



## Endogenous WNT Signaling Regulates hPSC-Derived Neural Progenitor Cell Heterogeneity and Specifies Their Regional Identity

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### SUMMARY

Neural progenitor cells (NPCs) derived from human pluripotent stem cells (hPSCs) are a multipotent cell population that is capable of nearly indefinite expansion and subsequent differentiation into the various neuronal and supporting cell types that comprise the CNS. However, current protocols for differentiating NPCs toward neuronal lineages result in a mixture of neurons from various regions of the CNS. In this study, we determined that endogenous WNT signaling is a primary contributor to the heterogeneity observed in NPC cultures and neuronal differentiation. Furthermore, exogenous manipulation of WNT signaling during neural differentiation, through either activation or inhibition, reduces this heterogeneity in NPC cultures, thereby promoting the formation of regionally homogeneous NPC and neuronal cultures. The ability to manipulate WNT signaling to generate regionally specific NPCs and neurons will be useful for studying human neural development and will greatly enhance the translational potential of hPSCs for neural-related therapies.

### INTRODUCTION

Neural progenitor cells (NPCs) and neurons derived from human pluripotent stem cells (hPSCs) could provide an unlimited source of cells for drug testing and cell-based therapies (Koch et al., 2009; Zhang et al., 2008). In addition, these cells provide a unique opportunity to explore complex neural development in a simplified and accessible system. Current protocols for differentiating hPSCs toward specific neuronal lineages result in a mixture of neurons from various regions of the CNS, which limits the use of these cells for cell-based therapies, disease modeling, and developmental studies that require uniform populations of neurons. However, the precise source of this heterogeneity in neuronal cultures has yet to be resolved.

Differentiation of stem and progenitor populations is largely governed by the heterogeneity present in these cultures, which ultimately determines their differentiation bias. For example, several studies have found subpopulations with distinct self-renewal and differentiation potentials in hematopoietic (Dykstra et al., 2007; Huang et al., 2007) and intestinal (Sangiorgi and Capecchi, 2008) stem cells. Likewise, heterogeneous expression of pluripotency-related transcription factors and other cell-surface markers bestows distinct lineage-specific differentiation propensities on hPSCs (Drukker et al., 2012; Hong et al., 2011; Nar-sinh et al., 2011; Stewart et al., 2006; Wu and Tzanakakis, 2012). In contrast, NPCs derived from hPSCs have been considered to be a homogeneous cell population, and it has been suggested that their differentiation to neuronal cultures can be biased and manipulated by altering culture

conditions (Dottori and Pera, 2008; Gaspard and Vanderhaeghen, 2010; Germain et al., 2010; Jiang et al., 2012; Liu and Zhang, 2011; Nat and Dechant, 2011; Peljto and Wichterle, 2011; Zhang, 2006). Our study challenges this simplistic view of neuronal differentiation in hPSC cultures.

We demonstrate that hPSC-derived NPCs, like other stem and progenitor populations, are heterogeneous and display a bias in their differentiation potential. Through the use of WNT reporter hPSC lines, we identified endogenous WNT signaling as a primary regulator of this heterogeneity in NPC and neuronal cultures. Flow cytometry (FC)-based purification and genetic assessment of reporter-expressing cell types revealed that the identity and differentiation potential of hPSC-derived NPCs are directly related to the level of endogenous WNT signaling present in these cell types. Through exogenous manipulation of WNT signaling, we were able to reduce NPC heterogeneity and generate cultures of regionally specific progenitors and neurons. Overall, this study demonstrates that WNT signaling plays an important role in deriving regionally homogeneous populations of NPCs and neurons, thereby greatly improving their scientific and therapeutic utility.

### RESULTS

#### Endogenous WNT Signaling Is a Major Source of Heterogeneity in NPCs Derived from hPSCs

It is well established that WNT signaling regulates the regional identity along the anterior-posterior (A/P) axis of

the developing CNS. To explore the possibility that WNT signaling exerts similar effects in a cell-culture-based system of neural development, we generated clonal human embryonic stem cell (hESC) lines (HUES9) carrying a stably integrated GFP reporter under the control of a WNT-responsive promoter, called TCF Optimal Promoter (TOP (Fuerer and Nusse, 2010) (Figure S1A available online). In undifferentiated hESCs, this reporter is inactive but expresses GFP upon stimulation with recombinant WNT3a (Figure S1B). In contrast to a previous study (Blauwkamp et al., 2012), none of our clones or the nonclonal pool expressed GFP in the absence of exogenous WNT3a. This likely reflects the heterogeneity among hESC lines, especially with respect to endogenous expression of WNT3 (Jiang et al., 2013). In a subsequent analysis we focused on one clone, clone 19 (hTOP-19), which exhibited robust GFP expression upon WNT3a stimulation (nearly 100%; Figure S1B), displayed a normal female karyotype of 46 chromosomes (Figure S1C), and responded to various concentrations of exogenously added WNT3a (Figure S1D) and chemical inhibitors of GSK3 $\beta$ , such as BIO (Figure S1E).

Upon differentiation of this WNT reporter line to NPCs (Brafman, 2014) (Figure S2A), we observed a heterogeneous pattern of GFP expression in the absence of any exogenously added WNT proteins (Figure 1A). Despite uniform expression of the pan-neural markers SOX1, SOX2, and NESTIN (Figures S2B and S2C), and cell morphology (Figure S2D), FC revealed that GFP expression peaked upon rosette formation and a stable population of GFP-positive (GFP<sup>+</sup>) cells persisted through subsequent NPC passages (Figure 1B). Addition of WNT3a to these NPC cultures resulted in uniformly high GFP expression, thereby demonstrating a homogeneous response to WNT signaling in this clonally derived population (Figure S1F). Furthermore, inhibiting endogenous WNT signaling with IWP2, a small molecule that acts on PORCN to block WNT processing and subsequent secretion (Chen et al., 2009), eliminated GFP expression, confirming that reporter expression was due to endogenous WNT signaling (Figure S1F).

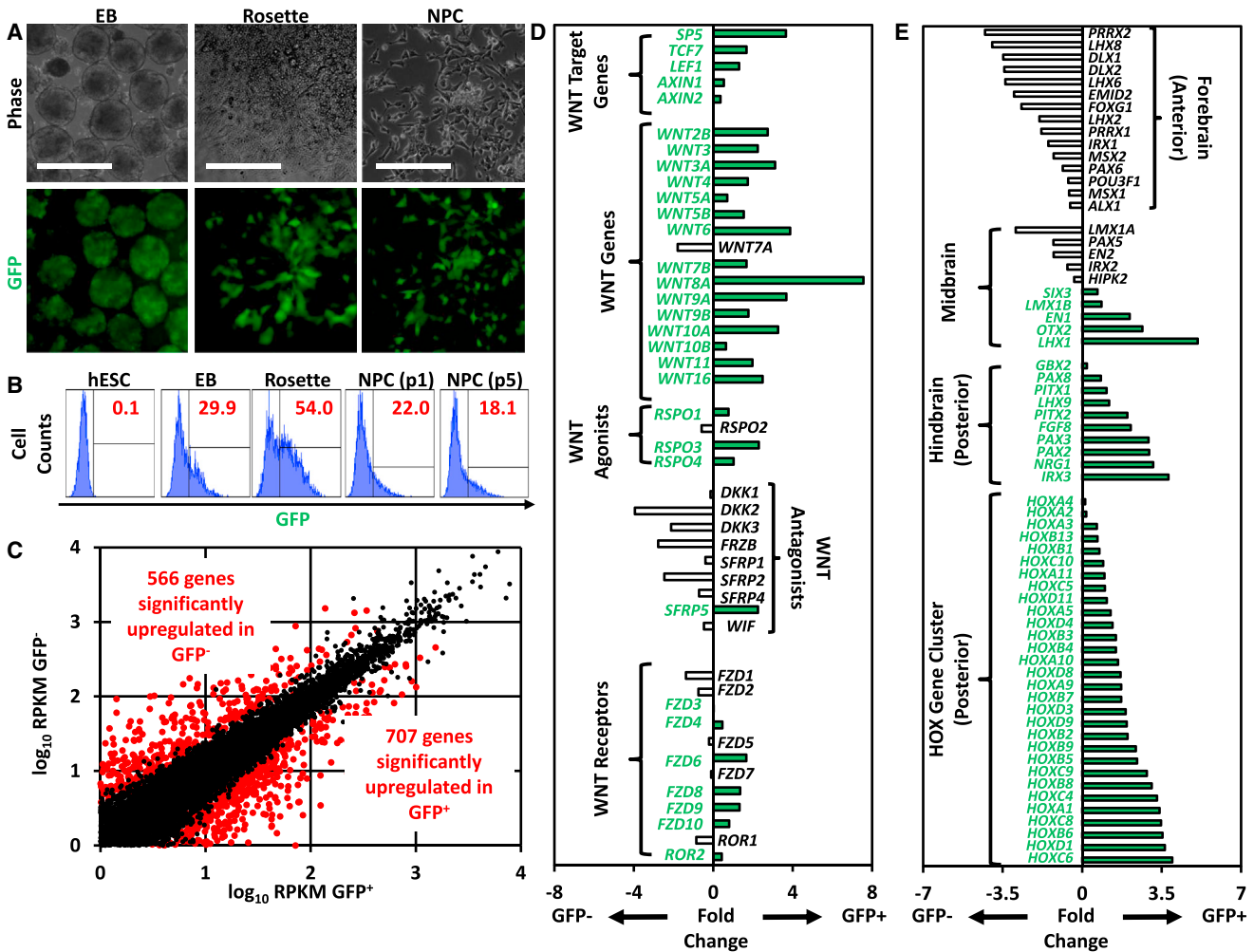
Consistent with this heterogeneous expression of WNT reporter activity and with previously published studies (Dottori and Pera, 2008; Gaspard and Vanderhaeghen, 2010; Germain et al., 2010; Jiang et al., 2012; Liu and Zhang, 2011; Nat and Dechant, 2011; Peljto and Wichterle, 2011; Zhang, 2006), we found that NPCs derived under this protocol exhibited significant heterogeneity with respect to regionally specific markers (Figures S2E–S2H) despite uniform expression of the pan-neural markers SOX1 and SOX2 (Figures S2B and S2C). For example, these NPCs expressed markers of all A/P regions, including the forebrain (*FOXG1* and *DLX2*), forebrain/midbrain (*OTX2*), midbrain/hindbrain (*EN1* and *IRX3*), and hindbrain/spinal cord (*HOXB4*) (Figure S2E). Moreover, single-cell analysis

by FC and immunofluorescence (IF) revealed that our NPC cultures heterogeneously expressed the regionally specific markers *FORSE-1* (Figure S2F; forebrain), *PAX6* (Figure S2G), and *HOXB4* (Figure S2H). Together, these data suggested that these in vitro NPC cultures, as in the developing embryo (Pevny et al., 1998; Uwanogho et al., 1995; Wood and Episkopou, 1999), are exposed to patterning cues that impart distinct regional identities and neuronal differentiation potentials. Additionally, we confirmed that these NPCs were able to differentiate to neurons (Figures S2I–S2K), including those with a GABAergic identity (Figure S2J), and glial cells (Figure S2K).

To investigate the extent to which the high degree of heterogeneity in A/P positional identity correlated with our observed heterogeneity in WNT reporter activity, we performed whole transcriptome RNA sequencing (RNA-seq) on sorted GFP<sup>+</sup> and GFP-negative (GFP<sup>-</sup>) NPCs from passage 5 NPC cultures (Table S1). Overall, we identified 1,273 genes with statistically significant differential expression between these two cell populations, with expression of 707 genes being elevated in the GFP<sup>+</sup> population and 566 genes elevated in the GFP<sup>-</sup> population (Figure 1C; Table S1). As expected, WNT target genes such as *SP5*, *LEF1*, and *AXIN2* were elevated in the GFP<sup>+</sup> population (Figure 1D; Table S2). Additional analysis of WNT pathway components revealed that expression of the majority of WNT proteins and agonists was higher in GFP<sup>+</sup> NPCs, whereas expression of WNT antagonists was higher in GFP<sup>-</sup> NPCs (Figure 1D; Table S2).

Furthermore, expression of genes with distinct domains of expression along the A/P axis segregated into these two cell populations (Figure 1E; Table S3). Specifically, we found that GFP<sup>-</sup> NPCs in clone hTOP-19 were enriched for forebrain/anterior-specific markers such as *LHX8*, *DLX2*, *FOXG1*, and *LHX2*. Conversely, the expression of hindbrain/posterior-related markers such as *GBX2*, *PITX2*, *FGF8*, and *IRX3* was increased in GFP<sup>+</sup> NPCs. Most notably, members of the *HOX* gene family, which are highly expressed in the hindbrain and spinal cord, were significantly upregulated in the GFP<sup>+</sup> cell population. Finally, various midbrain-associated genes such as *EN1*, *EN2*, *LMX1A*, and *LMX1B* were expressed in both populations. Collectively, this RNA-seq analysis establishes a correlation between cells that receive a WNT signal (i.e., the GFP<sup>+</sup> population) and posterior fates. In contrast, NPCs that do not receive an endogenous WNT signal input (i.e., the GFP<sup>-</sup> population) are biased toward an anterior identity.

To further characterize the diversity of cells expressing varying levels of GFP in the hTOP-19 NPCs, we used FC to isolate GFP<sup>HIGH</sup>, GFP<sup>MID</sup>, and GFP<sup>LOW</sup> populations (Figure 2A). After cell sorting, the specific level of GFP expression remained stable in subsequent culture (Figure 2B). As expected, expression of the WNT target gene *AXIN2* was



**Figure 1. NPCs Are Heterogeneous with Respect to Endogenous WNT Signaling**

(A) Phase contrast and fluorescent images of WNT reporter hESCs during neural differentiation (EB, scale bar, 500  $\mu$ m; rosette and NPC, scale bar, 200  $\mu$ m).

(B) FC of WNT reporter hESCs during neural differentiation. No detectable GFP signal was observed in hESCs, but upon differentiation to NPCs, a range of GFP expression patterns was observed. A population of GFP<sup>+</sup> cells endured through multiple NPC passages.

(C) Passage 5 reporter-expressing NPCs were separated by FC on the basis of GFP expression. A scatterplot of log<sub>10</sub> RPKM in GFP<sup>+</sup> and GFP<sup>-</sup> NPCs is shown. Genes with statistically significant differences in expression are shown in red.

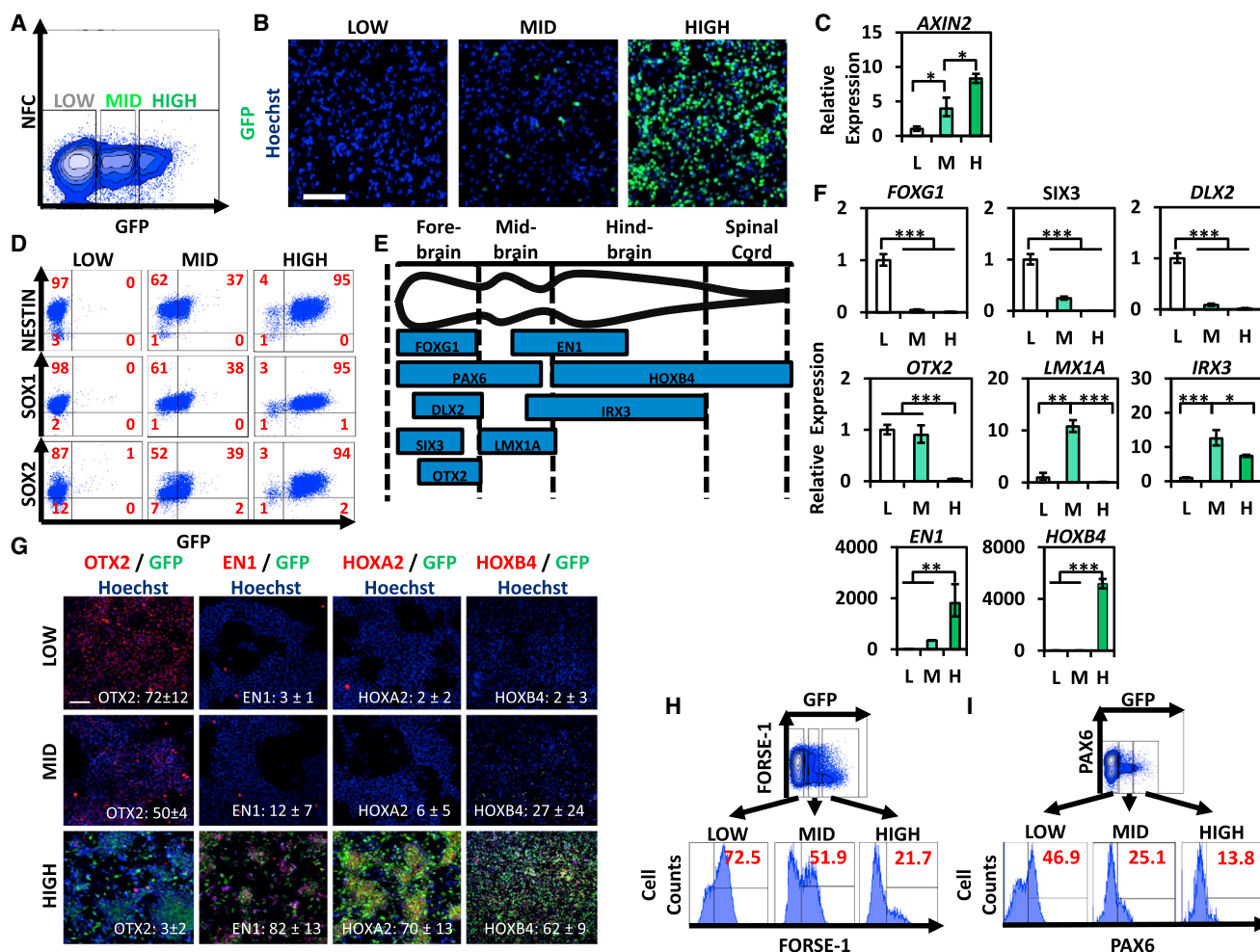
(D and E) Selection of differentially expressed genes highlighting differences in gene-expression patterns related to (D) WNT signaling and (E) A/P patterning and differentiation of the neural tube.

See also [Figures S1](#) and [S2](#) and [Tables S1](#), [S2](#), and [S3](#).

highest in the GFP<sup>HIGH</sup> cell population and lowest in the GFP<sup>LOW</sup> NPCs ([Figure 2C](#)). The three sorted cell populations expressed similar levels of the pan-neural markers SOX1, SOX2, and NESTIN ([Figures 2D](#), [S3A](#), and [S3B](#)), demonstrating that NPCs of varying endogenous WNT activity are homogeneous with respect to expression of pan-neural markers.

We examined the A/P expression profile ([Figure 2E](#)) of NPCs with different levels of endogenous WNT signaling by quantitative PCR (qPCR; [Figure 2F](#)), IF ([Figure 2G](#)), and

FC ([Figures 2H](#) and [2I](#)). Expression of the forebrain markers *FOXP1*, *SIX3*, and *DLX2* was highest in GFP<sup>LOW</sup> NPCs. Expression of *OTX2*, which is expressed at the forebrain-midbrain boundary, was similar in the GFP<sup>LOW</sup> and GFP<sup>MID</sup> populations but absent in the GFP<sup>HIGH</sup> NPCs. Expression of the midbrain marker *LMX1A* was highest in the GFP<sup>MID</sup> population. The midbrain/hindbrain boundary markers *IRX3* and *EN1* were expressed at high levels in both the GFP<sup>MID</sup>- and GFP<sup>HIGH</sup>-sorted cell populations. The hindbrain markers *HOXA2* and *HOXB4* were highly expressed



**Figure 2. Characterization of WNT Reporter-Expressing NPC Populations Reveals a Regional Bias**

(A) WNT reporter NPCs were divided into three populations on the basis of GFP expression:  $GFP^{LOW}$ ,  $GFP^{MID}$ , and  $GFP^{HIGH}$ . (B) Fluorescent images of GFP-sorted NPC populations. GFP expression remains stable after sorting and subsequent culture (scale bar, 100  $\mu$ m). (C) Gene expression of the WNT target gene *AXIN2* in GFP-sorted NPC populations (mean  $\pm$  SEM, n = 3 independent experiments). (D) FC of the pan-neural markers NESTIN, SOX1, and SOX2 in GFP-sorted NPC populations. (E) Schematic of areas of expression of key genes involved in A/P patterning of the developing neural tube. (F) Gene-expression analysis of A/P-related genes in GFP-sorted NPC populations (mean  $\pm$  SEM, n = 3 independent experiments). Populations were compared using Student's t test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. (G) IF of A/P-related markers in GFP expression in  $GFP^{LOW}$ ,  $GFP^{MID}$ , and  $GFP^{HIGH}$  (scale bar, 100  $\mu$ m). (H and I) FC of (H) FORSE-1 and (I) PAX6 expression in unsorted and sorted GFP-expressing NPC populations. Isotype controls used are listed in Table S5. NFC, nonfluorescing channel. See also Figure S3.

in the  $GFP^{HIGH}$  NPCs and diminished in both the  $GFP^{MID}$  and  $GFP^{LOW}$  populations. Finally, we performed FC on unsorted GFP-expressing hTOP-19 NPCs. This analysis demonstrated a negative correlation between GFP levels and expression of the anterior neural cell-surface marker FORSE-1 (Elkabetz et al., 2008) (Figure 2H). PAX6, which is expressed in the diencephalon and midbrain during early development, was expressed primarily in the  $GFP^{LOW}$  and

$GFP^{MID}$  cell fractions (Figure 2I). Together, these data suggest that the level of endogenous WNT signaling correlates with the positional identity of hESC-derived NPCs.

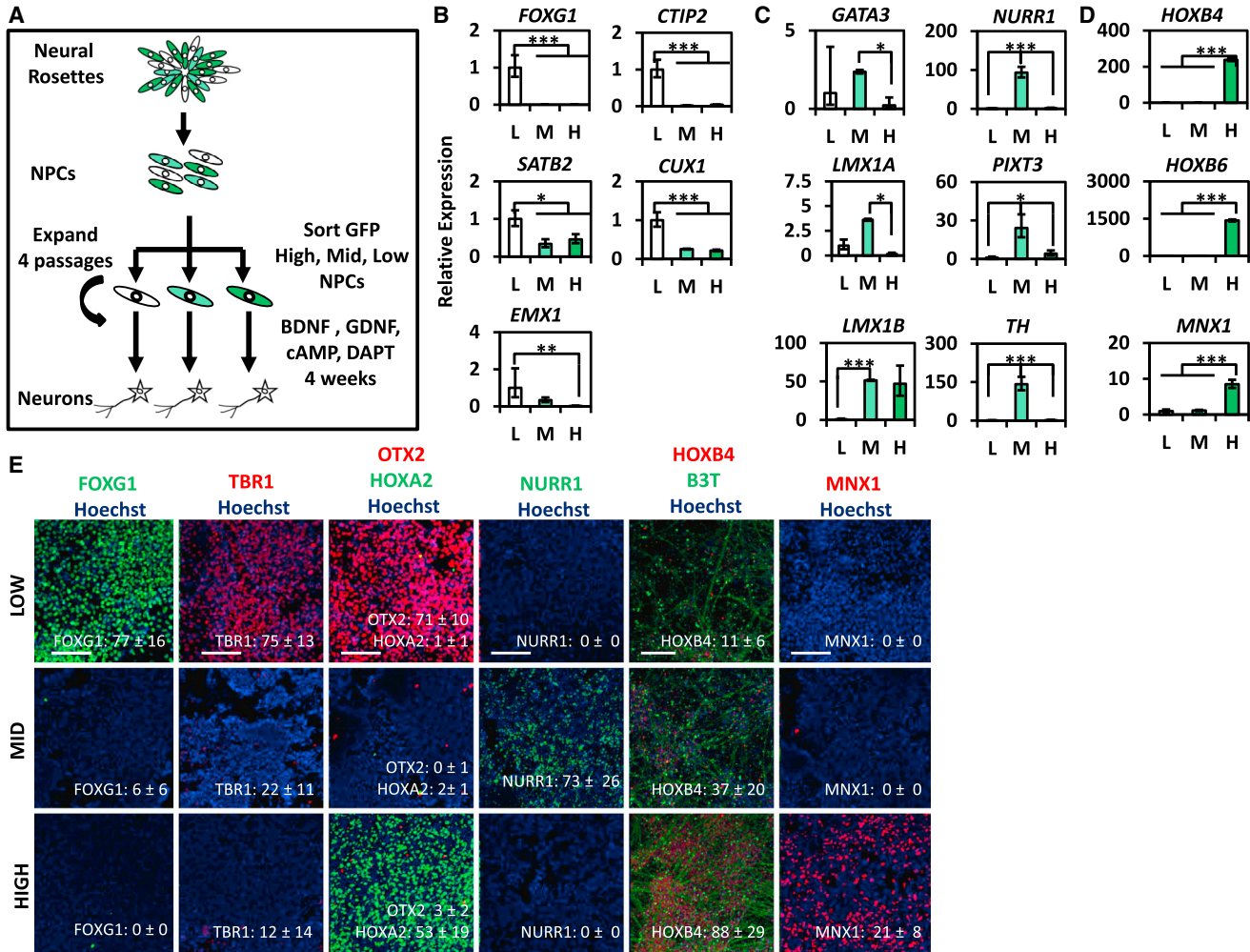
To determine whether the effect of endogenous WNT signaling on the regional patterning of NPCs was stable, we cultured sorted  $GFP^{HIGH}$  and  $GFP^{LOW}$  populations for ten passages (>50 days) and examined the expression of A/P-related genes (Figure S3C). Expression of *AXIN2*





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**Figure 3. Neuronal Differentiation Bias of Sorted GFP-Expressing NPC Populations**

(A) Schematic of the experimental protocol. FC was used to sort GFP-expressing NPCs into GFP<sup>LOW</sup>, GFP<sup>MID</sup>, and GFP<sup>HIGH</sup> populations. The sorted cell populations were expanded for four passages and then differentiated to neurons.

(B–D) Gene-expression analysis of (B) cortical/forebrain-, (C) midbrain-, and (D) hindbrain/spinal-cord-related neuronal genes in neurons derived from sorted GFP-expressing NPC populations (mean ± SEM, n = 4 independent experiments). Populations were compared using Student's t test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

(E) IF of cortical/forebrain-, midbrain-, and hindbrain/spinal-cord-associated markers in neuronal cultures differentiated from sorted GFP-expressing NPC populations (scale bar, 100 μm). L, GFP<sup>LOW</sup>; M, GFP<sup>MID</sup>; H, GFP<sup>HIGH</sup>.

See also Figure S4.

remained stable in the GFP<sup>HIGH</sup> NPCs, and *AXIN2* expression did not increase in the GFP<sup>LOW</sup> NPCs over ten passages (Figure S3D). During the course of ten passages, the forebrain markers *FOXG1*, *SIX3*, and *OTX2* were expressed in the GFP<sup>LOW</sup> NPCs, but not in the GFP<sup>HIGH</sup> NPCs (Figure S3E). Conversely, the hindbrain-associated genes *IRX3*, *EN1*, and *HOXB4* were stably expressed in the GFP<sup>HIGH</sup> NPCs, but were not elevated in the GFP<sup>LOW</sup> NPCs during the period of ten passages (Figure S3E). In sum, these data indicate that the positional identity of NPCs is stable during long-term culture.

### The Level of Endogenous WNT Activity Instructs the Neuronal Differentiation Potential

We wanted to determine whether the level of endogenous WNT signaling present in NPCs conferred a regional bias as they were expanded and subsequently differentiated to neurons in vitro. To address this issue, FC-purified GFP<sup>HIGH</sup>, GFP<sup>MID</sup>, and GFP<sup>LOW</sup> hTOP-19 NPCs were expanded for four passages and then differentiated to neurons (Figure 3A). After 4 weeks of differentiation, all three cell populations generated cells with a neuronal morphology (Figure S4A). Additionally, IF demonstrated

that each NPC population yielded similar numbers of MAP2<sup>+</sup> and B3T<sup>+</sup> neurons (Figures S4B and S4C). Importantly, gene expression (Figures 3B–3D) and IF analysis (Figure 3E) of neurons generated from these different WNT reporter NPC populations revealed distinct regional identities. Neurons generated from GFP<sup>LOW</sup> NPCs expressed the highest levels of *FOXP1* (a marker of neurons with telencephalic identity), *SATB2* (labels cortical neurons of layers II/III), *CTIP2* (expressed by striatal medium spiny neurons), *EMX1* (a marker for pyramidal neurons of the cerebral cortex), *CUX1* (expressed in layer IV-II late-born/upper-layer cortical neurons), and *TBR1* (labels cortical neurons, especially those associated with layer VI). By comparison, GFP<sup>MID</sup> NPCs differentiated into neurons with a midbrain phenotype, including expression of the midbrain GABAergic-associated marker *GATA3* and the midbrain dopaminergic (mDA)-related markers *LMX1A/1B* (regulate mDA progenitor proliferation, specification, and differentiation), *NURR1* (specifies neurotransmitter identity of mDA neurons), *PITX3* (regulates tyrosine hydroxylase [*TH*] expression in mDA neurons), and *TH* (the enzyme responsible for generation of L-DOPA, which is a precursor for the neurotransmitter dopamine). Finally, neurons differentiated from GFP<sup>HIGH</sup> NPCs expressed the highest levels of hindbrain/spinal-cord-associated genes, such as *HOXA2*, *HOXB4*, and *HOXB6*, as well as the motor neuron marker *MNX1* (also known as *HB9*).

### Exogenous Modulation of WNT Signaling Influences NPC Positional Identity and Reduces Heterogeneity in Neuronal Differentiation

The above analysis demonstrates that endogenous WNT signaling activity correlates with A/P regional identity of NPCs. To determine the extent to which WNT signaling is instructive in conferring regional bias, we used several methods to perturb WNT signaling during NPC generation. Specifically, we differentiated four independent hPSC lines (H9, HUES9, HES3, and RiPSC; Warren et al., 2010) to NPCs while activating or inhibiting WNT signaling with CHIR 98104 (CHIR, a potent inhibitor of GSK-3 $\beta$ ) and IWP2, respectively (Figure 4A). Activation of WNT signaling with CHIR treatment led to an increase in embryoid body (EB) size, whereas inhibition of endogenous WNT signaling through IWP2 treatment resulted in a decrease in EB size (Figures S5A and S5B), consistent with the known role of canonical WNT signaling in promoting proliferation in hESC-derived neurospheres (Davidson et al., 2007). CHIR-treated and IWP2-treated NPCs expressed levels of the pan-neural markers *SOX1*, *SOX2*, and *NESTIN* similar to those observed for untreated NPCs (Figures S5C–S5E).

Although there was no effect of WNT signal perturbations on the formation of *SOX1*<sup>+</sup>, *SOX2*<sup>+</sup>, and *NESTIN*<sup>+</sup>

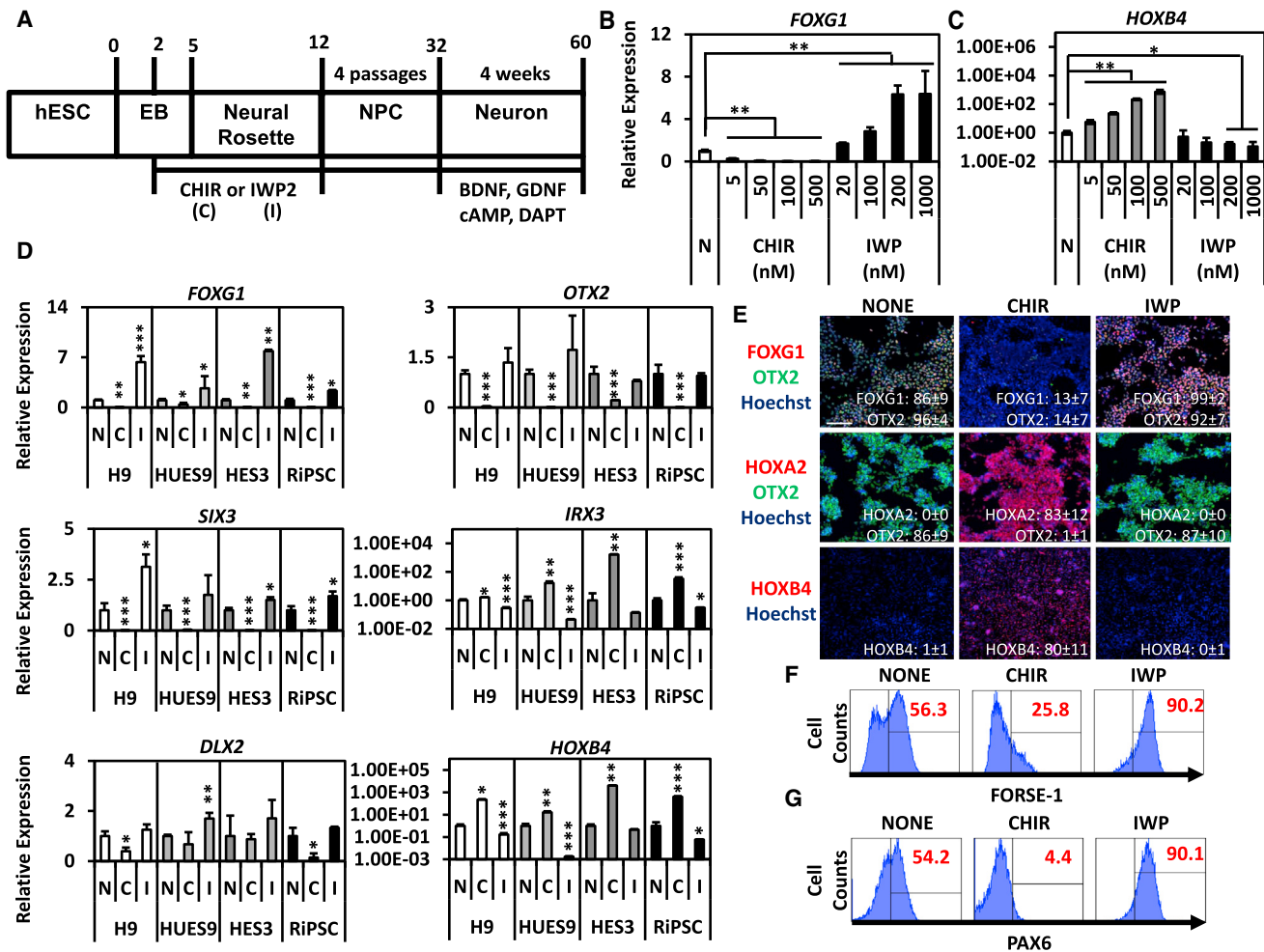
NPCs, CHIR or IWP2 treatment influenced the A/P expression profile of NPCs. Expression of the anterior marker *FOXP1* decreased in a dose-dependent manner with CHIR treatment, but increased with IWP2 treatment (Figure 4B). Conversely, the levels of the posterior marker *HOXB4* increased in a concentration-dependent manner with CHIR treatment, but decreased with IWP2 treatment (Figure 4C). The expression of A/P markers was also influenced by CHIR and IWP2 treatment across all hPSC lines tested (Figures 4D–4G). Specifically, expression of the anterior markers *FOXP1*, *FORSE-1*, *SIX3*, *DLX2*, *OTX2*, and *PAX6* was higher in IWP2-treated cells, whereas expression of the posterior markers *IRX3*, *HOXA2*, and *HOXB4* was higher in CHIR-treated cells. To eliminate the possibility that the posteriorizing effect of CHIR was not due to the activation of other signaling pathways that act through GSK3 (Jope and Johnson, 2004), we also generated NPCs in the presence of WNT3a (Willert, 2008). Similar to the effect of CHIR on A/P gene expression of NPCs, addition of WNT3a during NPC formation led to an increase in expression of the posterior genes *LMX1A*, *EN1*, *IRX3*, and *HOXB4*, and a decrease in expression of the anterior genes *FOXP1*, *SIX3*, *DLX2*, and *OTX2* (Figure S5F). These data demonstrate that exogenous activation or inhibition of canonical WNT signaling can be used to control the positional patterning of NPCs in differentiating hPSCs. Additionally, the observation that inhibition of endogenous WNT signaling with IWP2 during NPC generation reduced the expression of genes associated with posterior identity suggests that endogenous WNT signaling specifies a posterior identity, consistent with its known function in CNS development.

To examine whether patterning of NPCs imposed by exogenous WNT manipulation was stable, we cultured CHIR- and IWP2-treated NPCs in the absence of these exogenous signals for ten passages (>50 days) (Figures S6A and S6B). After ten passages, the expression level of the forebrain marker *FOXP1* remained unchanged in NPCs that were generated in the presence of IWP2 (Figure S6C). Along similar lines, the expression level of the hindbrain marker *HOXB4* remained constant over ten passages of the NPCs that were generated in the presence of CHIR (Figure S6D). Therefore, continued WNT pathway modulation during prolonged culture was not required to maintain the regional identity of NPCs. Furthermore, we examined the ability of NPCs to alter their positional identity after NPC formation. To test this, we treated regionally specified NPCs with CHIR or IWP2 for ten passages after NPC formation (Figures S6A and S6B). Addition of CHIR to anterior-specified NPCs (i.e., NPCs formed in the presence of IWP2) had no effect on *FOXP1* or *HOXB4* expression (Figures S6C and S6D). Likewise, IWP2 treatment of posterior-patterned NPCs (i.e., NPCs formed in the presence of



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**Figure 4. Exogenous Manipulation of WNT Signaling Reduces Heterogeneity in NPC Cultures**

(A) Outline of the experimental protocol. CHIR or IWP2 was added during day 2 of neural differentiation. CHIR- and IWP2-treated and untreated NPCs were expanded for four passages prior to differentiation to neurons.

(B and C) Gene-expression analysis of (B) *FOXG1* and (C) *HOXB4* in NPC cultures derived from H9 hPSCs in the presence of varying levels of CHIR and IWP2.

(D) A/P gene expression in 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9, HUES9, HES3, and RiPSC hPSCs (mean  $\pm$  SEM,  $n = 3$  independent experiments). Populations were compared with untreated (N) cells using Student's t test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

(E) IF of A/P-related markers in 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures (scale bar, 100  $\mu$ m).

(F and G) FC of (F) FORSE-1 and (G) PAX6 in 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures. Isotype controls used are listed in Table S5. N, none; C, CHIR 98014; I, IWP2.

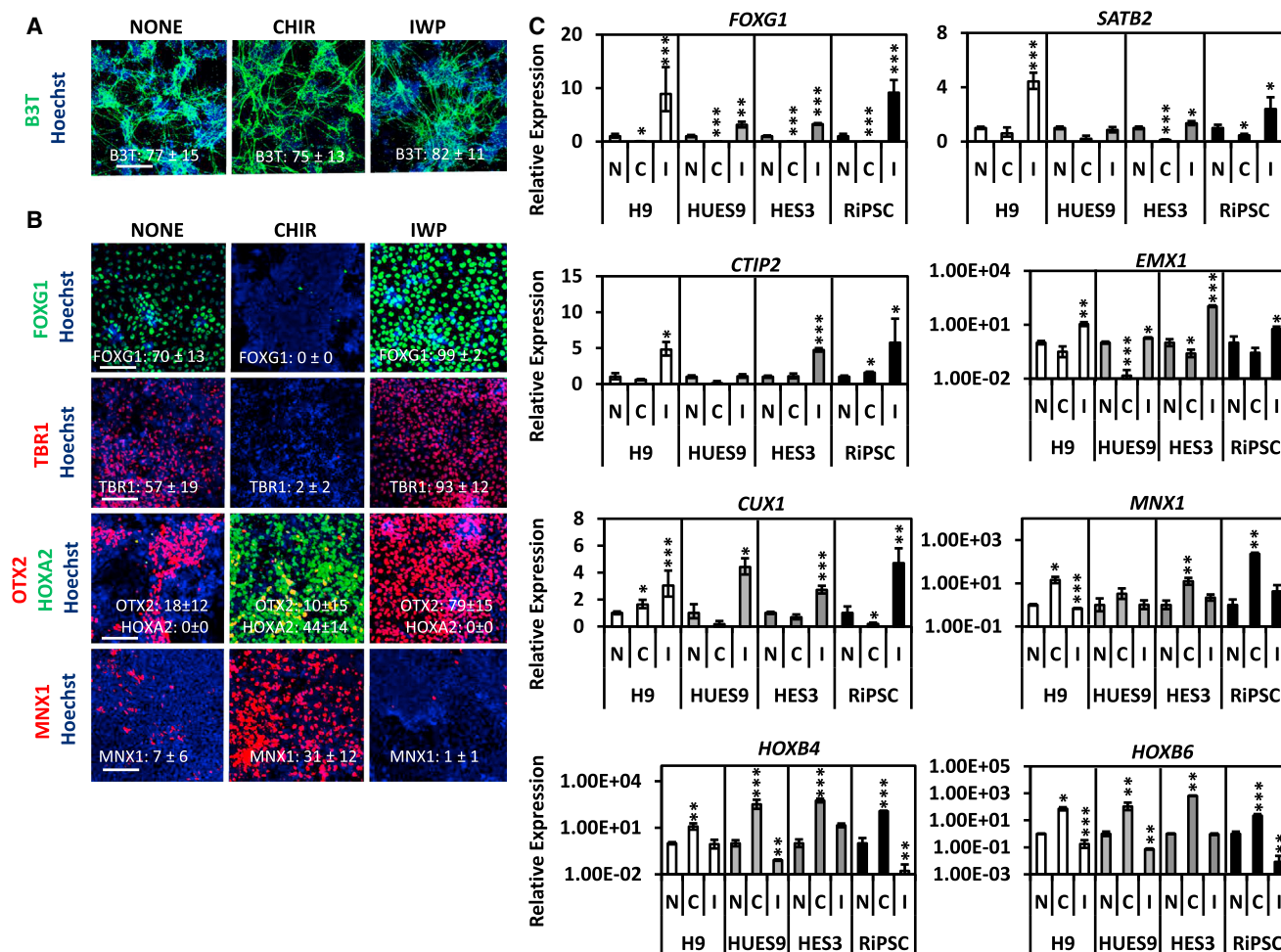
See also Figure S5.

CHIR) did not change their regional identity (Figures S6C and S6D). Collectively, these data suggest that the effect of exogenous WNT signaling on NPC patterning is imparted early during their generation and NPCs are unable to interconvert between positional identities during subsequent expansion.

We subsequently investigated whether NPCs generated with IWP2 or CHIR treatment retained their regional

phenotype upon differentiation to neurons. IWP2- and CHIR-treated NPCs were expanded for four passages and then subjected to the neuronal differentiation protocol. IF (Figure 5A) and gene expression (Figure S5G) revealed that IWP2- and CHIR-treated NPCs generated similar numbers of B3T<sup>+</sup> neurons compared with untreated NPCs. However, neurons generated from IWP2-treated NPCs expressed higher levels of the forebrain- and





**Figure 5. Analysis of Neurons Derived from CHIR- and IWP2-Treated and Untreated NPC Cultures**

(A) IF of mature neuronal markers B3T in neuronal cultures differentiated from 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9 hPSCs (scale bar, 100  $\mu$ m).

(B) IF of cortical-, forebrain-, midbrain-, hindbrain-, and spinal-cord-related neuronal genes in neurons differentiated from 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9 hPSCs (scale bar, 100  $\mu$ m).

(C) Expression of cortical-, forebrain-, midbrain-, hindbrain-, and spinal-cord-related neuronal genes in neurons differentiated from 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9, HUES9, HES3, and RiPSC hPSCs (mean  $\pm$  SEM, n = 4 independent experiments). Populations were compared with neurons differentiated from untreated (N) NPCs using Student's t test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

cortical-related neuronal markers FOXG1, TBR1, *SATB2*, *CTIP2*, *EMX1*, *CUX1*, and *OTX2* compared with neurons generated from CHIR-treated or -untreated NPCs (Figures 5B and 5C). On the other hand, neurons generated from CHIR-treated NPCs expressed higher levels of the hind-brain- and spinal-cord-specific markers HOXA2, HOXB4, HOXB6, and MNX1 (Figures 5B and 5C). Together, our results indicate that NPCs retain their regional identity over multiple passages and manifest that identity in differentiated neuron cultures.

Although we were able to generate NPC and neuron cultures with distinct anterior and posterior identities, we did

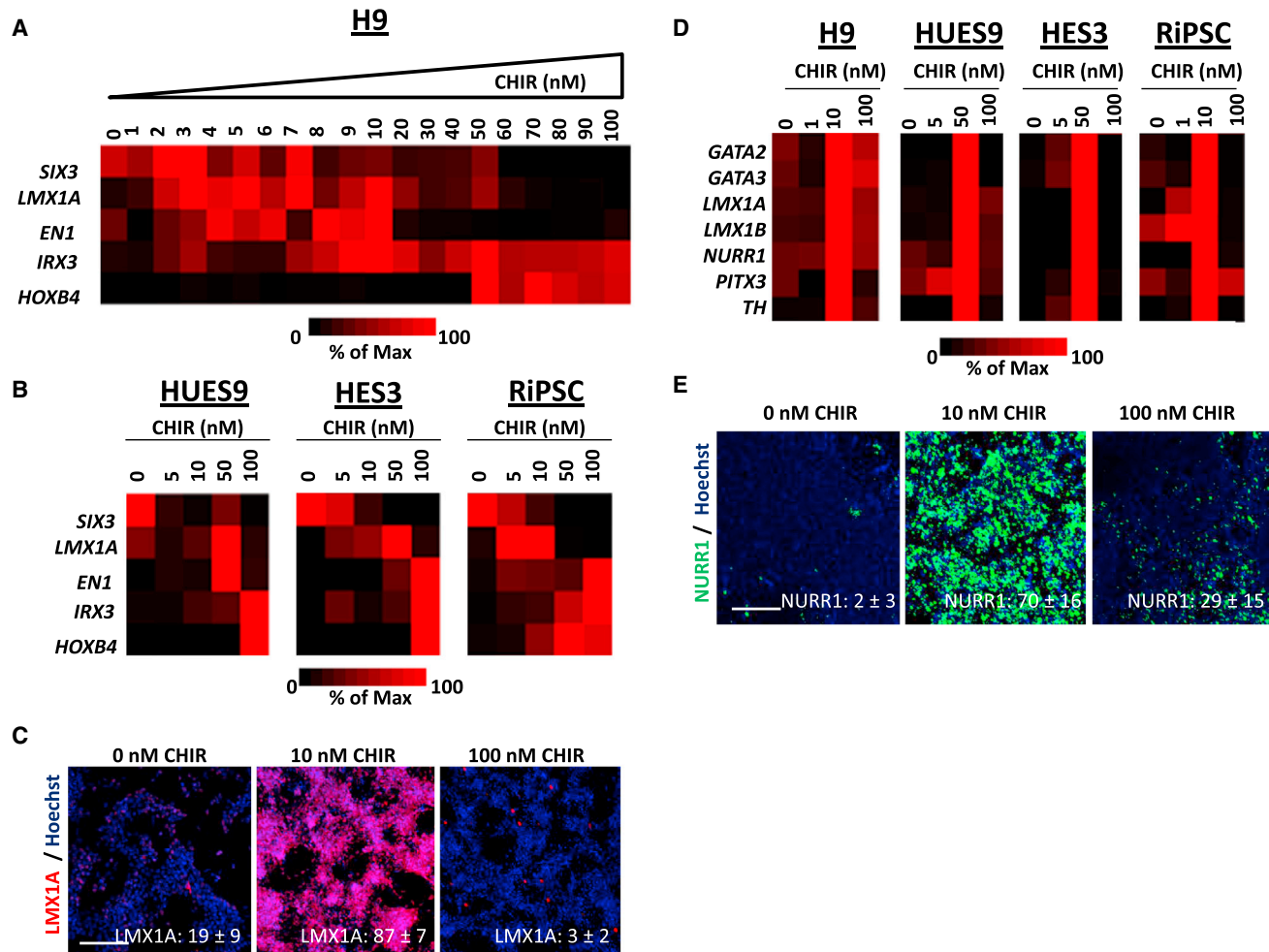
not observe cell populations expressing markers associated with a midbrain phenotype. We speculated that this notable absence of midbrain cell types may have been the consequence of endogenous WNT signaling activities that influence the extent to which exogenous WNT signaling imparts A/P identities. To eliminate the contribution of any endogenous WNT signaling during NPC generation and fine-tune the level of WNT signaling activity, we treated differentiating cultures (hPSCs to NPCs) with IWP2 simultaneously with increasing concentrations of CHIR. Analysis of the gene-expression profile of NPCs derived at various concentrations of CHIR revealed that we could





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**Figure 6. Specification of the Midbrain Neural Phenotype through Precise Exogenous Manipulation of WNT Signaling**

(A and B) Gene-expression analysis of A/P-related genes in NPCs generated from (A) H9 (mean,  $n = 3$  technical replicates) and (B) HUES9, HES3, and RiPSC (mean,  $n = 3$  independent experiments) hPSCs in the presence of 1,000 nM IWP2 and various concentrations of CHIR. The data are displayed in a heatmap where black corresponds to minimum expression levels and red corresponds to maximum levels. For each gene analyzed, the expression levels were normalized to the sample with the highest expression level.

(C) IF of LMX1A expression in NPCs generated from H9 hPSCs in the presence of 1,000 nM IWP2 and various concentrations of CHIR (scale bar, 100  $\mu\text{m}$ ).

(D) Gene-expression analysis of midbrain-related genes in neuronal cultures differentiated from NPCs treated with various CHIR concentrations (mean; H9,  $n = 3$  independent experiments; HUES9, HES3, RiPSC,  $n = 4$  independent experiments).

(E) IF of NURR1 expression in NPCs generated from H9 hPSCs in the presence of 1,000 nM IWP2 and various concentrations of CHIR (scale bar, 100  $\mu\text{m}$ ).

See also [Figure S6](#).

specify the A/P positional identity of NPCs by precisely controlling the level of exogenous WNT signaling ([Figures 6A–6C](#)). In all hPSC lines tested, induction of NPCs with a midbrain phenotype occurred at a narrow CHIR concentration range, with the precise CHIR range varying slightly between hPSC lines. CHIR concentrations significantly lower or higher than these optima led to the production of NPCs with an anterior/forebrain or hindbrain/spinal-cord phenotype, respectively.

We differentiated these NPCs of specific positional fate to neurons. Expression of the midbrain neuronal-related markers *GATA2*, *GATA3*, *LMX1A*, *LMX1B*, *NURR1*, *PITX3*, and *TH* peaked at a CHIR concentration of 10 nM for NPCs generated from H9 or RiPSC hPSCs, and a CHIR concentration of 50 nM for NPCs generated from HUES9 or HES3 hPSCs ([Figures 6D and 6E](#)). Together, these data indicate that by selecting the precise level of extrinsic WNT signaling in the context of suppressed endogenous



WNT signaling, we could generate hPSC-derived NPCs and neurons with midbrain characteristics.

## DISCUSSION

hPSCs and their derivative NPCs share the ability to self-renew indefinitely while retaining the potential to differentiate into mature cell types, although the potential of NPCs is restricted to cells of the CNS, including neurons, astrocytes, and oligodendrocytes. Our study demonstrates that NPCs are additionally restricted by their positional identity. As in development of the CNS, where establishment of the A/P axis early during neural tube formation confers a specific positional identity on naive neuroepithelial cells, NPCs acquire similar positional information from their microenvironment. Importantly, despite their apparent homogeneous appearance and lack of a clear 3D architecture, NPCs in cell culture acquire and stably maintain this A/P positional identity. In contrast to this stable identity of NPCs, where individual and heterogeneous states coexist and do not interconvert, ESCs exist in a metastable state in which individual cells exhibit oscillatory expression of transcription factors (Cahan and Daley, 2013; Cahan et al., 2010; de Souza, 2012; Galvin-Burgess et al., 2013; Narsinh et al., 2011; Stewart et al., 2006), correlating with a bias to either self-renew or differentiate. Importantly, we show that NPC positional identity and specific neuronal differentiation potential are retained over prolonged expansion (>50 days) in culture.

The WNT signaling pathway is a primary determinant in assigning an A/P positional identity to NPCs. This instructional cue is imparted early during NPC generation and once this identity is established, it is stable and cannot be altered through exogenous manipulation of the WNT pathway. Using a WNT reporter line, we show that endogenous WNT signaling is highly variable among individual cells as they acquire a NPC phenotype, with cells of posterior identity expressing WNT reporter activity. In addition to expressing markers of posteriorly fated NPCs, most notably genes of the HOX gene cluster, these cells also express multiple WNT ligands. In contrast, NPCs with anterior identity, as detected by a lack of WNT reporter activity, express multiple WNT antagonists. These differences in expression of WNT agonist and antagonist resemble those observed in the developing neural tube *in vivo*, with posterior tissues expressing WNT proteins and anterior tissues expressing WNT antagonists such as DKK1 and FRZB (Hashimoto et al., 2000; Leyns et al., 1997).

These opposing WNT signals generate an endogenous gradient of WNT activity, which divides the embryonic neural tube along the A/P axis into distinct progenitor domains, each of which gives rise to specific regionalized neu-

rons (Ciani and Salinas, 2005; Kiecker and Niehrs, 2001; Nordström et al., 2002). These progenitor domains have regionally specific gene-expression profiles and differentiation predispositions despite similar levels of expression of the pan-neural markers SOX1 and SOX2 (Pevny et al., 1998; Wood and Episkopou, 1999; Zappone et al., 2000). Here, we showed that NPCs exhibited a broad range of endogenous WNT activity that conferred specific regionalized fates despite comparable expression levels of SOX1 and SOX2, perhaps mimicking the same developmental events that are seen during early *in vivo* neural tube development. Therefore, the local WNT microenvironment tightly regulates the WNT activity status and hence the positional identity of NPCs.

A somewhat unexpected implication of these gene-expression patterns is that WNT signaling appears to be acting cell autonomously, with WNT signaling activity restricted to those cells expressing WNT genes. Although WNT signaling activity is present in a graded fashion in these NPC cultures, WNT proteins are acting in an autocrine rather than paracrine manner. Furthermore, expression of WNT antagonists may mute the response in cells near or adjacent to WNT secreting cells. A more careful analysis of this cell-based system will likely yield important mechanistic insights into the dynamic nature of WNT signaling during development.

This restricted WNT signaling activity observed in NPC cultures is consistent with the notion that WNT proteins act locally (Habib et al., 2013) and exhibit minimal, if any, extracellular diffusion. A recent study demonstrated that flies expressing an engineered membrane-tethered Wingless (a fly WNT protein) are viable and normally patterned, suggesting that the spread of Wingless is dispensable for patterning and growth (Alexandre et al., 2014). Similarly, in our cell-based system, WNT proteins act locally and do not signal to distant cells. In addition, expression of WNT antagonists in the WNT<sup>-</sup> populations may act to block paracrine WNT signaling activity. This local WNT activity is not the result of the physical separation of distinct WNT expressing domains, since this localized activity is retained in a mixed and seemingly homogeneous cell culture system.

While endogenous WNT signaling activity is a major source of heterogeneity among individual NPCs, exogenous manipulation of this signaling pathway can be exploited to impart specific positional identities to NPCs during their generation from hPSCs, thereby reducing cellular heterogeneity. Activation of WNT signaling with purified WNT3a protein or a GSK-3 $\beta$  inhibitor (CHIR98014) led to the generation of NPCs with a hind-brain/spinal cord identity, whereas inhibition of WNT signaling with a PORCN inhibitor (IWP2) to block endogenous WNT protein processing led to the generation of NPCs



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### WNT Regulation of NPC Regional Identity

with a forebrain phenotype. As shown in this and other studies (Li et al., 2009; Pankratz et al., 2007), in the absence of any WNT pathway manipulations, NPCs generated from hPSCs are generally biased toward an anterior fate, suggesting that endogenous WNT signaling in these culture systems is relatively low and insufficient to promote posterior fates. Consequently, ectopic activation of WNT signaling produces a prominent shift from an anterior to a posterior fate. In contrast, in the absence of WNT signaling (through IWP2 addition), the relative increase of anterior-related markers, though statistically significant, is less pronounced.

Although exogenous manipulation of WNT signaling can be used to reduce NPC heterogeneity, the window during which this manipulation is effective is limited. Specifically, we find that WNT signaling imparts positional identity early during NPC generation, likely during the rosette stage, in which the cell population most closely resembles the early developing neural tube. Once an NPC culture is established and propagated over multiple passages, A/P positional identity is stable and recalcitrant to exogenous manipulations of WNT signaling. Therefore, the identity, concentration, and timing of factors (e.g., WNTs) added during NPC generation are critical to produce homogeneous cultures.

Although WNT signaling plays a prominent role in A/P patterning of the neural tube, few studies have extensively examined the influence of WNT on the A/P positional identity of hPSC-derived NPCs and neurons. To date, most studies have relied on FGF8 (Yan et al., 2005; Yang et al., 2008) or retinoic acid (RA) (Dimos et al., 2008; Hu and Zhang, 2009; Li et al., 2005, 2008; Singh Roy et al., 2005) to generate posterior neural populations, such as midbrain dopaminergic and spinal cord motor neurons, respectively, from hPSCs. Similar to the approach we used in our study, two groups recently used a specific concentration of the GSK3 $\beta$  inhibitor CHIR99021 (an analog to the GSK3 $\beta$  inhibitor used in this study) to generate midbrain dopaminergic neurons from hPSCs (Kirkeby et al., 2012; Kriks et al., 2011). However, the ability of this compound to generate stable NPC populations from different areas of A/P axis was not extensively studied. We demonstrated that through precise chemical modulation of WNT signaling, we could control the A/P positional identity of hPSC-derived NPCs. Moreover, we demonstrated that these NPCs retained their positional specificity as they were differentiated to neurons *in vitro*. It should also be noted that previous studies (Hu and Zhang, 2009; Yan et al., 2005) have relied on the activation of SHH signaling in order to generate ventral neurons, such as mDA and motor neurons. In this study, we demonstrated that we were able to generate these ventral neuronal subtypes without exogenous modulation of SHH signaling. This suggests the possibility that endogenous SHH

signaling may regulate the dorsal-ventral (D/V) identity of hPSC-derived NPCs and neurons analogously to the manner in which endogenous WNT signaling regulates their A/P identity. Nonetheless, our study serves as proof-of-principle that modulation of developmental signaling pathways, such as WNT and SHH, can be exploited to refine the A/P and D/V identity of NPCs and neurons.

Our findings regarding the positional restriction of NPCs have important implications for the study and application of these cells. Several studies have described the transplantation of hPSC-derived NPCs into animal models of Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and spinal cord injury (Kim et al., 2013; Lindvall and Kokaia, 2010). However, few of these studies have described a successful long-term reduction in the symptoms associated with these disorders (Kim et al., 2013; Lindvall and Kokaia, 2010). Interestingly, several of these studies relied on NPCs generated by dual SMAD inhibition, which results in NPCs of an anterior telencephalic identity (Chambers et al., 2009), which may explain the lack of symptomatic improvement in animal models of disorders associated with the midbrain, hindbrain, and spinal cord. We speculate that future animal transplantation studies that utilize regionally specific and stably expandable NPCs, such as those generated in this study, will result in a significant improvement in the cognitive and motor deficits associated with these neurological disorders.

In summary, we determined that endogenous WNT signaling influences the heterogeneity, regional characteristics, and differential potential of hPSC-derived NPCs. In addition, we showed that precise exogenous modulation of WNT signaling during neural differentiation of hPSCs results in a homogeneous NPC population with a specific positional identity. Importantly, manipulation of endogenous and exogenous WNT signaling will allow for the development of defined methods for generating transplantable hPSC-derived NPCs for specific regions of the entire A/P axis of the neural tube. Furthermore, this study suggests that modulation of other developmental signaling pathways, including BMP, FGF, and SHH, can be exploited to further refine the positional identity of NPCs and neurons. In the future, these regionally specific NPCs will greatly enhance the translational potential of hPSCs for neural-related therapies.

## EXPERIMENTAL PROCEDURES

### Cells and Culture Conditions

Media compositions and sources for all cell lines are listed in the [Supplemental Experimental Procedures](#). All work with hPSCs was reviewed and approved by the Stem Cell Research Oversight Committee of the University of California, San Diego (project numbers 100210ZX and 090807ZX).





### Generation of WNT Reporter hESCs

The infection and generation of WNT reporter hESCs are described in the [Supplemental Experimental Procedures](#). The lentiviral construct that was used to generate the WNT reporter line contained a 7xTCF-eGFP construct and puromycin resistance gene (Fuerer and Nusse, 2010). High titer lentivirus was produced as previously described (Miyoshi et al., 1998; Zufferey et al., 1998). Clones were screened for robust TOP-GFP expression upon WNT3a stimulation and a normal euploid karyotype.

### NPC Generation, Expansion, and Differentiation

The methods used for the generation, expansion, and neuronal differentiation of hPSC-derived NPCs are described in the [Supplemental Experimental Procedures](#) and Brafman (2014).

### qPCR

Details regarding the methods used for qPCR are provided in the [Supplemental Experimental Procedures](#). RNA was isolated using TRIzol and reverse transcription was performed with the use of the qScript cDNA Supermix. qPCR was carried out using Taqman Probes (Table S4) and the TaqMan Fast Universal PCR Master Mix on a 7900HT Real-Time PCR machine. Gene expression was normalized to 18 s rRNA levels. All experiments were performed with three technical replicates.

### IF and FC

Detailed protocols for IF and FC are provided in the [Supplemental Experimental Procedures](#). The antibodies used are listed in Table S5. Quantification of images was performed by counting a minimum of nine fields at 20 $\times$  magnification. Image quantification of the data is presented as the average of these fields  $\pm$  SD.

### High-Throughput RNA-Seq

RNA-seq of RNA from TOP-GFP<sup>+</sup> and TOP-GFP<sup>-</sup> NPCs was performed as described in the [Supplemental Experimental Procedures](#), and differential gene-expression analysis was performed with TopHat and Cufflinks (Trapnell et al., 2012, 2013). Reads per kilobase per million mapped (RPKM) values were calculated for each gene and used as an estimate of expression levels. The raw RNA-seq data are provided in Table S1.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.10.004>.

### AUTHOR CONTRIBUTIONS

N.M., J.C., and D.A.B. designed and conceived the study. N.M., J.C., K.W., and D.A.B. performed experiments. N.M., J.C., K.W., T.G., and D.A.B. analyzed data. N.M., J.C., K.W., and D.A.B. wrote the manuscript.

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