Molecules and Cells

Generation of Isthmic Organizer-Like Cells from Human Embryonic Stem Cells

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The objective of this study was to induce the production of isthmic organizer (IsO)-like cells capable of secreting fibroblast growth factor (FGF) 8 and WNT1 from human embryonic stem cells (ESCs). The precise modulation of canonical Wnt signaling was achieved in the presence of the small molecule CHIR99021 (0.6 μM) during the neural induction of human ESCs, resulting in the differentiation of these cells into IsO-like cells having a midbrain-hindbrain border (MHB) fate in a manner that recapitulated their developmental course *in vivo*. Resultant cells showed upregulated expression levels of *FGF8* and *WNT1*. The addition of exogenous FGF8 further increased *WNT1* expression by 2.6 fold. Gene ontology following microarray analysis confirmed that IsO-like cells enriched the expression of MHB-related genes by 40 fold compared to control cells. Lysates and conditioned media of IsO-like cells contained functional FGF8 and WNT1 proteins that could induce MHB-related genes in differentiating ESCs. The method for generating functional IsO-like cells described in this study could be used to study human central nervous system development and congenital malformations of the midbrain and hindbrain.

Keywords: FGF, human pluripotent stem cells, isthmic organizer, neural differentiation, Wnt

INTRODUCTION

The secondary organizer, a specific group of cells that emerges during early embryonic development, can influence the identity of surrounding tissues (Kiecker and Lumsden, 2012). These cells have the ability to secret morphogens, thus specifying the fate of adjacent cells in a space- and time-dependent manner, and elaborating the development of a given tissue. The isthmic organizer (IsO) in the vertebrate central nervous system (CNS) has been comprehensively characterized. Located at the midbrain-hindbrain border (MHB) of the developing neural tube, the IsO influences the induction, proliferation, and differentiation of neural cells between the midbrain and hindbrain by secreting Wnt1 and fibroblast growth factor (FGF) 8 (Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The development of the IsO has been intensively studied in lower vertebrates, such as chick embryos. IsO development begins with anteroposterior (AP) patterning in the neural plate (Partanen, 2007), during which the canonical Wnt signal plays a crucial role in the establishment of the MHB (Ciani and Salinas, 2005; Kiecker and Lumsden, 2005; Wurst and Bally-Cuif, 2001). It is known that the position of the IsO is determined by the juxtaposition of two homeobox domain-containing transcription factors (Otx2 and Gbx2) (Simeone, 2000), followed by

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the expression of key transcription factors, such as En1, En2, Pax2, and Pax5, as well as Wnt1 and FGF8 in the MHB in a temporally and spatially controlled manner (Wurst and Bally-Cuif, 2001). Previous studies have shown that either the targeted ablation of Wnt1 or the aberrant expression of FGF8 will disturb the normal morphogenesis of the midbrain and hindbrain, and may cause cerebellar malformations (Crossley et al., 1996; McMahon et al., 1992), thus substantiating their pivotal roles in MHB formation. It has been shown that convergent Wnt and FGF signals precisely induce the formation of the IsO (Olander et al., 2006) and that the expression of both Wnt1 and FGF8 in the IsO can maintain the integrity of the MHB (Canning et al., 2007; Ciani and Salinas, 2005; Kiecker and Lumsden, 2005). On the other hand, the development of the IsO in higher mammals, like humans, remains unexplored, primarily due to the lack of an appropriate model system. Considering several congenital developmental defects that are relevant to the IsO (Barkovich et al., 2009; Basson and Wingate, 2013), there is a demand for a faithful model system in which to study the cellular and molecular mechanisms underlying MHB and IsO development.

In this study, we induced the differentiation of human ESCs into cells with characteristics of the IsO. Taking advantage of a neural differentiation technology, we attempted to regionalize human ESC-derived neural precursors (NPs) to the MHB by the precise control of canonical Wnt signaling. We also tested whether exogenous FGF8 was required for the generation of IsO-like cells. Finally, we characterized IsO-like cells by microarray analysis and assessed their functionality using lysates and conditioned media generated from these cells.

MATERIALS AND METHODS

Cells and culture conditions

Human ESCs (H9, WiCell Inc., USA) were cultured in human ESC medium composed of DMEM/F12 medium (Invitrogen, USA) supplemented with 20% Knockout-Serum Replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM beta-mercaptoethanol (Sigma, USA), and 4 ng/mL basic FGF (Peprotech, USA). For differentiation, embryoid bodies (EBs) were formed by mechanically detaching ESC colonies and culturing them in DMEM/F12:Neurobasal media (Invitrogen) (1:1), 1% N2 supplement (Invitrogen), and 2% B27 supplement without vitamin A (Invitrogen). On day 4 of differentiation, these EBs were plated onto Matrigel (BD Biosciences, USA)-coated dishes and cultured in the same medium except that the concentrations of N2 and B27 supplements were reduced by half (0.5%) for five days. During the first four days, 5 μM dorsomorphin (Calbiochem, USA) and 10 μM SB431542 (SB) (Sigma) were added to the medium to facilitate neural induction. To induce IsO-like cells, CHIR99021 (CHIR) at various concentrations (0-1.2 μM) (Calbiochem) and 100 ng/ml FGF8 (Peprotech) were added to the medium as described in Supplementary Fig. S1.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA was isolated using the Easy-Spin Total RNA Extraction kit (iNtRON Biotechnology, Korea). cDNA was synthesized from 1 μg total RNA using the PrimeScript RT Master Mix (Takara Bio, Japan). Transcript levels of each marker gene were quantified by real-time PCR using SYBR Premix Ex *Taq* (Takara Bio) and the CFX96 Real-Time System (Bio-Rad, USA). Ct values of target genes were normalized to those of β-actin. Normalized expression levels of target genes were compared using the ΔΔCt method (Pfaffl, 2001). Data are expressed as the mean relative expression level ± standard error of the mean (SEM) from at least three independent experiments. Sequences of primers used in qRT-PCR are listed in Supplementary Table S1.

Enzyme-linked immunosorbent assay

IsO-like cells and control cells (DMSO-treated and/or dorsomorphin + SB treated cells) were differentiated from human ESCs until day 7. After a thorough rinse with Dulbecco's phosphate-buffered saline (DPBS, Invitrogen) to avoid potential contamination with any exogenous factor added to the culture, cells were detached from the culture dish using a curved Pasteur pipette to spontaneously form spherical masses that were then cultured in media devoid of CHIR and FGF8. One day later, the same number of spheres were obtained from each experimental group and sonicated completely in lysis buffer. Supernatants were isolated from the cellular debris by centrifugation, and the concentration of total protein was measured using the Bradford protein assay. Human WNT1 and FGF8 kits (Cat. # CSB-EL026128HU and CSB-E15861h, CUSABIO Life-Science, Baltimore, MD, USA) were used to detect WNT1 and FGF8 protein levels in the cell lysates, following the manufacturer's protocol. Protein levels of WNT1 and FGF8 were quantified relative to total protein levels.

Microarray analysis

Ten micrograms of total RNA from each sample were collected and analyzed using a Human HT-12 Expression v.4.0 bead array (Macrogen, Korea). For clustering analysis, normalized data were narrowed down to 14,548 using a cutoff value that was based on a fail count of less ≤ 3. Gene Ontology (GO) analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery). Significantly upregulated and downregulated genes were compared against DAVID's GO FAT database to clarify their biological significance. *P* values were derived by Fisher's exact tests ($P < 0.01$; fold enrichment ≥ 2.0). Corrected *P* values were applied to multiple testing corrections using the Benjamini-Yekutieli method (Benjamini and Yekutieli, 2001). The accession number for the microarray data reported in this study is found in GEO: GSE104847.

Immunocytochemistry

Cells were fixed with 4% of paraformaldehyde at day 7, and permeablized with 0.1% of triton X-100 DPBS solution for 10 min and then blocked with 2% bovine serum albumin solution for at least 1 h. Then, cells were incubated with primary antibodies (mouse anti-WNT1 antibody, Abcam (ab91191), Cambridge, UK, 1:200; mouse anti-FGF8 antibody, Novus Biologicals (47109), Littleton, CO, USA, 1:50) for overnight at 4℃. After washing with DPBS, cells were exposed to a fluorescence-tagged secondary antibody (anti-mouse Alexa Fluor 488, Invitrogen, 1:500) for 1 h and mounted in DAPI-containing medium (Vector Laboratories, USA). IX71 microscope equipped with a DP71 digital camera (Olympus, Japan) was used to obtain images.

Functional assessment of IsO-like cells

Cell lysates were obtained using the same method described above. Cell lysates obtained from IsO-like cells and control cells were added to the culture after EB attachment at two different concentrations (1×, 96 μg/ml; 0.5×, 48 μg/ml) for four days. To collect conditioned media from IsO-like cells, human ESCs were differentiated for 6.5 days using the protocol described in Supplementary Fig. S1. These cells were then intensively washed with DPBS and cultured in new medium without supplementation containing either CHIR or FGF8. Two days later, the culture medium was harvested and concentrated using a 10K centrifugal filter (Millipore). After protein quantification using the Bradford assay, the concentrated and conditioned medium was added to the culture after EB attachment at two different concentrations (1×, 15.5 μg/ml; 0.5×, 7.75 μg/ml) for four days.

Statistical analysis

All data are presented as the mean ± SEM from at least three independent experiments. Statistical significance was evaluated using a two-tailed Student's *t* test or a one-way analysis of variance (ANOVA) when more than two groups were involved.

RESULTS

We induced neuroectoderm formation from human ESCs through the simultaneous inhibition of BMP and activin/nodal signals with the small molecule inhibitors dorsomorphin and SB (Kim et al., 2010). Human ESCs were cultured as EBs in chemically defined conditions and supplemented with dorsomorphin and SB for four days. EBs were allowed to attach onto Matrigel-coated dishes for induction of primitive NPs.

The early developmental program of the vertebrate CNS

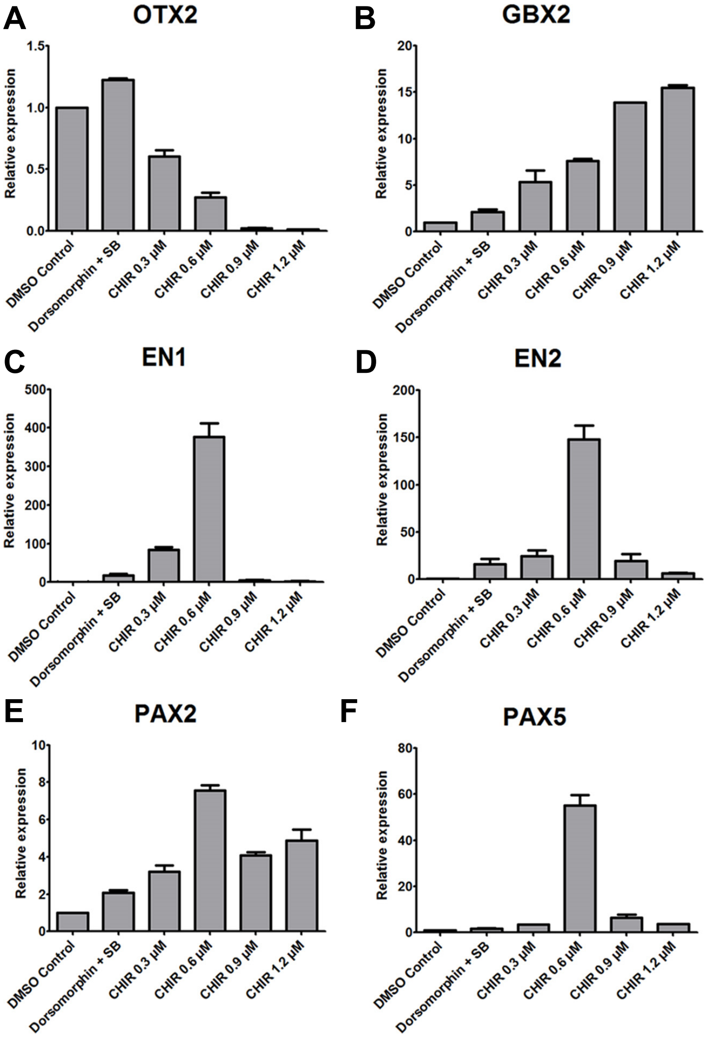

Fig. 1. Precise regulation of Wnt signaling induces key transcription factors of the IsO. Treatment with CHIR during neural induction of human ESCs reduced the expression of an anterior marker (*OTX2*, A) while increasing the expression of a posterior marker (*GBX2*, B) in a dose-dependent manner. All marker genes for the IsO such as *EN1* (C), *EN2* (D), *PAX2* (E), and *PAX5* (F) were highly expressed after treatment with 0.6 μM CHIR.

favors an anterior fate unless caudalizing cues are present (Stern, 2001). Mounting evidence has shown that NPs that are differentiated from human ESCs retain forebrain-like characteristics, supporting the notion of 'default differentiation' (Lupo et al., 2014). Therefore, induction of the IsO characteristics requires caudalization of NPs into the caudal midbrain/anterior hindbrain. It is known that AP patterning in the early vertebrate neural tube is established by graded Wnt signaling and that caudal neural cells are specified by high levels of Wnt ligand derived from paraxial mesoderm (Nordström et al., 2002). Therefore, we decided to modulate canonical Wnt signaling. Instead of using a natural Wnt ligand, a well-known GSK3β inhibitor (CHIR) was used to treat NPs as a Wnt signaling agonist, because it has been shown to be less toxic and a more potent activator of the Wnt/β-catenin pathway than other GSK3β inhibitors (Naujok et al., 2014).

Since graded Wnt signaling gives rise to neural cells with a distinctive regional identity along the AP axis (Nordström et al., 2002), we first determined the optimal concentration of CHIR for IsO generation. After treatment with CHIR at various concentrations (0-1.2 μM) for nine days, relative gene expression analysis revealed that neural cells tended to have a posterior fate with increased expression of *GBX2,* a posterior marker, and decreased expression of *OTX2*, an anterior marker, in response to increasing CHIR levels (Figs. 1A and 1B). Interestingly, the expression of *EN1,* an IsO-specific gene, increased as the CHIR concentration increased. The expression of *EN1* was maximized after treatment with 0.6 μM CHIR, with an approximate 300-fold increase over that in dorsomorphin + SB treatment control cells. However, it was drastically decreased after treatment with CHIR at concentrations higher than 0.6 μM (Fig. 1C). When expression levels of a different set of IsO-specific genes (*EN2*, *PAX2*, and *PAX5*) were examined, the highest expression levels of all genes tested were found in the group treated with 0.6 μM CHIR (Figs. 1D-1F). This finding suggests that exposure of human ESCs to 0.6 μM CHIR during neural induction is optimal for the development of IsO-like characteristics.

To further test whether this culture condition generates IsO-like cells, we examined the expression of two IsO markers: The *FGF8* transcript level was upregulated 3-fold by 0.6 μM CHIR compared to that in the dorsomorphin + SB group (Fig. 2A). However, *WNT1* expression was optimally upregulated by 0.3 μM CHIR (Fig. 2B). The expression of *WNT1* after treatment with 0.6 μM CHIR was lower than expected; this result might be due to the relatively strong activation of canonical Wnt signaling by CHIR treatment, which may negatively regulate endogenous *WNT1* expression via a negative-feedback mechanism (de Lau et al., 2014). Low *WNT1* expression may also be due to the possibility that *WNT* activation alone is insufficient for IsO induction. A previous study of chick embryo gastrulation showed that convergent Wnt and FGF signals could transform naïve forebrain cells into IsO-like cells (Olander et al., 2006). These results prompted us to test whether the addition of exogenous FGF8 could enhance the IsO-like properties of these cells. Surprisingly, the addition of 100 ng/mL FGF8 for five days in the presence of 0.6 μM CHIR increased *WNT1* expression 2.9 fold, along with a 2.6-fold upregulation of *PAX2* (Fig. 2C). Together, our results suggest that the precise modulation of canonical Wnt signaling can specify the regional identity of NPs to that of the MHB. In addition, co-treatment with FGF8 can induce IsO-like characteristics (Supplementary Fig. S1).

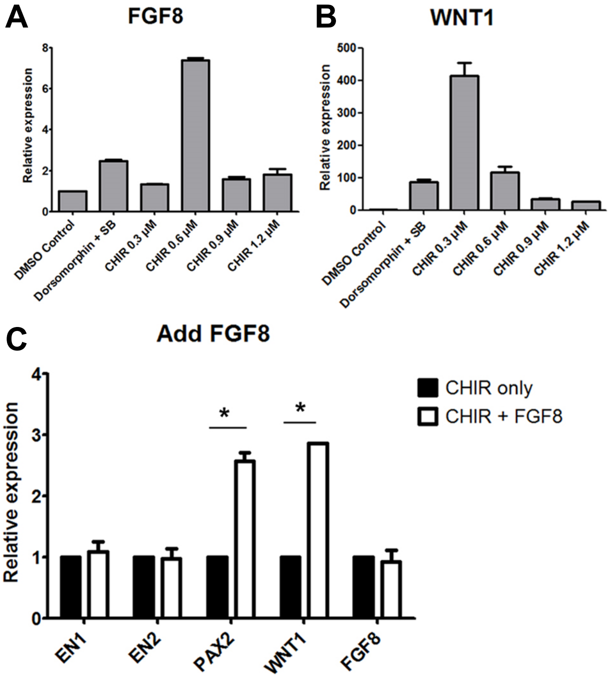

Fig. 2. Convergent Wnt and FGF signaling foster IsO characteristics. After treatment with 0.6 μM CHIR, expression of *FGF8* reached a maximum level (A), but *WNT1* expression was highest after treatment with 0.3 μM CHIR (B). Co-treatment with 100 ng/mL FGF8 in the presence of 0.6 μM CHIR increased the expression of *WNT1* by 2.9-fold along with a 2.6-fold upregulation of *PAX2* (C). *: $P < 0.05$ by Student's *t* test.

Next, we examined the time-course expression of various marker genes during the induction of IsO-like cells. Once differentiation was initiated, pluripotent markers, such as *OCT4* and *NANOG*, were rapidly downregulated to undetectable levels by five days under both DMSO-treated control and IsO induction conditions (dorsomorphin + SB + CHIR + FGF8) (Figs. 3A and 3B). The expression of *SOX1*, a pan-neural marker, was robustly increased after five days. Interestingly, IsO induction conditions resulted in the more rapid induction of *SOX1* expression than control conditions by day 7. After that time, *SOX1* expression decreased to a level similar to that under control conditions (Fig. 3C). *BF1*, a forebrain-specific gene, was never induced during the entire period under IsO induction conditions, whereas it gradually increased under control conditions as expected (Fig. 3D). The expression of MHB-specific genes, such as *EN1*, *EN2*, and *PAX2*, were all remarkably upregulated under IsO induction conditions by 300 to 12,000 fold compared to their basal expression levels under control conditions (Figs. 3E-3G). Interestingly, the expression levels of these genes demonstrated similar kinetics to those of *SOX1*, reaching

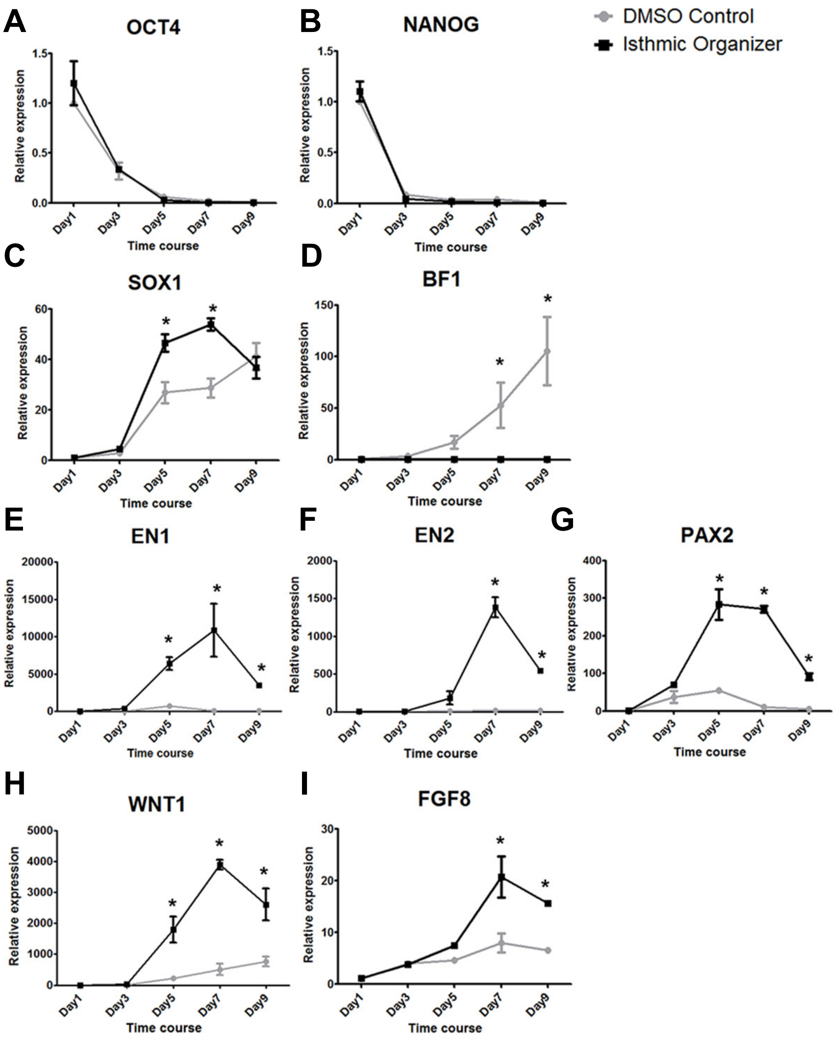

Fig. 3. Time-course gene expression of various markers under IsO induction conditions. Expression levels of pluripotency markers *OCT4* (A) and *NANOG* (B) were drastically diminished to undetected levels by day 5 in both control and IsO induction conditions. A pan-neural precursor marker (*SOX1*) was upregulated more rapidly in IsO conditions than in control conditions by day 7, after which its expression level did not significantly differ between the two conditions (C). Expression of forebrain marker *BF1* was not induced by IsO induction conditions but gradually increased under control conditions (D). Expression levels of IsO markers, including *EN1* (E), *EN2* (F), *PAX2* (G), *WNT1* (H), and *FGF8* (I), were all highly increased by day 7 in IsO conditions compared to those in control conditions. $*P < 0.05$ by Student's *t* test.

maximum levels on day 7. Unlike *SOX1*, however, the expression levels of markers for the MHB remained 100~500 fold higher under IsO induction conditions than under control conditions, even after day 7 (Figs. 3E-3G). Expression levels of *WNT1* and *FGF8*, indicators of IsO activity, were also enhanced under IsO induction conditions (100 fold and 2.7 fold, respectively, compared to control conditions); they also exhibited expression kinetics similar to MHB markers in that their expression levels both peaked on day 7 (Figs. 3H and 3I). When the expression kinetics of marker genes under IsO induction conditions were examined more closely, we noticed that the onset of *PAX2* expression preceded those of *EN1* and *EN2* (Fig. 3G vs. Figs. 3E and 3F). In addition, the expression of *EN1* was higher than that of *EN2* between days 3 and 5 (Figs. 3E vs. 3F). The ontogeny of gene expression in our differentiation conditions matched the dynamics of the gene expression patterns in the MHB *in vivo* (Wurst and Bally-Cuif, 2001). Collectively, the results of these time-course gene expression experiments suggest that treatment with CHIR and FGF8 during neural induction induces the differentiation of NPs into IsO-like cells, following the expected *in vivo* developmental trajectory. Given the temporal expression patterns of the various markers studied, we conclude that differentiation under our conditions for seven days is the best approach for obtaining a cell population with IsO characteristics (Supplementary Fig. S1).

To characterize IsO-like cells in greater detail, we analyzed global gene expression profiles. Microarray data were subjected to GO enrichment analysis using DAVID (Fig. 4A and Supplementary Table S2). We found that the most highly enriched transcripts were genes associated with MHB development, rostro-caudal neural tube patterning, neural tube development, and the Wnt receptor pathway. Consistent with our previous gene expression analysis, transcripts for ‘MHB development,’ including *WNT1* and *EN1*, were highly enriched more than 40 fold (Fig. 4A and Supplementary Table S2). In contrast, transcripts that were most robustly downregulated were those involved in forebrain and diencephalon development (*RAX, SIX3, FEZF2*, and *LHX2*) (Supplementary Table S2). These results strongly support the reliability of our differentiation conditions for inducing the production of IsO-like cells.

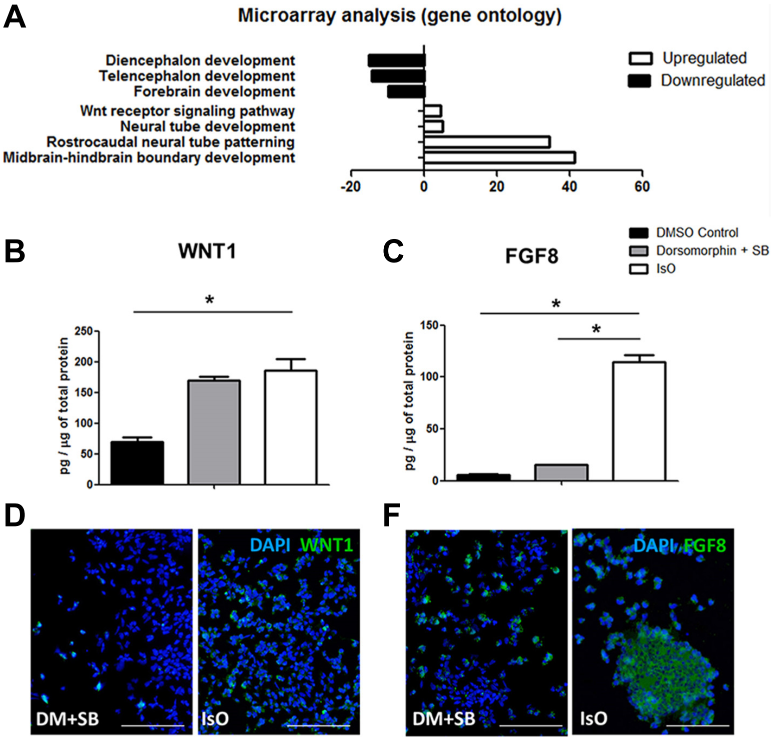

Fig. 4. Characterization of the hESC-derived IsO-like cells. (A) GO analysis of global gene expression in IsO-like cells on day 7. The dataset was normalized to the DMSO control. (B, C) Quantification of WNT1 and FGF8 protein in cell lysates by ELISA. IsO-like cells showed the highest protein levels of WNT1 and FGF8 among experimental groups. *$P < 0.05$ by one-way ANOVA. (D, E) Representative immunofluorescence images for WNT1 (D) and FGF8 (E) in dorsomorphin (DM) + SB condition and IsO inducing condition. Scale bars: 50 µm.

The key feature of the IsO is its ability to influence the fate of adjacent cells by producing functional WNT1 and FGF8 (Wurst and Bally-Cuif, 2001). To examine whether our differentiated IsO-like cells had such an activity, we first measured the levels of WNT1 and FGF8 in cell lysates on differentiation day 7 by ELISA. Protein levels of WNT1 were found to be 2.6 times higher in IsO-like cells than in DMSO-treated control cells (185.4 pg/µg total protein vs. 69.8 pg/µg total protein, respectively). There was a slight increase in WNT1 protein levels in IsO-like cells compared to those in dorsomorphin + SB treatment control cells (169.5 pg/µg total protein). However, this increase was not statistically significant (Fig. 4B). In contrast, protein levels of FGF8 in IsO-like cells were significantly higher than those in both DMSO-treated and dorsomorphin + SB treated cells (114.4 pg/µg total protein vs. 6.1 and 15.3 pg/µg total protein, respectively), consistent with its transcriptional levels (Figs. 2 and 4B and 4C). Immunocytochemical staining also revealed that many IsO-like cells were positively labeled by specific antibodies for WNT1 and FGF8 (Figs. 4D and 4E).

We hypothesized that if both WNT1 and FGF8 in cell lysates were functionally active, MHB gene expression might be induced in differentiating human ESCs. To test this hypothesis, we treated EBs with cell lysates at two concentrations (0.5× and 1×) after attachment on a Matrigel-coated dish. After four days of treatment, gene expression analysis showed that expression levels of *EN1*, *EN2*, and *PAX5* were significantly upregulated by IsO-like cell lysates compared to treatment with lysates from dorsomorphin + SB treated cells. More importantly, these genes were upregulated by IsO-like cell lysates in a concentration-dependent manner (Supplementary Figs. S2A, S2B, and S2D). *PAX2* was not differentially expressed (Supplementary Fig. S2C), which might be due to undefined effects from various factors contained in the lysate.

To further validate the IsO-like activity of these cells, we treated EBs with conditioned media. To our surprise, expression levels of all genes including *PAX2* were significantly increased in EBs cultured with conditioned media of IsO-like cells compared to those in control cells (Figs. 5A-5D). Although the fold increase of each gene was not as robust as that induced by lysate treatment [for example, 7.2 fold by conditioned media vs. 60 fold by *EN1* lysate (Fig. 5A); 24.8 fold by conditioned media vs. 60 fold by *PAX5* lysate (Fig. 5D)], these increases showed a clear dose-dependence, demonstrating an inductive effect by secretory factors in the conditioned media. Furthermore, a dose-dependent increase in *PAX2* expression further supports the specificity of such effects by secretory factors. Taken together, our data clearly demonstrate that the precise modulation of canonical Wnt and FGF8 signals can promote the differentiation of human ESCs into IsO-like cells, which exhibit gene expression profiles of the MHB with the ability to influence the expression of other cells via WNT1 and FGF8 production.

DISCUSSION

In this study, we describe a method for generating cells of the MHB with IsO activity from human ESCs. We found that a graded activation of canonical Wnt signaling during the neural induction of human ESCs transformed neural cells from a rostral to a caudal fate with antagonistic expression

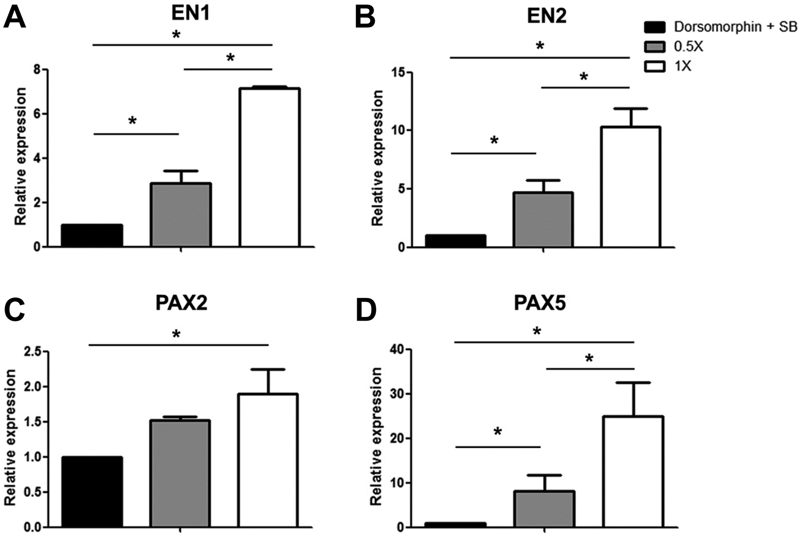

Fig. 5. Functional analysis of IsO-like cells. Functionality of IsO-like cells was evaluated by differentiating human ESCs in conditioned media. After two different concentrations (0.5×, 1×) of conditioned medium from IsO-like cells were used to induce the differentiation of ESCs in culture, quantitative gene expression analysis revealed that the expression levels of IsO markers (*EN1*, *EN2*, *PAX2*, and *PAX5*) were significantly increased by conditioned media of IsO-like cells in a dose-dependent manner compared to levels in dorsomorphin + SB treated cells. $*P < 0.05$ by one-way ANOVA.

of *OTX2* and *GBX2*. However, MHB formation still requires the synchronous expression of both *GBX2* and *OTX2* (Broccoli et al., 1999; Millet et al., 1999; Simeone, 2000) within a narrow range of canonical Wnt activator levels (0.3-0.9 μM CHIR) for precise AP patterning along the rostral neural tube (Kirkeby et al., 2012). Therefore, we first determined the optimal concentration of CHIR that was required for IsO induction and found it to be 0.6 μM. At this concentration, we found that induced cells exhibited gene expression patterns reminiscent of those of the MHB in the developing vertebrate neural tube (Figs. 1C-1F) (Rhinn and Brand, 2001). Our data also demonstrated the presence of a 'complementary loop' between Wnt1 and FGF8 during IsO induction, similar to that observed in animal models (Martinez et al., 1999), in which low expression levels of *WNT1* (after treatment with 0.6 μM CHIR) could be complemented by the addition of recombinant FGF8 (Fig. 2).

Our comprehensive analysis of the transcriptome determined that induced IsO-like cells exhibited gene expression profiles with features of the MHB. Most of the highly upregulated genes in IsO-inducing conditions were involved in midbrain and hindbrain formation and early neurogenesis (e.g., *WNT1*, *ASCL1*, *EN1*, and *WNT3a*). Interestingly, very few upregulated genes were related to the development of dopaminergic (e.g., *SLC6A3*) (Blaess and Ang, 2015) or serotonergic (e.g., *INSM1*) neurons (Kiyasova and Gaspar, 2011) (Supplementary Table S2), which suggest that IsO-like cells are in the initial stages of neural patterning. This suggestion is consistent with the fact that the IsO is the tissue that arises during the early stages of midbrain and hindbrain specification. More importantly, these cells produce and secrete functional WNT1 and FGF8 proteins that are capable of inducing MHB gene expression after exposure to differentiating human ESCs.

The ability to produce and secrete Wnt1 and FGF8 is a hallmark of the activity of the IsO as a local signaling center. Even though our differentiation conditions generated cells with such an activity, it is intriguing that the increase in the production of FGF8 was more prominent than that of Wnt1 (Fig. 4B-4C). Previously, FGF8 was shown to exhibit a partial organizing activity of the IsO in chick embryos, in which the implantation of FGF8-soaked microbeads into the hindbrain modulated gene expression similar to that of ectopically transplanted IsO tissue (Irving and Mason, 2000). Therefore, one may doubt that induction of MHB genes in differentiating human ESCs by IsO-like cell-conditioned medium might be solely attributed to the secretion of FGF8, which is reminiscent of the activity of the anterior neural ridge (ANR), another secondary organizer responsible for the maintenance of forebrain identity (Shimamura and Rubenstein, 1997). In this previous study (Irving and Mason, 2000), however, the molecular machinery for caudal regionalization was already present in the host cells. Therefore, FGF8 beads might be able to mimic the IsO. In contrast, differentiating ESCs acquire a rostral fate by default unless a caudalizing signal (i.e., Wnt) is present. If FGF8 is the primary organizing factor in the conditioned medium, it would foster a rostral fate as an 'ANR'-like activity. However, we found that the upregulation of MHB genes by the conditioned media of IsO-like cells is consistent with the activity of WNT1 secreted from IsO-like cells.

One important finding obtained from the ectopic transplantation experiment with IsO tissue in avian embryos was that the inductive effect of the IsO graft is always asymmetrical (Wurst and Bally-Cuif, 2001). In other words, new midbrain and hindbrain structures were induced in a polarized manner, depending on the rostrocaudal orientation of the IsO graft. Although the molecular basis underlying such an asymmetrical induction has not been clearly demonstrated, numerous genetic studies have provided evidence that genetic interactions between Otx2 and Gbx2 in IsO grafts could orchestrate the induction and maintenance of newly formed midbrain and hindbrain in the host, and may involve the locally restricted expression of Wnt1 and FGF8 in the rostral (Otx2-positive) and caudal (Gbx2-positive) regions, respectively (Wurst and Bally-Cuif, 2001). An asymmetric inductive effect is a key feature of the IsO. Unfortunately, our system was unable to recapitulate such activity, because *in vitro* differentiation, which relies on 2-dimensional (D) culture, might not allow cells to be spatially organized as a neural explant obtained from a developing chick embryo (Olander et al., 2006). A 3-D culture system often enables the self-organization of differentiating neural cells that is reminiscent of *in vivo* development (Eiraku and Sasai, 2012); we have observed that OTX2-positive forebrain cells and PAX2-positive midbrain cells are spatially segregated in defined regions when human pluripotent stem cells are differentiated into neural cells in a 3-D culture system (Supplementary Fig. S3). In this sense, it would be fascinating to test whether a recently introduced 3-D organoid culture (Lancaster et al., 2013) could create a spatially organized IsO-like structure with defined OTX2-GBX2 expression domains under our differentiation paradigm. Such a system may be able to faithfully recapitulate the *in vivo* development of the IsO and provide an advanced *in vitro* platform in which to study the molecular mechanisms underlying the genesis of the midbrain and hindbrain in humans.

In conclusion, our differentiation conditions, which involve the precise modulation of canonical Wnt signaling along with co-stimulation of FGF8 signaling during neural induction, give rise to IsO-like cells with gene expression profiles of the MHB and the ability to secrete functional Wnt1 and FGF8. We believe that our new model system can be used to study CNS development in humans and the etiology of congenital malformations related to the midbrain and hindbrain.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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