

## Notch Signaling Regulates Motor Neuron Differentiation of Human Embryonic Stem Cells

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### ABSTRACT

In the pMN domain of the spinal cord, Notch signaling regulates the balance between motor neuron differentiation and maintenance of the progenitor state for later oligodendrocyte differentiation. Here, we sought to study the role of Notch signaling in regulation of the switch from the pMN progenitor state to differentiated motor neurons in a human model system. Human embryonic stem cells (hESCs) were directed to differentiate to pMN-like progenitor cells by the inductive action of retinoic acid and a Shh agonist, purmorphamine. We found that the expression of the Notch signaling effector Hes5 was induced in hESC-derived pMN-like progenitors and remained highly expressed when they were cultured under conditions favoring motor neuron differentiation. Inhibition of Notch signaling by a  $\gamma$ -secretase inhibitor in the differentiating pMN-like progenitor cells decreased Hes5 expression and enhanced the differentiation toward motor neurons. Conversely, over-expression of Hes5 in pMN-like progenitor cells during the differentiation interfered with retinoic acid- and purmorphamine-induced motor neuron differentiation and inhibited the emergence of motor neurons. Inhibition of Notch signaling had a permissive rather than an inductive effect on motor neuron differentiation. Our results indicate that Notch signaling has a regulatory role in the switch from the pMN progenitor to the differentiated motor neuron state. Inhibition of Notch signaling can be harnessed to enhance the differentiation of hESCs toward motor neurons. *STEM CELLS* 2015;33:403–415

### INTRODUCTION

The Notch signaling pathway plays an essential role in maintenance of progenitor cell populations and in preventing their differentiation into mature progenies. Notch signaling is initiated when Notch receptor on one cell is activated by a ligand expressed on a neighboring cell. Upon activation, the Notch receptor intracellular domain is cleaved by Presenilin proteases of the  $\gamma$ -secretase complex and translocates to the nucleus to form a complex with CBF1/RBPj, Su(H), Lag-1 (CSL) and Master-mind (Maml) proteins [1–4]. This complex then activates expression of the Hes (Hes1 and Hes5) and Hey transcription factors, which repress the expression of proneural genes such as Neurogenin 1/2 and Ascl1, thereby inhibiting neuronal differentiation and maintaining neural progenitor cells [5].

In the developing spinal cord, Notch signaling has a prominent role both in maintenance of neural and glial progenitor cells and in regulation of specific neuronal fate decisions. Specific progenitor cells with distinct identities and fates are organized along the dorso-ventral axis of the neural tube in five domains, termed p0–p3 and pMN. Recently it was shown that the transcription factor Nkx6.1 plays an active role

in inducing the expression of the Notch ligand Dll1 in both the pMN and p2 domains and the resulting Notch signaling maintains the progenitor state in the distinct domains [6]. Accordingly, conditional knockout of Notch1 receptor results in a reduction of all neural progenitor subtypes in the ventral spinal cord [7].

Progenitor cells in the p0–p3 domains generate different classes of ventral interneurons, named V0–V3, respectively, whereas pMN progenitor cells at early stages of development appear to be committed to generate motor neurons (MNs). Later in development, they switch to produce oligodendrocytes [8–11]. pMN progenitor cells selectively express the bHLH protein Olig2, which is required to specify both motor neuron and oligodendrocyte cell identities [12, 13]. Olig2 primes pMN progenitor cells to become motor neurons by triggering the expression of Neurogenin 2 (Ngn2) bHLH protein. Coexpression of Olig2 and Ngn2 directs the pMN progenitors to leave the cell cycle and to become motor neurons, while progenitors in which Ngn2 expression is low remain as proliferative progenitors which are specified to an oligodendrocyte fate [13–15]. Ngn2 is repressed by Notch signaling, as was shown by the upregulation of Ngn2 expression in the ventral spinal cord of Notch1

conditional null mice [7]. In the pMN domain, Notch signaling acts to maintain the balance between progenitors that differentiate into motor neurons during the neurogenic phase and those that are preserved as a pool of presumptive progenitors for the gliogenic phase [16, 17].

It was shown that loss of the Notch signaling in the pMN domain increased motor neuron differentiation and results in a progressive depletion of the pMN progenitors over time. Conversely, activation of Notch signaling resulted in a reduction in motor neurons [6, 17]. In light of the potential role of Notch signaling during motor neurons development in animal models, we sought to study its role in human motor neuron development using human embryonic stem cells (hESCs) as a model system.

Human ESCs have been reported to generate spinal motor neurons in a pathway that recapitulates the steps of motor neuron differentiation *in vivo*. After initial neuralization, caudalization is induced by retinoic acid (RA) and ventralization by Shh morphogen [18–21]. In response to the Shh, hESC-derived pMN progenitors express Pax6, Nkx6.1, and Olig2 transcription factors similar to their *in vivo* counterparts, and can be further differentiated into early Hb9 expressing and to mature ChAT-producing spinal motor neurons.

Using BAC transgenic reporter lines, the Notch components Hes5 and Dll1 have been shown to be dynamically expressed during the differentiation of hESCs into motor neurons [22]. Hes5 is highly expressed in hESCs-derived neural progenitors and is downregulated during their differentiation into motor neurons. Conversely, Dll1 is expressed at low levels in neural progenitors and is upregulated upon their differentiation, being expressed in Hb9-positive motor neurons.

To better understand how the activity of Notch signaling controls differentiation of hESCs into motor neurons, the study we report here tested the functional relationship between the expression levels of the Notch downstream effector Hes5 and motor neuron differentiation. We show that in response to RA and the Shh agonist purmorphamine (PUR), hESC-derived neural progenitor cells are specified to generate pMN-like progenitor cells characterized by the expression of Olig2 and Ngn2. The neuralization and subsequent specification to pMN-like progenitors are concomitant with the induction of the expression of Hes5. However, further differentiation of the pMN-like progenitor cells into motor neurons is low, raising the possibility that Notch signaling inhibits their differentiation. Using the  $\gamma$ -secretase inhibitor DAPT to inhibit Notch signaling in differentiating pMN-like progenitor cells, we found that inhibition of Notch signaling downregulates Hes5 expression and enhances the differentiation of pMN-like progenitors into motor neurons. Conversely, over-expression of Hes5 in differentiating pMN-like progenitors inhibits subsequent differentiation into motor neurons. Still, in the absence of RA and PUR, inhibition of Notch signaling was not sufficient to direct the differentiation of the pMN progenitors toward motor neural fate, indicating a permissive rather than instructive role of Notch signaling in the process of differentiation toward spinal motor neurons.

## MATERIALS AND METHODS

### Cell Culture

Human ESCs (HES1 passages 21–33 with a normal karyotype) were cultured as described [23, 24].

For differentiation, hESC colonies were picked up by means of collagenase IV (1 mg/ml; GIBCO-BRL, Gaithersburg, MD, www.lifetechnologies.com), triturated into small, 50–100 cell clumps, and placed into ultralow adherent culture dishes (Thermo Scientific Nunc HydroCell, www.thermoscientific.com). For the first 4 days, cells were grown in neural stem cells (NSC) media, consisting of Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, www.lifetechnologies.com) and 2% B27 supplement with 20 ng/ml FGF2 (PeproTech, Inc., Rocky Hill, NJ) and 5  $\mu$ M SB431542 (SB; Selleck Chemicals LLC, Houston, TX, www.selleckchem.com). At day 14, neural spheres were switched to medium consisting of DMEM/F-12 and 1% N2 supplement with 1  $\mu$ M all-*trans* RA (Sigma-Aldrich, Saint Louis, MO, www.sigma-aldrich.com) and 1  $\mu$ M dibutyryl cAMP (Sigma). At day 21, the spheres were cultured in medium consisting of Neurobasal (Invitrogen) and N2 supplement with 1  $\mu$ M RA, 0.5  $\mu$ M PUR (Cayman Chemical, Ann Arbor, MI, www.caymanchem.com), and 1  $\mu$ M dibutyryl cAMP for a 3-week period. For differentiation, spheres were cut into small clusters and plated on poly-lysine/laminin-coated cover glasses for 1 week, in Neurobasal medium with 1% N2 supplement containing 0.25  $\mu$ M or 0.5  $\mu$ M RA, 0.125  $\mu$ M, or 0.25  $\mu$ M PUR, 10 ng/ml each brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1) (PeproTech, Inc., www.peproTech.com), and 1  $\mu$ M dibutyryl cAMP. DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester, dissolved in dimethyl sulfoxide (DMSO) (Sigma), was used at a final concentration of 1  $\mu$ M. DMSO was used as vehicle control.

### Lentiviral Constructs and Transduction

Human Hes5 (aa 1–167) was amplified using Phusion Hot Start Flex DNA polymerase (New England BioLabs, Inc., Ipswich, MA, www.neb.com) and cloned into pFLAG-CMV-2. FLAG-tagged Hes5 was cloned to SIN18.Cppt.hEF1 $\alpha$ .WPRES lentiviral vector. Empty lentiviral vector was used as control. Concentrated lentiviral stocks were prepared as described [23]. Neural spheres treated with 1  $\mu$ M RA and 0.5  $\mu$ M PUR for 19 days were cut into small clusters and incubated overnight with the concentrated viral supernatant, which was then replaced with fresh Neurobasal medium supplemented with RA and PUR. Two days later, the transduced cells were plated for differentiation as described above.

### Immunocytochemistry

Cells were fixed in 4% Paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained at room temperature with primary antibodies. Primary antibodies used in this study included antibodies against Ngn2 (Santa Cruz Biotechnology, Inc., Dallas, TX, www.scbt.com, 1:75), Goat Olig2 (R&D System, Inc., Minneapolis, MN, www.rndsystems.com, 1:75), mouse Olig2 (clone 211F1.1, Millipore Corporation, www.emdmillipore.com, 1:150), Islet-1 (Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, www.dshb.biology.uiowa.edu, 1:50), Lim3 (DSHB, 1:200), MNR2 or Hb9 (DSHB, 1:50), ChAT (R&D System, Inc., 1:300), FLAG (Sigma-Aldrich, 1:1,000), and DYKDDDDK Tag (Cell Signaling Technology, Inc., Danver, MA, www.cellsignal.com, 1:500). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, www.vectorlabs.com). Quantification was performed using ImageJ software (NIH, public

domain software) by measuring positive stained area relative to total DAPI. Quantifications are represented as a mean percentage of total DAPI  $\pm$ SD or SEM and are from at least 15 random fields captured in three or more independent experiments.

### PCR Analysis

Total RNA was extracted from cells at different stages along the differentiation into motor neurons, by means of TRIzol (Invitrogen). cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and random primers, according to the manufacturer's instructions (Promega Corporation, Madison, WI, [www.promega.com](http://www.promega.com)). RT-PCR was performed with Taq DNA Polymerase (Promega Corporation). Primers used are given in Supporting Information.

For quantitative real-time PCR, TaqMan Assays-on-Demand Gene Expression Products (Supporting Information data), TaqMan Universal PCR Master Mix, and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, [www.appliedbiosystems.com](http://www.appliedbiosystems.com)) were used. Large ribosomal protein P0 (RPLP0) was used as an internal reference for normalization.

### Statistical Analysis

All experiments were performed at least three times unless otherwise indicated. Data are presented as means  $\pm$  SD or SEM. Statistical significance was calculated using GraphPad Instat software using one-tailed unpaired Student's *t* test for comparison between two groups. A *p*-value of  $<.05$  was considered significant.

## RESULTS

### Derivation and Characterization of pMN-Like Progenitor Cells from hESCs

Induction of hESC differentiation toward motor neurons was previously described to progress through three sequential steps: neuralization, generation of caudal-ventral pMN progenitors, and differentiation into motor neurons. Based on similar principles, we used a modified four-stage protocol (Supporting Information Fig. S1) [18–20].

In stage 1, hESCs were induced to differentiate to neuroectoderm by culturing hESC clusters in the presence of SB431542 (activin receptor inhibitor) and FGF2 for 4 days followed by 10 days culturing in the presence of FGF2 only. At the end of this culture period, the clusters acquired a round morphology typical of neural spheres and were highly enriched for neural precursor cells expressing PSA-NCAM [25].

In stage 2, specification of these neural precursors toward caudal fate was induced by treating the spheres with RA (1  $\mu$ M). The spheres diameter were maintained below 300  $\mu$ m by gentle trituration to allow the penetration of RA. Following 1 week of treatment with RA, the neural precursors acquired a caudal fate as indicated by the upregulation of hox genes expression (Fig. 1A). The expression of Hox-c genes suggested that the neural progenitors acquired a rostral-cervical spinal cord identity [26].

It was previously shown that motor neurons differentiate from ventral progenitors of the pMN domain that coexpress Olig2 and Ngn2 [14, 15, 27–29]. In the developing spinal cord, the expression of Olig2 and Ngn2 is induced by Shh secreted from the floor plate and the notochord [26, 30]. To induce ventralization of the caudal progenitors generated upon RA treat-

ment, in stage 3 the spheres were cultured for 3 weeks in the presence of the Shh agonist PUR (0.5  $\mu$ M) in addition to RA. QRT-PCR analysis revealed that following 1 week caudalization with RA, Olig2 and Ngn2 expression levels in the progenitors were induced by 2.5- and 17-fold, respectively, while addition of PUR further induced both Olig2 and Ngn2 by 13- and 3.5-fold, respectively (Fig. 1B, 1C). Immunofluorescence staining revealed that upon the first week of RA treatment only few cells were immunoreactive with either anti-Olig2 (Fig. 1D) or -Ngn2 (Fig. 1E), while at the end of 3 weeks treatment with RA and PUR, 57% of the progenitors were stained positive for Olig2 (Fig. 1F) and 46% were stained positive for Ngn2 (Fig. 1G). Forty-four percentage of the cells coexpressed Olig2 and Ngn2 as demonstrated by coimmunostaining for these markers (Supporting Information Fig. S2). The expression of both Olig2 and Ngn2 is indicative of ventral pMN-like progenitors.

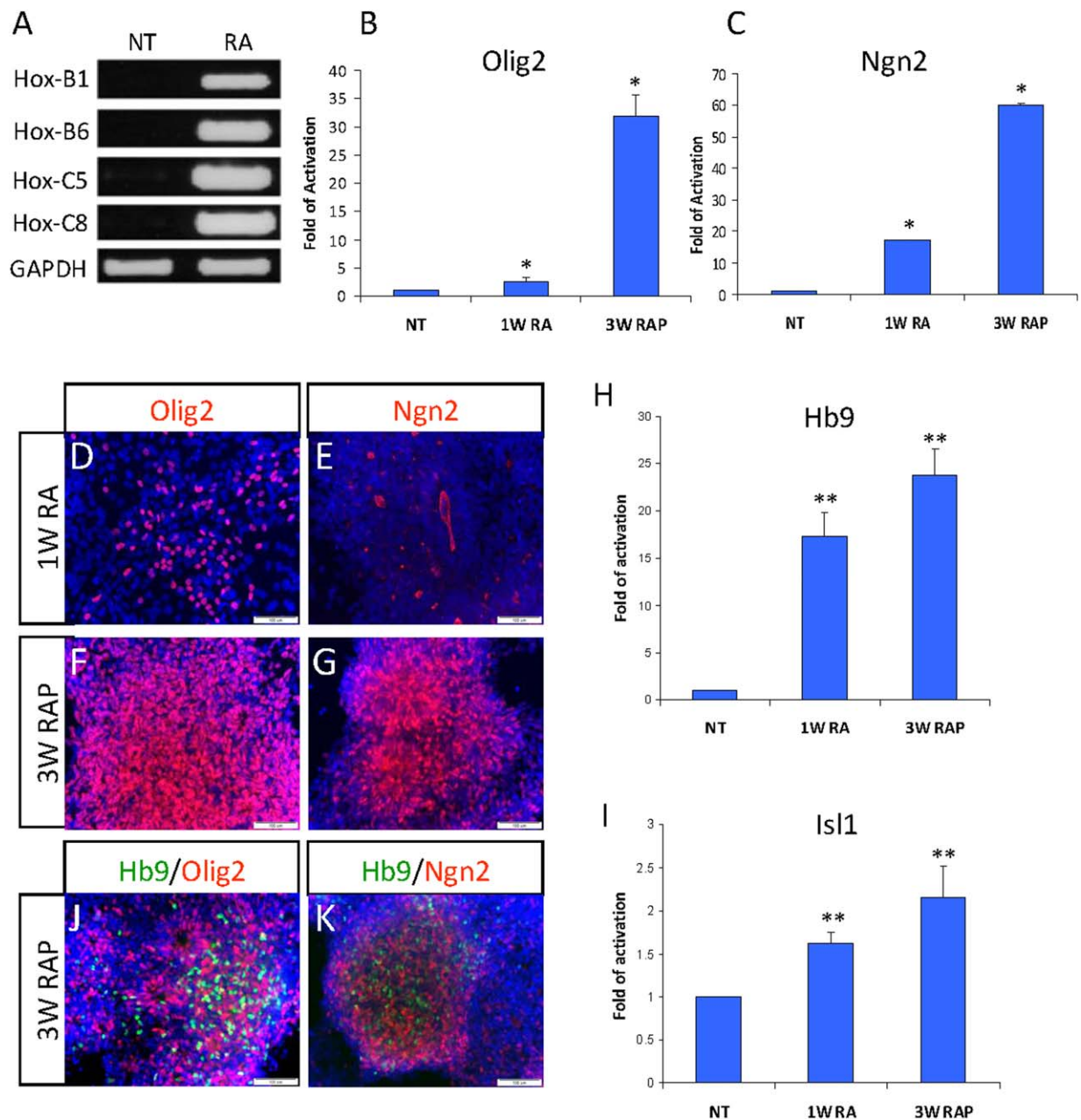
Analysis of motor neuron markers expression by qRT-PCR revealed that Hb9 gene was upregulated by 17-fold upon RA treatment and its expression was slightly increased (24-fold) in pMN-like progenitor cells upon addition of PUR (Fig. 1H). *Isl1* gene expression was induced by 1.6-fold in the caudal progenitors, and its expression was mildly increased in the pMN-like progenitors (Fig. 1I). However, coimmunostaining of the pMN-like progenitors for Hb9 and either Olig2 or Ngn2 revealed that while the majority of the cells expressed Olig2 or Ngn2, only a few cells were immunoreactive with anti-Hb9 (Fig. 1J, 1K). This observation suggested that at that stage the cells did not yet differentiate into motor neurons but remained as pMN-like progenitors that have the potential to differentiate into motor neurons.

### Differentiation of pMN-Like Progenitor Cells Toward Motor Neurons

In stage 4, the pMN-like progenitors were induced to differentiate into early motor neurons [Hb9(+), ChAT(-)] by plating on laminin for 1 week in differentiation medium (0.5-RP) that was supplemented with halved concentrations of RA and PUR (0.5  $\mu$ M and 0.25  $\mu$ M, respectively). The medium was further supplemented with dibutyryl cAMP and the neurotrophic factors BDNF, GDNF, and IGF-1. As a control, the progenitors were differentiated in the presence of DMSO vehicle combined with neurotrophic factors and dibutyryl cAMP.

Following 1 week of differentiation, the cells were analyzed for the expression of motor neuron markers *Isl1*, *Lhx3*, and Hb9. The transcriptional levels of *Isl1* and *Lhx3* were not significantly changed compared with the levels in the pMN progenitors (Fig. 2A, 2B). The expression level of Hb9 was significantly ( $p = .01$ ) increased by 9-fold in control cells treated with DMSO and by 12-fold in cells treated with RA and PUR (Fig. 2C). It appears that plating control cells on laminin promoted MN differentiation that was further mildly augmented by RA and PUR. The increase in Hb9 expression may be attributed to its activation by the upregulated expression of Ngn2 in pMN-like progenitors [31].

Immunofluorescence staining of the differentiating progenitors revealed 17% *Lhx3* and 12% *Isl1* positive cells in the presence of RA and PUR compared with 10% *Lhx3* and 5% *Isl1*-positive cells in the control ( $p < .01$ ) (Fig. 2D, 2D', 2E, 2E', 2G). Moreover, in the presence of RA and PUR, 22% of the cells were Hb9 positive compared with 5% positive cells in the control ( $p < .001$ ) (Fig. 2F, 2F', 2G). Still at this stage of differentiation 36% and 18% of the cells expressed Olig2 and Ngn2, respectively

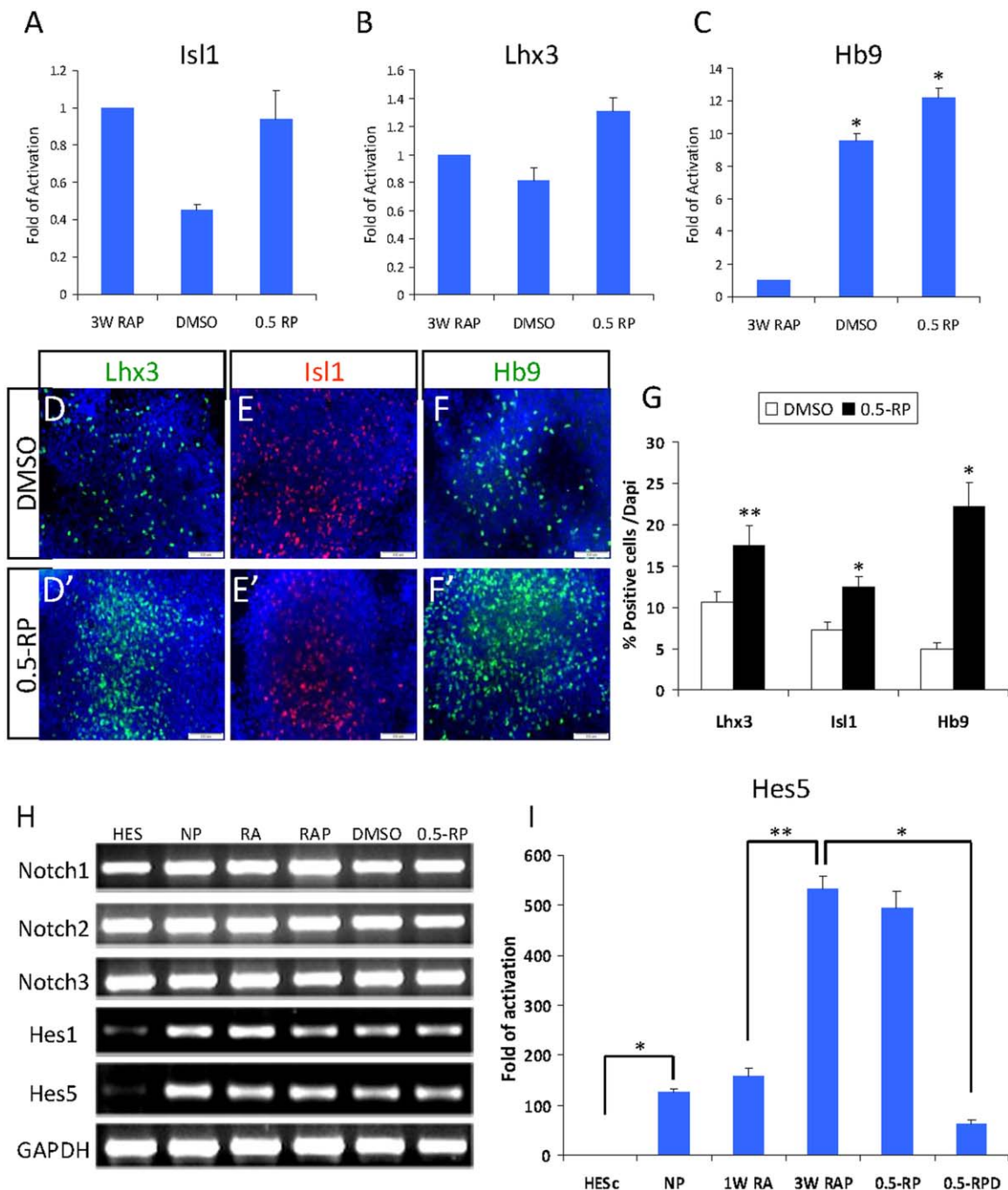


**Figure 1.** Caudalization and ventralization of neural progenitor cells. **(A)**: Hox genes expression in caudal progenitors grown for 1 week in the presence of 1  $\mu$ M retinoic acid (RA). RT-PCR analysis indicated induction of Hox genes in progenitors treated with RA compared with their nontreated counterparts (NT). GAPDH levels were used as quantitative reference. **(B, C)**: Expression of Olig2 and Ngn2 in caudal and ventral progenitor cells. Neural progenitors were grown for 1 week in the presence of 1  $\mu$ M RA followed by 3 weeks treatment with 1  $\mu$ M RA and 0.5  $\mu$ M purmorphamine to generate first caudal progenitors (1W RA) and then ventral pMN-like progenitors (3W RAP). QTR-PCR analysis of the expression Olig2 (B) and Ngn2 (C) genes in caudal (1W RA) and ventral (3W RAP) progenitor cells relative to the expression level in neural progenitor cells (NT). Expression level in neural progenitor cells was set as 1. Fold activation over control is derived from three experiments. Data are presented as mean + SD. \*,  $p < .01$  by Student's  $t$  test. **(D–G)**: Immunofluorescence images demonstrating the expression of Olig2 (D and F) and Ngn2 (E and G) in caudal (1W RA) and ventral (3W RAP) progenitor cells. Blue indicates DAPI stained nuclei. Scale bars = 100  $\mu$ m. **(H–K)**: Expression of motor neurons markers in caudal and ventral pMN-like progenitor cells. **(H, I)**: QRT-PCR analysis of the expression of Hb9 (H) and Isl1 (I) genes in caudal (1W RA) and ventral (3W RAP) progenitor cells relative to the expression level in neural progenitor cells (NT). Expression level in neural progenitor cells was set as 1. Fold activation over control is derived from three experiments. Data are presented as mean + SD. \*\*,  $p < .05$  by Student's  $t$  test. **(J, K)**: Immunofluorescence analysis of ventral progenitor cells (3W RAP) stained with antibodies to Hb9 (green) and Olig2 (red, J) or Ngn2 (red, K). Blue indicates DAPI stained nuclei. Scale bars = 100  $\mu$ m. Abbreviation: DAPI, 4,6-diamidino-2-phenylindole.

(Supporting Information Fig. S3). Taken together, these data suggest that RA and PUR promoted the differentiation of the pMN-like progenitors toward MN fate, albeit a proportion of the cells remained in the pMN-like progenitor state.

#### The Notch Downstream Effector Hes5 Is Upregulated Upon Differentiation of hESCs into Motor Neurons

It is well established that Notch signaling plays a key role in the maintenance of undifferentiated neural progenitors while



**Figure 2.** Expression of motor neuron markers upon differentiation of ventral pMN-like progenitor cells. Ventral pMN-like progenitor cells (3W RAP) were differentiated for 1 week in the presence of 0.5  $\mu$ M retinoic acid (RA) and 0.25  $\mu$ M purmorphamine (PUR) (0.5-RP) or in the presence of DMSO vehicle control. **(A–C):** QRT-PCR analysis of the expression of Isl1 (A), Lhx3 (B), and Hb9 (C) genes in progenitor cells differentiating in the absence (DMSO) or presence of RA and PUR (0.5-RP) relative to the expression levels in ventral pMN-like progenitor cells (3W RAP). Expression level in pMN-like progenitor cells was set as 1. Fold activation over control is derived from three experiments. Data are presented as mean + SD. \*,  $p = .01$  by Student's  $t$  test. **(D–G):** Immunofluorescence images demonstrating the expression of Lhx3 (D, D'), Isl1 (E, E'), and Hb9 (F, F') in cells differentiated in the presence of DMSO (D–F) or RA and PUR (0.5-RP, D'–F'). Blue indicates DAPI counter stained nuclei. Scale bars = 100  $\mu$ m. **(G)** Quantification of the percentage of Lhx3, Isl1, and Hb9 positive cells following differentiation in the presence of DMSO or 0.5-RP. Data derived from three experiments are represented as mean + SD. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's  $t$  test. **(H, I):** Notch signaling is active along the differentiation of neural progenitor cells into motor neurons. **(H)** RT-PCR analysis of the expression of Notch receptors and Hes genes in undifferentiated human embryonic stem cell (HES), NP, caudal progenitor cells (RA), ventral pMN-like progenitor cells (RAP), and progenitors differentiating in the absence (DMSO) or the presence of RA and PUR (0.5-RP). GAPDH levels were used as quantitative reference. **(I):** QRT-PCR analysis of the expression of Hes5 gene in undifferentiated hESCs, NP, caudal progenitor cells (1W RA), ventral pMN-like progenitor cells (3W RAP), progenitors differentiating in the presence of RA and PUR (0.5-RP) and progenitors differentiating in the presence of RA, PUR, and DAPT (0.5-RPD). Expression level in undifferentiated hESCs was set as 1. Fold activation over undifferentiated hESCs is derived from four experiments. Data are presented as mean + SD. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's  $t$  test. Abbreviations: NP, neural progenitor cells; DAPI, 4,6-diamidino-2-phenylindole.

preventing premature differentiation. To test whether Notch signaling prevents pMN-like progenitors to differentiate into postmitotic MNs, we tested whether Notch signaling is active in the differentiating pMN-like progenitors. We first assessed the expression of Notch receptors in hESCs and their differentiating progeny throughout the stages of the differentiation protocol. RT-PCR analysis showed that Notch receptors (Notch1, Notch2, and Notch3) were expressed at similar levels in hESCs as well as in neural precursors, caudal, and pMN-like progenitors and in the progenitors that were induced to differentiate (Fig. 2H). In contrast, low expression levels of the Notch downstream effectors, Hes1 and Hes5, were observed in hESCs and their expression was significantly induced upon differentiation into neural precursors, caudal, and pMN-like progenitors as well as differentiating progenitors (Fig. 2H).

Both Hes1 and Hes5 were shown to be expressed in the ventricular zone of the spinal cord in complementary domains. However, in Notch1<sup>-/-</sup> mice decreased expression of Hes5 but not Hes1 was observed in the ventral spinal cord [7, 32], suggesting that in the ventral spinal cord Hes5 is a direct target of Notch signaling while Hes1 could be regulated by other signaling pathways. Thus, we focused our analysis on Hes5 and tested its expression throughout the four differentiation stages by qRT-PCR.

Hes5 was dramatically induced (100-fold) upon differentiation of hESCs into neural precursors and a similar level of expression was maintained upon further differentiation to caudal progenitors. Following RA and PUR treatment and differentiation into pMN-like progenitors, a fourfold increase in Hes5 expression was observed. This expression level was maintained after 1 week of plating the progenitors for differentiation into MNs, in line with the maintenance of pMN-like progenitor state by a proportion of the cells (Fig. 2I). Collectively, these data suggest that Notch signaling is activated upon differentiation of hESCs toward pMN-like progenitors and that Hes5 may regulate the maintenance of pMN-like progenitors and the prevention of their differentiation.

### Inhibition of Notch Signaling Enhances the Differentiation of pMN-Like Progenitor Cells into Motor Neurons

To study whether Notch signaling prevents the differentiation of the pMN-like progenitors into motor neurons we tested whether its inhibition promotes motor neuron differentiation of the pMN-like progenitors. Notch signaling can be blocked pharmacologically by DAPT, which inhibits  $\gamma$ -secretase activity and prevents Notch receptor cleavage [33, 34]. To test whether DAPT inhibits Notch signaling during the differentiation of the pMN-like progenitors toward motor neurons, the progenitors were allowed to differentiate as before in the presence of RA and PUR combined DMSO or with DAPT (0.5-RP or 0.5-RPD, respectively). In the presence of DAPT, an eightfold decrease in Hes5 expression level was observed in the differentiating pMN-like progenitors, indicating that Notch signaling was inhibited (Fig. 2I).

Next, we tested whether the decrease in Hes5 expression was concomitant with an increased expression of motor neural markers. It was previously shown that specification of pMN progenitors into motor neuron fate is regulated by Ngn2 and Olig2, where progenitors that express both Olig2 and Ngn2 develop as motor neurons while those expressing only

Olig2 become oligodendrocytes [14, 15, 27, 29]. Neither increase nor decrease in Olig2 expression was detected in DAPT-treated cells, while a 1.5-fold increase in Ngn2 expression was observed (Fig. 3A). Similarly, immunostaining showed comparable levels of Olig2 either in the absence or presence of DAPT (Fig. 3B, 3B') while the expression of Ngn2 was increased in the presence of DAPT (Fig. 3C, 3C'). These results suggest that Notch inhibition promoted the differentiation of the pMN-like progenitors toward the motor neuron fate. In DAPT-treated cultures of differentiating pMN-like progenitors, the expression levels of Isl1 and Lhx3 were increased by 3- and 1.7-fold, respectively (Fig. 3D), indicating that inhibition of Notch signaling promoted the differentiation of the pMN-like progenitors toward motor neurons.

Ngn2 synergizes with Isl1 and Lhx3 to activate the expression of Hb9, which is specifically expressed in motor neurons [35–39]. In accordance with the increased expression of Ngn2, Isl1, and Lhx3, a threefold increase in Hb9 expression level was observed in DAPT-treated cells (Fig. 3E).

Immunostaining showed that in the presence of DAPT, Isl1 and Lhx3 expressions were detected in 30% of the differentiating cells compared with 12% and 17%, respectively, in the absence of DAPT (Fig. 3F, 3F', 3G, 3G', 3J). Hb9 expression was detected in 31% of the cells differentiating in the presence of DAPT compared with 20% in its absence (Fig. 3H, 3H', 3I).

The early motor neurons could be further matured into motor neurons by continuous culturing in suspension. Following 4–5 weeks culturing period in the presence of DAPT, the differentiated cells no longer expressed Hb9 while a substantial percent of the cells were stained positive to choline acetyltransferase (ChAT), which is exclusively expressed in motor neurons of the ventral spinal cord (Fig. 3I, 3I').

Moreover, when treatment with DAPT was combined with further lowering the concentrations of RA and PUR (0.25-RPD medium containing 0.25  $\mu$ M RA, 0.125  $\mu$ M PUR, and 1  $\mu$ M DAPT), 40% of the differentiating cells expressed Hb9 compared with 20% in the absence of DAPT (Fig. 3K, 3K'). Therefore, in subsequent experiments, we used the 0.25-RPD medium for differentiation of the pMN-like progenitors. Collectively, these data suggested that inhibition of Notch signaling in differentiating pMN-like progenitors resulted in an increased expression of motor neuron markers.

When the cells were allowed to differentiate in the presence of DAPT without RA and PUR, only 15% of the differentiating cells expressed Hb9 (data not shown). This finding suggested an instructive role of RA and PUR in directing the differentiation of the progenitors toward motor neurons, which was augmented when Notch signaling was inhibited.

### Over-Expression of Hes5 Inhibits the Differentiation of pMN-Like Progenitor Cells Toward Motor Neurons

Previous studies have identified Hes5 as a Notch target in the developing spinal cord, where it represses proneural genes such as Ngn2 [7, 40, 41]. To identify the downstream targets of Hes5 in the differentiating motor neurons, we used a lentiviral vector to over-express Flag-tagged Hes5 (FL-Hes5) in pMN-like progenitors just prior to their differentiation into motor neurons. Upon transduction of pMN-like progenitors with FL-Hes5 vector followed by differentiation in the presence of RA and PUR, an eightfold increase in Hes5 expression level was observed compared with control cells that were

transduced with a vector that did not include Hes5 (Vector, Fig. 4A). As was expected, FL-Hes5 transduced expression was not affected by DAPT (Fig. 4A), indicating a constitutive Hes5 expression in differentiating motor neurons, similar to constitutively active Notch signaling. To test the effect of constitutive Hes5 expression on motor neuron differentiation, we

analyzed the expression levels of motor neuron markers in FL-Hes5 over-expressing cells compared with vector-only expressing cells.

Ngn2 was previously shown to be a direct target for Hes5 repression [41]; accordingly in FL-Hes5 transduced cells, Ngn2 was no longer induced by RA and PUR compared with a 1.9-

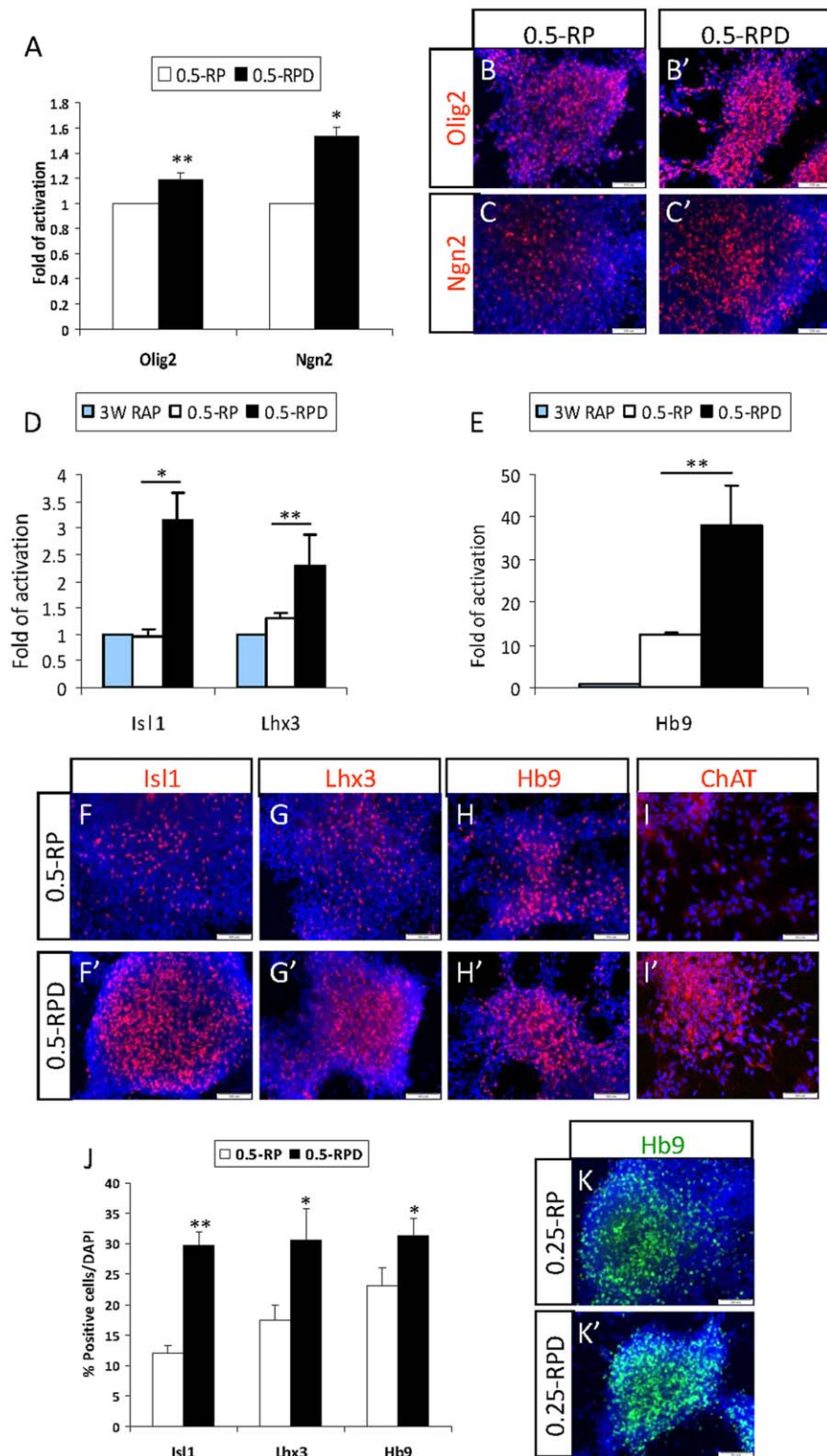


Figure 3.

fold induction in vector-only transduced cells (Fig. 4B, 0.25-RP). Moreover, in FL-Hes5 transduced cells Ngn2 expression was not induced in the presence of DAPT (Fig. 4B, 0.25-RPD), indicating a constitutive repression of Ngn2 expression in the differentiating pMN-like progenitors.

In vector-only transduced cells, RA and PUR induced a fourfold increase in Hb9 expression level, while only a twofold increase was observed in FL-Hes5 transduced cells (Fig. 4C, 0.25-RP). The expression level of Lhx3 in vector-only transduced cells was induced by 7.9-fold in the presence of RA and PUR, while only a twofold increase was observed in FL-Hes5 transduced cells (Fig. 4D, 0.25-RP). These data suggest that constitutively active Notch signaling interferes with RA and PUR to induce differentiation of pMN-like progenitors toward motor neuron fate. In the presence of DAPT, FL-Hes5 over-expression resulted in a 2.5-fold reduction in both Hb9 and Lhx3 expression levels compared with the expression levels in vector-only transduced cells (Fig. 4C, 4D; 0.25-RPD).

In contrast, in the presence of RA and PUR, Isl1 expression was similarly induced in both FL-Hes5 and vector-only transduced cells (1.7-fold) (Fig. 4E, 0.25-RP). In the presence of DAPT comparable expression levels of Isl1 (fourfold) were observed in either FL-Hes5 or vector-only expressing cells (Fig. 4E, 0.25-RPD). These results suggest that Hes5 does not directly regulate Isl1.

In support with the RNA expression data, immunostaining of FL-Hes5 transduced cells for the motor neuron markers revealed a dramatic decrease in differentiation of the pMN-like progenitors into motor neurons. In the absence of DAPT (Fig. 5A–5D', 0.25-RP), as low as 3%–5% of the FL-Hes5 transduced cells were stained positive for Hb9, Isl1, or Lhx3 compared with 10%–15% positive cells in vector-only transduced cells (5I–5K, 0.25-RP). In the presence of DAPT (Fig. 5E–5H', 0.25-RPD), 4%–6% of the FL-Hes5 transduced cells were stained positive compared with 20%–30% positive cells in vector-only transduced cells (Fig. 5I–5K, 0.25-RPD). Coimmunostaining for FL-Hes5 and Hb9 showed that Flag expressing cells did not express Hb9 (Supporting Information Fig. S4). Interestingly, while the level of Isl1 mRNA transcripts was not reduced by FL-Hes5 over expression (Fig. 4E), immunostaining analysis showed reduction in the percentage of Isl1 positive cells (Fig. 5D', 5H', 5K) suggesting post-transcriptional regulation of Isl1 as previously reported [42, 43].

Our data suggest that constitutive expression of FL-Hes5 in differentiating pMN-like progenitors inhibited their differentiation into motor neurons. These results suggest that Notch signaling maintains pMN progenitors and prevents them from differentiation toward postmitotic motor neurons.

### Inhibition of Notch Signaling Enhances Motor Neuron Fate Specified by RA and PUR

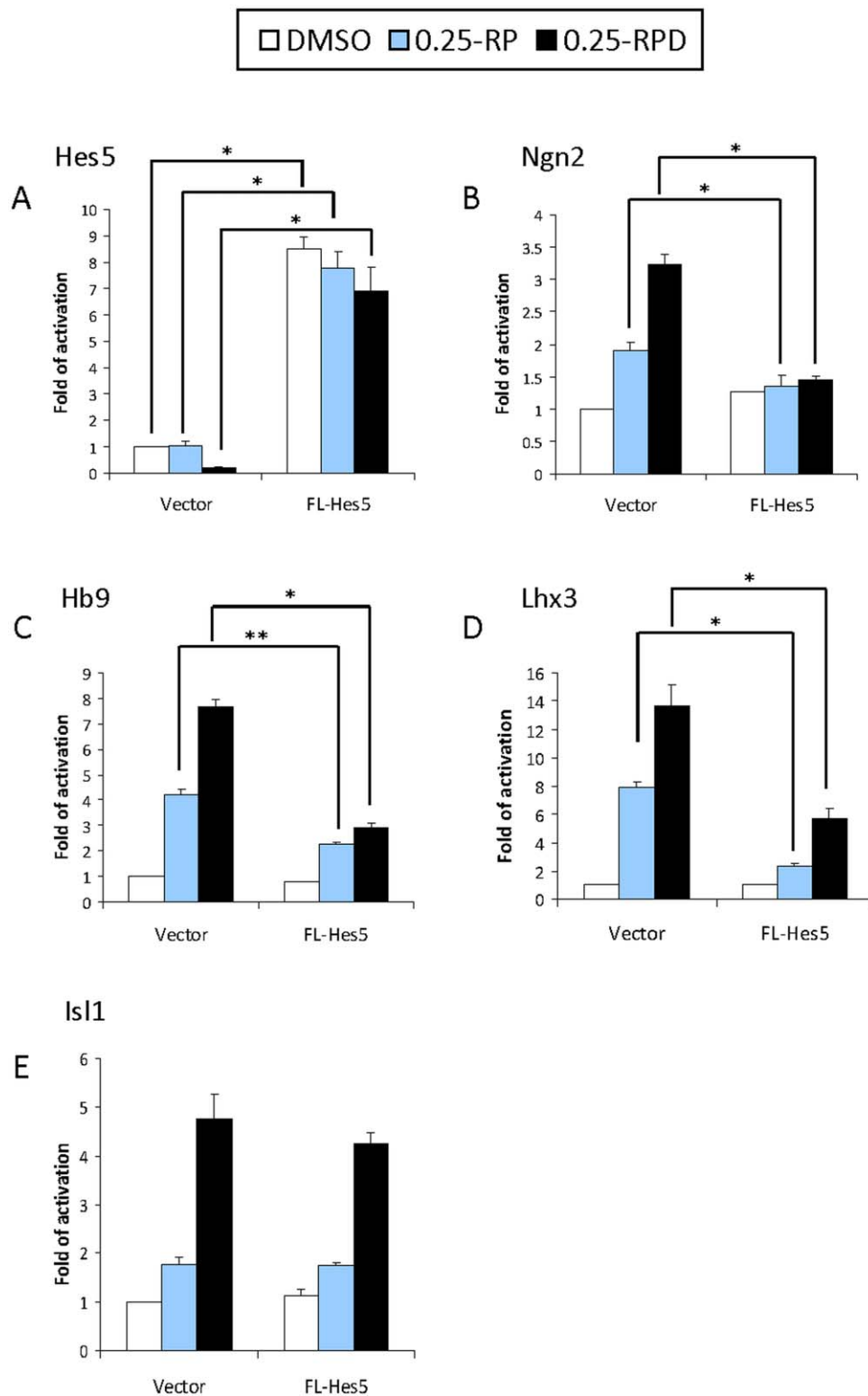
Lhx3 expression is not exclusive to motor neurons but is also characteristic to progenitors in the p2 domain of the developing spinal cord. The p2 progenitors express the Chx10 transcription factor in addition to Lhx3 and give rise to V2 interneurons [39]. To assess whether the pMN-like progenitors differentiate to a mixed population of motor neurons and interneurons, the differentiating cells were costained for Chx10, and either Lhx3 or Hb9. Strikingly, in the presence of RA and PUR, Chx10-expressing cells were not demonstrated in the absence or presence of DAPT (Fig. 6A–6B'), while at the same time cells expressing Lhx3 (Fig. 6A, 6A') or Hb9 (Fig. 6B, 6B') were observed as expected. However, when the cells were allowed to differentiate in the presence of DAPT without RA and PUR, we observed two cells populations: one co-expressed Chx10 and Lhx3 (Fig. 6C) and the other expressed either Hb9 or Chx10 (Fig. 6D), indicating differentiation into a mixed population of both interneurons and motor neurons. In the presence of DMSO vehicle, only a low percentage of the cells expressed Chx10, Lhx3, or Hb9 (Fig. 6C', 6D'). These results suggest that RA and PUR specify the pMN-like progenitors toward the motor neural fate while at the same time they prevent the differentiation toward the interneural fate. Moreover, inhibition of Notch signaling enhances the motor neural-directed differentiation induced by RA and PUR.

## DISCUSSION

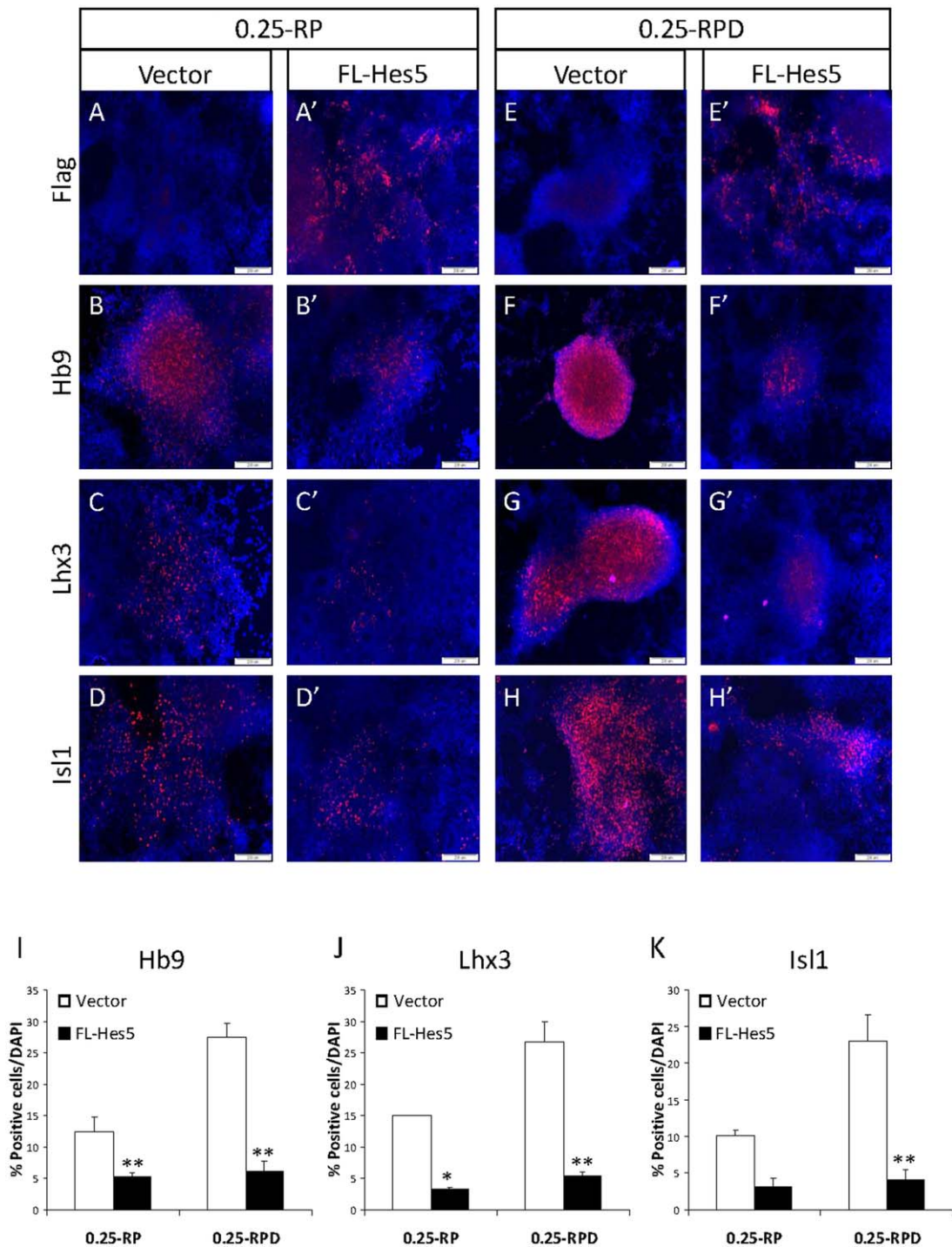
In this study, we show that Notch signaling has a role in motor neuron differentiation of hESCs. We demonstrate that Notch signaling is active during the differentiation of pMN-like progenitors inhibiting their maturation into motor neurons. Inhibition of Notch signaling at the stage of pMN-like progenitors differentiation by DAPT significantly enhances the

**Figure 3.** Inhibition of Notch signaling enhances the differentiation of pMN-like progenitor cells into motor neurons. pMN-like progenitor cells were differentiated for 1 week in the presence of 0.5  $\mu$ M retinoic acid (RA) and 0.25  $\mu$ M purmorphamine (PUR) combined with DMSO vehicle (0.5-RP) or with DAPT (0.5-RPD). **(A)**: QRT-PCR analysis of the expression of Olig2 and Ngn2 genes in pMN-like progenitor cells differentiating in the presence DAPT (0.5-RPD; black bars) relative to the expression levels in progenitor cells differentiating in the absence of DAPT (0.5-RP, white bars). Expression levels in progenitors differentiating without DAPT were set as 1. Fold activation is derived from four experiments. Data are presented as mean + SD. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's *t* test. **(B, C)**: Immunofluorescence analysis of Olig2 (B) and Ngn2 (C) expression in progenitor cells differentiated in the absence (0.5-RP, B, C) or presence of DAPT (0.5-RPD, B', C'). Blue indicates DAPI stained nuclei. Scale bars = 100  $\mu$ m. **(D, E)**: QRT-PCR analysis of the expression of Isl1, Lhx3 (D), and Hb9 (E) genes in progenitor cells differentiated in the presence of RA and PUR combined with DMSO vehicle (0.5-RP; white bars) or with DAPT (0.5-RPD; black bars) relative to the expression in pMN-like progenitor cells (3W RAP; blue bars). Expression level in pMN-like progenitors was set as 1. Fold activation is derived from four experiments. Data are presented as mean + SD. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's *t* test. **(F–H')**: Immunofluorescence images demonstrating the expression of Isl1 (F, F'), Lhx3 (G, G'), and Hb9 (H, H') in cells differentiated in the absence (0.5-RP, F–H) or in the presence of DAPT (0.5-RPD, F'–H'). Blue indicates DAPI stained nuclei. Scale bars = 100  $\mu$ m. **(I, I')**: Immunofluorescence images demonstrating the expression of ChAT in cells differentiating for 5 weeks in the presence of RA and PUR combined with DMSO vehicle (0.5-RP, I) or with DAPT (0.5-RPD, I'). Blue indicates DAPI stained nuclei. Scale bars = 100  $\mu$ m. **(J)**: Quantification of the percentage of cells stained positive for Isl1, Lhx3, and Hb9 following differentiation in the presence of 0.5  $\mu$ M RA and 0.25  $\mu$ M PUR with DMSO vehicle (0.5-RP; white bars) or with DAPT (0.5-RPD; black bars). Data derived from three experiments are presented as mean + SD. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's *t* test. **(K, K')**: Expression of Hb9 in cells differentiated in the presence of 0.25  $\mu$ M RA and 0.125  $\mu$ M PUR with DMSO vehicle (0.25-RP, K) or with DAPT (0.25-RPD, K'). Blue indicates DAPI stained nuclei. Scale bars = 100  $\mu$ m. Abbreviation: DAPI, 4,6-diamidino-2-phenylindole.





**Figure 4.** Over-expression of FL-Hes5 reduces the differentiation of pMN-like progenitor cells into motor neurons. pMN-like progenitor cells infected with FL-Hes5 lentiviral vector (FL-Hes5) or with an empty lentiviral vector (Vector) were differentiated for 1 week in the presence of DMSO vehicle (DMSO) or 0.25  $\mu$ M retinoic acid (RA) and 0.125  $\mu$ M purmorphamine (PUR) combined with DMSO vehicle (0.25-RP) or with DAPT (0.25-RPD). (A–E): QRT-PCR analysis of the expression of Hes5 (A), Ngn2 (B), Hb9 (C), Lhx3 (D), and Isl1 (E) genes in vector-only (Vector) and FL-Hes5 (FL-Hes5) infected progenitor cells differentiating in the presence of DMSO vehicle (DMSO; white bars) or in the presence of RA and PUR combined with DMSO vehicle (0.25-RP; blue bars) or with DAPT (0.25-RPD; black bars). Expression level in vector-only infected cells differentiating in the presence DMSO vehicle (DMSO) was set as 1. Fold activation is derived from four experiments. Data are presented as mean + SEM. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's  $t$  test.

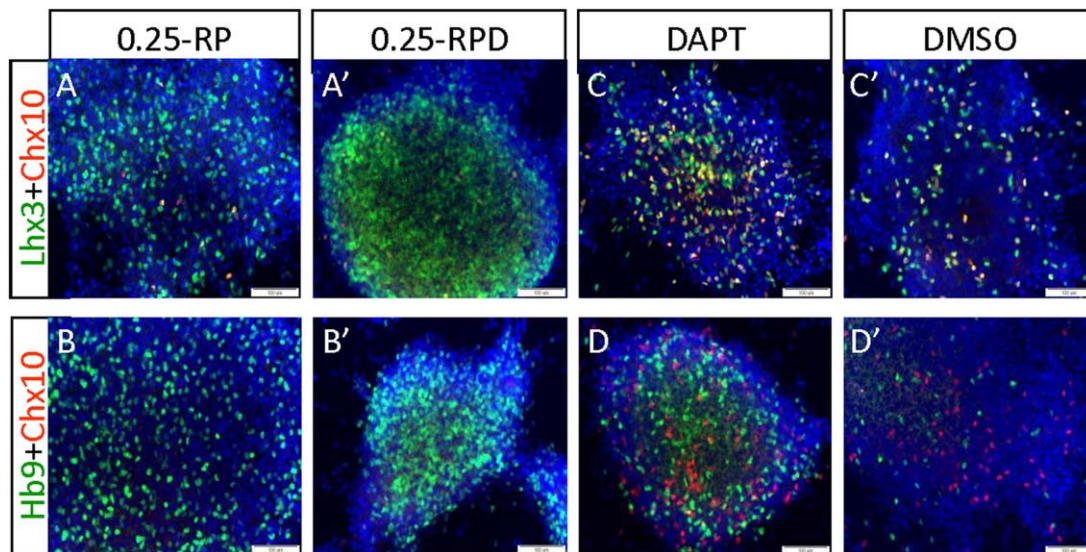


**Figure 5.** Immunostaining showing the expression of FL-Hes5 (A, A' and E, E'), Hb9 (B, B' and F, F'), Lhx3 (C, C' and G, G'), and Isl1 (D, D' and H, H') in vector-only (Vector, A–H) and FL-Hes5 (FL-Hes5, A'–H') infected progenitor cells, differentiating in the presence of retinoic acid (RA) and purmorphamine (PUR) combined with DMSO vehicle (0.25-RP, A–D') or with DAPT (0.25-RPD, E–H'). Blue indicates 4,6-diamidino-2-phenylindole stained nuclei. Scale bars = 200  $\mu$ m. (I–K): Quantification of the percentage of cells stained positive for Hb9 (I), Lhx3 (J), and Isl1 (K) in vector-only (white bars) and FL-Hes5 (black bars) infected progenitor cells following differentiation in the presence of RA and PUR combined with DMSO (0.25-RP) or with DAPT (0.25-RPD). Data derived from three experiments are presented as mean + SD. \*,  $p < .01$ ; \*\*,  $p < .05$  by Student's  $t$  test.

emergence of motor neurons, while forced expression of the Notch effector Hes5 inhibits motor neuron differentiation. Our data indicate that RA and Shh specify differentiating pMN-like progenitors to become motor neurons, and that inhibition of Notch signaling provides a permissive signal for

the differentiation of the specified progenitors toward motor neuron fate.

In line with previous publications [2, 44] we show that Notch signaling is activated upon early neural differentiation of hESCs as evidenced by induction of expression of its



**Figure 6.** Retinoic acid (RA) and pumorphamine (PUR) specify the differentiation of pMN-like progenitor cells toward motor neurons rather than interneurons. Immunostaining showing the expression of Chx10 (red) and Lhx3 (green) (A, A' and C, C') or Chx10 (red) and Hb9 (green) (B, B' and D, D') in pMN-like progenitor cells differentiating for 1 week in the presence of RA and PUR combined with DMSO vehicle (0.25-RP, A, B) or with DAPT (0.25-RPD, A', B') or in the presence of DAPT only (DAPT, C, D) or DMSO vehicle (DMSO, C', D'). Blue indicates 4,6-diamidino-2-phenylindole stained nuclei. Scale bars = 100  $\mu$ m.

downstream effector, Hes5, concomitant with neuralization. Intriguingly, we demonstrate that the expression level of Hes5 is further increased following specification of early neural progenitors into pMN-like progenitors by RA and the Shh agonist PUR. Moreover, it remains expressed at comparable levels in cultures of pMN-like progenitors under conditions favoring differentiation into motor neurons.

We provide evidence that Notch signaling inhibits the differentiation of the pMN-like progenitors toward motor neurons. Interfering with Notch signaling during the stage of their differentiation by DAPT leads to a marked decrease in Hes5 expression levels and enhances differentiation to motor neurons. Conversely, over-expression of Hes5 in differentiating pMN-like progenitors largely inhibits motor neuron differentiation.

The role of Notch signaling in the pMN domain of the developing spinal cord was previously studied in various animal models. Notch signaling was shown to preserve the undifferentiated state of pMN progenitors during the period of motor neuron generation for later differentiation to oligodendrocytes [16, 17]. Here, we used hESCs to efficiently generate pMN-like progenitors and study, for the first time, the role of Notch signaling during their differentiation into motor neurons in a humanized model system *in vitro*. We found that over-expression of Hes5 in the differentiating progenitors led to reduced expression levels of Ngn2, while the inhibition of Notch signaling in these progenitors resulted in an increase in the expression of Ngn2. These observations are consistent with a previous report of Ngn2 as a direct target for repression by Notch signaling [41]. Our data suggest that the inhibition of Notch signaling upregulates the expression of Ngn2 in differentiating pMN-like progenitors and promotes their differentiation into motor neurons.

Previously, it has been reported that inhibition of Notch signaling in mouse embryoid bodies (EBs) derived from *ptc1* null mutated ESCs, in which Shh is constitutively active, resulted in precocious loss of ventral neuronal precursors to

enhanced neuronal differentiation [45]. However, in these EBs, the inhibition of Notch signaling resulted in reduced expression of Olig2 and Nkx2.2, while in our pMN-like progenitors DAPT had no significant effect on Olig2 expression but rather increased the expression of Ngn2. Moreover, treating *ptc1* null EBs with Shh and DAPT increased *Isl1/2* expression but had no effect on Hb9 and Lhx3 expression. Hence, while precocious neuronal differentiation was observed in the absence of Notch signaling, motor neuron differentiation was not specifically augmented as observed in our results. Species-specific variation or differences in the methodology used may explain the dissimilar observations.

In a previous study, it was reported that in conditional Notch1 receptor null mice, more V2 interneurons are generated at the expense of earlier born motor neurons [7]. Our data indicate that the inhibition of Notch signaling *per se in vitro* is not sufficient to promote differentiation into a specific neuronal subtype. Inhibition of Notch signaling in the absence of RA and PUR resulted in differentiation of the pMN-like progenitors to a mixed population of motor neurons expressing Hb9 and V2 interneurons expressing Chx10 with no significantly enhanced differentiation to motor neurons or to V2 interneurons. This observation indicates that inhibition of Notch signaling provides pMN-like progenitors with a permissive rather than instructive signal for neuronal differentiation, while RA and Shh specify the progenitors toward motor neuron fate and block the differentiation into V2 interneurons. Furthermore, over-expression of Hes5 blocked the induction of Ngn2 expression and reduced the activation of motor neuron-specific genes by RA and PUR. Thus, it is possible that Notch signaling inhibits the differentiation of the pMN-like progenitors by interfering with RA and PUR in specification of the motor neuron fate.

We demonstrated that in addition to downregulating Ngn2 expression, over-expression of Hes5 in the differentiating pMN-like progenitors also reduced the expression levels of

Lhx3 and Hb9 genes. Previously, it was shown that Ngn2 is repressed directly by Hes5 [41]. However, whether Hes5 regulates the expression of Hb9 or Lhx3 through direct binding to their regulatory regions is currently unknown.

Previously it was shown that the MN<sup>E</sup> enhancer, which binds both bHLH proteins (Ngn2 and NeuroM) and Lim-HD proteins (Isl1 and Lhx3), regulates the expression of Hb9 gene [35, 36]. The bHLH proteins bind the two E-box consensus elements (CANNTG) included in MN<sup>E</sup> and synergize with the Lim-HD proteins to activate Hb9 expression. Thus, it is possible that Hes5 affects the expression of Hb9 indirectly by downregulating the expression of Ngn2.

Alternatively, it is possible that Hes5 targets the Hb9 enhancer directly. In support of this assumption, we identified within the MN<sup>E</sup> sequence an N-box consensus element (CACNAG) which was reported as a specific binding site for Hes5 [46]. Future analysis may indicate whether Hes5 binds directly to the MN<sup>E</sup> enhancer and regulates Hb9 expression. Recently it was reported that the expression of human Lhx3 gene is regulated by multiple enhancers, but no N-box element was identified in these regulatory elements, arguing against direct binding of Lhx3 regulatory regions by Hes5 [47].

The promotion of motor neuron differentiation by inhibition of Notch signaling may be used to enhance the yield of motor neuron differentiation protocols. Based on the inductive effect of RA and Shh signaling, our yield of motor neurons was 20%. Inhibition of Notch signaling doubled the yield of motor neurons. Inhibition of Notch signaling and improving the efficiency of motor neuron generation in vitro may be highly valuable for disease modeling, and high throughput screening assays for molecules that may have therapeutic value in motor neuron diseases [48].

## CONCLUSIONS

In conclusion, our data provide evidence for Notch signaling as an important mechanism in controlling the differentiation

of pMN progenitor cells into motor neurons. By upregulating the expression levels of Hes5, Notch signaling reduces the expression of Ngn2 as well as Hb9 and Lhx3, which are induced by RA and PUR and are required for directing the pMN progenitor cells toward motor neuron fate. The results argue for a model in which Notch signaling inhibits the differentiation of pMN progenitor cells by blocking the capacity of RA and PUR to induce the expression of key transcription factors that are required for specification and differentiation of the progenitor cells to motor neurons.

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## AUTHOR CONTRIBUTIONS

E.B.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; E.F.: financial support, manuscript writing, and final approval of manuscript; B.E.F.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

Benjamin Reubinoff is a founder, holds shares and is the Chief Scientific Officer (CSO) of CellCure Neuroscience Ltd. The focus of the company is the development of human embryonic stem cells for transplantation therapy in neurological and retinal degeneration disorders. The company does not fund the study presented in this manuscript and currently the company has no interest in the results of the study.

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