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Wnt signal activation induces midbrain specification through direct binding of the beta-catenin/TCF4 complex to the *EN1* promoter in human pluripotent stem cells

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Abstract

The canonical Wnt signal pathway plays a pivotal role in anteroposterior patterning and midbrain specification during early neurogenesis. Activating Wnt signal has been a strategy for differentiating human pluripotent stem cells (PSCs) into midbrain dopaminergic (DA) neurons; however, the underlying molecular mechanism(s) of how the Wnt signal drives posterior fate remained unclear. In this study, we found that activating the canonical Wnt signal significantly upregulated the expression of *EN1*, a midbrain-specific marker, in a fibroblast growth factor signal-dependent manner in human PSC-derived neural precursor cells (NPCs). The *EN1* promoter region contains a putative TCF4-binding site that directly interacts with the β -catenin/TCF complex upon Wnt signal activation. Once differentiated, NPCs treated with a Wnt signal agonist gave rise to functional midbrain neurons including glutamatergic, GABAergic, and DA neurons. Our results provide a potential molecular mechanism that underlies midbrain specification of human PSC-derived NPCs by Wnt activation, as well as a differentiation paradigm for generating human midbrain neurons that may serve as a cellular platform for studying the ontogenesis of midbrain neurons and neurological diseases relevant to the midbrain.

Introduction

The midbrain is a portion of the central nervous system that develops between the diencephalon and the hindbrain and contains numerous relay centers for neural circuits in voluntary motor control (substantia nigra), reward response (ventral tegmental area), transmission of auditory and visual information (inferior and superior colliculi), and regulation of sleep–wake stages (reticular

formation)¹. Because multiple types of neurons reside in the dorsal, medial, and ventral domains of the midbrain, including glutamatergic, γ -amino butyric acid (GABA)ergic, and dopaminergic (DA) neurons², malformation or functional impairment of midbrain neurons causes abnormal somatosensory information processing^{3,4}, reward responses⁵, and sleep control⁶, as well as neurological disorders such as Parkinson's disease⁷. Given the important roles in normal brain function and the diversity, the mechanisms of development and differentiation of midbrain-specific neurons from stem cells have been of great interest for decades⁸.

Wnt signal is one of the key regulatory pathways that determine the regional identities of neural cells in developing embryos^{9,10}, particularly those involved in

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specifying neural cells with posterior characteristics and implicated in midbrain morphogenesis¹¹. Deleting *Wnt1* resulted in the loss of the mid-hindbrain junction and failure in the midbrain and rostral hindbrain formation^{12–14}. In contrast, inhibiting Wnt signal by endogenous inhibitors (Dickkopf homolog-1 [Dkk-1] and Cerberus) was shown to specify forebrain regions in vertebrates^{15,16}. Moreover, Wnt ligands, depending on their concentration gradients, can either induce the expression of markers for posterior portions of the brain including engrailed 1 (En1), a midbrain marker¹⁷, and gastrulation brain homeodomain 2 (Gbx2), a hindbrain marker¹⁸, or suppress that of forebrain markers in explants of early avian embryos, suggesting that Wnt acts as a critical posteriorizing factor in vertebrates¹¹. The hypothesis that similar developmental programs can be applied to the human system has been supported by recent differentiation studies using human pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in which the endogenous level of the Wnt signal determined the regional identity along the anteroposterior (AP) axis¹⁹; moreover, stimulating Wnt signal could facilitate the derivation of DA neurons that retain features of midbrain origin^{20–22}. It was known that DA neurons arise from the floor plate of ventral midbrain, not of hindbrain²³. Thus, in attempts to induce authentic midbrain DA neurons, floor plate specification was first accomplished by early exposure of human PSCs to Sonic Hedgehog (SHH)²⁴. The differentiation strategy has been eventually evolved to combined modulation of both Shh and Wnt signals²⁰. Despite all the implication of the Wnt signal in AP patterning and midbrain specification in both animal and human systems, the detailed molecular mechanism underlying how the Wnt signal drives posterior fate during neural differentiation and how inhibitors of glycogen synthase kinase 3 (GSK3), a key enzyme mediating the canonical Wnt signal, facilitate midbrain specification during human stem cell differentiation remained unclear.

Fibroblast growth factor 8 (FGF8) is secreted from the isthmus organizer located in the mid-hindbrain junction and thus composes a regulatory network for proliferation, survival, and patterning in the midbrain region^{25,26}. It also acts as a key factor in inducing human PSCs into DA progenitors in cooperation with SHH because its expression in the developing embryonic brain is highly correlated with the region in which midbrain DA neurons arise²⁷. A previous study supported this idea by providing evidence that priming human ESC-derived neural precursor cells (NPCs) with FGF8 increased the number of cells with a midbrain fate in the culture²⁸. As such, the molecular mechanism by which FGF8 contributes to fate determination or regional identity of developing neural

cells in the midbrain area has been of interest in both developmental and stem cell biology.

Here we explored the role of canonical Wnt signal in AP patterning during neural differentiation of human PSCs. In particular, we investigated the molecular events during midbrain specification by Wnt activation while characterizing the interaction between the β -catenin/T-cell factor (TCF) complex and the *EN1* promoter region and exploring the potential role of FGF8 in the same developmental context. Lastly, we examined the differentiation potential of NPCs with a midbrain fate via small molecule-based regulation of the canonical Wnt signal pathway.

Materials and methods

Human PSC culture and differentiation

We routinely cultured the undifferentiated human ESC line WA09 (H9, p37-49, WiCell Inc., Madison, WI, USA), retrovirus-derived iPSCs (iPS-WT3)²⁹, and episomal vector-derived iPSCs (iPS-Epi3)³⁰, and obtained the homogenous population of NPCs from them through modified dual SMAD inhibition and neural rosette selection as described previously³¹. The NPCs were used as a cellular platform to test the effects of Wnt signal on regional specification. Small molecules including 0.1–2 μ M of 6-bromoindirubin-3'-oxime (BIO; Sigma-Aldrich, St. Louis, MO, USA), 10 μ M of 1-azakenpaullone (1-AKP; Sigma-Aldrich), and 5 mM lithium chloride (LiCl; Sigma-Aldrich) were used to modulate Wnt signal. We used 100 ng/ml FGF8b (PeproTech, Rocky Hill, NJ, USA) as a control agent for midbrain specification²⁸. To further differentiate NPCs into neurons, we mechanically detached the cell clumps and plated them after mild trituration on plates coated with Matrigel (Corning, Corning, NY, USA) and then cultured them in differentiation medium composed of DMEM/F12 supplemented with N2 (1 \times ; Invitrogen, Carlsbad, CA, USA), B27 supplement (1 \times ; Invitrogen), and various factors including 20 ng/ml glial cell line-derived neurotrophic factor, 20 ng/ml brain-derived neurotrophic factor, 2 ng/ml transforming growth factor β (TGF β) 3 (all from PeproTech), 200 μ M ascorbic acid, and 5 μ M forskolin (Sigma-Aldrich). In some experiments, we exposed NPCs to 200 ng/ml recombinant human SHH CII24 (R&D Systems, Minneapolis, MN, USA) and 100 ng/ml of FGF8b for a week before neuronal maturation.

Semi-quantitative and quantitative (real-time) RT-PCR analyses

We performed RT-PCR and quantitative RT-PCR (qRT-PCR) as described previously³¹; the primer sequences are listed in Supplementary Table 1. Briefly, we extracted total RNA using an Easy-Spin[®] Total RNA Purification Kit (iNtRON Biotechnology, Seoul, Korea),

and then converted 1 µg of RNA into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). QRT-PCR was performed using SYBR[®] Premix ExTaq[™] (TAKARA BIO Inc., Otsu, Japan) and the CFX96 Real-Time System (Bio-Rad). Beta-actin was used as an endogenous reference to calculate Ct values and relative expression levels ($2^{-\Delta\Delta C_t}$) of target genes. For semi-qRT-PCR, we performed PCR with the 2× EmeraldAmp[®] GT PCR Master Mix (TAKARA BIO Inc.) and 10 pmol of each primer (Supplementary Table 1). Amplified transcripts were normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific signal.

Lentiviral production for small hairpin RNA (shRNA)

For the β-catenin knockdown experiment, we obtained two shRNA sequences from Addgene (Plasmid #18803: pLKO.1 puro shRNA; #19761: pLKO.1.sh.β-catenin.1248; Cambridge, MA, USA). We constructed scrambled shRNA control using the HuSH[™] shRNA plasmid pGFP-C-shLent vector (OriGene, Rockville, MD, USA) and produced the viruses by transient transfection of 293T cells with lentiviral packaging plasmids (pLP1, pLP2, and pLP/VSVG; Invitrogen). We determined virus concentration using a Lenti-X Concentrator (Clontech, Mountain View, CA, USA) following the manufacturer's protocol, and we monitored knockdown efficiency by qRT-PCR and immunoblotting against β-catenin (see below).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, washed with PBS, and perforated with PBS containing 0.1% Triton X-100 for 10 min. Then, the samples were incubated with blocking buffer (PBS containing 2% bovine serum albumin) for 1 h. The cells were incubated with primary antibodies diluted in blocking buffer at 4 °C overnight. The following primary antibodies were used: rabbit anti-Nestin (1:1000, Millipore, Billerica, MA, USA), mouse anti-β-catenin (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-SOX1 (1:100, R&D Systems), mouse anti-EN1 (1:50, DSHB, Iowa City, IA, USA), rabbit anti-TUJ1 (1:1000, Covance, Richmond, CA, USA), goat anti-OTX2 (1:300, R&D systems), sheep anti-TH (1:300, Pel-Freez, Rogers, AR, USA), rabbit anti-TH (1:300, Pel-Freez), rabbit anti-glutamate (1:200, Sigma-Aldrich), rabbit anti-GABA (1:2500, Sigma-Aldrich), goat anti-LMX1A (1:200, Santa Cruz), rabbit anti-FOXA2 (1:300, Millipore), mouse anti-synaptophysin (1:1000, Millipore), and rabbit anti-MAP2 (1:500, Millipore). The samples were washed three times with PBS and then incubated with fluorescence-tagged secondary antibodies (Alexa Fluor 350, Alexa Fluor 488, and Alexa Fluor 594, 1:500, Invitrogen) in blocking buffer for 30 min at room

temperature. The cover slips were rinsed three times with PBS and mounted onto slides using mounting medium (Vector Laboratories, Burlingame, CA, USA). All images were obtained using a fluorescence microscope (DP71, FSX100; Olympus, Tokyo, Japan) or an LSM700 confocal microscope (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany).

Immunoblotting

Cells were washed once with ice-chilled PBS and lysed in a RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Roche Applied Science, Mannheim, Baden-Württemberg, Germany). Protein was quantified using the Bradford protein solution (Bio-Rad), and then 20 µg of protein samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Millipore). After incubation with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, the membrane was incubated with primary antibodies (mouse anti-β-catenin (Santa Cruz Biotechnology), mouse anti-EN1 (Abcam, Cambridge, UK), and mouse anti-β-actin (Sigma-Aldrich)) for 1 h at room temperature or overnight at 4 °C. The membrane was washed three times with TBS-T and then incubated in a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse secondary antibody for 30 min at room temperature. The membranes were washed with TBS-T three times and developed with the enhanced chemiluminescence system (Pierce, Rockford, IL, USA) according to the manufacturer's protocols.

Co-immunoprecipitation (Co-IP) assay

Total cell lysates were prepared by NETN lysis buffer (100 mM NaCl, 20 mM Tris-Cl, 0.5 mM EDTA, and 0.5% (v/v) NP-40) with a protease inhibitor cocktail (Roche Applied Science). Total protein was obtained from cell lysates by centrifugation at 12,000×g for 30 min at 4 °C and quantified using the Bradford protein solution (Bio-Rad). The protein samples were incubated with a mouse anti-TCF-4 antibody (Upstate, Billerica, MA, USA) overnight at 4 °C and then incubated with protein G-Sepharose beads (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for 1 h at 4 °C with rotation. The beads were collected by centrifugation, washed three times with the same lysis buffer, dissolved in sample buffer, and analyzed by SDS-PAGE followed by detection with mouse anti-β-catenin antibody (Santa Cruz Biotechnology).

Chromatin immunoprecipitation (ChIP) assay

For ChIP assays, cells were cultured with BIO for 3 days, at which point the cells were fixed in formaldehyde for 10 min, and then 0.125 M glycine was added to stop the reaction. The cells were washed twice with PBS. The ChIP

assays were performed with a ChIP kit (R&D Systems) according to the manufacturer's instructions. Briefly, the fixed cells were lysed and sonicated to shear the genomic DNA into 200–500 bp fragments. The cross-linked complexes (DNA–protein) were immunoprecipitated with anti-TCF4 antibody (Millipore) or normal goat IgG (Santa Cruz). ChIP-enriched DNA was quantified by qRT-PCR with primer pairs designed to flank the putative TCF-binding element. The primer sequences are listed in Supplementary Table 2.

Plasmid and reporter gene constructs

Expression vectors for Flag-tagged constitutively active β -catenin (β -catenin 4A; S33A, S37A, T41A, and S45A) and Myc-tagged wild-type TCF-4 were obtained from Addgene (plasmids #24204 and #16514). The human *EN1* promoter fragment (–4961 ~ –81) was amplified by PCR using ExTaq (TAKARA BIO Inc.) and human dermal fibroblast's genomic DNA as a template, and was subcloned into the pGL3-basic Luciferase Reporter Vector (Invitrogen). Cloning was performed using an In-fusion[®] HD Cloning Kit (Clontech Laboratories Inc., Mountain View, CA, USA). The primers were generated using SnapGene[®] software and are listed in Supplementary Table 3. The sequence of the *EN1* promoter was aligned with the sequence of human *EN1* (engrailed homeobox 1) on chromosome 2 (NCBI, NG_007123).

Transfection and luciferase reporter assays

For reporter assays, HEK 293 cells or NPCs were seeded into a 12-well culture plate and transiently transfected with 0.1 μ g reporter plasmid and 30 ng pRL-SV40 plasmid (Promega, Madison, WI, USA) containing the *Renilla* luciferase gene under the control of the Simian virus 40 promoter using polyethyleneimine (PEI, Sigma-Aldrich). For the over-expression of β -catenin and TCF4, cells were transfected with plasmid using PEI. Forty-eight hours after transfection, the cells were harvested, and luciferase activity was measured and normalized to *Renilla* luciferase activity. The amount of DNA in each transfection was kept constant by adding an appropriate amount of empty vector.

Electrophysiological recording

Electrophysiological recording of the differentiated neurons was conducted by whole-cell patch-clamp technique with an EPC 10 USB amplifier (HEKA Elektronik, Lamprecht, Germany). The differentiated neurons attached on the glass cover slips were transferred to a 0.5-ml recording chamber that was superfused continuously with a normal Tyrode solution by gravity at a rate of approximately 10 ml/min. The recording electrodes were manufactured from borosilicate glass capillaries (Sutter Instrument Company, Novato, CA, USA) using a PC-10

puller (Narishige Co., Tokyo, Japan), and the resistance of the pipettes was 4–6 M Ω when filled with the pipette solution described below. The cell membrane capacitance and series resistance were electronically compensated (typically 80%). After a giga-seal was obtained by negative suction, the cell membrane was ruptured by gentle suction to establish a whole-cell configuration. Data were filtered at 3 kHz, digitized at 10 kHz, and analyzed using Pulse ver. 8.67 (HEKA Elektronik) and Origin 6.1 software (MicroCal, Northampton, MA, USA). All experiments were performed at room temperature (21–24 °C). The membrane potentials and currents were recorded with the following normal Tyrode solution: 143 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.5 mM NaHPO₄, 10 mM glucose, and 5 mM HEPES. The pH level was adjusted to 7.4 with NaOH. The pipette solution contained 150 mM KCl, 1.0 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, and 2 mM Mg-ATP, and the pH level was adjusted to 7.2 with NaOH.

Statistics

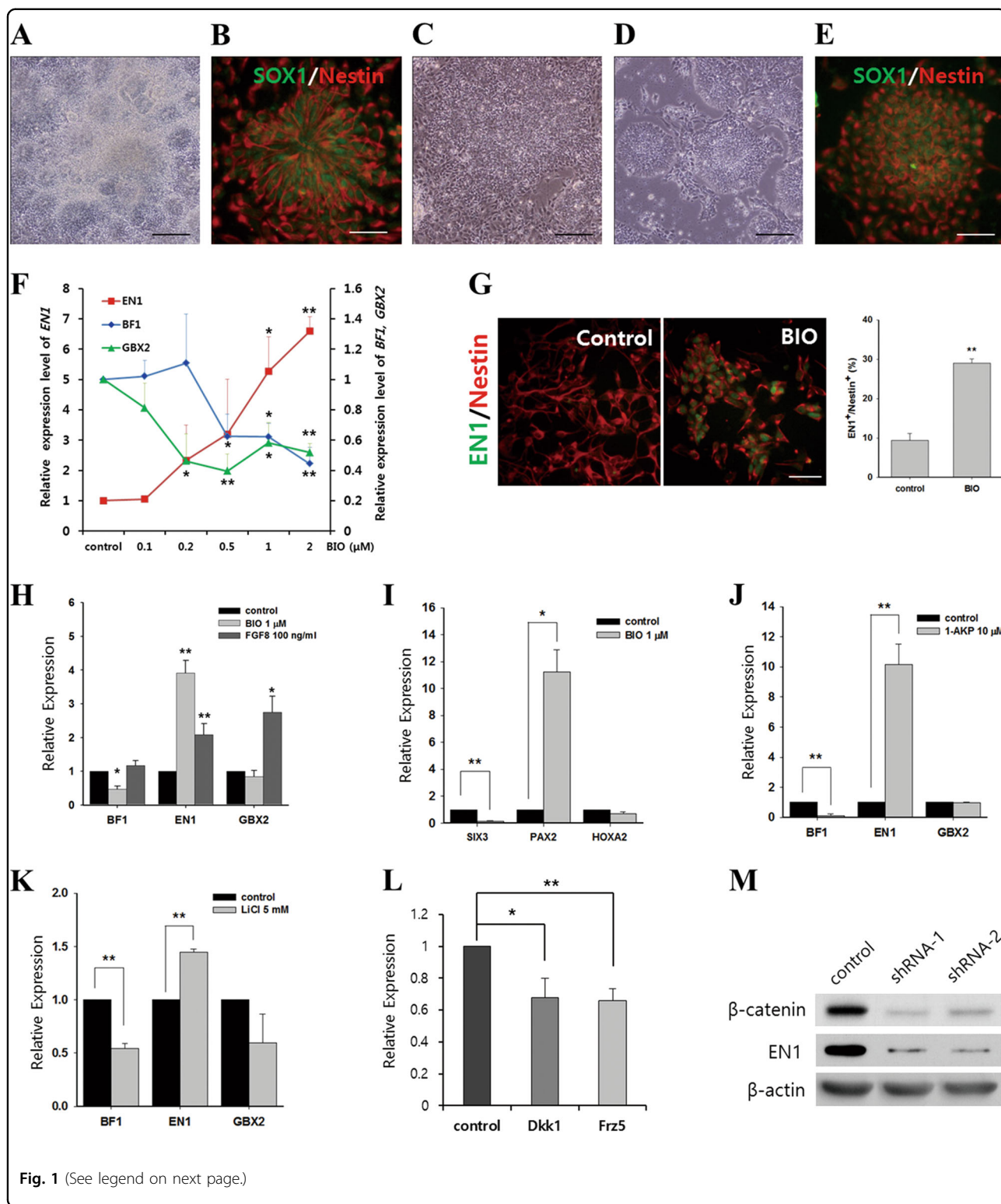
Values are expressed as the mean \pm S.E.M unless indicated otherwise. We used ANOVA and Student's *t* test using SPSS software version 12.0 to determine the statistical significance.

Results

Activating the canonical Wnt pathway induces midbrain-specific gene expression in human PSC-NPCs

To obtain a cellular platform for investigating the role of Wnt signal in AP patterning, we generated a NPC population from human PSCs through the simultaneous inhibition of the bone morphogenetic protein and TGF β signals with small molecules (dorsomorphin and SB431542)³¹. We observed extensive neural rosette formation in the center of the colonies 4–5 days after attachment of embryoid bodies that were exposed to dorsomorphin and SB431542 for 4 days (Fig. 1a). Mechanically isolated neural rosettes were highly positive for SOX1 and Nestin and maintained the typical morphology of the neural rosette during expansion (Fig. 1b, c). Because neural rosettes are an NPC population that can respond to extrinsic patterning cues directing regional specification^{32,33}, we used these cells to investigate cellular events during AP patterning by modulating Wnt signal pathway.

To test the potential effect of Wnt signal activation on AP patterning, we first treated human ESC-derived NPCs with a small-molecule GSK3 inhibitor, BIO, which is known to activate the canonical Wnt signal pathway^{34,35}, and then examined the expression of the following representative AP markers by qRT-PCR: brain factor 1 (*BF1*, a forebrain marker³⁶ also known as *FOXP1*), *EN1*, and *GBX2*. As a positive control culture condition, we



treated some cells with 100 ng/ml FGF8 because it was shown to induce midbrain-fated NPCs when treated at early stage of NPCs²⁸. Once treated with BIO, NPCs proliferated and formed compact colonies while

maintaining the expression of the NPC markers (Fig. 1d, e). Quantitative gene expression analysis following seven days of treatment revealed that *EN1* expression increased gradually in a dose-dependent manner up to five times at

(see figure on previous page)

Fig. 1 Activation of Wnt signal induces midbrain characteristics in human ESC-derived NPCs. **a** Efficient induction of neural rosette cells from human ESCs by co-treatment with dorsomorphin and SB431542. **b** Strong immunoreactivity for SOX1 and Nestin in neural rosette cells. Morphology of neural rosette cells expanding in either the absence (**c**) or the presence of 1 μ M BIO (**d**). **e** NPCs treated with BIO maintained immunoreactivity for SOX1 and Nestin. **f** Treatment with BIO upregulated *EN1* expression and downregulated expressions of *BF1* and *GBX2* in dose-dependent manner. **g** BIO treatment significantly increased the number of EN1-positive neural cells. **h** The inductive effect of BIO treatment on midbrain fate appeared to be more specific than that of FGF8. **i** Expression pattern of another set of regional markers (*SIX3* for forebrain; *PAX2* for midbrain; and *HOXA2* for hindbrain) supported the midbrain-biased fate of NPCs treated with BIO. Treatment with other known GSK3 inhibitors, 1-AKP (**j**) and LiCl (**k**), resulted in regionalization comparable to BIO treatment. **l** Treating NPCs with Wnt antagonists (100 ng/ml DKK-1 and 500 ng/ml frizzled-5) downregulated the endogenous level of *EN1* transcript in NPCs. **m** Immunoblot for β -catenin and EN1 protein after introduction of two different β -catenin-specific shRNAs (shRNA-1 and shRNA-2). EN1 protein level was directly downregulated by β -catenin knockdown. β -actin was a loading control. All data are expressed as mean \pm S.E.M. Statistical significance was estimated using Student's *t* test (**g**, **i**, **j**, and **k**) or one-way ANOVA (**f**, **h**, and **l**) from at least three independent experiments; **p* < 0.05, ***p* < 0.01. Scale bar: 200 μ m for **a**, **c**, and **d**; 50 μ m for **b**, **e**, and **g**

1 μ M, whereas the *BF1* and *GBX2* expression decreased as the BIO concentration increased (Fig. 1f). Although the *EN1* expression was gradually upregulated as the BIO concentration increased, detrimental effects on cell survival restricted us to use more than 2 μ M BIO for subsequent experiments (data not shown). Immunofluorescence staining supported the qRT-PCR results by showing an evident increase in the number EN1-positive cells in the BIO-treated group compared with the non-treated control (Fig. 1g). Interestingly, BIO treatment appeared to be more specific for inducing the midbrain marker than FGF8 treatment. The treatment with 1 μ M BIO increased *EN1* expression by two folds compared to 100 ng/ml FGF8 treatment which instead increased the level of *GBX2* transcripts with no change of *BF1* expression (Fig. 1h). Upregulation of both *EN1* and *GBX2* by FGF8 treatment might result from the fact that FGF8 affects the specification of both the midbrain and hindbrain²⁵. It was also intriguing that *BF1* expression was not affected by FGF8 treatment contradicting to the previous result²⁸ (Fig. 1h). The presence of SOX1-positive cells in the neural rosettes (Fig. 1b) and the previous finding that FGF8 suppresses the forebrain fate only in the “primitive” NPCs (paired box protein 6 [PAX6]-positive, but SOX1-negative)²⁸, indicate that a substantial number of the NPCs in our experiment are likely in the “definitive” (i.e., PAX6/SOX1-double positive) stage and thus less responsive to the activity of FGF8 in suppressing the forebrain fate. Nonetheless, BIO treatment potently increased *EN1* expression. This result suggests that BIO is a strong regionalizing cue and that our system stands valid to examine the effect of such regionalizing cue and its molecular mechanism.

Additional quantitative gene expression analysis with other AP markers, such as *SIX homeobox 3* (*SIX3*; forebrain³⁷), paired box gene 2 (*PAX2*; midbrain³⁸), and homeobox protein *HOXA2* (hindbrain³⁹), yielded similar results (Fig. 1i), supporting the midbrain-specific regionalization of human ESC-derived NPCs following BIO treatment. We also observed the same AP marker expression patterns after BIO treatment on both NPCs

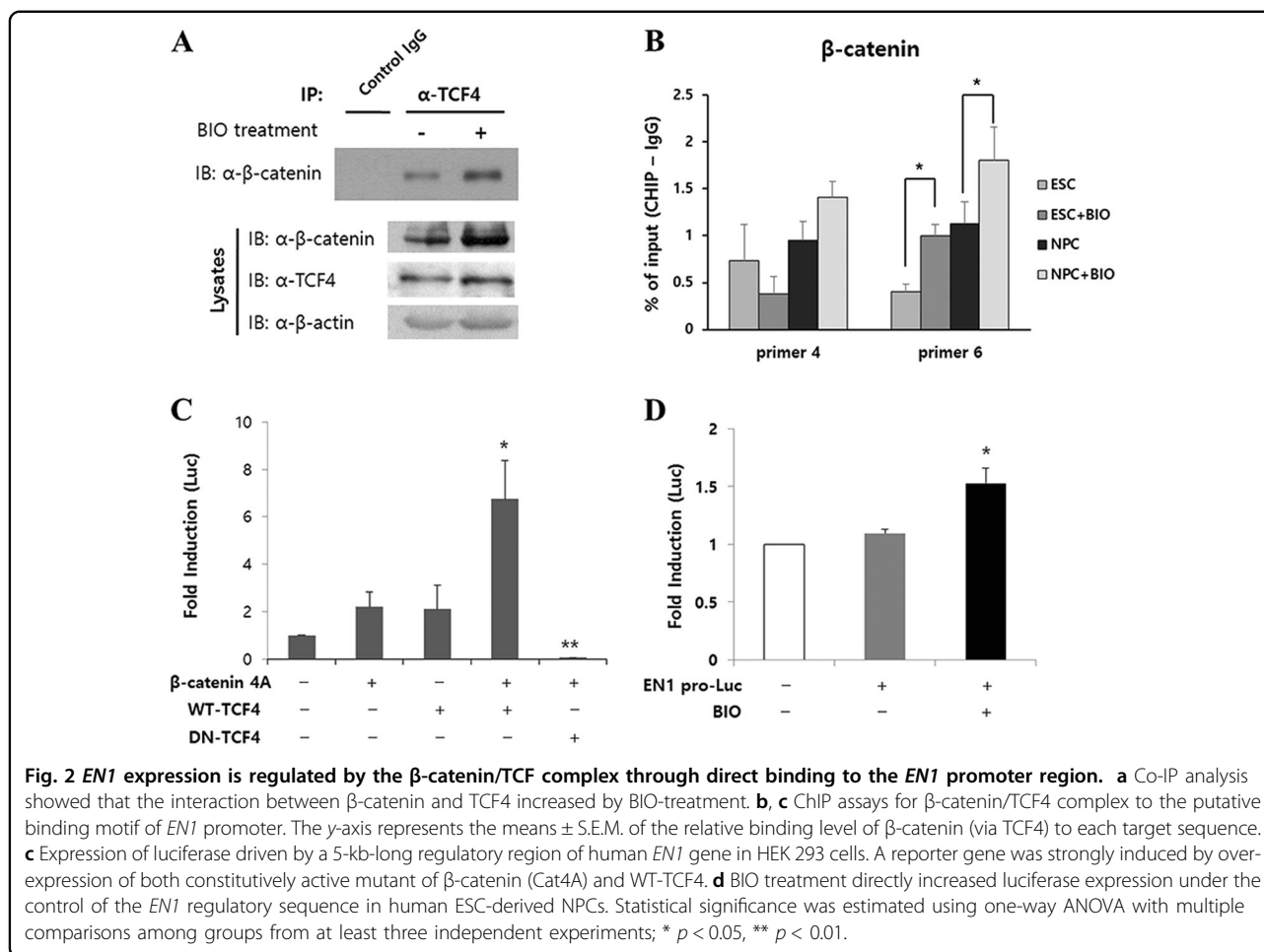
derived from two iPSC lines (iPS-WT3 and Epi3; Supplementary Fig. 1A-B and data not shown) and human ESC-derived NPCs cultured in suspension as described previously³¹ (Supplementary Fig. 1C), suggesting the robust effect of BIO on midbrain specification regardless of human PSC type or cultivation method.

To confirm whether the effect of BIO on midbrain specification was indeed through the activation of canonical Wnt signal, we tested other small molecules that inhibit GSK3 in different modes of action, such as 1-AKP⁴⁰ and LiCl^{41,42}. Exposing human ESC-derived NPCs to 1-AKP robustly increased *EN1* expression, whereas *BF1* expression level strongly decreased (Fig. 1j). LiCl treatment elicited similar gene expression patterns, although the fold changes in gene expression were lower than those of the other inhibitors (Fig. 1k). These data support that midbrain-specific gene expression results from the activation of canonical Wnt signal via GSK3 inhibition^{43,44}.

As another approach to investigate the involvement of the canonical Wnt signal pathway in the midbrain specification, we inhibited endogenous Wnt signal in the NPC culture and examined the *EN1* expression level. When the NPCs were cultured in the presence of Wnt signal antagonists such as DKK-1 or frizzled5-Fc (FZD-5), basal *EN1* expression was found to be significantly lower in both cases (Fig. 1l); significant diminution of *EN1* expression by FZD-5 was also found in iPSC-derived NPCs (Supplementary Fig. 2). Furthermore, knockdown of β -catenin by transducing NPCs with specific shRNAs downregulated EN1 protein level as well as β -catenin (Fig. 1m). Taken together, our results strongly suggest that the canonical Wnt signal pathway directly contributes to the expression of midbrain-specific genes in human PSC-derived NPCs.

The β -catenin/TCF complex binds directly to the EN1 promoter region

Previous genetic studies in *Xenopus* and mice provided evidence that ablation of *Wnt1* disrupts the AP axis, and that *Wnt1* regulates *EN1* expression in the earliest stages^{12,45–47}. These findings prompted us to ask whether



EN1 expression is directly regulated through binding of transcriptional modulators, downstream of the canonical Wnt signal, to its regulatory DNA elements in the human system. First, we verified that the interaction between β -catenin and TCF4, a well-known interacting partner of nuclear β -catenin, was increased by BIO treatment in human ESC-derived NPCs on Co-IP assay (Fig. 2a)

We next sought to determine the regulatory element around the *EN1* gene locus for the β -catenin/TCF complex binding. Because β -catenin lacks DNA-binding motifs, we searched putative binding sites for TCF4 using Patch 1.0 Transfac[®]. We identified eight candidates localized within a 5-kb region upstream of the human *EN1* transcription start site (Supplementary Fig. 3A, sites 1–8), but only one (site 6) out of the eight putative binding motifs was confirmed to be the consensus sequence for TCF4 (Supplementary Fig. 3B). To determine whether the β -catenin/TCF4 complex binds to the putative binding motif in response to the activation of the Wnt signal, we performed a ChIP assay using an antibody specific for β -catenin after treatment with BIO. In result, the β -catenin/TCF4 complex binding to site 6 was

markedly induced after BIO treatment in human ESC-derived NPCs, whereas binding to other sites with non-consensus sequences (site 4, for example) did not take place significantly (Fig. 2b). Interestingly, β -catenin/TCF4 complex binding to site 6 also increased markedly with BIO treatment in human ESCs (Fig. 2b). Our result demonstrates that β -catenin/TCF4 complex binding not only appears to increase with neural differentiation (ESC vs. NPC in Fig. 2b), but also increases in response to Wnt activation (NPC vs. NPC + BIO in Fig. 2b). These results suggest that the accessibility of regulatory elements situated in *EN1* gene depends on developmental stage as well as the recruitment of β -catenin/TCF4 complex to its regulatory element via Wnt activation.

In order to further examine whether the β -catenin/TCF4 complex directly induces *EN1* transcription, we performed the promoter activity assay using an *EN1* promoter-luciferase reporter plasmid in HEK 293 cells. A constitutively active mutant of β -catenin (Cat4A), wild-type (WT)-TCF4, and dominant-negative (DN)-TCF4 expression plasmid were transfected either alone or in combination and then the luciferase activity was

measured. Surprisingly, co-transfection of the Cat4A and WT-TCF4 dramatically increased transcriptional activity compared with transfection of the Cat4A or WT-TCF4 alone (Fig. 2c). In addition, we observed no transcriptional activity when we co-introduced Cat4A with DN-TCF4 (Fig. 2c). These findings indicate that β -catenin and TCF4 bind to the *EN1* promoter and induce the transcription as a complex. Finally, when the *EN1* promoter activity was tested in human ESC-derived NPCs, BIO treatment indeed slightly but significantly upregulated luciferase activity compared with the non-treated control group (Fig. 2d). Collectively, our data demonstrate that activating the canonical Wnt pathway induces *EN1* expression through direct binding of β -catenin/TCF to the *EN1* promoter region in human ESC-derived NPCs.

FGF signal is required for the induction of EN1 by Wnt signal activation

Previous evidence demonstrating the involvement of FGF signal in AP patterning and in inducing midbrain and rostral hindbrain cells^{9,26,46–49} prompted us to further investigate the role of FGF signal in midbrain specification by Wnt activation. As shown in Fig. 3a, we detected the endogenous expression of various FGF transcripts in the NPC culture. We also found that treatment of GSK3 inhibitor increased the expression of FGF8 as well as functionally related FGFs (FGF17, 18) (Fig. 3a), which is consistent with the previous findings in rodents that FGF8 expression is induced by the activation of the Wnt signal in the midbrain^{9,46,47}. To determine whether an endogenous FGF signal is required for basal *EN1* expression, we treated human ESC-derived NPCs with 2.5 μ M SU5402, an FGF receptor tyrosine kinase inhibitor, for 5 days. Gene expression analysis showed that blocking endogenous FGF signal significantly reduced *EN1* expression (Fig. 3b), indicating that endogenous FGF signal is at least partially responsible for the basal expression of *EN1*. To further examine the involvement of FGF signal in induction of *EN1* expression by Wnt activation, we treated NPCs with either recombinant FGF8 or SU5402 in the presence of BIO. After 5 days of treatment, qRT-PCR analysis revealed that co-treatment with FGF8 and BIO upregulated *EN1* expression more than treatment with BIO alone, but the increment did not reach statistical significance (Fig. 3c). However, when FGF signal is blocked by treatment with SU5402 in the presence of BIO, the increased *EN1* expression, attributed to endogenous plus BIO-induced FGFs, was abrogated (Fig. 3c). Immunoblot analysis showed a similar pattern of EN1 protein level to the results from qRT-PCR (Fig. 3d, e). Intriguingly, the total protein levels of β -catenin increased significantly in all BIO-treated groups compared with the untreated control but were unaffected by the presence of FGF8 or SU5402 (Fig. 3d, e). A recent study showed that

FGF receptor tyrosine kinase directly phosphorylates β -catenin at Tyr142 upon FGF binding, liberating β -catenin from the membrane and allowing for transcriptional activation in the nucleus⁵⁰. This result prompted us to examine whether FGF signal would influence on the subcellular localization of β -catenin under the active Wnt signal. Immunocytochemical analysis of β -catenin showed that β -catenin immunoreactivity was localized in the plasma membrane in the control group, but BIO treatment remarkably localized β -catenin in the nuclei (Fig. 3f, g). Moreover, FGF signal was unlikely to affect its translocation to the nucleus by Wnt activation (the BIO group vs. the BIO + FGF8 or BIO + SU groups in Fig. 3f, g). Taken together, our data suggest that FGF signal positively influences EN1 expression in human ESC-derived NPCs but in a manner independent of the nuclear translocation of β -catenin.

Differentiation potential of midbrain NPCs induced by BIO treatment

Next, we explored the differentiation potential of the midbrain-fated NPCs. After 1 week of BIO treatment, we differentiated the NPCs under neuronal maturation conditions (see Materials and Methods). After 3 weeks of neuronal differentiation, immunocytochemical analysis showed that $62.3 \pm 3.4\%$ of the total cells were positive for TUJ1 in the BIO-treated group, whereas $33.9 \pm 6.1\%$ of the cells turned positive for TUJ1 in the control group (Fig. 4a), possibly attributed to Wnt promoted neurogenesis⁵¹. As we expected, BIO-treated NPCs generated more TUJ1-positive neuronal cells that co-expressed EN1 ($67.6 \pm 6.6\%$) than the control group ($10.6 \pm 12.7\%$, $p < 0.05$; Fig. 4b). Notably, the majority of EN1-positive cells in BIO-treated group was also positive for orthodenticle homeobox 2 (OTX2) ($70.6 \pm 10.0\%$; Fig. 4b). Given that dual immunoreactivity of EN1 and OTX2 is indicative of midbrain fate,^{11,21} the majority of neurons expressing EN1 was indeed of midbrain character. Interestingly, glutamate (Glu)-positive putative excitatory neurons were the majority ($58.7 \pm 6.7\%$) among EN1-positive cells followed by GABA-positive ($16.7 \pm 5.3\%$) and TH-positive cells ($10.2 \pm 2.8\%$) (Fig. 4c). Considering the fact that glutamatergic excitatory and GABAergic inhibitory neurons mainly arise within the dorso-medial region whereas DA neurons are born in the most ventral region in the midbrain⁵², BIO treatment appeared to drive NPCs toward dorso-medial midbrain fates. To further explore whether the NPCs treated with BIO could also potentially generate neurons of the ventral midbrain (e.g., DA neurons), we treated NPCs with 200 ng/ml SHH and 100 ng/ml FGF8 for a week following BIO treatment. Immunostaining followed by quantitative analysis revealed that the treatment with SHH and FGF8 increased the proportion of TH-positive cells among EN1-positive cells ($20.9 \pm 2.8\%$)

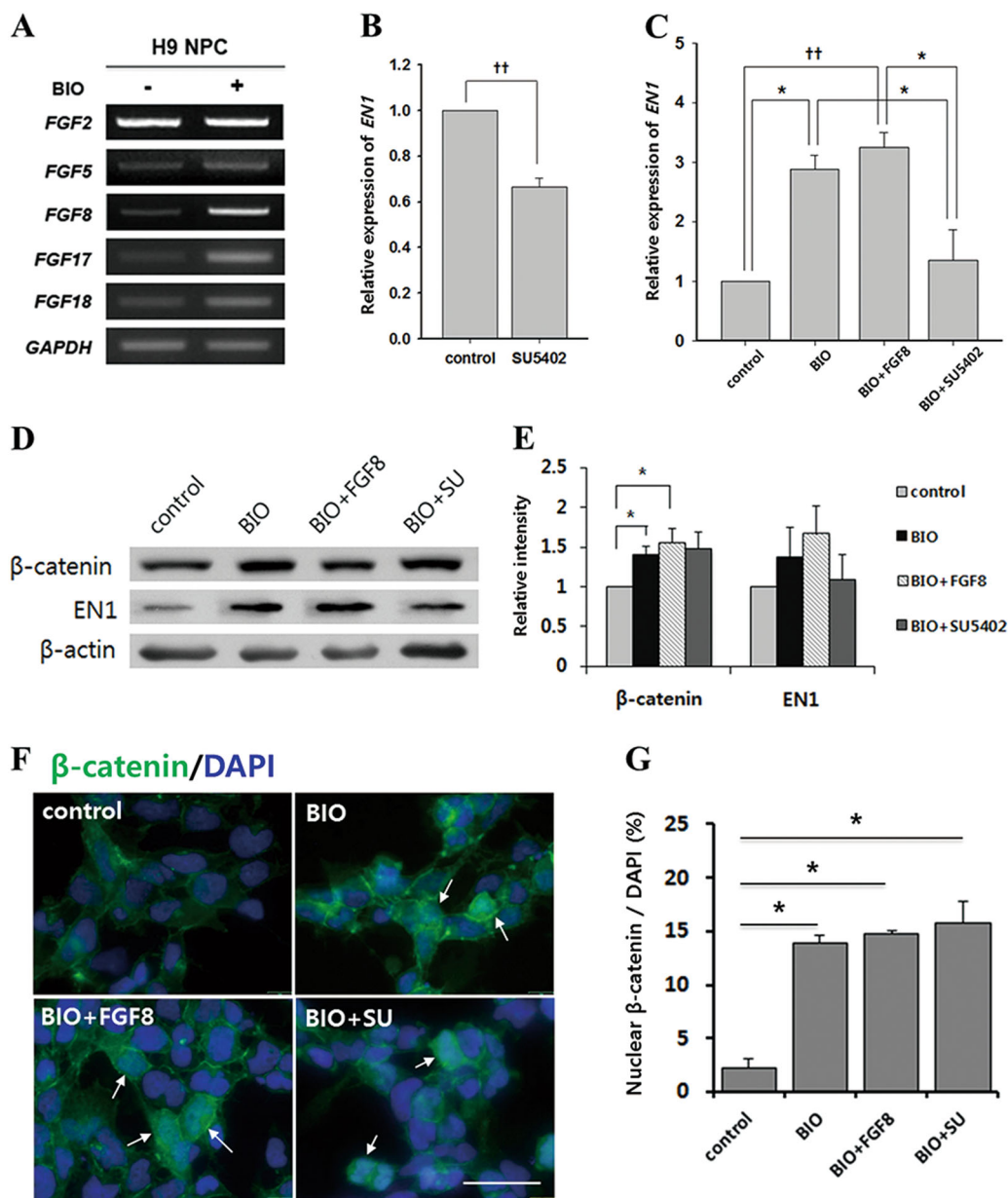


Fig. 3 FGF signal is required for induction of *EN1* by activation of Wnt signal. **a** Semi-qRT-PCR analysis showed endogenous and BIO-induced expression of various FGFs in human ESC-derived NPCs. **b** Inhibition of endogenous FGF signals by SU5402 significantly decreased basal *EN1* expression. **c** Co-treatment with BIO and FGF8 slightly increased *EN1* expression more than BIO alone whereas co-treatment of SU5402 with BIO abrogated the upregulation of *EN1* expression ($n = 4$). **d, e** Immunoblotting corresponded with the *EN1* expression in qRT-PCR ($n = 3$). **f, g** Nucleus localization of β -catenin was induced by Wnt activation, but not affected by FGF. White arrows indicate β -catenin in nucleus (scale bar: 20 μ m, $n = 3$). Statistical significance was examined by one-way ANOVA; $^{\dagger}p = 0.001$, $*p < 0.05$

while significantly decreasing the proportion of Glu-positive or GABA-positive cells ($4.8 \pm 3.0\%$ and $2.9 \pm 0.8\%$, respectively) (Fig. 4d vs. Fig. 4c). Intriguingly, further characterization of the TH-positive cells revealed that they rarely co-expressed LIM homeobox transcription factor 1 alpha (LMX1A), a determinant gene for midbrain DA neurons⁵³, or forkhead box protein A2 (FOXA2), a floor plate marker²³ (Fig. 4e, f, left). This result could be

attributed to the fact that authentic midbrain DA neurons originate from the floor plate of the midbrain²³ and the specification of floor plate fate is only efficient when human PSCs are exposed to an active form of SHH from the early stage of differentiation. Consistently, when we differentiated human ESCs using a previous method based on floor plate differentiation²⁰, most of the TH-positive cells strongly co-expressed LMX1A and/or FOXA2

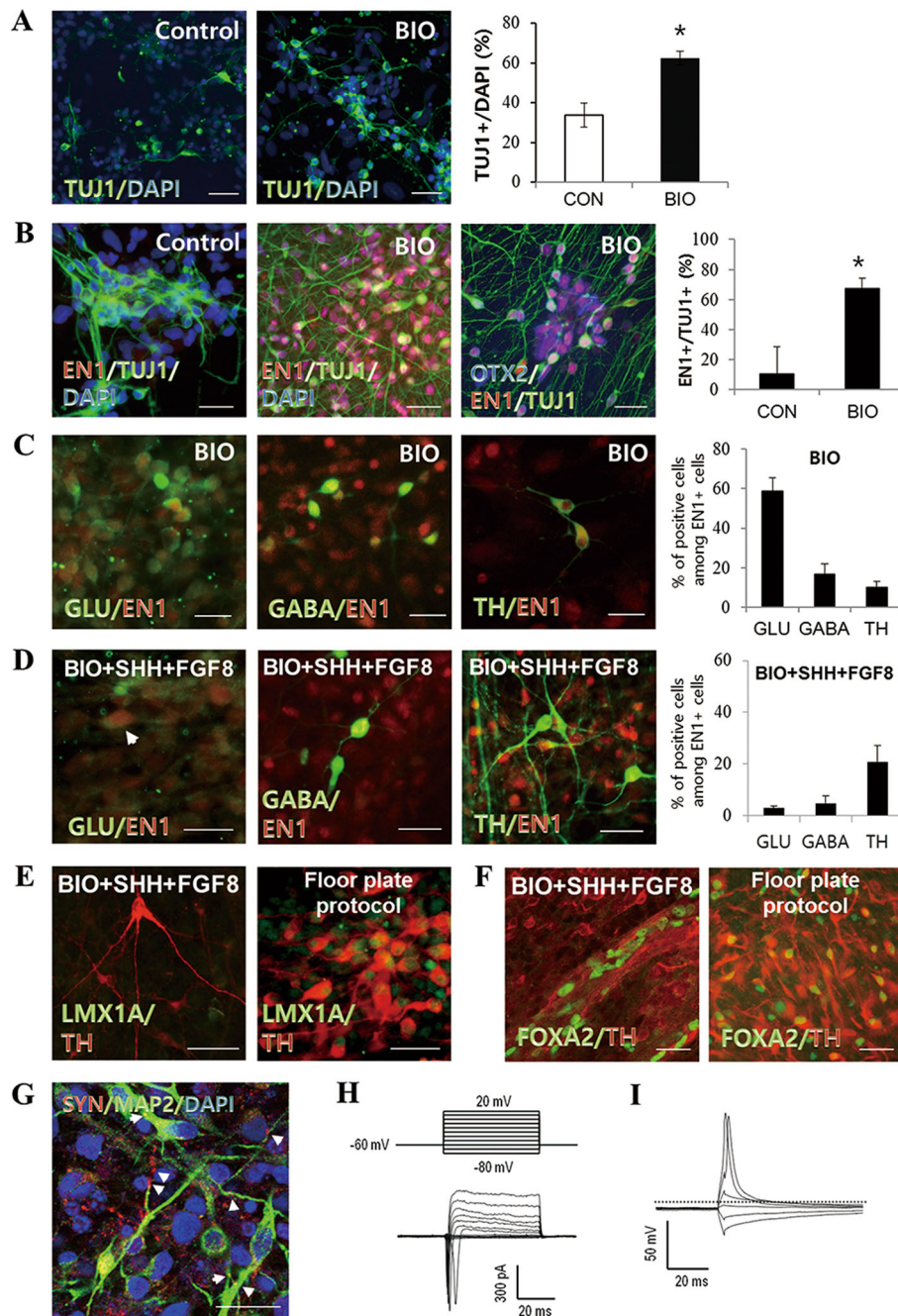


Fig. 4 Generation of functional midbrain neurons from the NPCs pre-exposed to BIO. **a, b** NPCs treated with BIO gave rise to TUJ1-positive and TUJ1/EN1 double-positive cells more than non-treated control NPCs. Note that the majority of EN1-positive neurons in BIO-treated group co-expressed OTX2 (**b**). **c** NPCs treated with BIO differentiated into various neuronal subtypes, primarily Glu-positive cells followed by GABA-positive and TH-positive cells. **d** The proportion of neuronal subtype cells differentiated by treatment with SHH and FGF8 following BIO. TH-positive cells were the most abundant, followed by GABA-positive and Glu-positive cells. **e, f** TH-positive cells generated by sequential treatment of BIO and SHH + FGF8 rarely co-expressed LMX1A or FOXA2 whereas TH-positive cells differentiated by floor plate-based protocol²⁰ were highly positive for both markers. **g–i** Neurons derived from NPCs treated with BIO were functional, as exemplified by the immunoreactivity of synaptophysin on dendrites (indicated by white arrows; **g**) and voltage-dependent membrane currents (bottom traces; **h**), depolarizing voltage steps (top traces; **h**) as well as evoked action potential by current injection (**i**). Bars in the graphs are presented as mean \pm standard deviation. Statistical significance was determined by Student's *t* test; **p* < 0.05. Scale bars: 20 μ m

(Fig. 4e, f, right). Thus, few TH-positive cells co-expressing LMX1A or FOXA2 might be resulted from an inefficient ventralization of NPCs because SHH was treated at a later stage (after BIO treatment) in our differentiation conditions.

Lastly, we assessed the functionality of neurons after the 3-week differentiation. As shown in Fig. 4g, microtubule-associated protein 2 (MAP2)-positive signals were colocalized with the immunoreactivity of synaptophysin on their dendrites or cell bodies, which indicates that these neurons have the potential to form synapses. We then tested the excitable electrophysiological property of the neurons. The resting membrane potential of the differentiated neurons ranged from -57 to -65 mV (-60.8 ± 1.1 mV, $n = 8$). The neurons exhibited voltage-dependent membrane currents that were elicited by 100-ms voltage steps between -80 and 20 mV from a holding potential of -60 mV (Fig. 4h, $n = 8/11$). Depolarizing voltage steps elicited both large outward potassium currents and fast inward sodium currents. In the current-clamp configuration, a short square current injection (3 ms) above the supra-threshold induced action potential in the neurons, and prolonged depolarizing current injections (500 ms) caused the neurons to fire a single action potential (Fig. 4i). These results indicate that the differentiated cells had characteristics of mature neurons.

Discussion

Wnt family proteins are expressed in the posterior region of vertebrate embryos during gastrulation, and accumulating evidence suggests Wnt signal as a specifying agent in caudal neural characterization^{11,13}. Previously, an elegant study provided direct evidence that Wnt signal, in combination with FGF signal, is responsible for inducing posterior characteristics in the neural cells of gastrulating chick embryos in a dose-dependent manner¹¹. A later report by the same group showed that cells with the molecular and functional characteristics of the isthmic organizer could be induced from naïve neural plate cells by a combination of Wnt and FGF⁵⁴.

In substantial agreement with findings from animal models, our data demonstrate that the activation of the canonical Wnt pathway by small-molecule GSK3 inhibitors induces a midbrain-like gene expression profile in human ESC-derived NPCs⁴⁹. Our data also show that inhibition of the canonical Wnt pathway by either a Wnt signal antagonist or β -catenin knockdown significantly reduces endogenous *EN1* expression, supporting that the canonical Wnt pathway is critical for the midbrain specification of human ESC-derived NPCs. Our data also recapitulate previous results from embryos of lower vertebrates with human ESC-derived NPCs by showing that (1) FGF signal is required for posteriorization by activation of Wnt signal in human ESC-derived NPCs⁵⁵

(Fig. 3c–e), and that (2) the activation of canonical Wnt signal by GSK3 inhibition elevates the expression of FGF8^{11,56} (Fig. 3a). These findings suggest that both signals cooperate in promoting midbrain-specific gene expression in human ESC-derived NPCs^{11,55,56} and that this cellular machinery is evolutionarily well preserved. Although the detailed molecular mechanism of how Wnt and FGF signals interact to induce *EN1* expression in human ESC-derived NPCs requires further study, our data clearly show that FGFs are necessary for induction of *EN1*, at least in a manner independent of the nuclear translocation of β -catenin (Fig. 3f, g). Moreover, our work provides molecular evidence that the β -catenin/TCF complex directly interacts with regulatory element of the *EN1* gene in human ESC-derived NPCs. Most importantly, we identified a potential β -catenin/TCF complex-binding site in the regulatory element of human *EN1* gene. As hypothesized, GSK3 inhibition resulted the enhanced binding of the β -catenin/TCF complex to the putative sites, by which canonical Wnt signal can directly increase the transcriptional level of *EN1*.

According to the recent studies from two independent groups, activating Wnt signal by applying a different GSK3 inhibitor, CHIR99021, during the neural differentiation of human ESCs induces NPCs to a posterior fate in a dose-dependent manner, and the strong activation of Wnt signal even drives cells to a hindbrain fate^{21,22}. In our experiment, however, Wnt signal activation by treatment with BIO (as well as with 1-AKP and lithium) only increased the midbrain specific genes but did not induce the hindbrain genes, even with higher dosage of BIO (>1 μ M). One possible explanation for this discrepancy would be the usage of the cells at different stages. Unlike the previous studies in which Wnt activation started at the initial stage of neural differentiation (day 0 ~ 3), we examined the effect of Wnt activation with the neural rosette cells, which is a much later stage than in previous studies. As other studies suggest, NPCs in different developmental stages exhibit variable responsiveness to regional patterning cues^{57,58}. Therefore, different temporal windows for patterning as well as specific dosages of the patterning cue may result in distinctive regional fates. It is likely that applying patterning cues during early stages of differentiation may be a better strategy if the purpose is purely obtaining neural cells with a particular fate at high efficiency.

It is interesting to note that NPCs treated with BIO predominantly generated glutamatergic and GABAergic neurons (Fig. 4c). In the mouse midbrain, glutamatergic and GABAergic neurons are interspersed in the dorso-ventral axis from the inferior and superior colliculi of the dorsal midbrain to the ventral midbrain including the substantia nigra and ventral tegmental area^{52,59}. Both subtypes found in various locations of the midbrain are

highly heterogeneous in morphological and neurochemical properties, and they participate in a variety of neural pathways including relaying somatosensory information and modulating voluntary movement³. Even though the distribution of midbrain glutamatergic and GABAergic neurons and the underlying molecular regulation in fate determination of both types have begun to be understood from mouse genetic studies, understanding the functional diversity and detailed differentiation mechanisms of human midbrain neurons is still on high demand owing to their implications in various neurological and psychological diseases^{52,59,60}. In this regard, our differentiation paradigm may provide an efficient system for generating a midbrain neuron model that can be used to study developmental mechanisms and roles in relevant neurological disorders.

In conclusion, we demonstrated that the canonical Wnt signal pathway regulates AP patterning during neural differentiation in human PSCs. We show that activating Wnt signal in human ESC-derived NPCs causes neural cells to exhibit midbrain characteristics. More importantly, our data demonstrate that Wnt signal regulates *EN1* transcription through direct binding of the β -catenin/TCF4 complex to the regulatory element of *EN1* promoter. Collectively, our finding provides mechanistic insights and technical advances in midbrain specification from human PSCs.

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Conflict of interest

The authors declare that they have no conflict of interest.

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