fate, more other morphogens such as WNTs, need to be applied on the base of dual-SMAD inhibition. Indeed, regional NPCs along A-P axis could be specified from hPSCs via dose-dependent activation of WNT signaling by GSK3β inhibitor CHIR combined with dual-SMAD inhibition (26). However, how GSK3β inhibition coordinates with other signaling to regulate early neural patterning in hPSCs remains unclear.

Here in this study, we investigated the molecular determinants that regulate neural patterning in hPSCs differentiation. We demonstrated that neural patterning is committed at very early stage of differentiation. The basal amount of OTX2 actively drive hPSCs into the “default” anterior fate right after the exit from pluripotency. Depending on WNT activation, CHIR treatment at the same stage temporally sustains NANOG and further represses OTX2 and switches the “default” rostral fate to the caudal one in later differentiation.

RESULTS

Generation of regionally specified forebrain and hindbrain NPCs from hPSCs

HPSCs could be differentiated into regionally neural cells by applying different morphogenetic cues, such as WNTs, FGFs or RA activation. (27-29). To investigate the molecular determinants on neural patterning, we optimized a monolayer and defined condition to induce neutralization of hPSCs by applying dual-SMAD inhibition combined with or without GSK3β inhibition (Figure 1A). Human ESCs, (30) or urine cells derived iPSCs (31) treated by dual-SMAD inhibition alone (SD, SB431542/Dorsomorphin) or combined with GSK3β inhibition (SDC, SB431542/Dorsomorphin/CHIR99021) showed obvious, but different morphology changes (Figure 1B). Consistently, we observed significant difference in expression of regionally specified neural markers between SD and SDC treatment cells (Figure 1C). The forebrain marker genes such as EMX2, OTX2 and FOXG1 were highly expressed in SD cells while the hindbrain marker genes like HOXB2, GBX2 and Lmx1b
were highly expressed in SDC cells. These data indicated that the SD triggered NPCs were rostral fate while the SDC NPCs were caudal fate. Consistently, pan-NPC marker genes, SOX2 and NESTIN were highly expressed in both SD and SDC NPCs. Notably, other neural factors, PAX6 and SOX1, also showed remarkable difference in expression between SD NPCs and SDC NPCs (Figure 1C). We further performed whole genome transcriptome analysis on SD and SDC NPCs. Pearson correlation analysis showed distinct expression profiling between SD and SDC NPCs (Figure 1D). The selected forebrain marker genes were highly expressed in SD NPCs while the hindbrain genes were highly expressed in SDC NPCs (Figure 1E). Furthermore, gene ontology analysis showed that the upregulated genes in SDC NPCs were more related to anterior/posterior region formation and hindbrain/spinal cord development while genes in SD NPCs were more related to forebrain and telencephalon development (Figure 1F). Several signaling pathways that were reported to be critical to regulate neural patterning, such as WNTs, SMADs, RA also showed different expression between SD and SDC NPCs (Table S3). For example, the expression level of WNT5A was relatively higher, while WNT5B was lower in SDC NPCs. Genes that are related to SMADs, WNTs and RA signal pathways were summarized in Table S3.

Immunostaining assay confirmed that both NPCs maintained in vitro are SOX2, NESTIN, and KI67 positive (Figure S1D) and could differentiate into astrocytes, and subtype neurons including GABAergic neurons, glutamatergic neurons, dopaminergic neurons and motor neurons (Figure S1E). Neurons differentiated from SDC or SD NPCs exhibit functional electrophysiological properties including robust but similar Na⁺ and K⁺ currents and repetitive action potentials (Figure S1F). In sum, we demonstrated that SD or SDC condition induced different regionally specific NPCs, i.e. SD induced forebrain specific NPCs while SDC induced NPCs closed to hindbrain region. Both SDC triggered caudal and SD triggered rostral NPCs hold the potency to differentiate various subtype neural cells.

**Rostral-caudal patterning occurs at early stage of neural differentiation**

The rostral neural fate is usually considered as a “default” fate in neural differentiation of hPSCs (32). We then have interests to investigate how and when GSK3β inhibition coordinates with dual SMADs inhibition to switch the “default” rostral fate to the caudal one. We firstly designed experiments to examine the timing of CHIR treatment to switch SD triggered rostral fate in hPSCs. In this experiment, CHIR was added or withdrew on day 2 or day 4 during SD or SDC treated differentiation (Figure 2A). Cell regional characters were checked at day 6. We chose PAX6 as a cell fate indicator to assess different treatments because it has been reported as a critical factor in fore-midbrain (33, 34) and is also suppressed by CHIR in our experiments (Figure S1A-C). Other regional marker genes, such as the forebrain markers, FOXG1, EMX2, OTX2; midbrain markers, Lmx1b, EN-1; hindbrain markers, GBX2, HOX42 and HOX82 were also examined by QPCR to confirm the different regional fate in different treatment (Figure 2B). As shown in Figure 2A (left panel), CHIR added as early at day 2 of SD treatment could significantly suppress PAX6 expression and switch the regional neural fate (Figure 2A, Group 2). However, applying CHIR at later time points, day 4, failed to affect the SD triggered rostral fate (Figure 2A-B, Group 3). On the other hand, CHIR treatment for the first 4 days could be sufficient to switch the SD triggered rostral fate to the caudal hindbrain one (Figure 2A-B, Group 5). To the contrast, CHIR treatment only for the first 2 days showed no effect on cell fate transition (Figure 2A, right panel, Group 4).
Furthermore, based on the analysis on several known critical factor for anterior-posterior (A-P) neural patterning, we found out OTX2 and GBX2, two known essential factors in fore-mid brain and hindbrain development (35-37), exhibited significantly differential activation between SD and SDC treated cells at 48h (Figure 2C and S2A), highlighting that Day2 is the critical time point for the irreversible rostral or caudal regional specification in neural differentiation of hPSCs.

**OTX2 dominantly triggers rostral fate differentiation when hPSCs exit pluripotency**

Our data showed that OTX2 and GBX2, while not other tested factors, exhibited significantly differential activation between SD and SDC treated cells at Day2, the critical timing window for neural patterning in differentiation (Figure 2B) indicates that they might play essential roles in neural patterning in hPSCs differentiation as reported in other model systems. To investigate the precise roles of these factors, we overexpressed OTX2, GBX2 or HOXB2 in hESCs through lentiviral approach (Figure 3A). Interestingly, hESCs with OTX2 over-expression displayed typical neural rosette-like phenotype, even were maintained in normal hPSCs medium that support self-renewal and suppress differentiation (Figure 3A). In contrast, hESCs with GBX2 or HOXB2 over-expression kept the undifferentiated morphology (Figure 3A). Consistently, pluripotent genes such as OCT4 and NANOG were suppressed in OTX2 over-expressed hESCs, but well maintained in GBX2 or HOXB2 over-expressed hESCs (Figure 3B). Though immunostaining and western blot, we confirmed that cells with OTX2 overexpression are positive for NPC markers, such as PAX6 and SOX2, while negative for OCT4 (Figure 3C, S2B). Similarly, neural ectoderm genes, while not other germ layer genes are highly activated in OTX2 over-expressed cells (Figure 3D). For regionally restricted neural marker genes, the forebrain genes, EMX2 and FOXG1, while not midbrain and hindbrain genes are significantly upregulated in OTX2 over-expressed hESCs (Figure 3E). These data demonstrated that OTX2 dominantly triggers neural differentiation of hPSCs towards the preferential forebrain fate.

To examine whether GBX2 could affect the regional cell fate at later neural differentiation, we triggered differentiation of GBX2 or HOXB2 expressed hESCs by SD. As shown, GBX2 over-expression significantly suppressed the forebrain genes such as PAX6 and OTX2 induced by SD treatment, while HOXB2 showed no similar suppression effect (Figure 3F). In all, our data indicate that both OTX2 and GBX2 are involved in A-P neural patterning in hPSCs differentiation, but function differently. OTX2 is a dominant trigger and could drive hPSCs into forebrain neural fate alone, while GBX2 could repress the forebrain fate and switch it to hindbrain at later differentiation stage.

**GSK3β inhibition sustains NANOG and represses OTX2 to switch the rostral-caudal fate decision.**

To investigate the role of GSK3β inhibition in neural fate decision, we examined the detailed expression of several critical factors during the first 2 days’ differentiation triggered by SD or SDC (Figure 4A). We identified that NANOG exhibit significantly higher expressions in SDC treated hPSCs than SD cells at 24 hours of differentiation (Figure 4A). OTX2 showed similar level between two treatments at 24h, but was dramatically suppressed at later time points in SDC treated cells (Figure 4A). Further by western blot, we confirmed that at 24h or 48h time points, certain level of NANOG protein was maintained in SDC while not SD treated hESCs (Figure 4B). These data indicate that the temporal expression of NANOG in SDC cells might play important roles in neural fate decision at early stage.

To determine the effect of NANOG in caudal induction, we prepared hESCs with over-
expression of **NANOG** through lentiviral approach. **NANOG** over-expressed hESCs maintained typical undifferentiated phenotype and showed no significant difference compared with control hESCs with overexpression of **GFP** (Figure 4C). We then treated them with SD or SDC to trigger rostral or caudal neural differentiation and examined expression of **OTX2** and **GBX2** at day 2. We showed that **NANOG** overexpression significantly suppressed **OTX2** expression in SD triggered rostral fate differentiation (Figure 4D). To the contrast, **GBX2** was not suppressed but even up-regulated in **NANOG** over-expressed cells during SDC induced caudal fate differentiation (Figure 4E). Because **GBX2** was reported to be direct target of WNT signaling (38) the activation of **GBX2** in CHIR treated cells might due to the activation of WNT signaling. Then through using WNT inhibitor (XAV939, 0.5 µM) (39), we confirmed that **GBX2** expression in CHIR treated cells is indeed WNT signaling dependent (Figure 4E). Similarly, the temporal expression of **NANOG** in SDC cells was also due to the activation of WNT signaling by CHIR (Figure 4F). In all, these data suggest that CHIR treatment temporary maintains **NANOG** at early stage via WNT signaling to repress **OTX2** and activate **GBX2** to initiate caudal fate.

Previous reports showed that **NANOG** also plays roles in primitive streak (PS) and mesendoderm differentiation in early mouse development (40-44). However, we failed to detect the significant up-regulation of known PS or mesendoderm genes, such as **T**, **MIXL1** and **EMOES** in SD and SDC treated cells, indicating a non-mesendoderm fate in SDC treated hPSCs (Figure 4G).

**NANOG** antagonize **OTX2** to balance the cell fate between “pluripotency” and “the default forebrain” in hPSCs

When examined the expression of **OTX2**, we found that certain amount of **OTX2** expression, while not **GBX2** could be detected in undifferentiated hPSCs (Figure 5A). The expression of **OTX2** in hPSCs might explain the “default” forebrain fate differentiation for hPSCs under non-optimal conditions to support self-renewal, for example, inhibition of SMAD signaling. We then performed double immuno-staining on **NANOG** and **OTX2** in both undifferentiated and SD or SDC treated hESCs (Figure 5B). Indeed, significant level of **OTX2** could be detected in undifferentiated hESCs and further increased in SD while not SDC treated cells (Figure 5B). Consistent to the data shown in Figure 4, low amount of **NANOG** could be detected in SDC while not SD treated cells (Figure 5B). Interestingly, we observed mutually exclusive pattern between **NANOG** and **OTX2** in some hESCs cells maintained in undifferentiated condition (Figure 5B, arrow), indicating a reciprocal antagonism between **NANOG** and **OTX2** in balancing the cell fate between “pluripotency” and “the default forebrain”. To further confirm the direct suppression of **OTX2** by **NANOG**, we examined the proximal region of **OTX2** promoter and identified couple known **NANOG** binding motifs (45) : CAAT and ATTA (Figure 5C). Further by ChIP assay, we showed that binding of **NANOG** on **OTX2** promoter region is significantly enriched not only in undifferentiated hESCs, but also in 24h of SDC differentiation, demonstrating that **NANOG** directly binds **OTX2** promoter region in hESCs, and especially in caudal initiation (Figure 5D). Taken together, we demonstrate that **NANOG** is an important regulator underlying rostral-caudal patterning at early stage of neural differentiation in hPSCs, as shown in the schematic diagram (Figure 5E).

**DISCUSSION**

Generation of expandable region-specific NPCs from hPSCs is important for either investigation on the molecular determinants of neural patterning or obtaining clinically relevant
region-specific NPCs for regenerative medicine. Neural patterning is a critical step in neural development as it starts to specify neural precursors with different functions. Studies on model systems such as mice, *Drosophila* etc., propose that neural patterning usually initiate with the specification on rostral-forebrain precursors (32, 46). The caudalising morphogens subsequently re-specify regional identity to establish other subdivisions of caudal neural precursors (47). Consistently, the rostral forebrain is also considered as a “default” cell fate during neural differentiation of hPSCs (19, 22, 23, 48). However, the detailed regulation and mechanisms underlying neural patterning at early stage has not been fully elucidated yet. Here, we show that *OTX2*, a known rostral marker gene exhibits relatively high level of expression in undifferentiated hPSCs and is a dominant trigger for hPSCs neural differentiation. GSK3β inhibition via CHIR at early stage of differentiation can switch the “default” rostral to the caudal fate. Mechanistically, we showed that NANOG was transiently sustained by CHIR at early differentiation stage through WNT signaling. Furthermore, NANOG served as a direct repressor for *OTX2* during differentiation to regulate the specification of the regional neural fate. Our findings provide new insight in understanding the molecular mechanisms underlying cell fate choice during neural morphogenesis, particularly in human model.

It has been reported that Wnt activation regulates the pluripotency in both human and mouse PSCs (49, 50) and NANOG is a key factor of pluripotency. We confirmed that NANOG was sustained by GSK3β inhibition at early stage of differentiation via WNT signaling (Figure 4E). Wnt signaling is also important to regulate neural patterning through forming morphogen gradients with other signal factors (7, 51-53). Our data then extend the role of NANOG in patterning the regional specified neural precursors at early stage of differentiation. Interestingly, OTX2 was also reported to interact with NANOG in different stage of development (45, 54, 55). Our findings in hPSCs model then extended these studies that NANOG and OTX2 form a mutual regulatory loop to regulate lineage decisions when hPSCs determine to exit pluripotency and initiate differentiation.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Neural Induction.*

Human embryonic stem cells (H1, H9, passage 40-50, Wicell, Madison, WI, USA) and iPSCs line (UC5C1, passage 15-25) were maintained on 6 well plates coated with matrigel (BD) in mTeSR1 (stem cell). The cells were passaged at a 1:4 split ratio each 3 or 4 days by 0.5mM EDTA-Na2 dissociation. Neural differentiation was performed as others previously described (12, 19). Pluripotent stem cells were passaged normally on matrigel, changed into neural induction medium (NIM) the following day. NIM contained N2B27 medium (DMEM/F12: Neuralbasal (1:1), 0.5x N2, 0.5x B27, 1% Glutmax (GIBCO), 1% non-essential amino acid (GIBCO) and inhibitors. SD-NIM includes 5µM SB431542 (sigma), 1µM Dorsomorphin (sigma), SDC-NIM includes 3µM CHIR99021 (sigma) also with SB431542 and Dorsomorphin. After 6 days of induction, cells were passaged on new matrigel plate using 0.2mg/ml dispase at a 1:2 ratio. Induction continued to day 14 changing NIM every two days. Cells were dissociated to small clumps and suspended in NSCM containing N2B27 medium and 20ng/ml EGF and 20ng/ml bFGF.

*Neural Differentiation in vitro.* SD NPCs and SDC NPCs were cultured in NSCM expanding in vitro in the form of single cell dissociated by accutase (Sigma). For pan-neural differentiation, NSC spheres were plated on matrigel-coated glass coverslips and cultured in N2B27 medium with BDNF, GDNF (all at 10 ng/ml, Peprotech) and 1µM cAMP (Sigma). Medium was changed every 2-3 days. Neuron
markers were examined by immunofluorescence after 4 weeks of differentiation.

**RNA Extraction and QPCR.** Trizol (Life technologies) was used to extract RNA according to manufacturer’s protocol. Real-time PCR was carried out using a CFX-96 Real-time PCR Detection System (BioRad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize measured transcript. Primer sequences are listed in supplemental information.

**Fluorescence-Activated Cell Sorting.** Human pluripotent stem cells or differentiated derivatives were dissociated into single cells using accutase, centrifuged and re-suspended with BD fixation buffer (catalog number, 554655) for 20 minutes at room temperature, subsequently washed in phosphate buffered saline (PBS), then permeabilized with dilute permeate buffer (BD, 11X,) for 10 minutes at 4°C. The straight marked antibody mouse anti-PAX6 (1:200, BD) and goat anti-T (1:200, R&D) were diluted with BD permeate buffer, incubated for 1 hour at room temperature, washed and suspended with PBS, then sorted using BD C6 analyzer. All primary antibodies information was listed in table S2.

**Immunostaining.** Cells were fixed in 4% PFA for 20 minutes at room temperature, and washed briefly for three times by PBS. Primary antibodies were diluted in buffer which containing 10% serum and 0.3% Triton X-100 in PBS. Then cells were incubated for 16-18 hours at 4°C. Primary antibodies were washed for three times by PBS. Second antibody diluting in PBS were incubated for 1 hour at room temperature. After washing 3 times by PBS, DAPI (0.2 ug/ml) was incubated for 10 minutes at RT. Then the coverslips were washed 2 times with PBS and mounted in Fluoromount-G (Life technologies).

**Western Blot and Phosphor-Western Blot analysis.** After remove medium, cells were washed using PBS. Then whole-cell protein was extracted by lysing cells in complete Lysis-M, an EDTA-free kit containing Complete Protease Inhibitor Cocktail or adding Phos-STOP (Roche, Indianapolis, IN, USA). Cell lysate was collected and centrifuged at 14,000 g for 5–10 min. The supernatants containing soluble proteins were used for further analysis. The extracted protein was fractionated by SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel and electroblotted onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were reacted with primary antibodies: GAPDH, PAX6 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), GSK3β mouse mAb (Cell Signaling Technology), phosphor-GSK3β (Ser9) rabbit mAb (Cell Signaling Technology), anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology), and anti-mouse IgG HRP-conjugated secondary antibody (Cell Signaling Technology). HRP was detected by Hyperfilm electrochemiluminescence (ECL, Invitrogen) and visualized with Gel Imaging Syste (SmartChemi™ II, Sagecreation).

**Electrophysiological analysis (patch-clamp recording).** Whole-cell patch-clamp recording techniques were used to analyze the physiological properties of induced NSCs-derived neurons in culture using a MultiClamp700B amplifier (Molecular Devices, USA). Individual coverslips transferred to the recording chamber were perfused with external solution containing (in mM): 145 NaCl, 3 KCl, 2 MgCl2, 3 CaCl2, 10 HEPES, and 10 glucose (pH with NaOH to 7.3). Patch pipettes with resistance between 8-10 MΩ were pulled from borosilicate glass and filled with intracellular solution with following composition (in mM): 136.5 K-glucanate, 0.2EGTA, 10HEPES, 5 NaCl, 1 MgCl2, 4 Mg-ATP and 0.3 Na-GTP, adjusted with KOH to pH 7.2, 285 osmol/l. The spontaneous postsynaptic current and current response to exogenous focal application of glutamate and GABA were recorded at a holding
potential of -70 mV Pressure ejection was used to deliver 1mM glutamate (10 p.s.i., 100ms) and 1 mM GABA (10 p.s.i., 100ms) through a puffing electrode (3-4 MΩ) placed near the recorded neuron. The patch pipette internal solution contained (in mM): The action potentials were elicited with injected current under the current clamp mode. Signals were digitized with a Digidata 1440, and acquired with pClamp 10 software. Off-line data analysis was performed using Clampfit 10 (Molecular Devices, USA).

**RNA-Sequencing.** Total of RNA was prepared with Direct-zol RNA MiniPrep kit (Zymo Research) following the manufacturer’s protocol. RNA was then purified, fragmented, reverse transcribed, labeled and amplified to generate sequencing-ready cDNA library with TruSeq RNA Sample Prep Kit (Illumina). A size selection step was included to purify cDNA libraries to enrich for 250-300 bp fragments instead of AMPure XP beads purification. The DNA was recovered from each gel slice using QIAquick gel extraction kit (QIAGEN). The cDNA library concentration was determined with QubitdsDNA HS Assay kit (Invitrogen). Additional sample concentrating step was included if the library concentration falls below required loading amount. The samples were run on MiSeq and Nexseq500 system with MiSeq Reagent Kits v2 (50 cycles) and NextSeq 500/550 High Output v2 (150cycles) (Illumina).

In data analysis, the correlation analysis, to avoid TPM divide by zero errors, log (0) errors, 1 is added to the TPM value and then log transform the expression value. In differential expression profiles analysis, the up-regulated genes in SDC NPCs and SD NPCs samples were those with fold change >2, the down-regulated genes in samples were those with fold change<1/2.

**Chromatin Immunoprecipitation (ChIP)-QPCR.** ChIP assays were performed as described elsewhere (56), with goat anti-NANOG and normal goat IgG. The sequences of all ChIP primers used in this study are given in Supplemental Table 2. The results were normalized to IgG control.

**Statistical analysis.** In general, experiments were done from three biological repeats. Data were presented as mean ± s.d. calculated using Prism. Data were compared by using standard or repeated-measures ANOVA. Pairwise comparisons were performed with the 2-tailed Student’s t test. *P<0.05; **P<0.01; ***P<0.001.

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Author contributions: G.P., Z.S. and B.L. conceived hypotheses and designed the experiments. G.P., Z.S. and X.Z. wrote the article. Z.S. and Y.Z. performed the experiments and generated data in all figures. In addition, L.W. and Y.G. provided assistance for research strategy; X.C. participated in experimental and analysis for Figure 1; H.W. completed and analyzed results of electrophysiology. Y.S. performed experiments for ChIP assay.

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FIGURE LEGENDS:

**Figure 1. Differentiation of forebrain or hindbrain NPCs from hPSCs.** (A) Scheme of neural differentiation protocols to induce forebrain and hindbrain NPCs from hPSCs. (B) Cell morphologic of hPSCs treated with SD or SDC at indicated time. Scale bars: 100 µm. (C) QPCR analysis on the
indicated marker genes in SD or SDC treated cells. (D) Pearson correlation on whole genome transcriptome between SDC NPCs and SD NPCs. The red points represent expression level of the indicated marker genes. R-value represents Pearson's Correlation Coefficient. (E) Box-plot on RNA level of selected marker genes based on RNA-seq data. (F) Gene ontology analysis on the differential expressed genes in SD and SDC treated cells. Yellow lines correspond to a twofold change.

**Figure 2. Commitment assay on rostral or caudal NPCs in hPSCs differentiation.** (A) CHIR was added or withdrew at indicated time points during SD induced neural differentiation of hPSCs. PAX6 was examined in cells from each group by FACS. (B) The expressions of other indicated marker genes in cells from each group were analyzed by QPCR. (C) OTX2 and GBX2 were the first responders on day 2 of differentiation.

**Figure 3. OTX2 play a dominated role in neural patterning during hPSCs differentiation.** (A) Cell morphology of hESCs with overexpressing OTX2, GBX2 or HOXB2. Scale bars: 100 µm. Expression levels of OTX2, GBX2 and HOXB2 were analyzed by QPCR. (B) Expressions of OCT4 and NANOG in the each indicated cell line. (C) Immunofluorescence results of PAX6, OCT4 and SOX2 in OTX2 overexpressed cell line. Scale bars: 50 µm. (D) and (E) QPCR analysis on the expression of indicated lineage genes in wild type hESCs and hESCs with OTX2 expression. (F) Expression of OTX2 and PAX6 under SD induction in GBX2 or HOXB2-overexpressed cells.

**Figure 4. GSK3β inhibition sustains NANOG and suppress OTX2 in hPSCs differentiation.** (A) QPCR analysis on the indicated genes in time course experiments of SD or SDC treated hPSCs differentiation. (B) Western blot analysis on NANOG and OTX2 in time course experiments of SD or SDC treated hPSCs differentiation at 24h and 48h. GAPDH served as the loading control. (C-D) Primitive strike markers, T, MIXL1 and EOMES were analyzed through FACS and QPCR. (E) Expression of WNT3A and NANOG in hESCs treated by SD, SDC, and SDC adding WNT inhibitor at 24h of differentiation. (F) Cell morphology and QPCR analysis of H1 ESCs with NANOG or GFP overexpression. Scale bar: 100um. (G) Expression of OTX2 in H1 hESCs with NANOG or GFP overexpression treated by SD or SDC. (H) Expression of GBX2 in hESCs with NANOG or GFP overexpression treated by SDC with or without WNT inhibitor.

**Figure 5. NANOG antagonize OTX2 to regulate neural patterning in hESCs.** (A) QPCR analysis on the expression levels of OTX2 or GBX2 in undifferentiated H1 hESCs and two iPSCs. (B) Dual immunostaining of NANOG and OTX2 in untreated, SD or SDC treated hESCs. Scale bar: 50um. (C) NANOG binding motifs (highlighted) in proximal region of human OTX2 promoter. Four pair of primers to detect NANOG bindings in ChIP assay are shown. (D) ChIP-QPCR assay to detect NANOG bindings in OTX2 gene region by the designed primer pares. Goat IgG serves as negative control. (E) The schematic diagram for the role of NANOG in fate decision during neural patterning in hESCs.
**Figure 1**

A) Day 0, Day 6, Day 14

- S/D: SB431542/Drosomophrin
- S/D/C: SB431542/Drosomophrin/CHIR99021

Gene profiling

- Forebrain, S/D NPC
- Hindbrain, S/D/C NPC

B) Day 0, Day 6, Day 14

- S/D
- S/D/C

C) Relative expression

| Gene          | S/D-NPC P1 | S/D/C-NPC P1 |
|---------------|------------|--------------|
| PAX6          | 0.000      | 0.000        |
| SOX1          | 0.005      | 0.005        |
| SOX2          | 0.2        | 0.2          |
| NESTIN        | 0.4        | 0.4          |
| EMTX2         | 1.2        | 1.2          |
| OTX2          | 1.5        | 1.5          |
| En1           | ***        | ***          |
| Lmx1b         | ***        | ***          |
| HOXA4         | ***        | ***          |
| HOXB2         | ***        | ***          |
| GBX2          | ***        | ***          |

D) Gene expression log2(TPM+1)

E) Gene expression log2(TPM+1)

F) Log fold change (log2)

- S/D: Up: 1313
- S/D/C: Up: 1398

Terms:
- midbrain-hindbrain boundary development
- regulation of epithelial cell proliferation
- Wnt receptor signaling pathway
- morphogenesis of an epithelium
- spinal cord association neuron differentiation
- tissue morphogenesis
- regulation of nervous system development
- spinal cord development
dorsal spinal cord development
hindbrain development
- cell morphogenesis
- cell morphogenesis involved in differentiation
- anterior/posterior pattern formation
- neuron differentiation
- embryonic morphogenesis
- cerebral cortex neuron differentiation
- epithelium development
- olfactory lobe development
- camera-type eye development
- pattern specification process
- forebrain regionalization
telencephalon development
- skeletal system development
- sensory organ development
- visual perception
- sensory perception of light stimulus
- regulation of cell proliferation
- forebrain development
Figure 3

A

Relative expression

B

Relative expression

C

D

Relative expression

E

Relative expression

F

Relative expression
Figure 5

A

Relative expression

0.000
0.005
0.010
0.015

H1

iPS1

iPS2

OTX2

GBX2

B

Relative NANOG enrichment to IgG

DAPI NANOG OTX2 Merge

S/D Day 2

S/D/C Day 2

OTX2

C

OTX2 promoter CHIP primers

Promoter

human OTX2 gene

NANOG binding site

D

Relative NANOG enrichment to IgG

H1

SD 24H

SDC 24H

E

S/D

OTX2

Forebrain

NANOG

S/D/C

WNT

GBX2

Hindbrain

hPSC

OTX2

NANOG
Antagonism between the transcription factors NANOG and OTX2 specifies rostral or caudal cell fate during neural patterning transition
Zhenghui Su, Yanqi Zhang, Baojian Liao, Xiaofen Zhong, Xin Chen, Haitao Wang, Yiping Guo, Yongli Shan, Lihui Wang and Guangjin Pan

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