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RESEARCH ARTICLE

Expression of *Neurogenin 1* in Mouse Embryonic Stem Cells Directs the Differentiation of Neuronal Precursors and Identifies Unique Patterns of Down-stream Gene Expression

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Background: Delineating the cascades of growth and transcription factor expression that shape the developing nervous system will improve our understanding of its molecular histogenesis and suggest strategies for cell replacement therapies. In the current investigation, we examined the ability of the proneural gene, *Neurogenin1* (*Neurog1*; *also Ngn1*, *Neurod3*), to drive differentiation of pluripotent embryonic stem cells (ESC). Results: Transient expression of *Neurog1* in ESC was sufficient to initiate neuronal differentiation, and produced neuronal subtypes reflecting its expression pattern *in vivo*. To begin to address the molecular mechanisms involved, we used microarray analysis to identify potential down-stream targets of *Neurog1* expressed at sequential stages of neuronal differentiation. Conclusions: ESC expressing *Neurogenin1* begin to withdraw from cycle and form precursors that differentiate exclusively into neurons. This work identifies unique patterns of gene expression following expression of *Neurog1*, including genes and signaling pathways involved in process outgrowth and cell migration, regional differentiation of the nervous system, and cell cycle. *Developmental Dynamics 242:230–253, 2013*. © 2013 Wiley Periodicals, Inc.

Key words: bHLH; BMP4; ES cell; FGF; microarray; neural crest cell; Neurod3; neural induction; neuronal differentiation; Ngn1; noggin; sonic hedgehog; Tet-on

Key Findings:

- Inducible expression of Neurogenin1 in ESC provides a novel model of neurogenesis.
- Pulsed expression of Neurogenin1 produces PNS and CNS neuronal sub-types.
- Neurog1 promotes differentiation of neuronal precursors that exclusively form neurons.
- This is the first description of the gene expression cascade downstream of *Neurog1*.

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INTRODUCTION

Vertebrate neurogenesis is a stepwise process in which the primitive ectoderm is first induced to form the neural ectoderm, which folds forming the neural tube. The neural tube is then patterned along the anterior–posterior (AP) and dorsal–ventral (DV) axes by morphogens that coordinate the expression of transcription factors that confer positional identity and/or promote neural differentiation. Proneural basic helix–loop–helix (bHLH) proteins comprise one such class of transcription factor essential during early neurogenesis (review, Bertrand et al., 2002). In *Drosophila*, the bHLH factors *achaete-scute* and *atonal* act

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as generic promoters of neuronal differentiation and neuronal subtype specification (Chien et al., 1996; Jarman and Ahmed, 1998). Vertebrate *atonal* homologs such as *Neurogenin 1* (*Neurog1*), *Neurogenin 2* (*Neurog2*; Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996) and *achaete-scute* homologs such as *Atoh1* (*Mash1*; Johnson et al., 1990) and *Ascl1* (*Math1*; Guillemot and Joyner, 1993) also promote neuronal differentiation both in vivo (Turner and Weintraub, 1994; Lee et al., 1995; Ma et al., 1996; Chung et al., 2002; Kim et al., 2004) and in vitro (Lo et al., 1998; Farah et al., 2000; Sun et al., 2001; Kanda et al., 2004; Satoh et al., 2010).

The expression of mammalian *achaete-scute* and *atonal* homologues within specific—largely nonoverlapping—regions of the developing central and peripheral nervous system (CNS and PNS) suggests roles in neuronal subtype specification that have been confirmed by loss- and gain-of-function studies. For example, *Neurog1* is expressed in the dorsal telecephalon where it appears to promote glutaminergic neuronal fates, *Atoh1* is expressed in the ventral telencephalon specifying γ-aminobutyric acid (GABA)ergic neurons (Fode et al., 2000; Parras et al., 2002; Kim et al., 2011), while *Neurog1* is expressed in the caudal ventricular zone of the rhombic lip, where it defines multiple GABAergic lineages (Dalgard et al., 2011). In the spinal cord, *Ascl1* is expressed in a dorsal stripe near the roof plate (Gowan et al., 2001), *Neurog1* is expressed in the ventral half and in a small region just below the roof plate, whereas *Atoh1* is found in the intervening domain (Sommer et al., 1996; Ma et al., 1997), where these transcription factors are thought to regulate neuronal phenotype by cross-inhibition (Briscoe et al., 2000; Gowan et al., 2001; Helms et al., 2005). Loss-of-function studies have shown that *Neurog1* is required for the development of dI2 dorsal spinal neurons, trigeminal and otic cranial sensory ganglia, and TrkA neurons of dorsal root ganglia (DRG) (Ma et al., 1997; Fode et al., 1998; Gowan et al., 2001). Gain-of-function studies have demonstrated that overexpression of *Neurog1* biases the migration of neural crest stem cells toward dorsal root sensory ganglia in vivo (Perez et al., 1999), whereas forced expression of *Neurog1* in dorsal neural tube progenitors and neural crest cells promotes their differentiation into sensory lineages (Lo et al., 2002). These data indicate that *Neurog1* is required for the development of sensory neuronal lineages in both the PNS and CNS; however, it is not clear whether *Neurog1* is itself sufficient to induce these lineages because the gain-of-function studies were conducted either in the embryo or in neural progenitors where the effects of morphogens and other instructive signals cannot be separated. While misexpression of proneural genes can produce ectopic neurogenesis in a variety of species (Quan and Hassan, 2005), relatively little is known regarding the molecular mechanisms involved or down-stream gene expression following bHLH gene expression. Because bHLH transcription factor expression is strongly affected by spatial and temporal context (Powell and Jarman, 2008), we used a gain-of-function approach in pluripotent embryonic stem cells (ESC) to determine the role of *Neurog1* in cell fate specification. ESC may be a particularly informative starting material because they have a bivalent chromatin structure with promoters poised for both lineage differentiation as well as for self-renewal (e.g., Boyer et al., 2006). Lineage specifying genes such as bHLH and paired-box family members may therefore control differentiation programs by directly affecting transcription and by narrowing differentiation choices by controlling chromatin.

The current investigation identifies potential down-stream targets of *Neurog1* including genes involved in cell cycle, cell migration and process outgrowth, and provides a source of neuronal precursor cells that remain sensitive to patterning molecules. Consistent with observations that *Neurog1* is present in cells about to withdraw from cycle and differentiate into layer-specific neurons (Kim et al., 2011), forced expression of *Neurog1* in ESC alters their cell cycle characteristics and is sufficient to initiate neuronal differentiation in the absence of other inducing factors. In fact, *Neurog1* expression was sufficient to overcome the inhibitory effects of LIF and serum proteins on ESC differentiation (Williams et al., 1988). In addition, *Neurog1* expression was also sufficient to generate both CNS and PNS neuronal subtypes typical of those dependent on *Neurog1* in vivo. Yet, the positional identity and neuronal differentiation potential of induced cells could be influenced by early acting patterning factors, suggesting that *Neurog1* promotes differentiation of neuronal precursors that can be influenced by the local microenvironment to subsequent regional and/or subtype-specific differentiation.

RESULTS

Inducible Expression of *Neurog1* in ESC

In the current investigation, we used the Ainv15 ESC line (Kyba et al., 2002) that expresses a "Tet-on" reverse tetracycline transactivator (rtTA) from the constitutively active ROSA26 locus, and a tet-inducible element with a LoxP targeting site and a truncated neomycin resistance cassette placed upstream of the HPRT locus. Site-specific integration of the targeting vector carrying cDNA for a gene of interest places the transgene downstream of the tet-inducible element and confers neomycin resistance, thereby allowing efficient selection of targeted cell lines. Once selected, cells are maintained in neomycin to ensure that the transgene does not undergo methylation or rearrangement, although this is unlikely at the HPRT locus (Wutz et al., 2002).

We targeted *Neurog1* to the inducible locus and obtained several G418 resistant colonies which were expanded to cell lines. Based on the reliability of this inducible system (Kyba et al., 2002; M. Kyba and G. Keller, personal communication) and our own analyses in which we observed no differences between the ESC lines in their differentiation capacity or response to the tetracycline analog doxycycline (data not shown), one line (N7) was selected for most subsequent experiments. As indicated in Figure 1A, robust *Neurog1* expression in N7 cells was induced within 12 hr of addition of

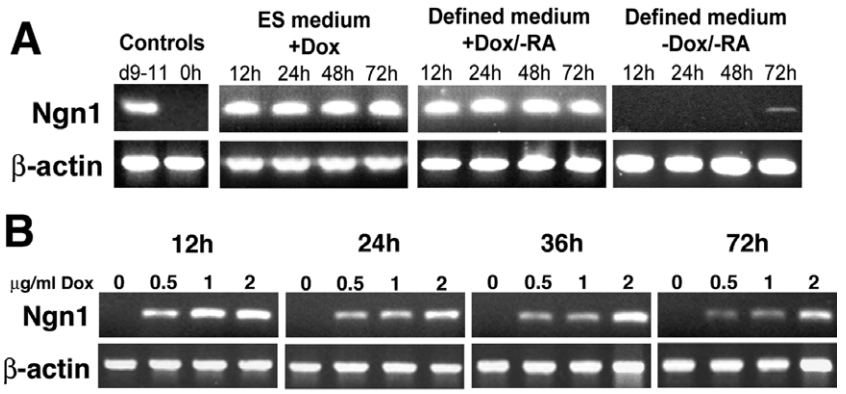


Fig. 1. Characterization of dox-inducible *Neurog1* embryonic stem cells (ESC). **A:** Cells cultured in either complete ES medium or in defined neural medium with 1 μg/ml doxycycline express *Neurog1* by reverse transcriptase-polymerase chain reaction (RT-PCR) within 12 hr of treatment, which was maintained 72 hr in culture. Cells cultured in defined neural medium without the addition of dox showed a very slight up-regulation of *Neurog1* after 72 hr. Controls include pooled cDNA from day 9–11 mouse embryos (d9–11) and N7 cells cultured in ES medium without dox (0 hr). **B:** N7 cells grown in serum free minimal medium (DMEM with 5% knockout serum replacement) with 0, 0.5, 1, or 2 μg/ml doxycycline express *Neurog1* in a dose-dependent manner. After 72 hr, cells not exposed to doxycycline show no expression of *Neurog1*.

doxycycline (dox), to monolayer cultures grown in either complete ES medium, which inhibits spontaneous differentiation (Williams et al., 1988) or in a defined neural medium, which supports neural differentiation of ESC in monolayer cultures. We did not observe any evidence of transgene silencing as *Neurog1* expression was maintained throughout the culture period. N7 cells cultured in complete (ES) medium without dox did not express *Neurog1* at 12, 24, 48, or 72 hr (Fig. 1A). N7 cells cultured in defined neural medium without dox were intolerant of these culture conditions often undergoing cell death by 72 hr (not shown). However, in cells that survived to 72 hr, it was possible to detect slight expression of *Neurog1* (Fig. 1A), likely due to stochastic neural differentiation and activation of endogenous *Neurog1* elicited by culture in these conditions. When cultured in a different serum-free medium containing 5% knock-out serum replacer (Fig. 1B), we observed no expression of *Neurog1* in the absence of dox, but, in the dox-treated groups, we could consistently induce expression of the *Neurog1* transgene in a dose-dependent manner. Overall, these data suggest that the N7 cell line provides a consistent and tightly regulatable means to assess the sequelae of *Neurog1* expression in ESC.

Expression of *Neurog1* in ESC Induces Neuronal Differentiation

Within 24 hr of inducing the *Neurog1* transgene, N7 cells exhibited overt neuronal morphologies including round, phase-bright somata and one or more long cytoplasmic processes when viewed with phase contrast microscopy. Cells cultured in complete (ES) medium or in defined neural medium (DM) with dox were fixed after 12, 24, 48, and 72 hr in culture followed by immunohistochemical localization of Neurog1 and neuronal tubulin (TuJ1 antibody). Virtually all of the cells cultured in the presence of dox were Neurog1$^+$ at each time point surveyed (Fig. 2), whereas those cultured without dox were largely Neurog1$^-$ (not shown), confirming the integrity of the tet-inducible locus. TuJ1 immunostaining demonstrated weakly reactive cells in both culture conditions as early as 12 hr following transgene induction, indicating the initiation of neuronal differentiation (Fig. 2A,B,I,J), although in defined medium there were occasionally cells that exhibited a more mature neuronal phenotype (Fig. 2A,B). In general, neuronal differentiation was robust in either culture condition after 72 hr of transgene induction, but differentiation progressed more rapidly in defined medium. After 72 hr, cell density was also considerably greater in cultures grown in complete medium. Overall, forced expression of *Neurog1* was sufficient to induce widespread neuronal differentiation.

Neurog1 Expression Reduces Proliferation and Increases G1 Phase of the Cell Cycle

Neurog1 has previously been reported to promote cell cycle exit (Farah et al., 2000), to increase G1/G0 and decrease S phase (Piao et al., 2012), but the kinetics and target genes are not known. To examine this directly, we first determined the growth characteristics of control Ainv15 and N7 cells in the presence and absence of doxycycline at 24-hr intervals. There was no significant difference in cell number between control Ainv15 ESC exposed to doxycycline and N7 cells grown without dox at any time point (Fig. 3A). Although there was no significant change in cell number at 24 hr of culture, by 48 and 72 hr of transgene induction, there was a significant decrease in the number of *Neurog1*-expressing N7 cells compared with uninduced N7 or control Ainv15 ESC ($P \leq 0.001$, Student's *t*-test).

To determine where in the cell cycle *Neurog1* expression affects proliferation, we cultured control Ainv15 and N7 cells±dox for 72 hr, labeled the cells with propidium iodide and used fluorescence activated cell sorting (FACS) to analyze cell cycle characteristics. Consistent with the proliferation data, there was little difference in the cycle characteristics of control Ainv15 ESC grown in the presence of doxycycline and N7 cells grown without dox: 36.8 and 38.1% of the cells were in G1, 50.8 and 50% in S, and 12.4 vs. 11.9% in G2/M in control+dox, and N7−dox cells, respectively (Fig. 3B). Induction of *Neurog1* expression had a significant effect on the number of cells in G1 phase; with 66.2% in G1, 22.9% in S, and 10.9% in G2/M. Cell death was similar in N7 ESC exposed to doxycycline (8.8±2.7%) and N7 not exposed to dox (8.2±0.5%), but only 4.0±0.8% in control Ainv15 ESC grown with doxycycline. These data demonstrate that induction of *Neurog1* expression in

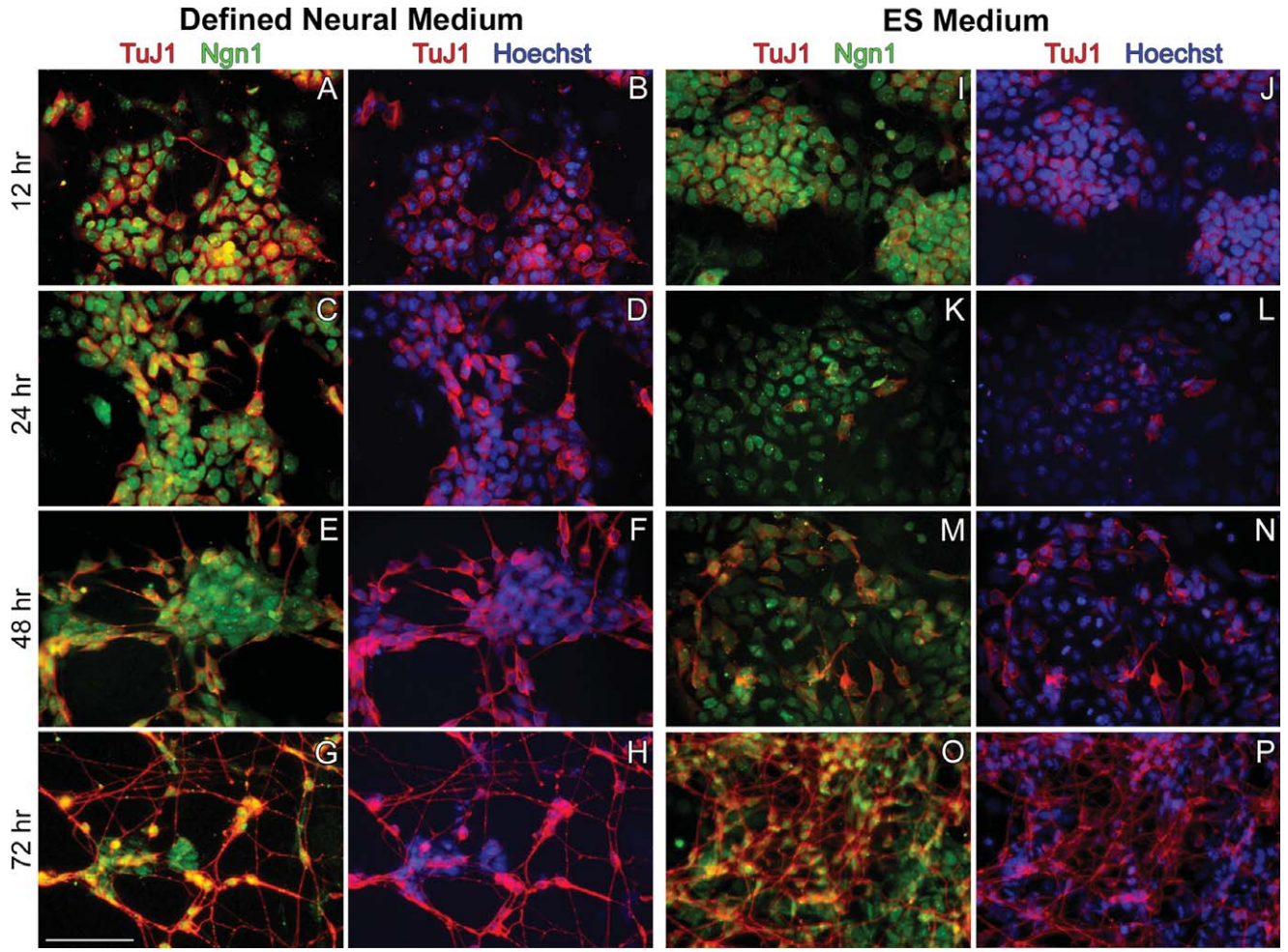


Fig. 2. Expression of *Neurog1* induces widespread neuronal differentiation of embryonic stem cells (ESC). **A–P:** Cells were cultured in defined neural medium (A–H) or in complete ES maintenance medium (I–P) supplemented with 1 μg/ml doxycycline. A,I: In both media, as early as 12 hr after dox induction, ESC expressed *Neurog1* (green). B,J: Expression of neuronal tubulin (TuJ1, red) illustrates the initiation of neuronal differentiation within 12 hr of dox treatment in both culture conditions. M,N: Cells grown in defined neural medium demonstrate overt neuronal differentiation (evidenced by neurite extension) as early as 12 hr after treatment whereas cells cultured in ES Medium do not exhibit morphological characteristics of mature neurons until 48 hr of treatment with doxycycline. C,D,K,L: These differences were maintained at 48 hr. By 72 hr, neuronal differentiation was extensive in both culture conditions. G,H,O,P: Cells grown in defined neural medium were highly enriched in neurons (G,H), whereas cultures grown in complete medium show the persistence of non-neuronal cells (O,P). Scale bar=100 μm.

ES cells slows proliferation by significantly increasing G1 and decreasing the proportion of cells in the S phase of the mitotic cycle.

Treatment With Retinoic Acid Posteriorizes Induced N7 Cells

To determine the anterior–posterior characteristics of the differentiating cells, and to determine their responsiveness to patterning factors, cells were grown in defined neural medium with and without 1 μm retinoic acid (RA). Expression of *Otx2*, a forebrain marker (Mallamaci et al., 1996), and *Hoxc6*, expressed in the rostral cervical spinal cord, were examined. In both culture media, cells expressed *Otx2* but not *Hoxc6* at all time points assayed, indicative of a forebrain phenotype (Fig. 4A). When N7 cells were cultured for 3 days in defined medium supplemented with 1 μM RA and dox, there was a significant reduction in *Otx2* expression and concomitant up-regulation of *Hoxc6* (Fig. 4A). Like previous studies of ESC (Wichterle et al., 2002), these findings suggest that N7 cells neuralized by forced *Neurog1* expression have an initial anterior (forebrain) phenotype that can be posteriorized by exposure to RA.

Expression of Dorsal and Ventral Markers Reflects *Neurog1* Expression In Vivo

Molecular markers of dorsal–ventral identity in the posterior neural tube have been well-characterized (reviewed in Jessell, 2000; Helms and Johnson, 2003; Helms et al., 2005; Fig. 4B); *Neurog1* is expressed in the ventral half and near the roof plate of the developing posterior neural tube (Sommer et al., 1996; Ma et al., 1997, Fig. 4B). We therefore sought to determine if induction of the *Neurog1* transgene could produce a similarly mixed population of

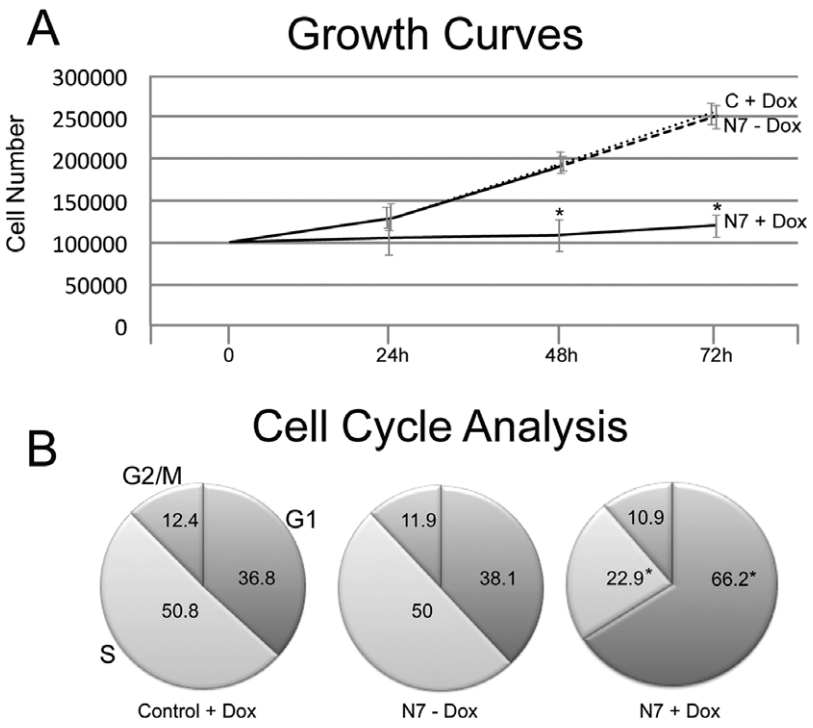


Fig. 3. Expression of *Neurogenin1* decreases proliferation and alters cell cycle characteristics. **A:** At 24 hr, there was no significant effect of transgene expression on cell number, but at 48 and 72 hr there were significantly fewer N7 cells grown with Dox, compared with either N7 cells alone (N7−Dox), or control Ainv15 cells with Dox (C+Dox). Means represent cell number from four biological replicates, 12 wells each. *$P \leq 0.001$, Students *t*-test. **B:** N7 (N7−Dox) and control Ainv15 (Control+Dox) cells were grown for 72 hr±Doxycycline, then subjected to cell cycle analysis. Induction of *Neurog1* expression (N7+Dox) significantly increased G1 and decreased S phase compared with cells absent transgene induction. *$P \leq 0.001$, Student's *t*-test.

dorsal and ventral subtypes following treatment with retinoic acid. We examined induced cell cultures grown in ESC medium, in which an indeterminate amount of RA was likely present in the fetal bovine serum constituent (Napoli, 1986), and in defined neural medium supplemented with 1 μM RA. When assayed by reverse transcriptase-polymerase chain reaction (RT-PCR), induced cultures expressed markers associated with the dorsal (*Pax3*, *Pax7*), ventral (*Nkx2.2*, *Nkx6.1*), and middle (*Dbx2*) thirds of the developing neural tube (Fig. 4C), which generally correspond to regions of *Neurog1* expression in vivo. Differences in the timing of expression were observed between the proteins assayed; e.g., *Dbx2* expression was evident within 12 hr of inducing the *Neurog1* transgene, although it was not expressed by undifferentiated ESC, while robust *Pax7* expression was not observed until 48 hr after induction. Of interest, in subsequent experiments, Pax7 and Nkx6.1 were rarely co-expressed (Fig. 7), indicating the presence of distinct dorsal and ventral subtypes. Thus, forced expression of *Neurog1* in ESC produces a population of precursors that approximately recapitulates the in vivo pattern of *Neurog1* expression.

Forced Expression of *Neurog1* Produces CNS and PNS Neurons

The distribution of CNS and PNS phenotypes was determined after 5 days of culture with and without the addition of RA. Cells were grown in defined medium plus dox to induce the *Neurog1* transgene for 72 hr then switched to a medium supplemented with 5% knockout serum replacer (SRM5) without dox for 48 hr, as these conditions increased cell survival. After 5 days, there was extensive neuronal differentiation as evidenced by TuJ1 immunoreactivity (Fig. 5A); a subset of these cells (Fig. 5B) was also positive for peripherin, with a Tuj1+:peripherin+ ratio of approximately 21:1 (Table 1). To obtain quantitative data, we scored only peripherin+ somata, so these data likely underestimate the total, as there were many peripherin+ fibers whose cell bodies were outside the field of view. Some neurons expressed Islet1 (Fig. 5C), and, invariably, these cells were also Brn3a+ (Fig. 5D), an expression pattern typical of sensory ganglia (SG) of the PNS as well as of dI3 dorsal interneurons in the CNS (Gowan et al., 2001). There were also many Brn3a+/Isl1− cells (Fig. 5C,D); an expression pattern that characterizes dI-1, 2, or 5 interneurons in the CNS (reviewed in Helms and Johnson, 2003). Nearly all (approximately 97%) of the peripherin$^+$ cells were also Brn3a$^+$ and had long, well-developed processes and unipolar or bipolar morphologies (Fig. 5E,F)—a pattern most consistent with a SG neuronal phenotype. Of interest, there was little statistically significant difference in marker expression in groups exposed to RA vs. those not exposed to RA, although the overall number of neurons present as evidenced by TuJ1 immunoreactivity was slightly higher in the RA-treated cultures as was the number of peripherin+ neurons (Table 1).

Induced Cells Respond to Dorsal–Ventral Patterning Factors

Because forced expression of *Neurog1* in ESC is sufficient to promote both dorsal and ventral neural phenotypes, we wished to determine if the resulting neuronal cells could be patterned by exogenous factors. We cultured N7 cells in defined medium with dox and RA for the first 3 days and then changed the culture media to a serum replacement medium (SRM) with RA but not dox, to enhance cell survival. To test the effects of exogenous factors, media were supplemented with Sonic hedgehog (Shh), bone morphogenetic protein-4 (BMP4), or Noggin (Nog) recombinant proteins from the beginning of each experiment. RT-PCR analysis indicated that Shh

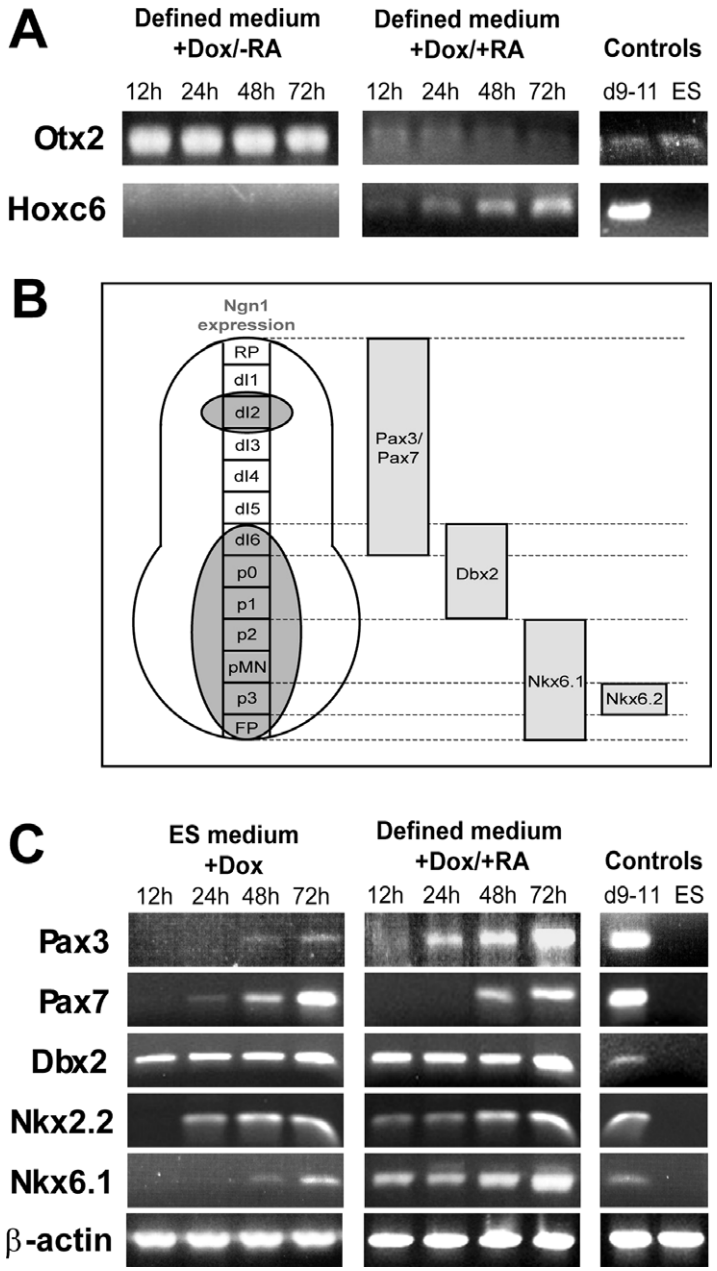


Fig. 4. Expression of dorsal and ventral markers reflects *Neurog1* expression in vivo. **A:** Cells induced in the absence of retinoic acid (RA) demonstrate an anterior neural phenotype (expression of *Otx2*), whereas a marker of spinal cord (*Hoxc6*) was not expressed. Cells treated with 1 μM RA show diminished *Otx2* expression and increased expression of *Hoxc6* indicating that the anterior–posterior identity of the neuronal precursors can be modulated by treatment with RA. **B:** *Neurog1* is expressed in the ventral half of the developing neural tube, in the roof plate and neural crest-associated structures such as dorsal root ganglia. *Pax3* and *Pax7* are expressed in the dorsal half of the cord, *Dbx2* in the middle third, *Nkx6.1* and *Nkx2.2* in the ventral spinal cord. **C:** With differentiation in either complete ES medium (in which some RA is likely present in the serum), or in defined neural medium supplemented with 1 μM RA, expression of dorsal (*Pax3* and *Pax7*), intermediate (*Dbx2*), and ventral (*Nkx2.2* and *6.1*) markers were observed in RT-PCR.

treatment induced expression of the ventral marker, *Nkx6.1*, and suppressed expression of the dorsal marker, *Pax7* (Fig. 6A). Exposure of cells to either high (25 nM) or low (2.5 nM) concentrations of Shh, resulted in a dose-dependent expression of ventral markers. On day 1, cultures grown in either high or low levels of Shh expressed higher levels of *Nkx6.1* compared with those without Shh. At day 3, *Nkx6.1* expression levels were similar between Shh-treated and untreated cultures, but by day 5, cells grown in the high Shh condition expressed higher levels of *Nkx6.1*. Expression of the intermediate dorsal–ventral marker, *Dbx2*, was reduced by treatment with Shh in a dose-dependent manner. Treatment with Shh also resulted in the expression of Islet1, which is associated with motor neurons in the ventral spinal cord, and of nestin, expressed by neural ectoderm and neural stem cells.

Treatment with BMP4 (5 nM) drastically reduced CNS neural differentiation as evidenced by down-regulation of all three CNS neural progenitor markers (*Pax7*, *Dbx2*, and *Nkx6.1*) (Fig. 6A). RT-PCR analysis confirmed expression in BMP4 exposed cultures of *GATA-4*, *Brachyury*, and *Claudin6*, markers of endoderm, mesoderm, and epidermal ectoderm respectively (not shown). BMP4-treated cultures also showed a marked increase in the expression of *Snail*, a marker of EMT and early neural crest cells and of *peripherin*, expressed in the PNS and motor neurons (Fig. 6A), although we did not observe many overtly neuronal cells in these cultures. Taken together, these data support the recognized role of BMPs as neural antagonists in the early gastrula stage embryo as well as during the early stages of mouse ESC differentiation. However, the expression of neural crest and PNS markers suggests that, in the context of forced *Neurog1* expression, BMPs can act instructively to promote neural crest and PNS phenotypes (e.g., Aihara et al., 2010).

To address the possibility that endogenous BMPs might influence the neuronal differentiation observed in our cultures, we treated cell cultures with low (2 nM) or high (5 nM) levels of the BMP antagonist, Noggin (Nog), in combination with low or high Shh treatment. Consistent with previous observations, Shh-treated cultures expressed low levels of the dorsal marker, *Pax7* (Fig. 6B). Of interest, *Pax7* expression in Nog-treated cultures was higher than untreated controls. However, as we observed that Nog treatment appeared to enhance neuronal differentiation and/or survival such that there were many more neurons and primitive neural

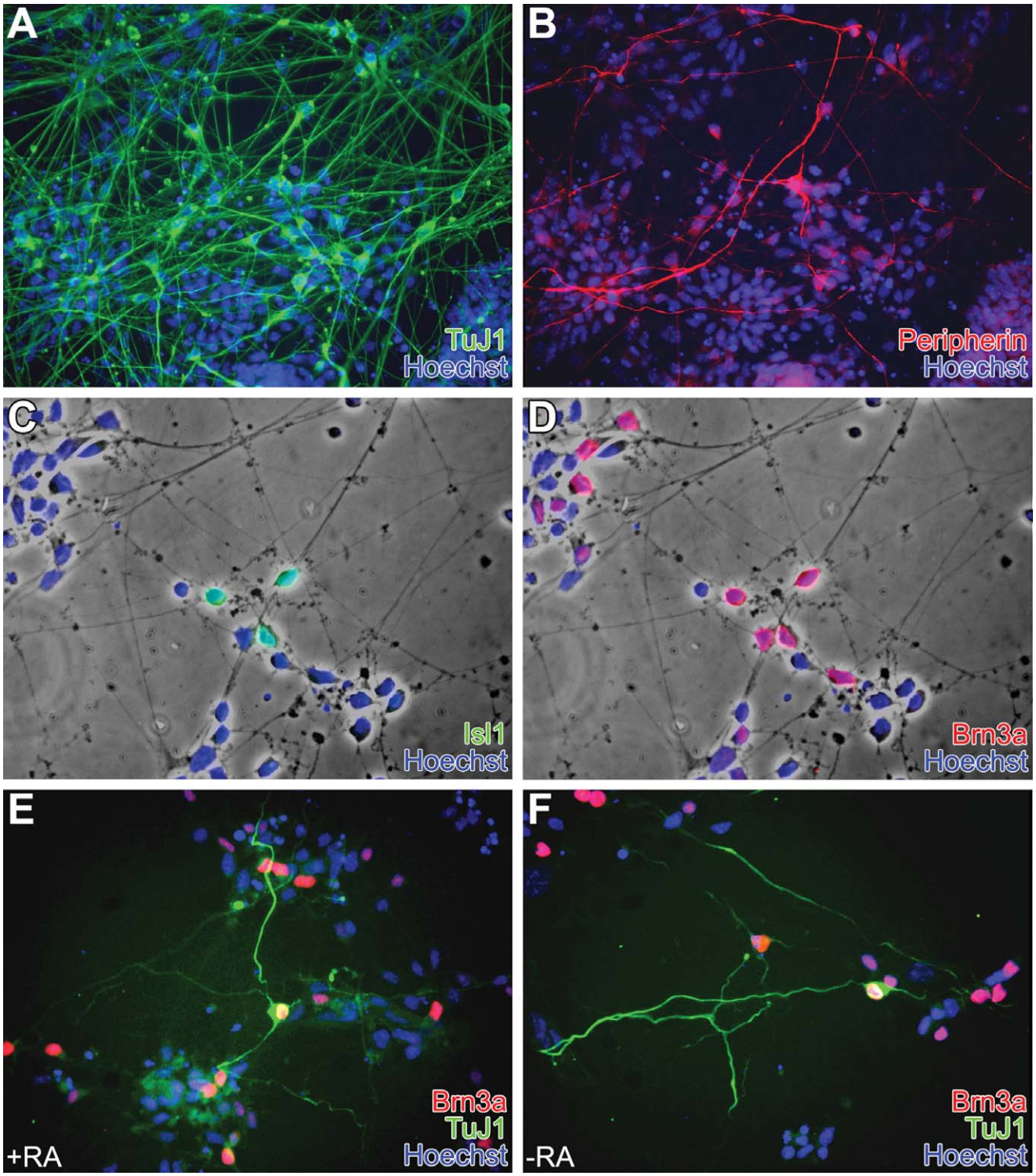


Fig. 5. Induced N7 cells form central nervous system (CNS) and peripheral nervous system (PNS) phenotypes. N7 cells were grown for three days in defined neural medium in the presence of Dox, followed by two days without Dox±1 μM RA. **A,B:** N7 cultures grown with RA for 5 days exhibit widespread neurogenesis. Some TuJ1+ neurons were also Peri+. Similar results were observed in RA-free cultures (not shown), although there was a slight attenuation in the overall number of neurons observed (see text and Table 1). **C,D:** Cells cultured in defined medium with RA for 5 days illustrating the presence of Isl1+ and Brn3a+ neurons. Isl1+/Brn3a+ are co-expressed in dorsal interneurons (lamina 3), and in sensory ganglia; Isl1-Brn3a+ expression is typical of dI- 1, 2, or 5 in CNS (dI-2 precursors are normally Neurog1+); Isl1+/Brn3a- expression is typical of CNS motor neurons. Most Isl1+ cells were Brn3a+, but not all Brn3a+ cells were Isl1+. **E,F:** Cells cultured +RA (E) or −RA (F) for 5 days illustrating Peri+/Brn3a+ (SG phenotype) cells. The overall frequency was similar in +RA and −RA-treated cultures. Nearly all cells that were Peri+ were also Brn3a+.

TABLE 1. Distribution of Neuronal Markers[a]

TuJ1/Peripherin staining	+RA	−RA	+RA vs. −RA *P*=
%TuJ1+	69.2±1.2	63.7±1.5	<0.001
%Peripherin+ somata	3.5±0.3	2.9±0.2	0.022
TuJ1+: Peripherin+	20.9±1.6:1	22.7±1.3:1	0.143
Brn3a/Peripherin staining			
%Peripherin+ somata	3.4±0.4	2.9±0.2	0.032
%Brn3a+	17.8±1.0	16.1±1.0	0.063
% of Peri+ also Brn3a+	96.9±2.4	96.8±2.5	0.4755
Brn3a/Isl1 staining			
%Brn3a (total)	18.1±0.9	16.7±1.1	0.067
Brn3a+/Isl1+	7.4±0.7	6.5±0.5	0.065
Brn3a+/Isl1−	11.3±0.8	10.3±1.2	0.168
%Isl1+ (total)	7.4±0.6	6.5±0.5	0.095
Isl1+/Brn3a−	0.2±0.1	0.1±0.1	0.191

[a]N7 cells were grown for three days in defined neural medium in the presence of Dox, followed by two days without Dox in defined neural medium±1 μM retinoic acid (RA). At least 1000 cells from 10 randomly selected fields in three replicate dishes were scored for immunoreactivity to the markers indicated and divided by the total cell number in each field determined by Hoechst nuclear staining. The data presented are the overall means for each treatment group±SEM across the three replicates. *P* values are based on results of analysis of variance, followed by pairwise Student's t-test.

"rosettes" present in Nog-treated groups compared either with controls or cultures treated with Shh alone (not shown). Because the enhanced *Pax7* signal could be due to the increase in neuronal differentiation, we examined the expression of Nkx6.1 and Pax7 using immunohistochemistry (Fig. 7) after 5 days of differentiation. Very few cells co-expressed Pax7 and Nkx6.1, indicating that the cultures contained a mixed population of precursor cells that could be characterized as distinctly "dorsal" (i.e., Pax7+) or "ventral" (i.e., Nkx6.1+). After quantifying the Pax7 and Nkx6.1-positive populations, we then applied the Tuj1 antibody to assess overall neuronal differentiation and found little difference between the treatment groups. Moreover, the Tuj1+ neurons were generally neither Pax7+ nor Nkx6.1+, further confirming that these markers labeled precursors rather than more mature neurons (Fig. 7, right panel). In cultures not treated with Shh or Nog, 46.1% (± 1.0) of cells were Pax7 positive, while 14.4% (± 0.4) were Nkx6.1 positive (Fig. 8). Treatment with either Shh or Nog at low or high levels resulted in a significant reduction in Pax7 immunoreactivity and a corresponding increase in the number of Nkx6.1-positive cells. We also observed more ventral phenotypes in Shh-treated groups compared with Nog treatment, and the ventralizing effect appeared to be dose-dependent. Of interest, co-application of Shh and Nog at either dose produced more ventral phenotypes than either factor alone, suggesting that these two molecules may act synergistically to promote ventral phenotypes. Altogether, the data suggest that forced expression of *Neurog1* in ESC produces a population of neuronal precursors that remain responsive to extrinsic patterning signals.

Neural Induction by Means of Forced *Neurog1* Expression Can Be Influenced by Fibroblast Growth Factor Signaling

The role of fibroblast growth factor (Fgf) signaling in neural induction and ESC differentiation is unresolved, with some studies suggesting that Fgf signaling is indispensable in neural induction both in vivo (Streit et al., 2000) and in ESC in vitro (Ying et al., 2003a). To assess the role of Fgf signaling during neuronal differentiation of ESC in the context of *Neurog1* expression, we exposed N7 cells in defined medium to a 12-hr pulse of 5 nM SU5402, which targets the kinase domain of FGFR1 and therefore abrogates all signaling by this receptor (Mohammadi et al., 1997). We treated cells at three intervals: 12 hr before the addition of dox (pre-dox), concurrent with the addition of dox (simultaneous), and 12 hr following the addition of dox (post-dox); the control group received dox 12 hr after plating (Fig. 9A). Cells were then cultured for 72 hr in defined neural medium containing dox then assayed for the expression of Sox3, a marker of neural ectoderm and neuronal precursors, TuJ1 labeling early neurons, or Oct4, expressed in undifferentiated ESC. Media changes and monitoring by phase microscopy occurred at 12, 24, 36, and 48 hr at which time we observed no differences in cell death (as evidenced by nonadherent cells observed in the plates and trypan blue staining of withdrawn media) across any of the treatment groups compared with controls. Consistent with either a requirement for Fgf/ERK signaling for ESC differentiation (Kunath et al., 2007), or alternatively, that Fgf/ERK signaling prevents dedifferentiation to a more primitive ES-like state (Greber et al., 2010), there were considerably more Oct4-expressing cells (53.4±5.4%) in the pre-dox group (Fig. 9B). Neuronal differentiation as evidenced by Tuj1 immunoreactivity was strikingly reduced in the pre-dox group (16.7±6.2%) and the number of Sox3+ precursors (66.3±7.4%) was higher compared with the other treatment groups. Simultaneous inhibition of Fgf signaling with *Neurog1* induction resulted in many fewer Oct4+ ESC (18.4±2.3%), and slightly more TuJ1+ neurons (28.1±1.9%) compared with the pre-dox group. The number of Sox3+ precursors was slightly reduced in the simultaneous group (63.5±6.6%) compared with the pre-dox group, but the difference only approached statistical significance (*P*=0.06). In the post-dox group, very few Oct4+ ESC remained (5.5±3.5%). There was also a slight, but

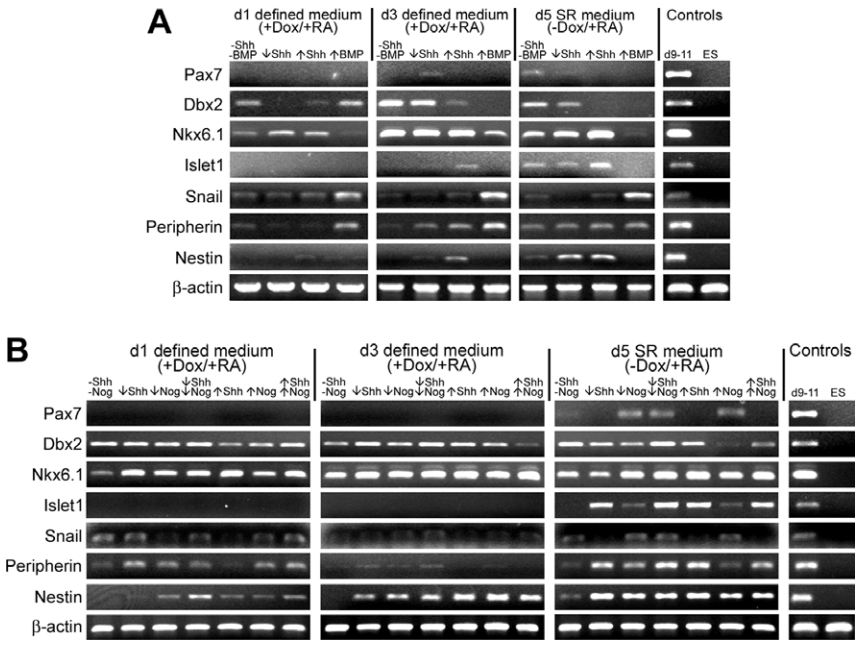


Fig. 6. Induced N7 cells respond to patterning factors. N7 cells were cultured for 3 days in defined neural medium with 1 μg/ml doxycycline (+ Dox) and then switched to serum replacement (SR) medium without Dox for 2 additional days of culture. Media were supplemented with 1 μM retinoic acid (RA) and growth factors as indicated for the entire time in culture. Samples from noninduced N7 cells grown in complete medium (ES) and pooled RNA from d9, 10, and 11 embryos (d9–11) show expected expression of all markers assayed. β-*actin* is included as a positive control. ↓Shh=2.5 nM, ↑Shh=25 nM, ↑BMP=5 nM BMP4, ↓Nog=2 nM, ↑Nog=5 nM. **A:** Sonic hedgehog (Shh) and bone morphogenetic protein-4 (BMP4) treatment: Cells induced in the presence of either low or high doses of Shh show reduced expression of both dorsal (Pax7) and intermediate (Dbx2) neural tube markers and increased expression of a ventral marker (Nkx6.1) compared with untreated cells. In Shh-treated cultures, down-regulation of *Dbx2* and up-regulation of *Nkx6.1* and *Islet1*, were dose-dependent. Shh treatment also resulted in up-regulation of the neural stem cell-associated intermediate filament protein, *Nestin*, by d5 of culture. BMP4-treated cultures show up-regulation of *Snail*, a marker of premigratory neural crest, and *Peripherin*, a peripheral neuronal marker, and abrogation of all other neural markers assayed. **B:** Shh and Noggin treatment: Cells were induced in the presence of combinations of Shh and a BMP antagonist, Noggin (Nog). In general, all treatment groups showed increased expression of *Nkx6.1* compared with untreated cells. Expression of *Pax7* increased in Nog-treated groups, likely due to an overall increase in the number of neural progenitors as evidenced by increased expression of *Nestin*. Cultures treated with high doses of Shh and Nog show no expression of *Pax7*, indicating that these cultures are ventralized by Shh treatment. Shh-treated cells also show increased expression of *Islet1* and *Peripherin*, markers both present in ventral motor neurons, and diminished expression of *Snail*. High doses of Nog also reduced expression of the intermediate neural tube marker *Dbx2*, suggesting a role for BMP signaling in establishing this lineage.

statistically significant ($P \leq 0.05$), reduction in the number of Sox3+ precursors (58.8±3.9%) in the post-dox group compared with either the pre-dox or simultaneous treatment groups, and a concomitant increase in the number of mature neurons (40.5±6.2%). Controls receiving no SU5402 showed a dramatic overall increase in differentiation as there were few Oct4+ cells remaining (0.7±1.0%), and a significant reduction in the number of Sox3+ precursors (35.9±6.4%). The number of TuJ1+ neurons in the control group was much higher than any of the treatment groups (64.6±4.7%), suggesting that the reduction in Sox3+ cell numbers was likely the result of increased differentiation of the Sox3+ precursor pool into (Tuj1+/Sox3−) neurons. Overall, these data indicate that the induction of Sox3+ neuronal precursors in the context of *Neurog1* expression is largely Fgf-independent, but there is a temporal window within which neuronal differentiation can be held in check by Fgf-mediated persistence of the Oct4 pluripotency pathway.

Microarray Analysis

After 24 hr of transgene induction, 1,384 genes were significantly differentially expressed; 951 up-regulated. At 48 hr, 5,894 genes were significantly altered (2,856 up-regulated); increasing to 7,205 (3,263 up-regulated) after 72 hr of doxycycline exposure. Gene ontology classification analysis identified significant alterations in the expression of genes associated with: nervous system development (48%), development (24%), metabolism (4%), cell cycle (4%), signaling (4%), and cellular organization (4%). Supplementary Table S1, which is available online, summarizes the results of functional annotation clustering of genes significantly up- and down-regulated at 24, 48, and 72 hr of transgene induction. There were significant changes in the expression of genes involved in neurogenesis, in process outgrowth and cell migration, those involved in the regional development of the nervous system, in cell cycle, as well as genes involved in ESC homeostasis.

We carried out KEGG pathway analysis to map alterations in signaling pathways and molecular functions and identified sequential induction of genes in pathways involved in axon guidance, cancer and melanogenesis (Supp. Table S2). By 72 hr of transgene induction, 19 pathways were activated, including: ErbB, Neurotrophin, Wnt, Mapk, Focal adhesion, Calcium, and Insulin signaling pathways. After 72 hr, only one pathway, cysteine and methionine metabolism, was significantly down-regulated.

Consistent with our RT-PCR results, *Neurog1* was up-regulated 74-, 74-, and 42.7-fold at 24-hr intervals compared with uninduced cells. Other bHLH genes were also induced: *Neurod4* increased by 33-, 206-, and 127.8-fold; *Neurod1* increased 9.7-, 11.5-, and 10.2-fold, while *Neurog2* was up-regulated by: 0-, 4.4-, and 3.8-fold (Table 2). Pluripotency factors expressed by undifferentiated ESC: *Eed*, *Eras*, *Fbxo15*, *Foxd3*, *Klf2*, *Klf4*, *Nanog*, *Pou5f1 (Oct4)*, *Sox2*, *Zic3*, were significantly down-regulated with differentiation. Genes associated with neurogenesis including: *Ebf2*, *Ebf3*, *Elavl3*, *Elavl4*, *Fabp7*, *Hes5*, *Lhx2*, *Ncam1*, *Nhlh1*, *Nhlh4*, *Pax3*,

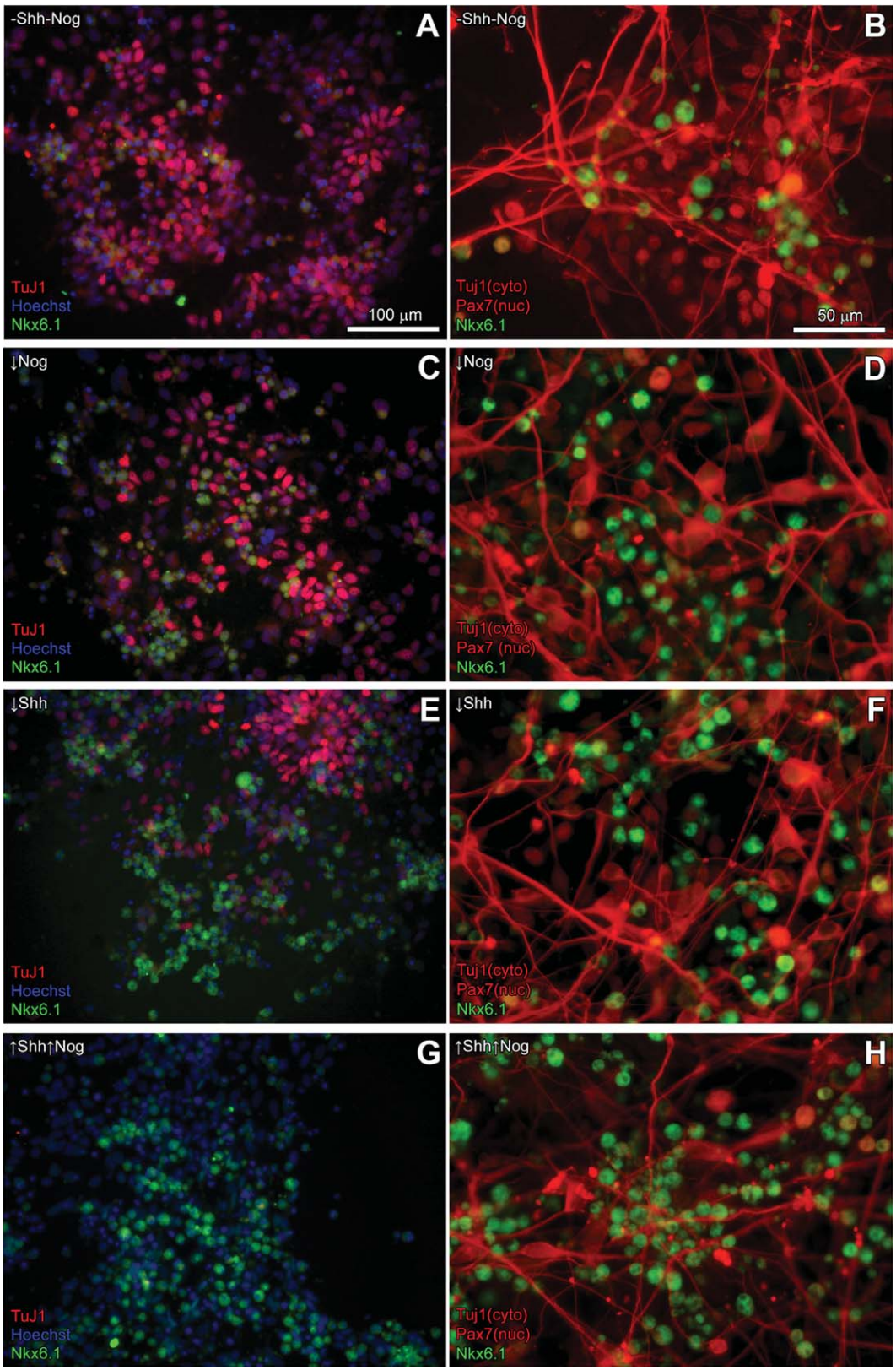
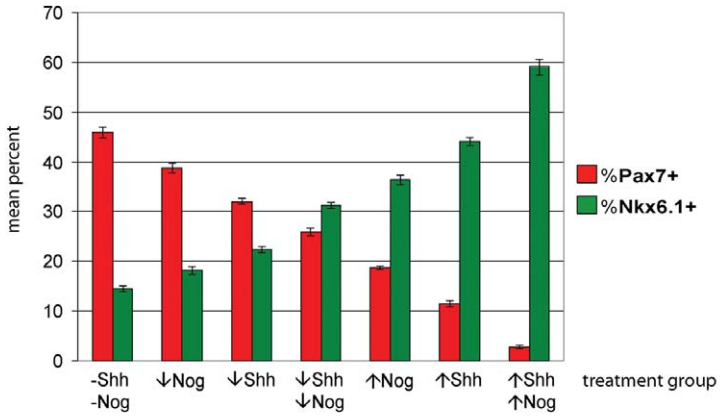


Fig. 7. Shift in dorsal–ventral phenotypes with Sonic hedgehog (Shh) and Noggin (Nog) treatment. Left panels: Immunostaining with Nkx6.1 (green nuclei) and Pax7 antibodies (red nuclei) indicate that more Pax7+ precursors are present in the absence of or at low doses of Shh and/or Nog. Higher doses of Shh and/or Nog reduce the number of Pax7+ cells observed and generate more Nkx6.1+ cells (not shown), and the application of high doses of Shh and Nog together (↑Shh↑Nog) resulted in a dramatic increase in Nkx6.1+ cells and a concomitant decrease in the number of Pax7+ cells. Total cell number in each field was determined by Hoechst nuclear staining (blue). Scale bar=100 micrometers. Right panels: After cell counts, cultures were stained with Tuj1 to distinguish neurons (red cytoplasm), which are largely Pax7- (red nuclei) and Nkx6.1- (green nuclei) as might be expected for markers that are normally down-regulated with differentiation. Scale bar=50 micrometers.



Treatment Group:	-Shh -Nog	↓Nog	↓Shh	↓Shh/ ↓Nog	↑Nog	↑Shh	↑Shh ↑Nog
%Pax7+	46.1±1.0	38.8±0.8	31.9±0.4	26.0±0.6	18.5±0.2	11.7±0.5	3.1±0.3
%Nkx6.1	14.4±0.4	18.5±0.6	22.8±0.5	31.4±0.5	37.1±0.8	44.0±0.4	59.4±1.6

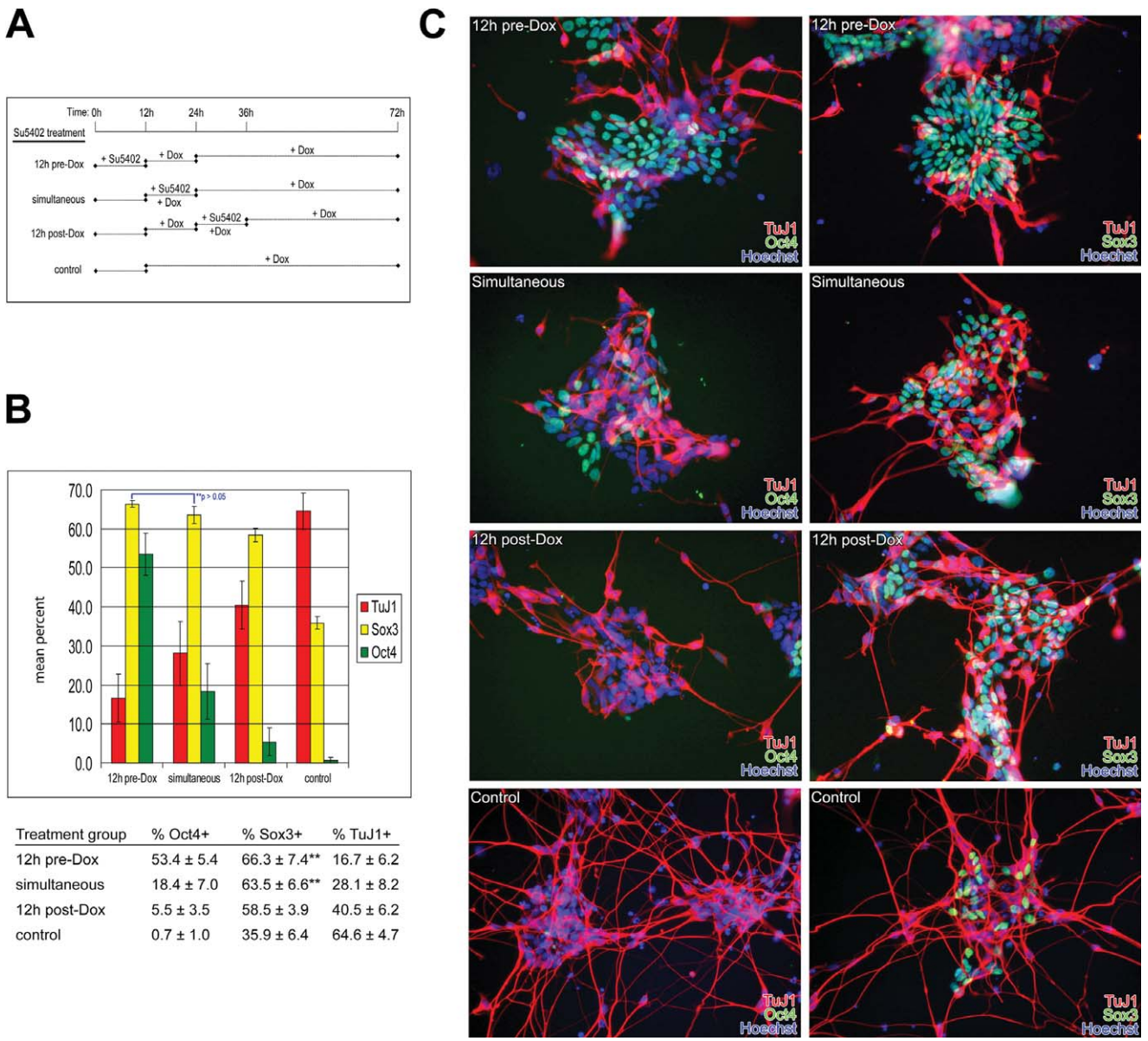
Fig. 8. Quantification of dorsal–ventral phenotypes with Sonic hedgehog (Shh) and Noggin (Nog) treatment. There was a dose-dependent decrease in the percentage of Pax7+cells and an increase in the percentage of Nkx6.1-positive cells following Nog and Shh treatment. Differences observed between all treatment groups were statistically significant ($P \leq 0.05$, analysis of variance, followed by pairwise Student's *t*-test). n=1,000 cells from 10 random fields from two replicate cultures. ↓Shh=2.5 nM, ↑Shh=25 nM, ↓Nog=2 nM, ↑Nog=5 nM.

Pax6, *Reln*, *Zic1*, were induced, while the negative regulator of neurogenesis *Rest* (RE1-silencing factor), was down-regulated. Many genes expressed in the ventricular zone (Fu et al., 2009) and by neural stem cells including *Dll1*, *Dcx*, *Prom1*, *Reln*, *Coup-TFI* and *II* (Naka et al., 2008), and *Hes5* (Hatakeyama et al., 2004; Basak and Taylor, 2007) were expressed, as were genes associated with cell cycle exit and differentiation including *Ebf2,3* (Pozzoli et al., 2001; Garcia-Dominguez et al., 2003). *Id4*, *Pax3*, *Pax6*, *Sox3*, *Sox4*, and *Sox11* (Bergsland et al., 2006) were also increased. Factors involved in process outgrowth and cell migration including ephrins and their Eph receptors, semaphorins and plexins, were induced by *Neurog1*. Of interest, epithelial genes including junctional proteins and cell–cell adhesion molecules such as E-Cadherin (*Cdh1*) were down-regulated whereas N-Cadherin (*Cdh2*) expression significantly increased.

There were also significant increases in the expression of genes associated with regional neuronal cell types, including telencephalon-associated genes *Elavl4*, *Fabp7*, *Foxd1* and *Foxg1*. *Lhx2*, which plays a critical role in cortical patterning (Mangale et al., 2008; Chou et al., 2009) was particularly strongly induced by *Neurogenin1*. Several factors associated with ventral neural fates, e.g., striatal (*Foxp2*), midbrain (*Pcdh8*), and midbrain dopaminergic neurons (*Foxa2*, *Ebf3*) were also induced. Hindbrain markers *Hoxa2* and *Hoxb2* were significantly induced, as was *Hoxd9*, which is expressed in thoracic lateral motor neuron columns (Dasen et al., 2005). Other genes associated with a motor neuron phenotype such as *Irex3* and *Isl1* were also significantly increased. Genes expressed in cephalic placodes including *Eya1*, *Ebf2*, *Fbxo2*, *Six1*, *Netrin1* and its receptors *DCC* and *Unc5C* were up-regulated as were genes associated with neural crest cells, including *Ednrb* and *Ret*. Few genes associated with glial differentiation (Fu et al., 2009) were identified, and genes that inhibit oligodendrocyte or astrocyte differentiation, e.g., *Hes5* (Liu et al., 2006) and *Id4* (Marin-Husstege et al., 2006) were induced.

Activation or silencing of lineage-specific sets of genes during development is controlled by transcription factors as well as by epigenetic regulators of chromatin structure. Because ESC have a chromatin configuration that is "poised" for lineage differentiation (e.g., Boyer et al., 2006), selective induction of chromatin regulators associated with other lineages, or removal of repressive marks on neuronal promoters/enhancers could drive neuronal differentiation. We examined the expression of transcripts encoding proteins involved in chromatin modification including SWI/SNF, polycomb family members, HDACs, etc., and found minimal (< three-fold) changes. There were three exceptions that are of potential interest. The first is in Forkhead box (Fox) gene expression. Foxa proteins are considered "pioneering" in that they open chromatin to allow modifiers access to chromatin, Foxp factors function as classic transcription factors recruiting enzymes to regulate gene expression, while Foxo proteins appear to do both (Lalmansingh et al., 2012). Following *Neurog1* expression, *Foxa2* was increased 21.2- and 13.7-fold at 48 and 72 hr, and *Foxp2* was increased 62.4- and 53.8-fold at similar time points, suggesting a role in neuronal lineage differentiation. Second, *Phc2*, a polycomb group member expressed in germinal zones of the nervous system (Kim et al., 2005), was induced 7.1-, 8.9-, and 8.3-fold. Finally, the histone methyltransferase, *Suv39h1* was down-regulated 35.7- and 17.6-fold at 48 and 72 hr. *Suv39h* proteins have been shown to oppose *Ring1* to coordinate early lineage decisions in the blastocyst (Alder et al., 2010), and to interact with Smads to coordinate BMP-induced gene repression and lineage differentiation (Frontelo et al., 2004). Somewhat surprisingly, given a recent study demonstrating that *Ezh2* is required to inhibit transcription of non-muscle lineage genes in satellite cells (Juan et al., 2011), we did not observe alterations in either the ubiquitous *Ezh1* or in *Ezh2* associated with proliferating tissues (Margueron et al., 2008). Future interrogation using ChIP and knock-down will explore these possibilities more directly.

Microarray analysis also identified a complex pattern of expression of genes involved in cell cycle control. At 24 and 48 hr, cluster analysis did not identify any significant clusters containing cell cycle related genes, but by 72 hr of transgene induction, three clusters identified down-regulated genes involved in cell cycle. Cluster 3 (Enrichment Score=7, $P \leq 5.0 \times 10^{-9}$, Benjamini-Hochbert corrected) contained genes involved in cell cycle progression, Cluster 4 (Enrichment



Treatment group	% Oct4+	% Sox3+	% TuJ1+
12h pre-Dox	53.4 ± 5.4	66.3 ± 7.4**	16.7 ± 6.2
simultaneous	18.4 ± 7.0	63.5 ± 6.6**	28.1 ± 8.2
12h post-Dox	5.5 ± 3.5	58.5 ± 3.9	40.5 ± 6.2
control	0.7 ± 1.0	35.9 ± 6.4	64.6 ± 4.7

Fig. 9. Neuronal differentiation by means of forced *Neurog1* expression is affected by fibroblast growth factor (Fgf) signaling. **A:** Treatment groups. N7 cells were plated in defined medium (time=0 hr) and exposed to a 12 hr pulse of 5 nM SU5402. One group (preinduction) was treated with SU5402 at the time of plating, after which the medium was exchanged for fresh medium supplemented with 1 μg/ml doxycycline (+Dox) for the remainder of the culture period. The second group (simultaneous) was plated in defined medium for 12 hr followed by addition of SU5402 and Dox. At 24 hr, the medium was exchanged for fresh medium supplemented with Dox for the remainder of the culture period. The final group (post-induction) was plated in defined medium for 12 hr followed by addition of Dox. At 24 hr, a 12-hr pulse of SU5402 was administered, and, at 36 hr, the medium was exchanged for fresh medium supplemented with Dox for the remainder of the culture period. **B:** Oct4/Sox3/TuJ1 cell counts. There was a decrease in the number of Sox3+ cells between the preinduction and simultaneous treatment groups that was not significant (**P=0.058), while the decrease in the number of Sox3$^+$ cells in the post-dox group was slight but significant compared with the other treatment groups. There was a striking increase in TuJ1 expression with delayed SU5402 treatment and a corresponding decrease in the number of Oct4+ cells, while the number of Sox3+ cells observed dropped only slightly with delayed treatment. Untreated controls demonstrated markedly increased neuronal differentiation, a concomitant reduction in neuronal precursor cells, with very few cells expressing Oct4. At least 1,000 cells from 10 randomly selected fields in two replicate dishes were scored for Oct4, Sox3, and TuJ1, and analyzed by pairwise Student's *t*-test. **C:** Oct4/Sox3/TuJ1 immunohistochemistry. The cell counts described above were obtained from cell cultures that were fixed after 72 hr and co-stained with TuJ1 and Oct4 antibodies (left panels) or TuJ1 and Sox3 antibodies (right panels). Nuclei are labeled with Hoechst 33258. Blocking FGF signaling resulted in fewer neurons (TuJ1$^+$) in a manner depending on the timing of treatment. Control cells receiving no SU5402 show robust neuronal differentiation.

Score=5.5, $P \leq 3.1 \times 10^{-9}$, corrected) identified genes involved in chromosome organization, sister chromatid, and centromere structure, while Cluster 7 (Enrichment Score=4.9, $P \leq 2.3 \times 10^{-6}$, corrected) identified genes involved in chromosome organization (Supp. Table S2).

Because the ESC population is relatively homogeneous (compared with brain tissue) it is possible to examine the sequential expression/repression

TABLE 2. Microarray Expression Analysis[a]

Gene	UniGene #	24h	48h	72h
Pluripotency Factors				
Eed	Mm.380914	–	−3.6	−4
Eras	Mm.250895	−4.9	−34	−66
Fbxo15	Mm.28369	–		−14
Foxd3	Mm.4758	–	−12	
Klf2	Mm.26938	–	−28	−41
Klf4	Mm.4325	−8.4	−16	−23
Nanog	Mm.440503	−4.7	−23	−33
Pou5f1	Mm.17031	−4.8	−27	−38
Sox2	Mm.65396	–		−2.7
Tert	Mm.10109	–		−3.5
Zic3	Mm.255890	−2.2	−5.5	−10
Neural Differentiation				
Ascl1	Mm.136217	–		
Brn2	Mm.129387	–		7.2
Dclk1	Mm.393242	–	41	120
Dcx	Mm.12871	8.2	194	359
Ebf2	Mm.319947	95.7	159	96.3
Ebf3	Mm.258708	47.7	186	282
Ednrb	Mm.229532	–		195
Elavl3	Mm.390167	–	30.9	35
Elavl4	Mm.3970	–	75	144
Fabp7	Mm.3644	49.4	201	444
Gfap	Mm.1239	–		
Isl1	Mm.42242	10.1	89.3	197
Lhx2	Mm.142856	54.8	145	192
Myt1	Mm.458718	–	24	66.2
Ncam1	Mm.4974	–		6.8
Nefh	Mm.298283	−2.3	−2.4	−2.6
Nefl	Mm.1956	−2.7	4.2	23.4
Nefm	Mm.390700	–		258
Neurod1	Mm.4636	9.7	11.5	10.2
Neurod4	Mm.10695	33	206	128
Neurog1	Mm.266665	74.4	74	42.7
Neurog2	Mm.42017	–	4.4	3.8
Nhlh1	Mm.2474	27.4	102	105
Nhlh2	Mm.137286	–	110	204
Nr2f1	Mm.439653	–	90.1	198
Nr2f2	Mm.158143	–	74.9	106
Nrxn1	Mm.480021	–	76.3	228
Olig1	Mm.39300	–	–	
Olig2	Mm.37289	–	–	
Olig3	Mm.156946	4.5	19.6	
Pax3	Mm.1371	20.2	177	98.3
Pax6	Mm.33870	–	10.8	23.1
Prom1	Mm.6250	–	10	0
Rein	Mm.425236	–	40.8	43.3
Rest	Mm.28840	−4.5	−6.4	−16
Ret	Mm.57199	–	96.8	251
Runxlt1	Mm.4909	–	5.6	19.3
Soxl1	Mm.41702	4.2	34.7	43.1
Sox3	Mm.35784	9.9	15.7	11.9
Sox4	Mm.240627	6.1	13.2	18
Tubb3	Mm.40068	–	7.7	8.7
Zeb1	Mm.3929	–	6.4	6.5
Zfp238	Mm.480309	6.6	11.1	7.8
Zic1	Mm.335350	53.4	40.7	31
Neural Patterning				
Eya1	Mm.250185	–	7	11.6
Foxa2	Mm.938	–	21.2	13.7
Foxd1	Mm.347441	–	11.1	23.9
Foxg1	Mm.390496	–		13

TABLE 2. *Continued*

Gene	UniGene #	24h	48h	72h
Foxp2	Mm.332919	–	46.7	438
Hoxa1	Mm.197	24.3		
Hoxa2	Mm.131	–	18	24.4
Hoxa4	Mm.439647	–	21.9	
Hoxb1	Mm.890	10.7	16.5	
Hoxb2	Mm.281153	33.5	92.7	79.4
Hoxb4	Mm.3546	22.6	43.3	45.6
Hoxd9	Mm.26544	–		10.6
Irx1	Mm.316056	–	4.2	6.5
Hoxb2	Mm.281153	33.5	92.7	79.4
Hoxb4	Mm.3546	22.6	43.3	45.6
Hoxd9	Mm.26544	–		10.6
Irx1	Mm.316056	–	4.2	6.5
Irx3	Mm.238044	8.5	39.9	42.6
Isl1	Mm.42242	10.1	89.3	197
Neural Patterning				
Lhx2	Mm.142856	54.8	145	192
Onecut1	Mm.303355	18.3	13	27.3
0necut2	Mm.234723	8	121	185
Six1	Mm.4645		5.2	15.5
Cell Surface				
Cdh1	Mm.35605		−8.9	−11
Cdh2	Mm.257437		18.3	31.8
Dab1	Mm.289682		2.9	5.4
Dcc	Mm.167882		61.5	114
Efna1	Mm.15675			–
Efna5	Mm.7978		5.2	6.6
Efnb1	Mm.3374	12.7	2.9	–
Efnb2	Mm.209813		8.3	9.6
Epha1	Mm.133330			−34
Epha2	Mm.2581		−6	−6
Epha3	Mm.1977		−4.3	–
Epha4	Mm.400747	−2.2	2.2	2
Epha5	Mm.137991		18	43.7
Ephb1	Mm.22897			–
Ephb2	Mm.250981		2.6	3.7
Esrrg	Mm.89989			98
Itga1	Mm.317280		−4.8	–
Itga5	Mm.16234			−3.1
Itga6	Mm.225096		−2.5	4.6
Netrin1	Mm.39095	−3.2	14.2	–
Ntrk1	Mm.80682		11.4	–
Ntrk3	Mm.421361		6.2	22
Pcdh8	Mm.390715	10.5	155	108
Plxna1	Mm.3789		3	2.3
Plxna2	Mm.2251	7.1	64.1	58.1
Plxnb1	Mm.53862		2.8	2.5
Plxnc1	Mm.256712		10	11.6
Plxnd1	Mm.3085		5.9	6.9
Robo1	Mm.310772		4.6	6.3
Sema3c	Mm.5071			12.8
Sema3d	Mm.89313			13.8
Sema4a	Mm.439752		−5.4	−22
Sema4b	Mm.275909			−2.5
Sema4c	Mm.29558			9.9
Sema4f	Mm.270543			6.4
Sema5a	Mm.260374	−3.5	4.3	4
Sema6a	Mm.40909			4.7
Sema6d	Mm.330536	11.3	37.7	44.9
Slit1	Mm.40322		8.6	–
Slit2	Mm.289739		3.5	8.1
Synpr	Mm.37515		47.5	145

TABLE 2. *Continued*

Gene	UniGene #	24h	48h	72h
Unc5c	Mm.24430		12.2	31.8
Signaling				
Bmp1	Mm.27757	2.7	5.8	4.7
Bmp4	Mm.6813	−6.2		−8.1
Dil1	Mm.4875	15.8	8.3	4.2
DII3	Mm.12896	19.9	20.5	5.5
DII4	Mm.143719	58.2	56	18.8
Dner	Mm.292560		12	23
Fgf4	Mm.4956	−4.7	−48	−8.1
Fgf5	Mm.5505			–
Fgfl2	Mm.7996		20.9	30.6
Fgfl3	Mm.7995			5.2
Fgfl5	Mm.3904		3.6	–
Fgfl7	Mm.12814	−13	−92	−56
Frzb	Mm.427436		46.3	35.9
Gli1	Mm.391450			−15
Gli2	Mm.273292	−2.9		−3.8
Gmnn	Mm.12239		−2.7	−3
Hes5	Mm.137268	21	385	203
Hes6	Mm.280029	17.9	7.5	2.6
Hhat	Mm.145857	3.6	5.2	4.7
Id2	Mm.34871		2.9	–
Id3	Mm.110	−11		–
Id4	Mm.458006	5.8	20.7	14.8
Igflr	Mm.275742		3.1	–
Igf2bp3	Mm.281018		3.4	2.3
Igfbp2	Mm.141936		−2.6	−4.9
Igfbp4	Mm.233799		25.4	13.5
Igfbp5	Mm.405761		18.1	25.3
Igfbp7	Mm.233470	−3.2		−4.9
Signaling				
Igfbpl1	Mm.3919	9.4	67.1	58
Inhbb	Mm.3092	−12	−44	−131
Jag1	Mm.22398	–	6.2	3.8
Nik	Mm.9001	–	–	2.1
Nodal	Mm.57195	−2.2	−14	−22
Notch1	Mm.290610		2.8	
Notch2	Mm.254017	3	2.7	
Notch4	Mm.173813	–	−4	
Ntrk1	Mm.80682	–	11.4	
Ntrk2	Mm.130054	–	–	
Ntrk3	Mm.33496	–	6.2	22
Sfrp2	Mm.19155	–	–	12.4
Smad1	Mm.223717		–	4.2
Smad2	Mm.391091		–	2.1
Smad3	Mm.7320	2.8	2.8	
Smad4	Mm.100399	–	–	
Smad7	Mm.34407	−5.1	−8.7	−6.6
Socs2	Mm.4132	−2.1	−4.4	−17
Socs6	Mm.91920	–	4.7	5.2
Stat1	Mm.277406	3.9	–	
Stat3	Mm.249934		−2	−2
Cell Cycle				
G1/S checkpoint				
Cdkn1a	Mm.195663	–	5	3
Suv39h1	Mm.9244	2.9	−35.7	−17.6
Trp53	Mm.222	–	−3.3	−5.5
G1/S				
Btg2	Mm.392646	3.6	4	
Cables1	Mm.40717		2.3	2.7
Ccnd1	Mm.16110	−2.2	−6.1	−6.9
Ccne1	Mm.16110	–	−6.1	−6.9

TABLE 2. *Continued*

Gene	UniGene #	24h	48h	72h
Cdca5	Mm.23526	–	−3.1	−3.7
Cdkn1a	Mm.195663	–	5	3
Ccnd2	Mm.333406	–	3	6
Gspt1	Mm.325827	–	2.1	2.1
S				
Brca2	Mm.236256		−2.8	−3.4
Cdt1	Mm.21873	–	−2.8	−3.7
Pola1	Mm.1923	–	−2.5	−3
Skp2	Mm.35584	−2.5	−2.2	−2.3
G2/M Checkpoint				
Cdk1	Mm.281367	–	–	
Cdt1	Mm.21873	–	−2.8	−3.7
Pola1	Mm.1923		−2.5	−3
Tipin	Mm.196219		−2.6	−3.4
G2/M				
Birc5	Mm.8552	–	−3.4	−5
Ccnb2	Mm.22592	–	–	−3.1
Cdc25b	Mm.38444	4.6	–	
Cdkn1a	Mm.195663	–	5	3
Gadd45g	Mm.281298	9.6	–	
Gpc1	Mm.297976		3.6	3.9
Hmga2	Mm.157190		–	3
M				
Aurka	Mm.249363	–	−2.9	−3.7
Birc5	Mm.8552	–	−3.4	−5
Bub1b	Mm.29133	–	−4.2	−4.8
Ccdc99	Mm.194368	–	−2.4	−3
Ccnb1	Mm.260114		−3.3	−4.5
Ccnb2	Mm.22592		–	−3.1
Ccng2	Mm.3527	3.7	6.2	5.6
Cdc20	Mm.289747	–	−3	−4.2
Cep55	Mm.9916	–	−6.1	−6.3
Ercc61	Mm.31911	–	−4.9	−6.6
F630043A04Rik	Mm.30173	–	−2.3	−3.9
Fbxo5	Mm.197520	–	−2.9	−4
Hells	Mm.57223		−3.5	−4
Incenp	Mm.29755		−2.7	−3.1
Ndc80	Mm.225956	–	−2.6	−3.3
Nek2	Mm.33773	–	−2.8	−4
Nup43	Mm.299887	–	−3.3	−3.7
Plk1	Mm.16525	2	−2.5	−3.5
Rcc2	Mm.253	–	−4.4	−5.1
Spc24	Mm.295642	–	−3	−3.3
Tipin	Mm.196219		−2.6	−3.4

[a]Fold changes in gene expression following 24 hr, 48 hr, and 72 hr of transgene induction.

of transcripts involved in cycle regulation (Table 2). Positive regulators of cell cycle including: *Birc5*, *Id3*, *Skp2* were down-regulated, and negative regulators: *Btg2*, *Ccng2*, *Ebf2,3*, *Gadd45g*, *Gspt1*, *Hipk2*, *Prmt2* were up-regulated with transgene expression. However, other positive regulators were stimulated, including: *Ccnd1*, *Ccnd2*, *Cdc25b*, *Cyr61*, *Gpc1*, *Hmga2*, *Tead2*. Others were strongly changed initially by transgene induction but then were reduced by 48 or 72 hr, including: *Cdc25b*, *Gadd45g*, and *Id3*. Of interest, the tumor suppressor *Trp53*/*p53* was down-regulated significantly both at 48 and 72 hr of induction. *P53* plays major roles in apoptosis, cell cycle arrest, and by means of its downstream targets *Gadd45g* and *p21* (*Cdkn1a*) which is up-regulated at all time points, which functions as a G1 regulator to produce growth arrest.

We also compared gene expression in *Neurog1*-expressing cells at 24 vs. 48, 48 vs. 72 and 24 vs. 72 hr time points. Overall, microarray analysis of changes in gene expression between 24 and 48 hr identified sets of genes expressed in neural stem cells, neural crest and placodes. Comparing 24 and 72 hr patterns, increasing numbers of mature markers including channel genes, neurotransmitter/receptor genes were identified, consistent with our previous observations that they exhibit mature axon potentials (Reyes et al., 2008).

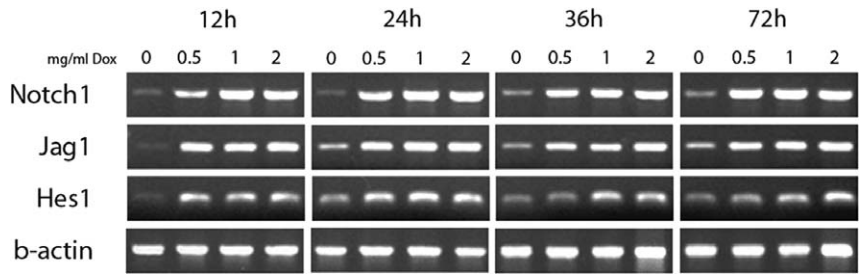


Fig. 10. Induced N7 cells express Notch-Delta pathway members in a dose-dependent manner. Cells cultured in defined neural medium with 0, 0.5, 1, or 2 mg/ml doxycycline up-regulate the *Notch1* receptor and its ligand *Jagged1*, as well as the downstream target of Notch activation, *Hes1*. Up-regulation of genes associated with Notch-Delta signaling is correlated with the expression of *Neurog1*, providing further evidence of an interaction between *Neurog1* and the Notch-Delta signaling pathway, as observed in microarray analysis.

To validate the microarray data, we chose to examine Notch-Delta pathway members, as they have previously been suggested to be targets of *Neurog1* (Ma et al., 1997). As early as 12 hr after doxycycline treatment, expression of Notch pathway members was strikingly up-regulated, remaining high during the 72 hr culture period. Over time in control cultures there was a gradual increase in expression of pathway members, *Notch1*, *Jag1*, and *Hes1* as neuronal differentiation increased (Fig. 10). Consistent with these observations, at 24 hr, array analysis identified *Notch2* (three-fold), *Hes5* (21) and *Hes6* (17.9) as up-regulated (Table 2). By 48 hr, many pathway members including *Notch1* (2.8), *Notch2* (2.7), *Notch4* (-4), *Jag1* (6.2), *Hes5* (384), *Hes6* (7.5), *Dner* (12) were identified. At 72 h, *Jag1* (3.8) and *Hes5* (203.2), *Hes6* (2.6), and *Dner* (23) were up-regulated.

Microarray data have been deposited in GEO, accession numbers: GSE42883, GSM1052734-GSM1052745.

DISCUSSION

Inducible Expression of *Neurog1* in ESC Provides a Novel Model of Neurogenesis

ESC provide a powerful model system to study the mechanisms of cell fate specification and lineage segregation at a stage in early development not readily accessible for manipulation. Studies of directed ESC differentiation typically rely on differentiation in embryoid bodies and/or exposure to stromal cell derived factors or to non-specific morphogens such as RA. While these approaches can produce populations enriched with neurons (e.g., Zeng et al., 2011), undefined factors and endogenous cytokines present in EBs, media containing serum, or stromal cell co-culture, complicate efforts to understand the role of specific signaling pathways in lineage segregation. Differentiation of ESC in monolayer cultures in defined media minimizes these effects (e.g., Ying et al., 2003b) and, if combined with forced expression of a lineage restricted gene, presents a unique opportunity to tease out the interactions of particular genes and growth factors in early development. An additional advantage of the inducible gene expression paradigm is the ability to maintain transgene expression following implantation (Reyes et al., 2008). Here, we demonstrate that expression of *Neurog1* in ESC is sufficient to induce neuronal differentiation even in conditions that normally maintain pluripotency (adherent culture at high density with serum and LIF), in defined media where the presence of morphogens can be more precisely controlled.

Neurog1 Expression in ESC Generates Representative Neuronal Subtypes and Neural Crest Derivatives

Consistent with the pattern of Neurog1 expression in defined domains of both the CNS and PNS (Ma et al., 1996; Sommer et al., 1996; Murray et al., 2000; Kim et al., 2011; Takano-Maruyama et al., 2012), expression of Neurog1 in ESC is sufficient to generate neuronal precursors and mature cell types representative of these expression domains (Figs. 4, 5). In fact, this paradigm may represent a reliable method to obtain sensory neurons, which have been difficult to generate from embryonic stem cells (Sasai, 2005). We observed a significant number of neurons that expressed Brn3a, a POU homeodomain transcription factor associated with sensory lineages in both the CNS and PNS. In the CNS, Brn3a+ sensory lineages arise throughout the anterior–posterior extent of the dorsal alar plate in the neural tube. In the posterior spinal cord, the alar plate generates Brn3a+ dorsal interneurons, of which the dI2 commissural lineage is specifically Neurog1-dependent (Gowan et al., 2001), whereas rostral domains of Brn3a and Isl1 expression are present in the tectum and tegmentum of the midbrain (Fedstova and Turner, 2001). A 4 kb enhancer has been shown to drive reporter gene expression to the midbrain, hindbrain and spinal cord (Nakada et al., 2004), while a second 0.8 kb enhancer drives expression to cells fated to become interneurons in the ventral spinal cord (Quinones et al., 2010). The presence of peripherin+ cells in our cultures presents the intriguing possibility of having generated motor neurons which express this marker (Escurat, et al., 1990). However, these cells almost invariably co-expressed Brn3a, which has not been reported in motor neurons, but is more suggestive of PNS lineages. Regarding glial differentiation, we did not observe any up-regulation in glial markers in either our microarray analyses (Table 2) or by RT-PCR or immunohistochemistry (not shown). Moreover, our previous work similarily failed to detect GFAP+ cells in vivo or in vitro (Reyes et al., 2008), consistent with observations that Neurog1 promotes neurogenesis while inhibiting gliogenesis (Sun et al., 2001) and detailed fate mapping studies in which Neurog1 was found to be exclusively associated with neuronal lineages (Kim et al., 2011).

In the PNS, *Neurog1* has been shown to be instructive for sensory neurogenesis (Perez et al., 1999; Lo et al., 2002) and required for the generation of proximal cranial sensory

ganglia and TrkA neurons of dorsal root ganglia (Ma et al., 1997), whereas *Atoh1* is associated with development of adrenergic autonomic ganglia (Guillemot et al., 1993). Correspondingly, we failed to detect tyrosine hydroxylase, a marker of adrenergic neurons in any of our cultures (not shown) but instead found many peripherin+ neurons that invariably co-expressed Brn3a (Fig. 5E,F), a phenotype representative of both cranial and dorsal root sensory ganglia (Gowan et al., 2001). A possible caveat to this interpretation is that Brn3a (Nadal-Nicolas et al., 2009) and peripherin (Tajika et al., 2004) are also co-expressed in retinal ganglion cells, but other markers of retinal ganglion cells such as *Chx10*, *Dlx1*, *Dlx2*, and *Brn3b* (de Melo, et al., 2003) were not significantly up-regulated in our microarray analysis, making this phenotype less likely.

Derivation of neural crest from ESC typically is a multistep process that requires sequential exposure to multiple growth and differentiation factors (Mizuseki et al., 2003; Aihara et al., 2010; Kawaguchi et al., 2010). Our findings suggest that *Neurog1* expression can direct ESC toward Ret+, Ednrb+ neural crest fates, and induces expression of Sox4 and Sox11 required in early sympathetic ganglia (Potzer et al., 2010), in the absence of exogenous instructive signals. However, our cultures contained a mixture of CNS and PNS types, and it remains to be determined what factors in addition to *Neurog1* expression mediate the choice between these lineages.

ESC Expressing *Neurog1* Can be Patterned Along the Anterior–Posterior and Dorsal–Ventral Axes and Influenced by Fgf Signaling

Expression of *Neurog1* prompted early expression of forebrain (*Otx2*, *Hoxa1*) as well as midbrain (*Engrailed1*, not shown) markers, strong induction of *Hoxb2* but not of more posterior markers including *Hoxc6*. However, treatment of these cultures with RA promoted expression of Hoxc6 and down-regulation of anterior markers. These data suggest that these cells possess an intrinsic anterior character that can be posteriorized by RA- mediated signals (Irioka et al., 2005). Because these cultures lack other cell types, these results clearly demonstrate that *Neurog1*-expressing neuronal precursors possess an anterior character in the absence of confounding "mesendodermal" signals.

Based on previous gain-of-function studies, we expected that *Neurog1* expression in ESC would produce specific subtypes of neurons independent of any endogenous patterning molecules. However, we were surprised to find that induced cells were quite responsive to dorsal–ventral (DV) patterning molecules (Wilson and Maden, 2005) such that more ventral phenotypes were observed in cultures treated with Shh, and this effect could be augmented by addition of Noggin protein. RA treatment alone also produced ventral phenotypes. In addition, noggin treatment accelerated and BMP treatment suppressed neurogenesis in our cultures. Together, these data indicate that ESC forced to express *Neurog1* progress through a stage where they remain receptive to patterning cues.

Recent evidence indicates that FGFs may have an independent and indispensable role in the induction of neural fate in vivo (Streit et al., 2000; Wilson et al., 2000; Lanner and Rossant, 2010) and in the neuronal differentiation of ESC (Ying et al., 2003a). Fgf signaling can maintain self-renewal of ESC (Sterneckert et al., 2010; Staviridis et al., 2010), and is required for cell cycle exit and the transition to lineage differentiation (Kunath et al., 2007). Other studies have demonstrated that Fgf signaling is essential for the progression of ESC to an epiblast state (Stavridis et al., 2010); preventing dedifferentiation to a more primitive ICM-like state (Greber et al., 2010) and inhibiting neural differentiation (Greber et al., 2010; Jaeger et al., 2011). Our data suggest that expression of *Neurog1* in ESC is sufficient to generate Sox3+ progenitors independent of Fgf signaling (Fig. 9B). However, the reduction in the percentage of neurons in the preinduction and simultaneous induction treatment groups, and striking increase in the number of Oct4+ and Sox3+ cells in the earlier treatment groups, suggests that the initial specification of Sox3+ precursors in the context of *Neurog1* expression does not require Fgf signaling. However, there is a window where inhibition of Fgf signaling interferes with the ability of these precursors to differentiate into mature neurons. It has been suggested that inhibition of Fgf signaling can revert epiblast stem cells to an Oct4+ ESC state (Greber et al., 2010). However, we failed to detect up-regulation of epiblast markers such as *Fgf5*, but we did see markers of neural ectoderm such as *Pax 6* (Table 2), suggesting that neuronal differentiation by means of *Neurog1* expression likely bypasses an epiblast state. Nonetheless, the Oct4 pathway appears to be initially intact in these cells and abrogation of Fgf signaling reengages ESC programs that inhibit neuronal differentiation.

ESC Neuralized by *Neurog1* Expression May Progress Through a Notch-Sensitive Neuronal Precursor Stage

Although our immunohistochemical analyses indicated that all of the cells in our cultures express Neurog1 (Fig. 2), not all matured into neurons after 3 or even 5 days in culture, similar to previous observations in embryonal carcinoma (EC) (Farah et al., 2000; Kim et al., 2004) and ESC (Kanda et al., 2004). Although primitive neural stem cells appear relatively insensitive to Notch signaling, definitive neural stem cells may be maintained in an undifferentiated state by activation of the Notch pathway (Tropepe et al., 2001), like neuronal precursors, whose differentiation into mature neurons can be held in check by the Notch-Delta pathway (reviewed in Louvi and Artavanis-Tsakonas, 2006). We observed that induction of *Neurog1* results in transient expression of the Notch ligand, *Jag1* (Fig. 10; Table 2), and of *Dll1,3,4* (Table 2), as well as up-regulation of *Hes1*, *Hes5*, and *Hes6*, repressor-type bHLH factors that are downstream targets of the Notch signaling pathway. The Notch target gene *Hes5*, required for NSC maintenance (Basak and Taylor,

2007; Hatakeyama et al., 2004), was strongly induced by *Neurog1*. In fact, the up-regulation of these repressor-type bHLHs is more likely the effect of *Jag1* expression in neighboring cells than a direct effect of *Neurog1* expression. Thus, ESC expressing *Neurog1* may progress through a Notch-sensitive stage in which cells at the edge of rosettes escape inhibition to differentiate into neurons, and then repress neuronal differentiation in neighboring cells.

Gene Expression Patterns Down-stream of *Neurog1*

Because *Neurog1* is expressed early in the neurogenic cascade at the time of lineage commitment/determination (Blader et al., 1997), we used microarray analysis to identify transcripts expressed down-stream of *Neurog1*. Many of the genes strongly induced by *Neurog1* are expressed in the stratifying neural ectoderm at the time of cell cycle exit (Fu et al., 2009); coupling *Neurogenin1* expression with cell cycle exit and neuronal differentiation (Kim et al., 2011). Other candidate transcription factors such as *Sox4* and *Sox11*, which are known to play a role in the downstream effects of proneural bHLH factors, were strongly induced, while genes associated with ESC maintenance, and inhibitors of neuronal differentiation were down-regulated. In addition, genes associated with astrocyte and oligodendrocyte differentiation were not identified or were down-regulated.

Genes associated with neuronal cell migration were strongly induced by *Neurogenin1*, including the microtubule associated protein doublecortin (*Dcx*), *Cdn2*, reelin, CoupTF factors (*Nr2f1*, *Nr2f2*; Tripodi et al., 2004), as well as *EphA5* previously identified as down-regulated in *Neurog2:Neurog1* double mutant mice (Mattar et al., 2004). There was also a striking switch in expression of genes associated with an epithelial morphology including junctional proteins, as well as E-Cadherin (by ESC) to N-Cadherin by differentiating neurons. These data suggest that *Neurog1* expression activates a program of neurogenesis that includes cell surface changes, induction of genes involved in cell migrations, transcription factors and cell cycle modulators.

In a screen of *Ngnr1* and *NeuroD* targets in *Xenopus*, Seo et al. (2007) identified similar sets of target genes: 47% of the genes induced by *Ngnr1* over-expression in *Xenopus* were identified in our analysis, while 43% of the genes induced by *NeuroD* were present in our screen. Somewhat surprisingly, all of the seven core regulatory factors suggested to promote Neurogenin-mediated neurogenesis: *Ebf2,3*, *Hes6*, *Myt1*, *Nhlh1*, *Neurod1*, *Neurod4*, and *Runx1t1* (Pang et al., 2011) were significantly increased in our cells. An in silico analysis to identify targets of *Neurog2* (dorsal telencephalon) vs. *Ascl1* (ventral telencephalon) (Gohlke et al., 2008) identified only *Dcx*, *Elavl4*, *Nrarp*, in common with our *Neurog1* down-stream targets; and one, *Fzd5*, was down-regulated significantly in our analysis. Of interest, constitutive over-expression of *NEUROG1* in a cell line derived from human fetal telencephalon identified 588 potential target genes; 27.7% of them were identified in this analysis at 72 hr. These represent a very different population as telencephalon progenitor cells expressed transcripts associated with glial cells as well as with neurons (Satoh et al., 2010). Similarly, expression of transcripts in the hindbrain neural ectoderm on E11.5—when Purkinje progenitors are developing—in *Neurog1*−/− embryos identified 31/117 transcripts also present in our analysis. These data suggest that ultimately, subtype specificity is likely controlled by combinations of bHLH proteins and region-restricted transcription factors (particularly homeodomain proteins), which in combination with noncoding RNAs, may regulate each other or stabilize a neurogenic program.

Other candidates that may act in concert with bHLH factors to restrict lineage progression are chromatin regulators. The ESC faces the unique problem of maintaining both self-renewal and pluripotency, which it may solve with a unique bivalent chromatin structure in which enhancers of lineage-specific genes are associated with specific methylation marks that repress lineage gene expression "poised chromatin", while pluripotency genes maintain an "active" chromatin through successive rounds of division. These modifications are lost with differentiation (Boyer et al., 2006), but many are reset with somatic cell reprogramming (Creyghton et al., 2010). In fact, it has been suggested that because many lineage-specific transcription factors belong to large families that recognize a common binding sequence, e.g., the E box, additional mechanisms, such as chromatin modification, must exist to limit the sites available for transcription factor binding (Conerly et al., 2011). Our microarray analysis identifies several transcripts encoding chromatin modifying factors as possible targets of *Neurog1*. These factors may function to restrict binding of non-neuronal lineage factors during *Neurog1* driven differentiation, and may ultimately suggest methods for direct reprogramming of adult cells to neurons. Additional work will examine direct interactions and use shRNA to probe their roles in the context of *Neurog1* expression.

Neurog1 Alters Cell Cycle Progression

Although the proneural bHLH genes *Atoh1* (*Math1*), *Asc1* (*Mash1*), *Neurog1/2* promote cell cycle exit and expression of neuronal genes, the mechanisms involved and target genes are largely unknown. Recently two large-scale analyses of gene expression downstream of *Ascl1* (Castro et al., 2011) and *Atoh1* (Machold et al., 2011) identified transcripts involved in cell cycle exit and sequential phases of neural differentiation. While it is not surprising that proliferating granule cell precursors maintained *Atoh1* and cell cycle genes, in the current investigation we also identified genes involved in positive regulation of the cell cycle. Of the 95 cycle-related genes expressed by *Mash1*+ rhombic lip precursors, 17 were also present in *Neurog1* over-expressing ESC, including: *Aurka*, *Birc5*, *Brca2*, *Bub1b*, *Ccnd1*, *Ccnd3*, *Cdc20*, *Cdca2*, *Cdca5*, *Cdt1*, *E2f8*, *Fbxo5*, *Gmnn*, *Hells*, *Jub*, *Nek2*, *Trpt53/p53*. Downstream of *Ascl1* (Castro et al., 2011) only five were genes were common to *Neurog1* cells:

Ccnd1, *Ccd3*, *Cdkn1a/p21*, *Plk1*, *Gadd45g*.

Gadd45g, a strong inhibitor of cell cycle progression (e.g., Mak and Kultz, 2004) was strongly induced by *Neurog1* at 24 hr. *Ebf3* was particularly strongly up-regulated by *Neurog1* (48, 162, 182-fold at 24-hr intervals); it has previously been reported to be downstream of *NeuroD* (Pozzoli et al., 2001). *Ebf* genes inhibit cell cycle progression at G1/S, and couple cycle exit and neural differentiation (Garcia-Domingues, 2003); over-expression decreases proliferation consistent with our observations. Neuron-specific activators of the cell cycle progression inhibitor Cdk5 (*Cdk5r1/p35* and *Cdk5r2/p39*), which are responsible for producing the unique neuronal cell cycle, were also increased. Other genes such as *E2f8*, which is induced at G1/S and acts as a repressor of cycle progression by means of p53 (Christensen et al., 2005), were down-regulated in *Neurogenin1*-expressing cells. *Cdc20*, which has previously been associated with increased neural plasticity (Conway et al., 2007), was also decreased in N7 cells following transgene induction. This work demonstrates that the neurogenic bHLH genes affect transcripts involved in cell cycle progression, neural differentiation, migration and apoptosis, and may reflect the presence of a population of proliferative progenitors in the *Neurog1*-expressing cells.

ESC, like the early epiblast, spend most of their time in S phase and have an attenuated G1 (Burdon et al., 2002; White et al., 2005), possibly to minimize exposure to differentiation factors present in the local microenvironment (Orford and Scadden, 2008). Lengthening of G1, which occurs with differentiation of the epiblast and ESC and is characteristic of somatic cells, was also observed following expression of *Neurogenin1*. Whether a cell exits cycle is determined at the G1 checkpoint, and exit from cycle is required for many cell fate decisions, consistent with the presence of a population of proliferative precursors in these cultures.

The ability to induce expression of *Neurog1* in ESC constitutes a straightforward and powerful model system that provides insight into the complex interplay of signaling molecules and transcription factors that shape the two-cell embryo into the thousands of mature cell types present in the adult PNS and CNS, and increases our understanding and eventual control of normal development, birth defects and tumor formation. Ultimately, elucidation of the molecular histogenesis of the nervous system will also improve our understanding of the factors that promote ectopic neurogenesis in several neurodegenerative conditions (Curtis et al., 2003). In addition, primitive precursors such as those that can be generated by the N7 cell line may be useful for cell transplantation or as a resource to identify down-stream targets involved in activation of a program of neurogenesis.

EXPERIMENTAL PROCEDURES

Targeting Vector Construction and Generation of Inducible *Neurog1* Cell Lines

A *Neurog1* insert from pCS2-ND3 (M. McCormick) was ligated with an *Eco*RI-*Not*I fragment from pI_2R (Clontech, Palo Alto, CA) containing an IRES-DsRed2 cassette into the pLox targeting vector to generate the targeting construct, pLox-N1-I_2R. To generate targeted cell lines, 1.0×10^6 Ainv15 ESC (Kyba et al., 2002) were plated in a 60-mm dish and co-transfected 24 hr later with 1 μg each of pLox-N1-I_2R and pSalk-Cre (M. Kyba) using Lipofectamine/Plus Reagent (Invitrogen) in serum-free DMEM. After 3 hr, the transfection medium was replaced with complete ES medium. The following day, the cells were split to a 150-mm dish in complete ES medium (see below) containing 350 μg/ml G418 (Invitrogen). After 10 days in selection, 12 colonies remained, from which we were able to establish 7 independent cell lines. Three of these lines were selected for additional analysis to confirm targeted integration of the *Neurog1* transgene at the tet-inducible locus, stable expression of rtTA, doxycycline-inducible expression of *Neurog1*, and uniform neuronal differentiation after 1 and 3 days of culture in defined neural medium (described below) with 1 μg/ml doxycycline (Supp. Fig. S1). We observed no differences between the cell lines and we therefore selected one of the lines, N7, to carry out most subsequent experiments.

Cell Culture and Neural Differentiation

Ainv15 ESC were cultured on 0.1% gelatin coated substrates in complete ES medium (ESM) consisting of high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% ES-tested fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, or Harlan Bioproducts, Indianapolis, IN), 10^{-4} M β-mercaptoethanol (Sigma, St. Louis, MO), 0.224 μg/ml L-glutamine (Invitrogen), 1.33 μg/ml HEPES (Invitrogen), and 1,000 units/ml human recombinant LIF (Oncogene Research Products, San Diego, CA). N7 cells were maintained in ESM plus 350 μg/ml G418 (Invitrogen) and 1.5 μg/ml Puromycin (Sigma). For neural differentiation, cells were plated at 5.0×10^4 cells/cm^2 in gelatin-coated 6- or 12-well plates in ESM or in defined medium supplemented with 1 μg/ml doxycycline (Sigma), and media changed every day. Defined neural medium consisted of 80% F-12/DMEM and 20% Neurobasal media with 10 μl/ml MEM pyruvate, 8 μl/ml N2 supplement, and 4 μl/ml RA-free B27 supplement (all media components from Invitrogen). Where indicated, media were supplemented with 1 μM all-*trans*-retinoic acid (Sigma). For long-term culture, defined medium was replaced after 3 days with doxycycline-free serum replacement medium (SRM5) consisting of high glucose DMEM supplemented with 5% knockout serum replacer (Invitrogen), 10^{-4} M β-mercaptoethanol, 0.224 μg/ml L-glutamine, and 1.33 μg/ml HEPES to enhance cell survival. Where indicated, recombinant growth factors (all from R&D Systems, Minneapolis, MN) were added to freshly prepared media at the following concentrations: 25 nM (high) or 2.5 nM (low) mouse Shh; 5 nM human BMP4; and 5 nM (high) or 2 nM (low) Noggin-F_c chimera. To examine the role of FGF signaling, cultures were exposed at

intervals to 5 nM SU5402 (Calbiochem) for 12 hr to block FGFR1 signaling.

Proliferation and Cell Cycle Analysis

To analyze the effects of transgene induction on proliferation, Ainv15 and N7 ESC were plated at 1×10^5 cells/well in 12-well plates in triplicate (with four biological replicates) in complete ES medium±2 μg/ml doxycycline. After 24, 48, and 72 hr, cells were disaggregated using trypsin and counted in a Coulter counter (Becton Dickinson).

For cell cycle analysis, cells were plated in triplicate at 2×10^5 in six-well plates in complete ES medium±2 μg/ml doxycycline. After 72 hr, cells were fixed in cold absolute ethanol, suspended in PBS and DNAse free RNase A (Sigma) at a final concentration of 20 μg/ml. Samples were incubated at 37° for 30 min and DNA labeled with 0.1 mg/ml propidium iodide (Sigma). Analysis was done in a FACS Calibur using Cell Quest Pro MAC 9.0 and cell cycle analysis done with Mod Fit LT Mac 3.1 SP3.

RT-PCR

RNAs were extracted from lysates of cells treated with TRIzol reagent (Invitrogen), DNAsed, and quantified by spectrophotometry. Control RNA samples were obtained from N7 cells cultured in ES medium and from pooled samples of gestation day 9, 10, and 11 mouse embryos. RNAs (1 μg each) served as templates in reverse transcription reactions with oligo-dT primers, and 1/20 of the single-strand cDNA products were used in each PCR amplification. General PCR conditions were 94°C/3 min, 94°C/30 s, 53–63°C/1 min, 72°C/1 min for 25–40 cycles. Specific information regarding PCR primer sequences and reaction conditions is provided in the on-line supplementary material. The PCR products (10 μl each) were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, visualized on a ultraviolet transilluminator and digitally photographed using a Bio-Rad Gel Documentation system. Primers and conditions are summarized in Supplemental Table 3.

Immunohistochemistry

To examine cell type-specific and positional markers, cells were fixed in 2% paraformaldehyde for 15 min at 25°C, then stored in PBS at 4° before processing for immunohistochemistry (IHC). Briefly, cells were permeabilized and nonspecific antibody binding blocked using host serum before incubation with primary antibodies at 4° overnight. Cells were washed, then exposed to secondary antibody conjugated to Cy3 or FITC (Jackson ImmunoResearch Laboratories) for 30 min at 25°C. Nuclear staining for total cell number counts was achieved by incubating cells with 1 μM Hoechst 33285 (Sigma) for 2 min. Cells were washed, then mounted using Prolong Gold (Molecular Probes/Invitrogen) and 25 mm coverslips. Cells were examined, counted and photomicrographs imported into Photoshop using a Leica DMIRB inverted microscope and Olympus digital camera.

Primary antibodies included: Sox3 (Mike Klymkowsky; 1:1,000) expressed by in neural ectoderm and neuronal precursors (Zhang et al., 2003), Tuj1 (Covance MMS-435P; 1:250) which recognizes a neuron-specific class III β-tubulin expressed very early in neuronal differentiation and persisting in mature neurons (Moody et al., 1987), peripherin (7C5, AbCam ab4573, 1:1,000) which is expressed in the PNS, Brn3a (Eric Turner, 1:1,000) expressed in sensory neurons, tyrosine hydroxylase (Chemicon AB5968, 1:1,000) expressed in adrenergic neurons, Oct4 (Santa Cruz sc-8628, 1:100) expressed in undifferentiated ES cells. Dorsal–ventral markers included: Pax7 (Developmental Studies Hybridoma Bank supernatant, 1:20) labeling dorsal neural progenitors, Nkx6.1 (N15, Santa Cruz sc-15027, 1:100) expressed in ventral progenitors, and Islet 1 which recognizes Islet 1 and Islet 2 (Developmental Studies Hybridoma Bank, 39.4D5, 1:100) labeling ventral motor neurons and dorsal root (sensory) ganglia, or Dbx2 expressed in the middle zone of the neural tube (Agalliu and Schieren, 2009).

For quantitative analysis of the cell types present, cell counts were performed on 10 microscopic fields at ×20 from at least two replicate cultures, counting at least 1,000 cells per replicate. For each field, the number of a given cell type was divided by the total number of cells present as determined by Hoechst nuclear staining to calculate a percentage for each field which was then summed to obtain a mean percentage for each replicate. The data presented are the overall mean±SEM across all replicates. Data were analyzed using ANOVA, Students *t*-, or Chi-square test where appropriate (with Bonferroni correction as necessary for multiple comparisons); a P value of≤ 0.05 was considered significant.

Microarray Analysis

RNAs were extracted as described above from triplicate samples (cells differentiated in three independent experiments) of control N7 cells (-Dox) at 24-hr culture intervals and N7 cells exposed to doxycycline for 24, 48, and 72 hr. RNA was hybridized to Affymetrix Mouse 430 2.0 arrays (Santa Clara, CA, http://www.affymetrix.com) by the NIH NINDS arraying core at TGen (http://www.tgen.org). The signal intensity of each array was normalized to a mean of 150 by robust multiarray averaging (RMA), then GeneSpring (Agilent) software was used to select genes with≥two-fold change between groups. At each time point, N7 cells (−Dox) were used as the reference for comparison with +Dox samples. Welch t-tests were then carried out to identify significant differences between controls and *Neurogenin1*-expressing cells. Gene expression was also compared in +Dox (and −Dox control) samples at sequential time points (e.g., 24 vs. 48 hr, 48 vs. 72 hr, and 24 vs. 72 hr of transgene induction). Benjamini-Hochbert false discovery corrections were performed on each comparison to reduce the number of false-positive results. Finally, Gene Ontology, Functional Annotation Clustering, and KEGG pathway analyses were carried out using DAVID V 6.7 (Huang et al., 2009).

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