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RESEARCH ARTICLE

Notch signaling regulates the differentiation of neural crest from human pluripotent stem cells

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ABSTRACT

Neural crest cells are specified at the border between the neural plate and the epiderm. They are capable of differentiating into various somatic cell types, including craniofacial and peripheral nerve tissues. Notch signaling plays important roles during neurogenesis; however, its function during human neural crest development is poorly understood. Here, we generated self-renewing premigratory neural-crest-like cells (pNCCs) from human pluripotent stem cells (hPSCs) and investigated the roles of Notch signaling during neural crest differentiation. pNCCs expressed various neural-crest-specifier genes, including *SLUG* (also known as *SNAI2*), *SOX10* and *TWIST1*, and were able to differentiate into most neural crest derivatives. Blocking Notch signaling during the pNCC differentiation suppressed the expression of neural-crest-specifier genes. By contrast, ectopic expression of activated Notch1 intracellular domain (NICD1) augmented the expression of neural-crest-specifier genes, and NICD1 was found to bind to their promoter regions. Notch activity was also required for the maintenance of the premigratory neural crest state, and the suppression of Notch signaling led to the generation of neural-crest-derived neurons. Taken together, we provide a protocol for the generation of pNCCs and show that Notch signaling regulates the formation, migration and differentiation of neural crest from hPSCs.

KEY WORDS: Human embryonic stem cells, Human induced pluripotent stem cells, Neural crest, Notch signaling

INTRODUCTION

Neural crest cells are found in all vertebrate embryos, and this transient cell population can differentiate into neurons and glial cells of the peripheral nervous system, skin melanocytes, craniofacial cartilage, dentin, odontoblasts and alveolar bone (Dupin et al., 2007). Early disruption of neural crest development can lead to a variety of congenital disorders known as neurocristopathies, whereas disruption at later stages can contribute to cancers such as melanoma (Etchevers et al., 2006; Kokta et al., 2013). Neural crest cells originate from neuroectoderm at the border between the neural plate and the epiderm (Meulemans and Bronner-Fraser, 2004), and they are marked by the expression of genes that are specific for the neural-plate border, such as *DLX5*, *MSX1*, *MSX2* and *ZIC1*. Later, during the neural-tube folding process, neural crest cells remain within the neural folds and subsequently localize inside the dorsal portion of the neural tube. These premigratory neural crest cells express specifier genes, such as *SNAIL* (also known as *SNAI1*), *SLUG* (also known as *SNAI2*), *SOX10* and *TWIST1* (LaBonne and Bronner-Fraser, 2000; Mancilla and Mayor, 1996). Following the formation of the neural tube, premigratory neural crest cells undergo an epithelial-to-mesenchymal transition (EMT) and become migratory neural crest cells. Migratory neural crest cells delaminate from the neuroepithelium and migrate away from the neural tube to various embryonic tissues prior to giving rise to specialized cell types.

In vivo, the specification of neural crest cells from the embryonic ectoderm is directed by multiple signaling pathways including the BMP, FGF, Wnt, TGF-β and Notch pathways (Stuhlmiller and García-Castro, 2012). The canonical Notch pathway has five ligands (Delta-1, -3 and -4 and Jagged-1 and -2) that interact with four receptors (Notch-1–Notch-4) (Perdigoto and Bardin, 2013). Upon ligand–receptor binding between adjacent cells, the Notch receptors are cleaved, and the resulting cytoplasmic Notch intracellular domain (NICD) translocates into the nucleus and forms a transcription activator complex with a DNA-binding protein, CBF1 (Schroeter et al., 1998). The complex then binds to the consensus sequence (C/T)GTGGGAA (Tun et al., 1994), and activates the transcription of target genes (Hsieh et al., 1996; Jarriault et al., 1995). Notch-mediated lateral inhibition prevents neurogenesis and, in some cases, has been shown to promote neural crest formation and development (Humphreys et al., 2012; Mead and Yutzey, 2012). Notch is required for the differentiation of neural-crest-derived vascular smooth muscle and the maintenance of enteric nerve cells (Okamura and Saga, 2008). Nevertheless, the role(s) of Notch signaling in the specification and differentiation of the human neural crest lineage have not been reported.

Human pluripotent stem cells (hPSCs) are widely used to study the molecular mechanisms underlying early human development. The differentiation of hPSCs follows hierarchical signaling cascades that also regulate embryonic development (Hay et al., 2008; Wu et al., 2010). Protocols to derive neural crest cells from hPSCs are available (Lee et al., 2007; Menendez et al., 2011), but they produce migratory neural crest cells [marked by the expression of *p75* (also known as *NGFR*) and *HNK1* (also known as *CD57* and *B3GAT1*)]. Both premigratory and migratory neural crest cells are able to generate neuronal and non-neuronal

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descendants (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988); however, migratory neural crest cells will undergo progressive lineage specification towards terminally differentiated cell types, which might restrict their usefulness for research purposes (Hari et al., 2012; Schwarz et al., 2009). Therefore, premigratory neural crest cells would be ideal for studying the signaling pathways that control the specification of neural crest fate.

In this paper, we report a protocol for the differentiation and long-term maintenance of premigratory-neural-crest-like cells (pNCCs) from hPSCs. Furthermore, we use the protocol to investigate the crucial roles of Notch signaling in the commitment of hPSCs to a neural crest fate, the maintenance of the premigratory neural crest state and the differentiation of premigratory neural crest cells upon Notch inhibition.

RESULTS

Derivation of pNCCs from hESCs

Capturing the early phase of the differentiation of the neural crest lineage is an important step in revealing the signaling requirements for commitment to the neural crest cell fate. To generate a naïve premigratory neural crest cell type, we modified a previous differentiation protocol that used Wnt-mediated activation together with BMP inhibition (Menendez et al., 2011), by replacing StemPro human embryonic stem cell (hESC) medium with N2B27 neural differentiation medium and withdrawing TGF-β inhibition. After 10 days of induction, these cells robustly expressed neural-crest-specifier genes, including *PAX3*, *TWIST1*, *SLUG*, *MSX2*, *HAND2* and *SOX10* (Fig. 1A–C), whereas the expression of the pluripotency genes *OCT4* and *NANOG* was undetectable (supplementary material Fig. S1). Of note, our neural crest differentiation protocol specifically induced pNCCs but not neural progenitor cells, as indicated by the lack of expression of neural marker genes [*PAX6*, *NGN2* (also known as *NEUROG2*) and *SOX1*] after 10 days of differentiation in conditions that induce neural crest (supplementary material Fig. S1). Similar neural crest induction was also observed in induced pluripotent stem cells (iPSCs) (supplementary material Fig. S2). The pNCCs sustained the expression of neural crest marker genes and neural crest differentiation capacity in long-term culture (after more than 20 passages), suggesting that the induction conditions can also maintain pNCC identity (supplementary material Fig. S3). pNCCs stained homogeneously positive for PAX3, SLUG, SOX10 and TWIST1; however, only a few of them were positive for AP-2α, a marker of migratory neural crest cells (Fig. 1B). The efficiency of the derivation of clonal pNCC lines (Fig. 1D) was 78.1%±2.4% (±s.d.). These pNCCs possessed the typical differentiation capacity of neural crest cells, as shown by a series of differentiation experiments. Mesenchymal lineage derivatives were obtained after 2 weeks of directed differentiation with commercially available kits. The resulting adipocytes exhibited lipid-droplet-containing cells and were stained by Oil Red O, whereas chondrocytes and osteocytes were detected by Alcian Blue and Alizarin Red, respectively (Fig. 1E). When pNCCs were cultured in N2B27 medium without Wnt activation or BMP inhibition, neural-like cells were spontaneously formed in some areas. These cells were positive for Tuj1 and peripherin, and a few of them became GFAP-positive glial cells (Fig. 1F).

During neural crest differentiation, we found that the Notch signaling pathway was activated, as shown by an increased level of Notch1 intracellular domain (NICD1) protein, but not NOTCH1 protein (Fig. 1G). In addition, we observed a significant upregulation in the expression of the Notch effector genes *HES1* and *HES5*, as well as the gene encoding a Notch ligand, *JAG1*, during pNCC commitment (Fig. 1H).

Notch is indispensable for the differentiation of neural crest from hESCs

Notch signaling has been reported to suppress neural differentiation and neurogenesis (Geling et al., 2004). To establish the role of Notch in neural crest differentiation, we performed loss- and gain-of-function experiments. First, we inhibited Notch signaling by using either a chemical inhibitor (DAPT) or *JAG1* knockdown during the pNCC differentiation process. We applied DAPT at two different concentrations, 10 μM and 20 μM, for 10 days and collected RNA samples for gene expression analyses. At day 10, the expression of neural-crest-specifier genes (*DLX5*, *PAX3*, *SLUG*, *SOX10* and *TWIST1*) was strongly suppressed by both concentrations of DAPT, whereas the expression of the neural genes *NGN2* and *NGN3* was upregulated (Fig. 2A). The fate switch from neural crest to neural lineage was explicitly shown by the detection of NGN2- and NGN3-immunopositive cells following DAPT treatment for 10 days (Fig. 2B,C). We then confirmed the effect of Notch inhibition by silencing the expression of *JAG1*, a crucial ligand for Notch signaling that induces cell differentiation (Ba et al., 2012), and which was significantly upregulated in pNCCs (Fig. 1H). The *JAG1* knockdown hESC clones displayed ~60% reduction in the mRNA level of *JAG1*, leading to a substantial reduction in JAG1 protein expression, as validated by western blotting (Fig. 2D). The cells with lowered *JAG1* gene expression displayed diminished expression of all the neural-crest-specifier genes that were studied, whereas a neural marker, *NGN2*, was induced (Fig. 2E). In addition, the *JAG1*-deficient cells failed to produce neural crest derivatives (Fig. 2F). These data suggest that Notch activation is required for the formation of pNCCs by two possible mechanisms; (1) the activation of the expression of neural-crest-specifier genes and/or (2) the repression of the expression of neural-lineage genes (Cornell and Eisen, 2002).

We next ascertained the role of Notch signaling in the specification of neural crest cells by comparing global gene expression profiles of pNCCs in the presence or absence of Notch inhibition (20 μM DAPT) at day 10 of differentiation (Fig. 3A–D; all raw data are available at GEO, accession number GSE53203). The 20 most highly up- and down-regulated genes are listed in supplementary material Tables S1 and S2. Gene expression profiles were clustered as neural crest/neural-related genes (Fig. 3A), Notch-related genes (Fig. 3B) and lineage-specific genes (supplementary material Fig. S4). Several well-known neural crest lineage genes *(BMP2*, *CDH1*, *DLX5*, *HAND1*, *ID1*, *KAL1*, *PAX3*, *SNAIL2*, *WNT3A*, *SOX9* and *TWIST1*) were suppressed by DAPT (Fig. 3A). By contrast, a number of neural lineage genes *(NGN1*, *NGN2*, *MASH1 NEUROD1* and *PAX6*) were upregulated when DAPT was applied (supplementary material Fig. S4). Some of these observed differences in gene expression were confirmed by real-time PCR (Fig. 3C). Of note, untreated pNCCs expressed various cell-surface markers (e.g. *CD44*, *ITGB2* and *CDH1*; Fig. 3A) that could be advantageous for cell isolation. Besides lineage-specific genes, we analyzed the effect of DAPT on genes implicated in the Notch signaling pathway (Fig. 3B). As expected, we found that a number of Notch genes, for example *NOTCH1*, *NUMBL*, *JAG1*, *JAG2*, *HES1*, *HES5*, *LFNG* and *NCSTN*, were suppressed upon DAPT

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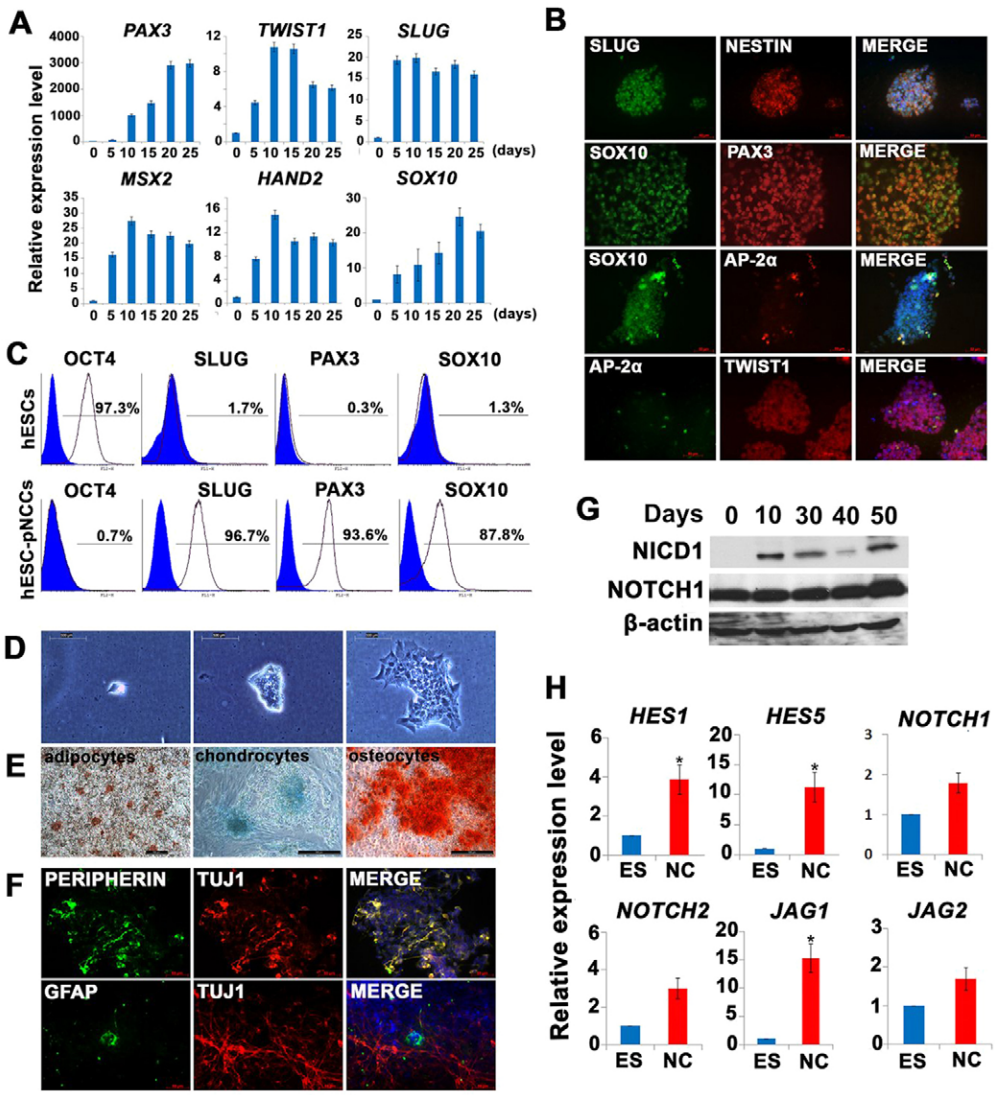

Fig. 1. Notch signaling is activated during neural crest differentiation. (A) Induced expression of neural-crest-specifier genes was assessed by using real-time PCR at different time-points during the differentiation. Fold changes were calculated relative to hESCs, after normalization to cyclophilin G. The data are presented as the mean±s.d.; $n=3$. (B) The expression of PAX3, Nestin, TWIST, SLUG and SOX10 was assessed by using immunostaining. Only a fraction of the cells was positive for AP-2α, a marker of migratory neural crest cells. (C) The purity of the pNCC population was evaluated by flow cytometric analyses. PAX3, SLUG and SOX10 staining was evident in nearly all cells whereas none of the cells were positive for OCT4, a marker of undifferentiated hESCs. Blue, IgG control; white, specific antibody. (D) Differentiation of clonally derived pNCCs to neural crest derivatives. Representative phase-contrast images of the clonal derivation of pNCCs. pNCCs were plated at a density of 1 cell/well onto 96-well plates and were cultured in neural-crest-induction medium. Scale bar: 500 µm. (E) Phase-contrast images of adipocytes, chondrocytes and osteocytes derived from clonally derived pNCCs. Adipocytes are recognized as lipid droplet-containing cells stained red with Oil Red O, chondrocytes as blue Alcain-blue-stained cells and osteocytes as red Alizarin-Red-stained cells. Scale bars: 100 µm (left); 500 µm (middle and right). (F) The differentiation of pNCCs to neural lineages was confirmed by peripherin, Tuj1 and GFAP immunostaining. (G) Activation of the Notch pathway during neural crest differentiation was proved by the presence of NICD1, as shown by western blotting. (H) The induction of Notch-related genes was measured in hESCs (ES) and pNCCs (NC) by using real-time PCR. The data are presented as the mean±s.d.; $n=3$; $^*P<0.05$ (Student's *t*-test).

treatment (Fig. 3B). Pathway analysis revealed that several additional factors that are known to be linked to Notch signaling were affected by DAPT treatment during neural crest specification. Among these, there were several novel potential genes that might be regulated by Notch, such as *CER1*, *FOSB*, *FOXC1* and *IGFBP4* (Fig. 3D).

NICD1 positively controls the expression of neural crest genes

We next tested one of the possible mechanisms by which Notch could augment the expression of neural-crest-specifier genes. We first constructed a constitutive expression vector for human NICD1 (Fig. 4A) and validated its activity by performing both a *HES1* promoter assay and a *CBF1* responsive element (*CBF1RE*)-luciferase activity assay. When transfected into HEK 293 cells, NICD1 significantly activated the *HES1* promoter and *CBF1RE*-luciferase activity (Fig. 4B). We then stably transfected hESCs and iPSCs with this construct and confirmed the overexpression of NICD1 by western blotting (Fig. 4C). To investigate the effect of NICD1 overexpression on neural crest differentiation, we followed the expression profiles of neural-crest-specifier genes at various days during the differentiation (Fig. 4E,F). Cell

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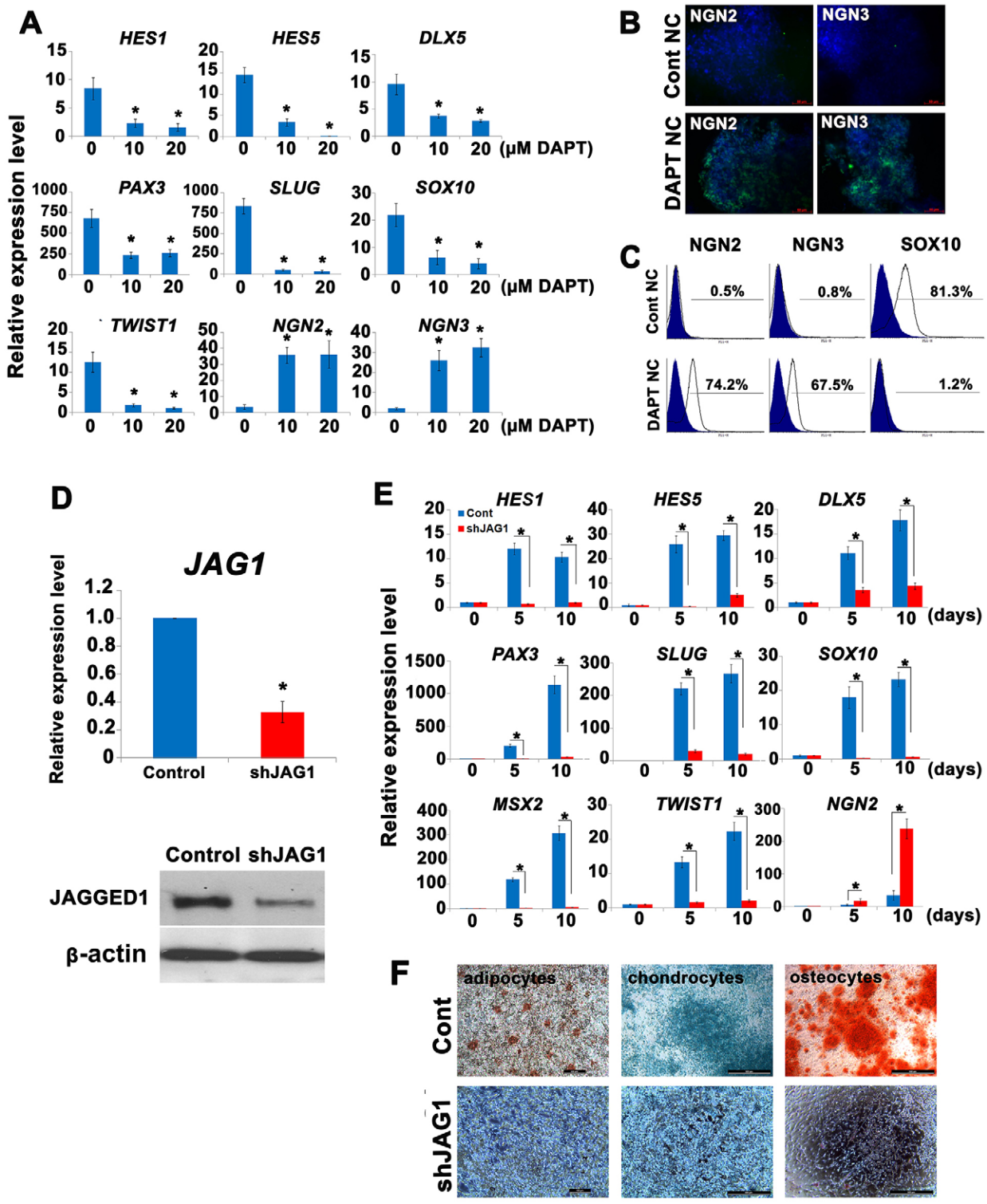

Fig. 2. See next page for legend.

morphology and the expression of pluripotency-associated genes in undifferentiated NICD1-overexpressing hESCs were normal (Fig. 4D). Upon differentiation towards pNCCs, the expression of the neural-crest-specifier genes *PAX3*, *TWIST1*, *SLUG* and *DLX5* was dramatically upregulated in NICD1-overexpressing cells in two hESC clones (data from one representative clone is shown) and in one iPSC clone (Fig. 4E). The expression of these genes was amplified almost tenfold in NICD1-overexpressing cells at day 10 as compared with control cells (i.e. without NICD1 overexpression). In addition, we detected TWIST1-immunopositive cells and cells that were doubly positive for SOX10 and PAX3, and SLUG and SoxE as soon as day 5 of NICD1 overexpression, whereas these proteins were undetectable in control cells at this time-point (Fig. 4F). This result raised the

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Fig. 2. Inhibition of Notch signaling prevents commitment to the neural crest fate. (A) Supplementation with DAPT (10 μM or 20 μM) inhibited the expression of neural-crest-specifier genes, as assessed at day 10 of neural crest differentiation. By contrast, the expression of neural genes (*NGN2* and *NGN3*) increased significantly. Fold changes are relative to undifferentiated hESCs after normalization to cyclophilin G. (B) Immunostaining of NGN2 and NGN3 in hESCs that were differentiated in neural-crest-induction medium with (DAPT NC) or without (Cont NC) 20 μM DAPT for 10 days. Clear positive staining is observed only after DAPT treatment. Scale bars: 50 μm. (C) Flow cytometric analysis of NGN2, NGN3 and SOX10 in control (Cont) and DAPT-treated pNCCs indicated a loss of SOX10-positive cells, whereas most of the cells became NGN2 and NGN3 positive. (D) Real-time PCR and western blotting analyses of hESCs that were stably transfected with a construct expressing *JAG1* shRNA (shJAG1) confirmed the knockdown of *JAG1* mRNA and protein expression (>60% mRNA reduction). (E) The knockdown of *JAG1* resulted in significantly reduced expression of neural-crest-specifier genes, whereas *NGN2* expression was induced at day 5 and 10 of neural crest differentiation as compared with wild-type hESCs. (F) Phase-contrast images of adipocytes, chondrocytes and osteocytes derived from control and *JAG1*-knockdown hESC-derived pNCCs. No mesenchymal differentiation could be detected in *JAG1*-knockdown cells. Adipocytes are recognized as red lipid-droplet-containing cells stained by Oil Red O, chondrocytes as blue Alcain-blue-stained cells and osteocytes as red Alizarin-Red-stained cells. Scale bars: left 100 μm, middle and right 500 μm. The data are presented as the mean±s.d.; $n=3$; $^*P<0.05$ (Student's *t*-test).

possibility that NICD1 might directly bind to the regulatory regions of neural-crest-specifier genes. We therefore investigated the binding capability of NICD1 by using chromatin immunoprecipitation (ChIP). We focused on the 5′ promoter regions of neural-crest-specifier genes that were previously found to be induced by NICD1. The DNA regions under investigation contained the recognition sequence of the NICD1–CBF1 complex (Fig. 4G) (Tun et al., 1994). Significantly, NICD1 protein bound to the regulatory regions of neural-crest-specifier genes in pNCCs, but not in hESCs (Fig. 4H). *HES1* and *HES5*, the Notch effector genes, were used as positive controls. These results suggest that NICD1 acts as a direct activator of the transcription of neural-crest-specifier genes and thereby promotes the differentiation of neural crest from hESCs.

Notch signaling modulates the fate of pNCCs

During early fetal development, neural crest cells migrate along the medial and lateral pathways around the developing somites, and simultaneously undergo progressive restriction of cell fates (Bronner and LeDouarin, 2012; Theveneau and Mayor, 2012). Notch is known to preserve the stem cell state in various tissues (Maillard et al., 2008), and FGF8 is recognized as a chemotactic signal for the migration of neural crest cells (Sato et al., 2011). In our wound-healing experiments, pNCCs were not able to migrate, even when the medium was supplemented with 100 ng/ml FGF8 (Fig. 5A). By contrast, when both FGF8 (100 ng/ml) and DAPT

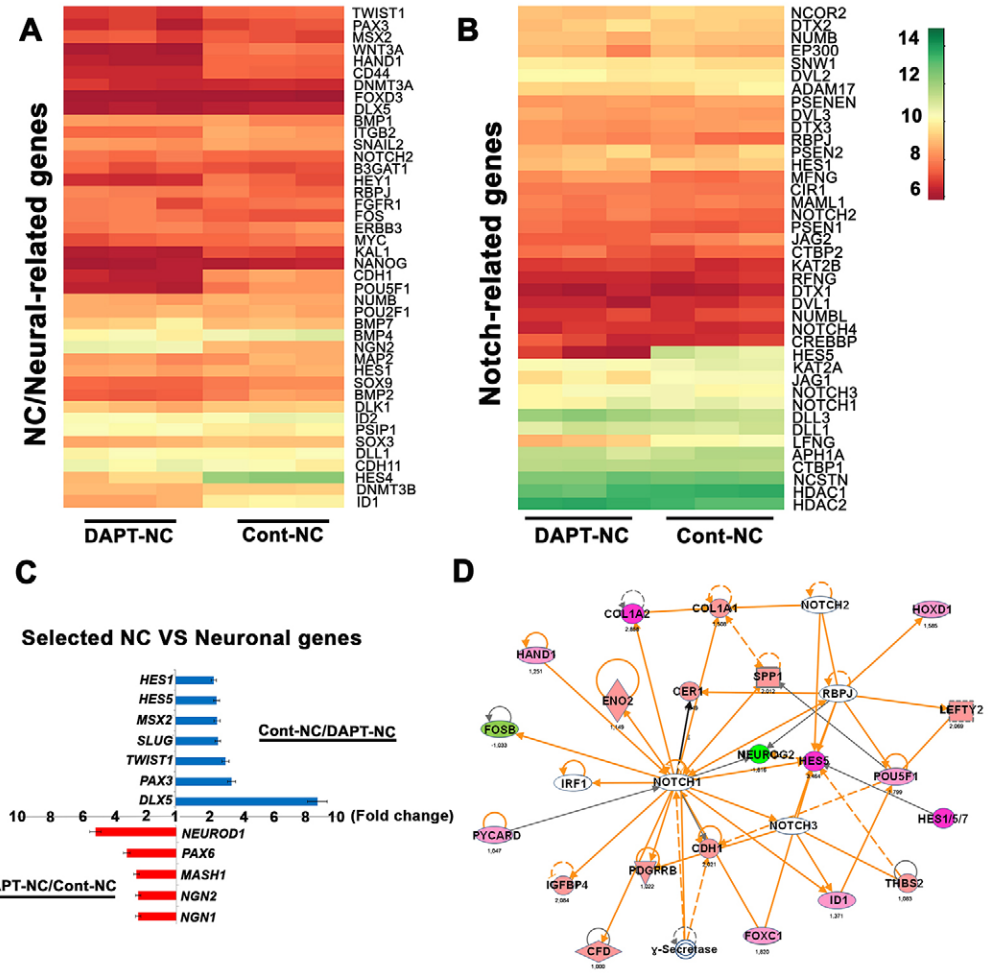

Fig. 3. Global gene expression analysis of control and DAPT-treated pNCCs. Three independently replicated sample sets were used for the analysis. (A) Microarray results for neural crest (NC)/neural-related genes. (B) Microarray results for Notch-related genes. Green, upregulated transcripts; red, downregulated transcripts; DAPT-NC, DAPT-treated pNCCs; Cont-NC, control pNCCs. (C) The comparative expression level from microarray samples was validated by real-time PCR. The data are presented as the mean±s.d.; $n=3$. (D) Notch pathway analysis showing the signaling network that is controlled by Notch during neural crest differentiation. Red indicates higher expression and green indicates lower expression in control neural crest cells when compared with neural crest cells grown in the presence of DAPT.

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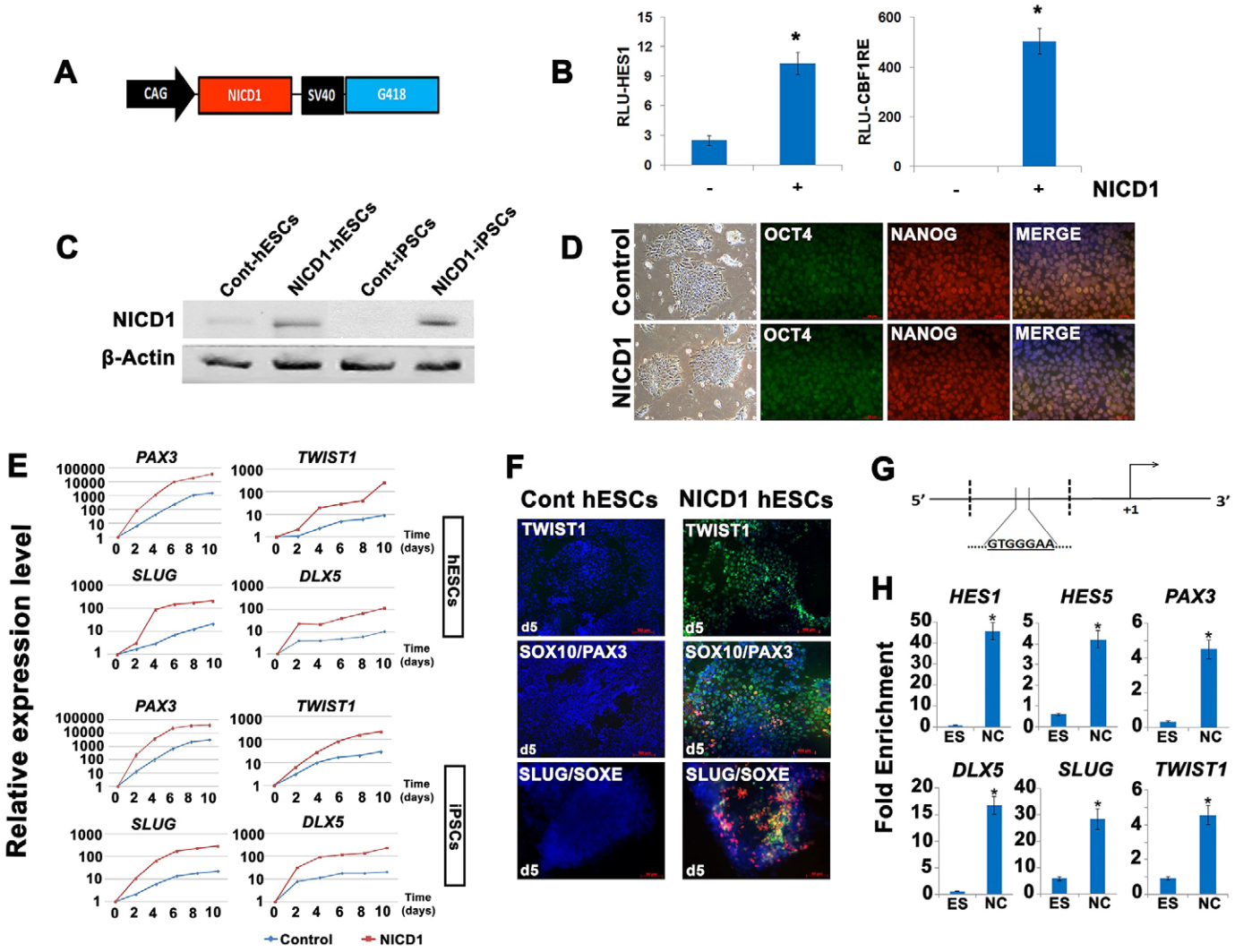

Fig. 4. Notch directly controls the expression of neural-crest-specifier genes. (A) A schematic of the human NICD1 overexpression construct. (B) Luciferase activity assays in HEK 293 cells showed the dramatic activation of the *HES1* promoter and CBF1-responsive element (*CBF1RE*) reporters by NICD1-overexpression, showing the functionality of the transfected construct. (C) Western blotting confirmed the stable overexpression of NICD1 in human ESCs and iPSCs. (D) Phase-contrast images of control and NICD1-overexpressing hESCs. The ectopic NICD1 expression did not affect the properties of undifferentiated hESCs. Immunostaining of OCT4 and NANOG is shown. (E) Quantitative real-time PCR analyses showed faster and higher induction of neural-crest-specifier genes in NICD1-overexpressing cells than in control cells during the neural crest differentiation. The data are presented as the mean expression level from three experiments. (F) Immunostaining of TWIST1, SOX10 (green) and PAX3 (red), and SLUG (green) and SOXE (red) at day 5 in control hESCs (left) and NICD1-overexpressing hESCs (right) during neural crest differentiation indicated that the neural-crest-specifier proteins could be detected earlier in NICD1-overexpressing cells as compared with expression in control cells. (G) A schematic of a representative promoter region with the NICD1-binding sequence shown. The studied DNA fragments contained the recognition sequence for the NICD1–CBF1 complex. (H) ChIP was performed in hESCs (ES) and pNCCs (NC) with an anti-NICD1 antibody, and the NICD1-bound DNA fragments were amplified with specific primers. The data are normalized to the IgG input value and presented as the mean±s.d.; $n=3$; *$P<0.05$ (Student's *t*-test).

(20 μM) were added, the cells moved across the wound in 24 hours (Fig. 5A). Simultaneously, *AP-2α*, *HNK1* and *NGFR*, markers of migratory neural crest cells, were upregulated. The induction was seen only when both FGF8 and DAPT were applied, but not if FGF8 was given alone (Fig. 5B,C). Upon the treatment of pNCCs with FGF8 and DAPT for 3 days, the number of AP-2α-immunopositive cells substantially increased from 5.7%±0.8 to 52.7%±2.2 (±s.d.; Fig. 5D), and a similar increase was seen in the number of HNK1-positive and NGFR-positive cells (Fig. 5E). *In vivo*, neural crest cells give rise to neurons in the peripheral nervous system (PNS) and are also a source of sensory neurons (Cimadamore et al., 2011; Raible and Ungos, 2006). Interestingly, when pNCCs were treated with 20 μM DAPT alone for only 3 days, the cells appeared as filamentous neurons throughout the culture dishes (Fig. 6C). This appears to be a simple way to induce the neuronal differentiation of neural crest cells without supplementation with neurotrophic factors, as suggested previously (Lee et al., 2007; Menendez et al., 2011). Simultaneously, NICD1 was completely abolished, in accordance with a significant suppression of *HES1* and *HES5* expression (Fig. 6A). DAPT-treated pNCCs significantly upregulated the expression of *DBH* and *PHOX2B* (markers of neural-crest-derived neurons), and the neuronal genes *MASH1*, *NEUROD1* and *NGN2* (Fig. 6B). In addition, we detected cells that were doubly positive for

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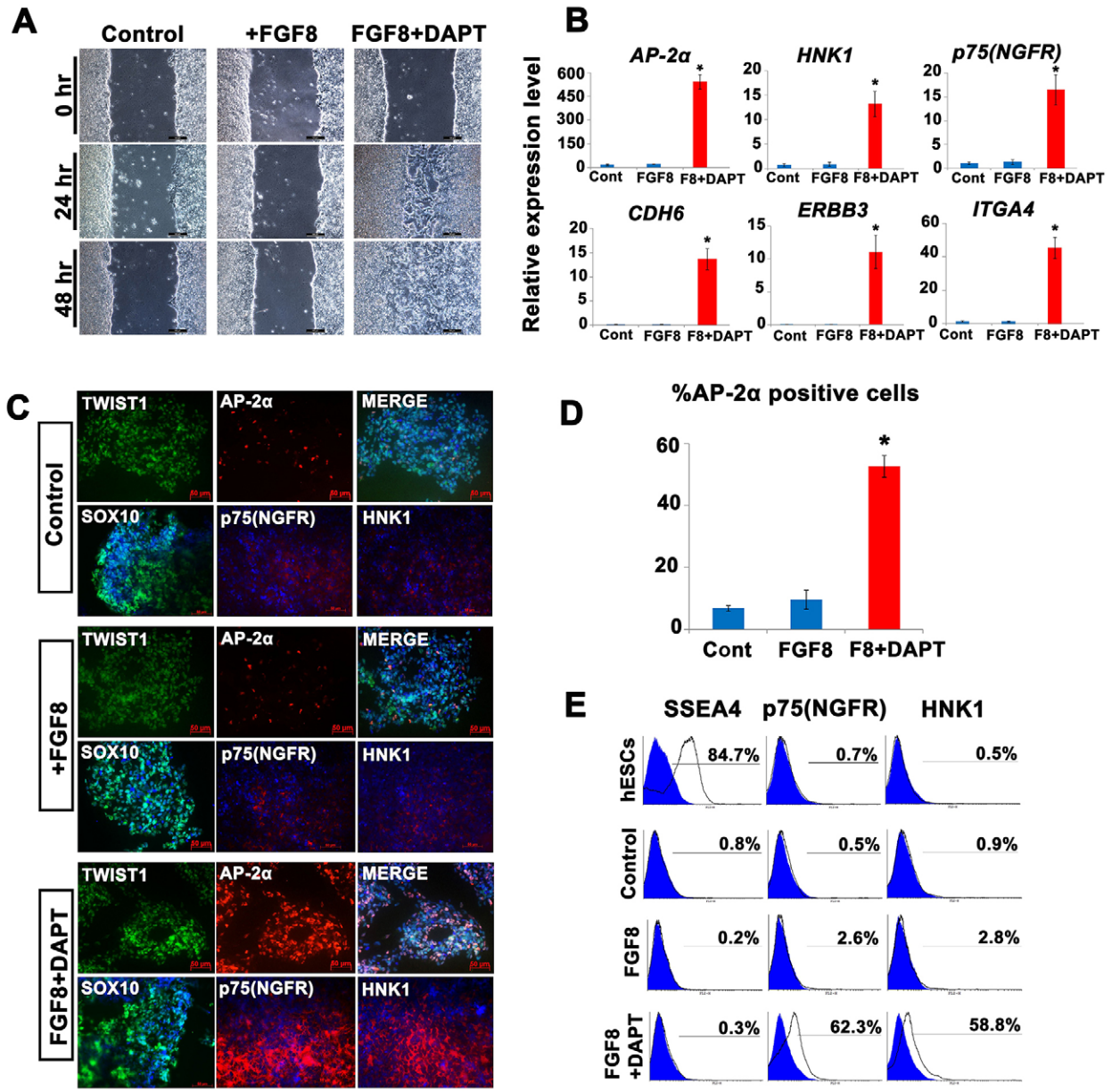

Fig. 5. A combination of Notch inhibitor (DAPT) and FGF8 induces migration of pNCCs. (A) Wound-healing assays of untreated (control) pNCCs and pNCCs treated with either FGF8 alone or FGF8 and DAPT. Without the combination of FGF8 and DAPT, pNCCs did not migrate, whereas after treatment with FGF8 and DAPT the cells rapidly filled the wound. Scale bars: 100 µm. Co-treatment with FGF8 and DAPT induced the expression of marker genes of migratory neural crest cells as shown by real-time PCR (B) and immunocytochemistry (C). (D) Following FGF8 and DAPT treatment, a significant increase in the number of AP-2α-positive cells was observed. (E) Flow cytometric analyses of SSEA4 (a hPSC marker), p75 (NGFR) and HNK1 was performed for hESCs, control pNCCs and pNCCs treated with FGF8 alone or FGF8 plus DAPT. Blue, IgG control; white, specific antibody. The data are presented as the mean±s.d.; $n=3$; *$P<0.05$ compared with control pNCCs (by Student's *t*-test).

peripherin and Tuj1, which marked neural-crest-derived peripheral neurons (Fig. 6C). Some of these cells showed peripheral sensory neuron (positive for BRN3A and peripherin) and autonomic neuron (positive for PHOX2B and Tuj1) phenotypes (Fig. 6C). Quantitatively, the proportions of DAPT-treated pNCCs that became BRN3A-positive cells and PHOX2B-positive cells were 39.8%±3.1 and 13.4%±2.7, respectively (Fig. 6D).

DISCUSSION

Previous protocols for generating neural crest cells from pluripotent stem cells have resulted in cellular phenotypes and gene expression patterns consistent with migratory neural crest cells (Lee et al., 2007; Menendez et al., 2011). However, the migration of neural crest cells from the neural tube to peripheral tissues is rapidly followed by sequential differentiation of the cells according to instructions received from the surrounding tissues (Wilson et al., 2004). In this study, we have developed a protocol to derive a more naïve pNCC form from hPSCs. These pNCCs can easily be maintained with the potential to differentiate towards neural crest derivatives (Bronner-Fraser and Fraser, 1988). Our differentiation protocol efficiently generates pNCCs, but not neural lineage cells, from hPSCs. By employing hESC-derived

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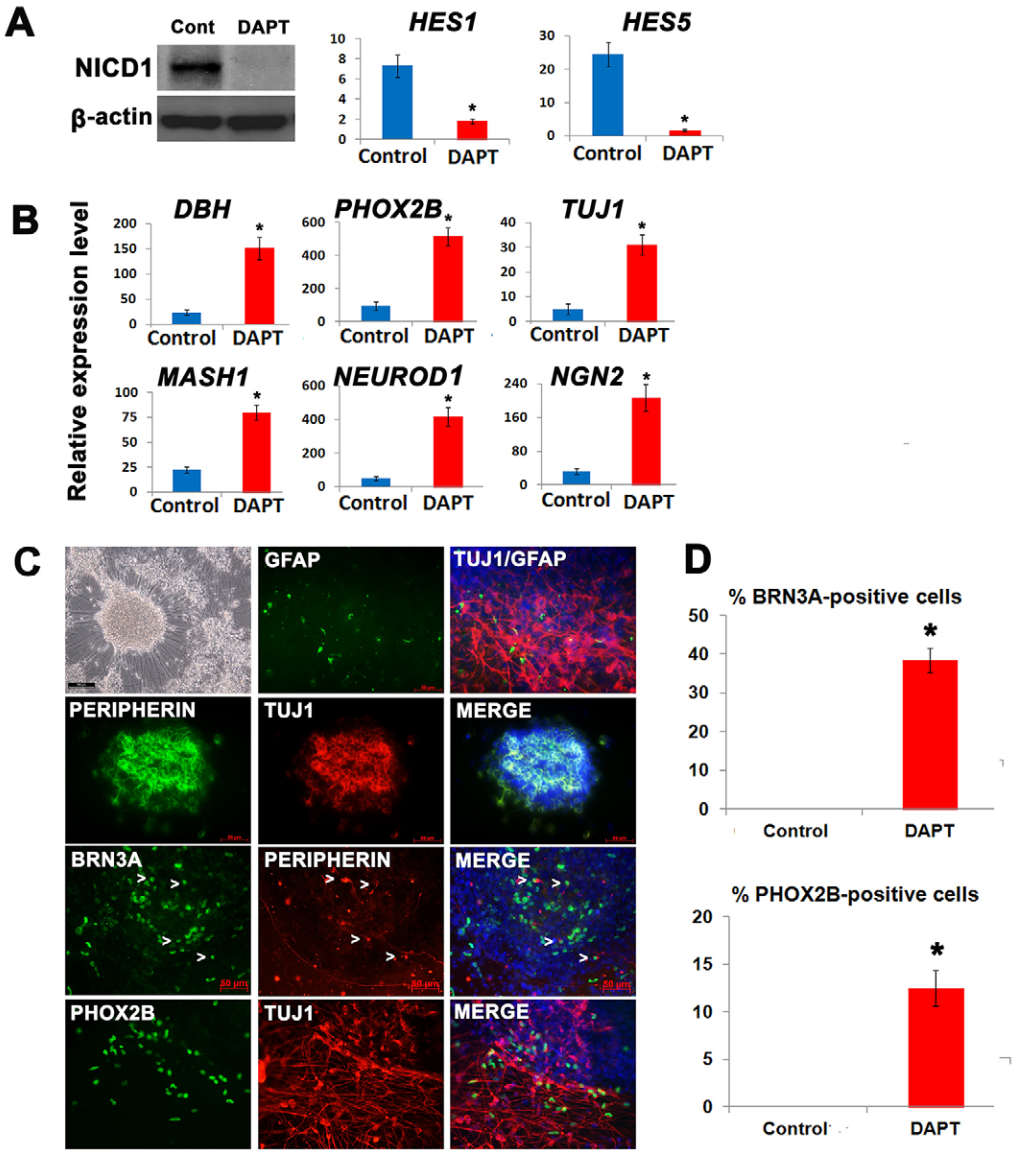

Fig. 6. Neural-crest-derived neurons are generated upon inhibition of Notch signaling. (A) Western blot of NICD1 and real-time PCR of the Notch target genes *HES1* and *HES5* in hESC-derived pNCCs following treatment with the Notch inhibitor DAPT. (B) The expression of neural genes was significantly upregulated in DAPT-treated pNCCs. Relative expression levels were measured by using real-time PCR, and the fold changes were calculated relative to hESCs, after normalization to cyclophilin G. (C) Phase-contrast imaging and immunostaining of pNCCs that were treated with DAPT for 3 days. Treatment with DAPT resulted in the generation of a mixture of neural cell lineages. Immunostaining was performed for GFAP, TUJ1, peripherin, BRN3A and PHOX2B. Arrowheads indicate the co-staining of BRN3A and peripherin, suggesting the formation of peripheral sensory neurons. (D) The percentages of BRN3A-positive and PHOX2B-positive cells were determined from cells visualized using the fluorescent microscope. The data are presented as the mean±s.d.; $n=3$; $^*P<0.05$ (Student's *t*-test).

pNCCs, we show that Notch signaling regulates various fate decisions of neural crest cells on their way from early commitment to migration and terminal differentiation.

The backbone of our neural crest differentiation protocol comes from the protocol reported by Menendez and colleagues (Menendez et al., 2011), but there are two major differences that could explain how pNCCs, rather than migratory neural crest cells, were generated in this study. Firstly, dorsomorphin and 1-azakenpaullone were used to modulate BMP and Wnt signaling instead of Noggin and Bio, respectively. Dorsomorphin blocks BMP signaling at the receptor level and, thus, has a wider action on BMP signaling than Noggin, which specifically binds to and inhibits a distinct set of BMP proteins (Vogt et al., 2011; Zimmerman et al., 1996). Also, besides inhibiting BMP type I receptors (also known as ALK receptors), dorsomorphin inhibits the activity of various other kinases and, hence, modulates cell physiology through many signaling pathways (Vogt et al., 2011). In addition, we did not block the activin–TGF-β pathway with SB431542, because this pathway has been shown to be crucial for neural crest differentiation and proliferation (Nakazaki et al., 2009; Wurdak et al., 2005). Secondly, we used N2B27, not only N2, as an additive to the basal medium. B27 contains an enriched mixture of insulin and several antioxidant substances that might modulate the promotion, maintenance and survival of pNCCs (Chen and Sulik, 1996; Ngamjariyawat et al., 2012). hESC-derived pNCCs are an important tool for studying both the early events of commitment to the neural crest lineage in humans and the specification of the neural crest fate. Importantly, our protocol is applicable to human iPSCs as well (supplementary material Fig. S2), and should now allow both the investigation of the mechanisms underlying neurocristopathies and the screening of candidate drugs for potential therapeutic interventions.

The specification of neural crest fate from hESCs requires modulatory actions of multiple signaling pathways (Lee et al., 2007; Menendez et al., 2011). Our results, for the first time in the context of hPSCs, demonstrate multiple roles for Notch signaling during neural crest development and fate specification. Firstly, Notch signaling is activated during neural crest differentiation. This is seen in the induction of transcription of the Notch effectors *HES1* and *HES5*, and the expression of NICD1 that was sustained during the induction of neural crest. Notch signaling possibly specifies hESCs towards a neural crest fate by

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modulating both intrinsic factors and extrinsic signals (Endo et al., 2002; Geling et al., 2004). Previous studies have shown that Notch signaling is normally inactive in undifferentiated pluripotent stem cells, but that it is required for hESC lineage commitment (Noggle et al., 2006; Yu et al., 2008). Our results now extend this to neural crest differentiation as well. Secondly, the overexpression of NICD1 clearly augmented the expression of neural-crest-specifier genes (Fig. 4E). Although a recent study reports the genome-wide characterization of cis-regulatory elements occupied by AP-2α and NR2F1 in hESC-derived neural crest cells (Rada-Iglesias et al., 2012), it has been unclear whether NICD1 directly binds to the regulatory regions of neural-crest-specifier genes and regulates their expression. Our ChIP analyses show that NICD1 binds to 5′ promoter regions of *DLX5, PAX3*, *SLUG* and *TWIST1* (Fig. 4H), suggesting a direct positive role of NICD1 on the expression of neural-crest-specifier genes. In line with this finding, NICD1 has been shown to directly regulate other neural-crest- and Notch-related processes. The overexpression of NICD1 alone is sufficient to convert melanocytes into neural crest stem-like cells (Zabierowski et al., 2011), and NICD1 interacts with β-catenin to upregulate Notch target genes (Jin et al., 2009). Thirdly, the inhibition of Notch signaling during neural crest induction, either by treatment with DAPT or by silencing *JAG1*, suppressed neural-crest-specifier gene expression and caused the cells to switch their fate towards a neural identity. One possible mechanism by which Notch signaling promotes the commitment of hPSCs to the neural crest lineage is through the Notch effector genes *HES1* and *HES5*, which activate the expression of neural crest genes (i.e. *PAX3*, *SLUG*, *SOX10* and *TWIST1*) and suppress proneural genes (i.e. *NGN2* and *NGN3*) (Conboy and Rando, 2002; Imayoshi et al., 2008; Niessen et al., 2008). Besides being direct targets of NICD1 (Fig. 4), neural-crest-specifier genes can be indirectly regulated by Notch. For instance, in the *Xenopus* embryo, *Hairy2* acts to regulate *Bmp4*, which in turn induces the expression of *Xmsx1* and *Xslug* in cranial neural crest cells (Glavic et al., 2004), underscoring an alternative layer of Notch signaling in controlling neural crest fate. Our microarray data revealed several newly identified Notch targets, the expression of which is affected by DAPT treatment during neural crest differentiation – for instance *APLNR*, *COL1A2*, *CDX1*, *IFITM1*, *LGALS1* and *NDRG1* (supplementary material Table S2). Of note, our data suggest a significant role for *JAG1* in neural crest development, supported by the fact that *Jag1* knockout mice display semicircular canal hypoplasia (Humphreys et al., 2012; Tsai et al., 2001), a phenotypic feature found in neurocristopathies, such as Waardenburg (Sznajer et al., 2008) and CHARGE syndromes (Bajpai et al., 2010).

Notch signaling is crucial for maintaining the progenitor state in several tissue-specific stem cells (Hilton et al., 2008; Mourikis et al., 2012). We accordingly hypothesized that the inhibition of Notch would promote the migration and maturation of pNCCs as seen *in vivo* (Okamura and Saga, 2008). Indeed, treatment of pNCCs with FGF8, a chemotactic signal for neural crest cells (Sato et al., 2011), and concomitant inhibition of Notch pathway by using DAPT, induced pNCCs to migrate and to express *NGFR* and *HNK1*, markers of migratory neural crest cells (Betters et al., 2010; Thomas et al., 2008). The resulting cells expressed other markers of migratory neural crest cells as expected, including *AP-2α*, *CDH6*, *ERBB3* and *ITGA4* (Fig. 5B,C). *In vivo*, both premigratory and migratory neural crest cells are able to generate neuronal and non-neuronal derivatives (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988). However, migratory neural crest cells differentiate into less potent progenitor cells after initiation of the migratory phase (McKeown et al., 2003). pNCCs derived by our protocol, therefore, serve as a self-renewing and expandable source of neural crest cells for future use. Finally, we found that, when DAPT was applied, pNCCs differentiated into neural-crest-derived neurons, and a substantial number of cells became peripheral sensory neurons (Fig. 6C). Furthermore, upregulation of sympathetic neuron genes (*DBH* and *PHOX2B*) and proneural genes (*MASH1*, *NEUROD1* and *NGN2*) was also detected in DAPT-treated pNCCs (Fig. 6B,C). These findings support the anti-neuronal differentiation role of Notch, found in the central nervous system (CNS) and sensory neurogenesis (Lassiter et al., 2010; Nelson et al., 2007; Shimojo et al., 2008). The important role of Notch signaling in controlling the commitment, migration and differentiation of neural crest cells, as found in our study, is well supported by earlier experiments in mouse embryos, in which Notch signaling promotes the proliferation of neural crest cells and gliogenesis, while inhibiting neuronal differentiation (Mead and Yutzey, 2012). Furthermore, our findings suggest that pNCC neurogenesis is prevented by Notch signaling that might act through the negative regulation of neuronal genes, such as *MASH1* and *NGN*s (Lo et al., 2002). Taken together, our data suggests that Notch activity preserves the progenitor state and prevents pNCCs from undergoing neuronal differentiation.

In conclusion, we describe a protocol for the generation of pNCCs from human ESCs and iPSCs. We show that Notch signaling is indispensable for neural crest differentiation in humans, and hypothesize that altered Notch signaling might underlie aberrant neural crest development (High et al., 2007). Based on our findings, we propose a model in which Notch signaling plays distinct roles during neural crest differentiation from hPSCs (Fig. 7). This model is in line with previous studies in *Xenopus* embryos, where Notch signaling permits the induction of neural crest by activating the transcription factor *Hairy2*,

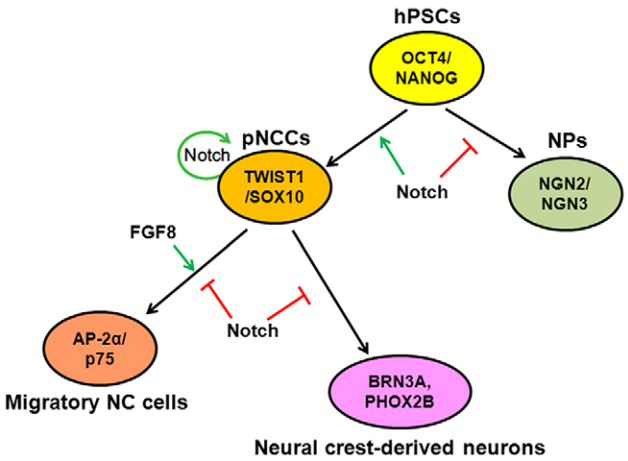

Fig. 7. Schematic summary of the proposed role of Notch signaling during neural crest differentiation from hPSCs. Notch is required for the commitment of hPSCs to the neural crest lineage. Notch activity also supports the self-renewal of the pNCC population, and the inhibition of Notch signaling leads to maturation and differentiation of pNCCs. In the absence of Notch, hPSCs undergo neural differentiation. hPSCs, human pluripotent stem cells; pNCCs, premigratory neural-crest-like cells; NPs, neural progenitor cells.

which subsequently maintains undifferentiated neural crest cells (Glavic et al., 2004; Nichane et al., 2008).

MATERIALS AND METHODS

Culture and maintenance of human pluripotent stem cells and DNA transfections

H9 hESCs (WiCell) and Hel11.4 iPSCs (University of Helsinki) were cultured on Geltrex-coated plates with StemPro complete medium (all from Invitrogen) and were routinely propagated by using a combination of collagenase IV treatment and mechanical dissociation. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) as described previously (Noisa et al., 2010). Briefly, confluent cells were split in a 1:3 ratio with 0.02% EDTA (Sigma) into a Geltrex-coated culture dish 24 hours before transfection. G418 selection was applied 48 hours after transfection at 200 µg/ml for 3 weeks, and surviving colonies were picked individually to a 24-well plate and expanded.

shRNA-mediated knockdown of *JAG1* and overexpression of human NICD1

Effective siRNA sequences for *JAG1* were obtained from a previous report (Choi et al., 2008). The target sequences of *JAG1* and scrambled siRNA are 5′-AGGATAACTGTGCGAACATC-3′ and 5′-GGGCGTC-GATCCTAACCGG-3′, respectively. These sequences were annealed and cloned into the pSuperior-Neo plasmid, driven by the H1 promoter (OligoEngine). This shRNA system was previously shown to have a high knockdown efficiency in hESCs (Zafarana et al., 2009). The human NICD1 sequence was obtained from Addgene [plasmid 17623 (Yu et al., 2008)] and was subcloned into a vector containing the CAG promoter and a G418 resistance cassette.

Neural crest, neural lineage and mesenchymal derivative differentiation

The neural crest differentiation protocol was modified from a previous report (Menendez et al., 2013). When confluent, hESCs were passaged by using 0.02% EDTA in phosphate-buffered saline (PBS, Sigma) into a Geltrex-coated dish, 24 hours before changing to the differentiation medium. The neural crest lineage was induced by using N2B27 medium, which was supplemented with 2 µM dorsomorphin (BMP inhibitor, Sigma) and 5 µM 1-azakenpaullone (GSK3-β inhibitor, Sigma). The medium was changed every other day until the cells were confluent. hESC-derived pNCCs were dissociated into single cells with TrypLE (Invitrogen) and maintained in the same differentiation medium. pNCCs were directed towards the neural lineage by the addition of 20 µM DAPT (Sigma) into neural-crest-inducing medium. Osteocyte, adipocyte and chondrocyte differentiation from pNCCs was performed by using the StemPro Osteogenesis Kit, StemPro Chondrogenesis Differentiation Kit and StemPro Adipogenesis Differentiation Kit (all Invitrogen), respectively, according to the manufacturer's instructions. Neural differentiation from hESCs was performed under the same conditions as neural crest differentiation, except that 1-azakenpaullone was withdrawn.

Immunocytochemistry

Cells were fixed at room temperature by using 4% paraformaldehyde for 10 minutes. Non-specific proteins were blocked by incubation in PBS containing 10% fetal bovine serum (Sigma) and 0.1% Triton X-100 for 1 hour at room temperature. The cells were then treated with primary antibodies overnight at 4℃. After washing with PBS, the cells were incubated with fluorescently labeled secondary antibody for 45 minutes and finally mounted on coverslips by using Vectashield mounting medium with DAPI (Vectorlabs). SOX*E* (Schmidt et al., 2003) and SOX10 (Stolt et al., 2003) antibodies, developed by Michael Wegner, were obtained from the University of Erlangen-Nuremberg, Germany. All antibodies used in this study are listed in supplementary material Table S5.

Flow cytometry

Cells were detached from culture vessels by using trypsin-EDTA solution and were washed with PBS. The cells were then stained on ice with antibodies against cell-surface antigens and were analyzed on a FACScan (BD Biosciences) using CELLQUEST software (BD Biosciences). Between 10,000 and 20,000 cells were acquired for each sample. For antibodies against nuclear proteins, the cells were fixed with 4% paraformaldehyde for 15 minutes, permeated with 100% ethanol for 2 minutes and treated with 10% goat serum for 15 minutes after washing with PBS. Cells were then incubated with the primary antibody for 30 minutes and secondary antibody for 30 minutes prior to FACScan analysis.

Migration assay

A wound-healing assay was used to measure cell migration. Confluent sheets of cells were scored with a 10-µl pipette tip to make a wound, and cell migration was observed after 24 and 48 hours under a light microscope.

Microarray expression profiles

Total RNAs were extracted from the samples by using the All Prep DNA/RNA/Protein Mini kit (Qiagen). The quality of the RNAs was assessed by using a 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent). The samples were processed for the transcriptome analysis with Illumina Human HT-12 Expression BeadChip according to the manual provided by Illumina. The raw data was pre-processed using the lumi package in R (Du et al., 2008). Pre-processing included background correction, variance stabilization and quantile normalization. Duplicate probe sets were removed using the genefilter package in R by retaining the probe set with the highest interquartile range. Present and absent calls for the probe sets were obtained by using the detection *P*-values. A probe set was defined to be present if it had a detection *P*-value of <0.01. Differential expression analysis was performed using an unpaired moderated *t*-test as implemented in limma in R (Smyth, 2004). A gene was considered to be differentially expressed if the Benjamini-Hochberg adjusted *P*-value was <0.05 and the log fold change was <-1 or >1. The GEO accession number for the microarray gene expression data reported in this paper is GSE53203.

Reverse transcription and real-time PCR

Total RNA was extracted using the RNA Spin II kit (Macherey-Nagel) according to the manufacturer's instructions. Briefly, first-strand cDNA was synthesized from 2 µg of total RNA by using SuperScript III reverse transcriptase (Invitrogen) with oligo dT primer (Invitrogen) in a volume of 20 µl. Each PCR included 1% of the resulting cDNA in a 20 µl mixture containing 10 µl of SYBR Green Taq solution (Sigma) and 5 µl of 2 µM primer mix. PCR reactions were performed in a Corbette thermal cycler (Qiagen) for 40 cycles of 95℃ for 15 seconds, 60℃ for 30 seconds and 72℃ for 30 seconds. Non-reverse-transcribed RNA was used as a negative control. The relative expression levels of target genes were calculated by calibrating their CT values to that of the housekeeping gene (cyclophilin G, also known as *PPIG*) and normalizing to the expression level in hESCs (as the reference sample). The primer sequences are listed in supplementary material Table S3.

Chromatin immunoprecipitation

Chromatin immunoprecipitation and real-time PCR were performed using the Agarose ChIP Kit as described in the manufacturer's protocol (Thermo Scientific, Pierce). Briefly, 2×10^6 cells were crosslinked by the addition of formaldehyde into the medium to a final concentration of 1% for 10 minutes at 37℃. After crosslinking was quenched by the addition of glycine to a final concentration of 0.125 M for 5 minutes at room temperature, cell extracts were prepared and then digested by using micrococcal nuclease (MNase) treatment at 37℃ for 15 minutes before adding the stop solution. Fragmented chromatin was mixed with 5 µg of anti-NICD1 antibody (Cell Signaling Technology, #4147) and 5 µg of normal rabbit IgG (provided with the kit), and was left on a rotating wheel overnight at 4℃. 20 µl of Protein-A/G-plus–agarose beads were added and incubated for 3 hours at 4℃. After elution of the immune complexes, DNA was extracted and resuspended in 50 µl DNA elution buffer, of which 2 µl was used for real-time PCR analysis with specific

primers. The fold-enrichment method was used to determine the level of enrichment of NICD1 at a particular region of DNA, taking into account the total chromatin (input) and any non-specific binding (normal rabbit IgG). The primer sequences are listed in supplementary material Table S4.

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Competing interests
The authors declare no competing interests.

Author contributions
P.N., T.T. and T.R. designed the study. P.N., C.L., K.K. and H.C. performed experiments. P.N., R.L., H.L., T.T. and T.R. analyzed data. R.L. and T.O. provided resources. P.N., K.L., T.T. and T.R. prepared the manuscript.

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