Correction

CELL BIOLOGY


The authors note that they omitted a reference to an article by Wang et al. The complete reference appears below.

Additionally, the authors note that on page 19245, left column, third full paragraph, lines 1–6, “WA09 (WiCell), RUES1, RUES2 (A. Brivanlou, The Rockefeller University, New York) hESCs, and the hiPSC lines Fib2-iPS4 and Fib2-iPS5 (George Daley, Children’s Hospital, Boston) were cultured on Geltrex-coated plates (Invitrogen) in chemically defined media containing Heregulin β (10 ng/mL), Activin A (10 ng/mL), LR-Igf (200 ng/mL), and Fgf2 (8 ng/mL) as described previously (24)” should instead appear as “WA09 (WiCell), RUES1, RUES2 (A. Brivanlou, The Rockefeller University, New York) hESCs, and the hiPSC lines Fib2-iPS4 and Fib2-iPS5 (George Daley, Children’s Hospital, Boston) were cultured on Geltrex-coated plates (Invitrogen) in chemically defined media containing Heregulin β (10 ng/mL), Activin A (10 ng/mL), LR-Igf (200 ng/mL), and Fgf2 (8 ng/mL) as described previously (25).”


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Wnt signaling and a Smad pathway blockade direct the differentiation of human pluripotent stem cells to multipotent neural crest cells

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Neural crest stem cells can be isolated from differentiated cultures of human pluripotent stem cells, but the process is inefficient and requires cell sorting to obtain a highly enriched population. No specific method for directed differentiation of human pluripotent cells toward neural crest stem cells has yet been reported. This severely restricts the utility of these cells as a model for disease and development and for more applied purposes such as cell therapy and tissue engineering. In this report, we use small-molecule compounds in a single-step method for the efficient generation of self-renewing neural crest-like stem cells in chemically defined media. This approach is accomplished directly from human pluripotent cells without the need for coculture on feeder layers or cell sorting to obtain a highly enriched population. Critical to this approach is the activation of canonical Wnt signaling and concurrent suppression of the Activin A/Nodal pathway. Over 12–14 d, pluripotent cells are efficiently specified along the neuroectoderm lineage toward p75

Hnk1+ Ap2+ neural crest-like cells with little or no contamination by Pax6+ neural progenitors. This cell population can be clonally amplified and maintained for >25 passages (>100 d) while retaining the capacity to differentiate into peripheral neurons, smooth muscle cells, and mesenchymal precursor cells. Neural crest-like stem cell-derived mesenchymal precursors have the capacity for differentiation into osteocytes, chondrocytes, and adipocytes. In sum, we have developed methods for the efficient generation of self-renewing neural crest stem cells that greatly enhance their potential utility in disease modeling and regenerative medicine.

Results

Activation of the Wnt Pathway Redirects Neural Progenitors Toward a Neural Crest Fate. Human pluripotent cells can be efficiently differentiated into Pax6+ NPCs by simultaneous inhibition of Activin A/Nodal and BMP signaling with SB 431542 and Noggin, respectively (Fig. 1 A and B) (12). Although Pax6+ Sox1+ Sox2+ NPCs predominate in cultures where Smad signaling is blocked, relatively minor amounts of p75

Hnk1+ neural crest cells are also

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The authors declare no conflict of interest.

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generated under these conditions (Fig. 1B and Fig. S1), which is consistent with previous findings (12).

The signaling requirements that determine a NCSC versus a NPC fate have not been previously defined in culture. In the absence of a specific method that allows for the generation of highly enriched cultures of NCSCs, FACS isolation of Hnk1⁺ p75⁻ cells from NPC cultures has been the method of choice to obtain hESC-derived neural crest cells (18). Because canonical Wnt signaling performs known roles in promoting neural crest formation in vertebrate development (3–5), we asked whether concomitant activation of Wnt signaling combined with global Smad inhibition would more efficiently divert early neuroectoderm away from a NPC fate toward a neural crest-like identity. This was initially tested by addition of (2′Z,3′E)-6-bromoindirubin-3'-oxime (BIO), a small-molecule inhibitor of glycogen synthase kinase 3 (GSK3) that acts as a Wnt mimetic in a variety of contexts (19, 20). Addition of BIO to hESC cultures efficiently activates the canonical Wnt pathway, as indicated by the activation of a β-catenin–dependent luciferase reporter (Fig. S2). Concurrent Smad inhibition combined with activation of Wnt signaling (+BIO) after 12 d

Fig. 1. hESC (WA09) differentiation to neuroprogenitor cells is inhibited by Wnt signaling. (A) Schematic summarizing differentiation of pluripotent cells into neural progenitor cells and neural crest cells together with markers for the two cell types. (B) Treatment of hESCs with SB 431542 (20 μM) and Noggin (500 ng/mL) promotes differentiation into Pax6⁺ cells but concurrent treatment with BIO suppresses this and generates p75⁻ Pax6⁺ neural crest-like cells. (Scale bar, 100 μm.) (C) Flow cytometry showing that Dickkopf (Dkk) decreases the p75bright population in NPC cultures generated by treatment with SB 431542 and Noggin. (D) Real-time PCR data for Ap2 and Pax6 from p75dim or p75bright sorted cells (from C). Activation of the canonical Wnt pathway by (E) GSK3 inhibition with BIO (0.1–2 μM) or by (F) addition of Wnt3a (1–50 ng/mL) promotes the formation of p75bright Hnk1bright cells in a dose-dependent manner. Isotype controls are shown in red, and positive cells are shown in blue. The percentage of double p75⁺ Hnk1⁺ cells is shown in each graph in E and F.
severely inhibited the formation of Pax6+ cells while markedly increasing the percentage of p75+ cells (Fig. 1B). These cultures lost markers for pluripotent cells, such as Nanog and Oct4 (Fig. S3). Addition of Dickkopf (Dkk), a Wnt antagonist, severely reduced the low level of p75bright cells present in NPC cultures obtained by treatment with Noggin and SB 431542 (Fig. 1C). p75bright cells expressed high levels of the neural crest marker Ap2 but low levels of the NPC marker Pax6 (Fig. 1D). p75dim cells, on the other hand, expressed high levels of Pax6 transcript but low levels of Ap2, indicating that p75dim and p75bright cells represent NPC and neural crest-like populations, respectively (Fig. 2). These data indicate that the minor population of p75bright neural crest-like cells in NPC cultures has a requirement for Wnt signaling (discussed in further detail below).

To establish a role for Wnt signaling in the specification of hESC-derived neural crest-like cells, we performed dose–response experiments where the GSK3 inhibitor BIO or Wnt3a were added to cultures treated with SB 431542 and Noggin. Increasing the amount of BIO (0–2 μM) or recombinant Wnt3a (0–50 ng/mL) increased the proportion of Hnk1bright p75bright cells in a dose-dependent manner (Fig. 1E and F). As the concentration of BIO was reduced, the proportion of p75dim cells increased, indicating that cells were being directed to a Pax6+ p75dim fate (Fig. 1C). This was confirmed by comparing p75dim and p75bright cells that were generated following treatment with an intermediate concentration of BIO (0.5 μM). Quantitative RT-PCR analysis shows that p75dim cells expressed elevated levels of Pax6 and Sox2 but low levels of Ap2, p75, and Slug transcripts (Fig. S4A). Conversely, p75bright cells show elevated levels of Ap2, p75, and Slug transcripts but low levels for Sox2 and Pax6. Immunostaining also confirmed that Sox2 expression showed a dose-dependent response to BIO when Smad signaling was suppressed, consistent with cells being directed away from a neural progenitor fate toward a neural crest-like identity at higher concentrations (Fig. S4B). As expected, the pluripotent cell markers Nanog and Oct4 were lost over a broad range of BIO concentrations in the presence of SB 431542 (Fig. S4C). Elevating Wnt signaling in the context of low Smad activity therefore directs cells away from a Pax6+ p75dim population to a neural crest-like fate.

Fig. 2. hESC differentiation to neural crest cells requires Wnt signaling and is antagonized by Activin A and BMP pathways. (A) Flow cytometry analysis of WA09 hESCs treated as indicated for 15 d. Cells were analyzed by probing with antibodies for p75 and Hnk1. The percentage of double negative and positive cells is indicated in the bottom left and top right, respectively, of each graph. (B) Immunocytochemistry and bright field (Lower Right) of WA09 hESCs treated with BIO and SB 431542 for 12 d. Cells were probed with antibodies as indicated: p75, Pax6, Ap2, Hnk1, and DAPI (DNA). (Scale bar, 100 μm.) (C) RT-PCR transcript analysis of hESCs and NCSCs (passage 10) treated with BIO and SB 431542. Transcript levels were normalized to Gapdh control. Assays were performed in triplicate and are shown as ±SD. (D) Schematic illustration of the signaling requirements for neural crest differentiation from hESCs.
Neural crest-like cells. Addition of SB 431542, BIO, and Noggin had no major impact (Fig. 24), indicating that active suppression of BMP signaling is not required under our conditions. This can be explained by the low level of basal BMP-dependent Smad1,5,8 activity in these cells (Fig. S5). Addition of BMP4 to SBio-treated cultures, however, suppressed the transition to an Hnk1\textsuperscript{bright} p75\textsuperscript{bright} state showing that BMP signaling antagonizes this pathway (Fig. 24). Recombinant Wnt3a can substitute for BIO when combined with SB 431542, but BIO and SB 431542 by themselves are ineffective.

To characterize the Hnk1\textsuperscript{bright} p75\textsuperscript{bright} cell population in further detail, immunostaining was performed using antibodies for neural crest markers p75, Ap2, Hnk1, and the NPC marker Pax6. This showed that >90% of SB 431542 and BIO (SBio)-treated cells were positive for neural crest markers, but <5% expressed Pax6 (Fig. 2B). Elevated levels of Ap2, p75, Sox9, Sox10, Pax3, Brn3, and Zic1 transcripts further show that the p75\textsuperscript{+} population generated with SBio is closely related to authentic neural crest cells (Fig. 2C). hESC-derived NCSCs could be maintained as a stable, self-renewing population over extended periods of culture in SBio-containing media (25 consecutive passages). Similar results were obtained when different hESC lines and hiPSCs were treated with SBio-containing media (Figs. S6 and S7). We conclude that activation of canonical Wnt signaling combined with low Smad2,3 and Smad1,5,8 activity are strict requirements for the efficient generation of neural crest-like cells from hESCs (Fig. 2D).

Multilineage Differentiation of hESC-Derived Neural Crest Cells. The neural crest is a multipotent population of cells arising from the neural ectoderm in vertebrate embryos, capable of forming a diverse array of cell lineages. To characterize the developmental potential of hESC-derived NCSCs described in this report, we asked if these cells could differentiate into lineages previously shown to be generated by hESC-derived neural crest-like cells (15). The experiments designed to answer this question were performed on WA09-derived NCSCs that had been self-renewed for >10 passages to establish that the developmental potential of these cells was retained over time. First, we confirmed that NCSCs have the capacity for neural differentiation as previously described (12).

**Fig. 3.** Peripheral neurons derived from hESC-derived (A, C, and D) and hiPSC-derived (B) neural crest-like stem cells. BIO and SB 431542-treated NCSCs were differentiated to peripherin\textsuperscript{+} β-tubulin\textsuperscript{+} cells for 14 d after switching to N2-based neural differentiation media. Fixed cells were then probed with antibodies for peripherin and β-tubulin. DNA was detected by staining with DAPI. (Scale bar, 100 μm.)

Next, we performed further analysis to define the signaling requirements required to efficiently specify hESC-derived Hnk1\textsuperscript{+} p75\textsuperscript{+} neural crest-like cells. Addition of SB 431542, BIO, and Noggin (SBioN) generated a highly enriched Hnk1\textsuperscript{+} p75\textsuperscript{+} population but, unexpectedly, omission of Noggin had no major impact (Fig. 24), indicating that active suppression of BMP signaling...
After culture in media containing a mixture of factors (BDNF, GDNF, NGF, neurotrophin-3, and dbcAMP) that promote neural differentiation (18), ~75% of cells expressed β-tubulin and a similar number expressed peripherin/neurofilament 4 (Fig. 3 A), indicative of peripheral neurons. Similar results were obtained with two other hESC lines (RUES1 and RUES2) and hiPSCs (Fig. 3 B–D).

Neural crest cells can also form mesenchymal precursor cells in vitro (15, 21). By culturing cells in media containing 10% FBS (21, 22), we confirmed that SBio-generated NCSCs can be efficiently converted to a cell type with mesenchymal properties over a 4-5 day period (Fig. 4 A and B). Mesenchymal cells produced were highly enriched for mesenchymal stem cell markers such as CD73, CD44, CD105, and CD13 but lost expression of p75 (Fig. 4 A and D). We also showed that hESC-derived mesenchymal cells could be converted into smooth muscle, chondrocytes, osteocytes, and adipocytes (Fig. 5). Many of these observations were repeated using NCSCs derived from other hESC lines (RUES1, RUES2) and from hiPSCs (Figs. S8 and S9). In summary, neural crest-like cells generated by our efficient one-step method using small-molecule compounds is capable of multilineage differentiation. This is comparable to the developmental potential of neural crest cells isolated from NPC cultures by FACS sorting reported previously (15, 18).

**In Vivo Potential of hESC-Derived NCSCs.** To assess the in vivo activity of SBio-derived neural crest, cell aggregates labeled with the DiO cell tracer were implanted into Hamburger-Hamilton (HH) stage 8–10 chicken embryos (n = 26) along the boundary between the neural and nonneural ectoderm at the level of the forming forebrain and midbrain (Fig. 6A). Of the 19 embryos in which aggregates remained in place, migrating cells were observed in 13. Seventy-two hours after injection, fluorescently labeled cells were observed in the head and pharyngeal regions (Fig. 6B), including the cranial ganglion (Fig. 6 C–J). The identity of cells was confirmed by staining with the human-specific nuclear antigen antibody (hNA; Fig. 6E). To confirm the developmental potential of injected NCSCs and their ability to generate peripheral neurons in vivo, we assessed hNA-positive cells for expression of the neural markers Tuj1 or peripherin. Double hNA/Tuj1-positive cells were observed in small clusters throughout the head mesenchyme (Fig. 6 G–J). hNA/peripherin-positive cells were also found in the mesenchyme and incorporated into host cranial ganglia (Fig. 6 C–F). The injected cells therefore migrate and differentiate into peripheral neurons in vivo, which is consistent with the expected characteristics of neural crest cells.

**Discussion**

Several reports have described the generation of neural crest progenitor cells from human pluripotent cells. These involve coculture on PA6 or M5 feeder layers (15, 17), differentiation through an embryoid body stage (23), and differentiation along a neuroectoderm pathway using inhibitors of the Smad pathway (18). The latter represents a culture system primarily designed to generate Pax6+ NPCs. Minor amounts of p75+ neural crest cells produced in this system are likely to be a consequence of signaling heterogeneities in the culture dish. None of these approaches represents a guided approach to specifically generate neural crest cells and, as a major downside, require a cell-sorting step to isolate highly enriched neural crest cell populations. This is obviously a significant obstacle that must be circumvented for the utility of neural crest cells to be fully realized in an experimental and cell therapy setting. This report describes a guided differentiation strategy specifically for the purpose of generating neural crest cells without significant amounts of other ectoderm-derived lineages. At the molecular level, neural crest cells generated by our method...

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**Fig. 5.** Differentiation of NCSC-derived mesenchymal cells. (A) Schematic showing the lineages capable of being formed from neural crest-derived mesenchymal cells in culture. (B) Bright-field picture (Left) after differentiation into calponin+ smooth muscle actin (SMA) smooth muscle cells. (C) Oil red O-stained adipocytes and (Right) a bright-field image of adipocytes showing oil droplets. (D) Osteocytes produced by differentiation of neural crest-derived mesenchymal cells, detected by staining with Alizarin Red and alkaline phosphatase (AP) staining. (E) Differentiation of mesenchymal cells to chondrocytes, as detected by staining with Alcian Blue. (Scale bar, 100 μm.)

**Fig. 6.** In vivo migration and differentiation of WA09 hESC-derived NCSCs. (A) DiO-labeled cells at time of injection and (B) 48 h later showing cell migration. (C–F) Immunocytochemistry and bright-field images of the same microscopic field 72 h after injection showing cells that had incorporated into a cranial ganglion area and differentiated to peripheral neurons. Cells were probed with antibodies for peripherin and hNA and counterstained with DAPI (DNA). (Scale bar, 50 μm.) (G–J) Images of the same microscopic field showing a cluster of human NCSCs (hNA-positive) in the head mesenchyme adjacent to the neural tube. Many of the cells are also Tuj1-positive. (Scale bar, 20 μm.)
is indistinguishable from that generated by other methods (15, 18) and, importantly, displays a similar differentiation potential.

The rationale for our directed-differentiation approach is based on the known roles of canonical Wnt signaling in neural crest formation during vertebrate development (3–5). The signaling conditions for neural crest progenitor specification from hESCs and hiPSCs involve inhibition of GSK3, an antagonist of Wnt signaling, and activation of Activin A/Smad signaling with SB 431542. BMP4 antagonized neural crest specification, but inhibitors such as Noggin were not required due to the low basal level of Smad1,5,8 signaling in our system. The activation of Wnt signaling was sufficient to divert cells from a Pax6

NPC fate to a p75

neural crest cell fate, both of which require low levels of global Smad signaling. Wnt briefly controls a molecular switch that determines definitive ectoderm fates arising from human pluripotent cells in culture. These results indicate that suppression of Wnt signaling with Dkk, for example, may be a useful approach to reduce the number of contaminating neural crest cells from NPC cultures.

In summary, we describe a method for directed differentiation of human pluripotent cells toward a neural crest fate. This method is highly efficient and cost-effective and precludes formation of contaminating Pax6

NPCs. Removing the need for FACS-assisted purification provides a platform from which the basic biology of neural crest cells can be better understood and better applied to disease modeling. This approach also establishes a starting point for the generation of neural crest cells at a scale that can be used in applications in tissue engineering and regenerative medicine.

Materials and Methods

Stem Cell Culture. WA09 (WiCell), RUES1, RUES2 (A. Brivanlou, The Rockefeller University, New York) hESCs, and the hiPSC lines Fib2-IP54 and Fib2-IP55 (George Daley, Children's Hospital, Boston) were cultured on Geltrex-coated plates (Invitrogen) in chemically defined media containing Heregulin (10 ng/mL), Activin A (10 ng/mL), LR-lf (200 ng/mL), and Fgf2 (8 ng/mL) as described previously (24).

Neuroprogenitor Cell, Neural Crest, and Mesenchymal Cell Differentiation. NPC differentiation was performed as described (18). Briefly, cells were plated on Geltrex-coated plates in defined media without Activin A, supplemented with 20 μM SB 431542 (Tocris) and 500 ng/mL Noggin (R&D Systems) for 11 d with or without 2 μM of BIO (GSK3 inhibitor IX, Calbiochem) or 15 μg/mL Dkk (R&D Systems). For direct neural crest differentiation, cells were plated at a density of 1 x 10

5
cells/cm

2 in defined media lacking Activin A and supplemented with 2 μM BIO and 20 μM SB 431542 (SB media). Media was replaced every day. Additional experiments were performed by adding 25 ng/mL Wnt3a, 500 ng/mL Noggin or 5–50 ng/mL BMP4 (R&D Systems) to SB media. For peripheral neuron differentiation, neural crest cells were plated in poly-ornithine/laminin or Geltrex-coated four-well chamber slides. The following day, SB media was switched to DMEM/F12 supplemented media with BDNF (10 ng/mL), GDNF (10 ng/mL), NT3 (10 ng/mL), ascorbic acid (200 μM), and dbcAMP (0.5 mM). Cells were grown for 10–14 d and assayed by immunocytochemistry. For mesenchymal differentiation, neural crest cells were cultured in media containing 10% FBS and passed every 4–5 d. Osteocyte, adipocyte, and chondrocyte differentiation was performed according to manufacturer directions using StemPro Osteogenesis Kit, Stem-Pro Chondrogenesis Differentiation Kit, and Stem-Pro Adipogenesis Differentiation Kit (Invitrogen, respectively).

Immunocytochemistry and Flow Cytometry. Cells were fixed in 4% paraformaldehyde in PBS and stained with the primary antibodies listed in Table S1. Secondary antibodies were Alexa Fluor 488- and Alexa Fluor 555-conjugated (Invitrogen). DNA was visualized by staining with DAPI. For flow cytometry, 1 million live cells dissociated with Accutase were resuspended in PBS and incubated for 30 min on ice with primary conjugated antibodies (Table S1). Unconjugated p75 (neuropilin-receptor) and Hnk1 antibodies were detected with Alexa Fluor 633- and Alexa Fluor 488-conjugated secondary antibodies, respectively. Cells were analyzed using the CyAN ADP (Beckman Coulter) and FlowJo software.

Real-Time PCR and Western Blot Analysis. RNA was extracted using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was used for cDNA synthesis using the Script cDNA Kit (BioRad). Ten nanograms of cDNA were used for real-time PCR with Taqman assays (Applied Biosystems) in a Myq real-time PCR iCycler (BioRad). Each sample was analyzed in duplicate, and gene expression was normalized to GAPDH. For Western blot analysis, hESCs were plated in defined media and then switched to SB media alone or with addition of 5–50 ng/mL BMP4 (R&D Systems) or 500 ng/mL Noggin for 3 or 6 h. After protein extraction with RIPA buffer, ~30 μg of total protein extract were loaded in 12% SDS gels, transferred to nitrocellulose membranes, and probed with antibodies for pSmad1,5,8, Smad1 (Cell Signaling Technologies), and Cdk2 (Santa Cruz Biotechnology).

In Ovo Transplantation. Neural crest cells were passaged with Accutase and then seeded on agarose-coated plates to form small-sized aggregates. Three days later, cell aggregates were labeled with the fluorescent dye Dio (3,3’-dio-ladecylxocularcyanine perchlorate) (Invitrogen) as described (24). A slit was cut into HH stage 8–10 chicken embryos in ovo at the junction of the nonneural ectoderm and the forming neural tube, and a small Dio-labeled cell aggregate was inserted and positioned under a fluorescence stereomicroscope. For further details, see SI Materials and Methods.

ACKNOWLEDGMENTS. This work was supported by Grant HD049647 from the National Institute of Child Health and Human Development (to S.D.), by Grant GM75334 from the National Institute for General Medical Sciences (to S.D.), and by Grant P41RR018502 from the National Center for Research Resources (to S.D.).


Supporting Information

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SI Materials and Methods

Luciferase Assay. Cells were transfected with Top-Flash or Fop-Flash and Renilla plasmids (1) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The next day media was changed to either complete defined media (untreated) or defined media lacking Activin A supplemented with (2′Z,3′E)-6-bromoindirubin-3′-oxime. Forty-eight hours after the transfections, β-catenin activity was measured following instructions for the Dual-Luciferase Reporter Assay System (Promega) on a Synergy2 microplate reader (Bio-Tek).

In Ovo Transplantation. Egg windows were sealed with transparent tape and eggs were incubated for 48–72 h, after which embryos were evaluated for the presence of fluorescent cells. Embryos were photographed to document the localization of DiO-labeled cells and then fixed in freshly prepared 4% paraformaldehyde overnight at 4 °C. Embryos were rinsed three times in PBS, incubated in 30% sucrose/PBS overnight at 4 °C, and embedded in OCT compound (Tissue-Tek) and frozen in a bath of dry ice/isopentane. Sections (8–12 μm) were cut and processed for immunofluorescence detection of human nuclear antigen (hNA) (1:100; Chemicon), β-tubulin isotype III (Tuj1) (1:400; Sigma), or peripherin (1:200; Santa Cruz Biotechnology). Sections were dried at room temperature for 10 min, rehydrated in PBS, and permeabilized in PBS, 0.2% Tween 20 for 15 min. Sections were incubated with primary antibody diluted in 1% BSA/PBS and 0.1% Triton X-100 at 4 °C overnight in a humid chamber, washed three times for 10 min in PBS (0.2% Tween 20), and then incubated with a 1:200 dilution each of Cy2-conjugated goat anti-mouse IgG1 (hNA) and Cy3-conjugated goat anti-mouse IgG2b (Tuj1) or donkey anti-goat IgG (peripherin) at 37 °C for 1.5 h. Sections were washed twice in PBS, 0.2% Tween 20, and finally in PBS. Some sections were stained with DAPI (5 μg/mL) in PBS for 10 min before applying a coverslip using Prolong Gold (Invitrogen). Fluorescence was visualized and photographed on a Zeiss AXIO microscope.


Fig. S1. WA09 human embryonic stem cells were cultured in neural progenitor cell differentiation media supplemented with SB 431542 and Noggin for 14 d. Cells were then fixed and stained with antibodies for Pax6, Sox1, and Sox2. DNA was detected by staining with DAPI. (Scale bar, 100 μM.)

Fig. S2. Luciferase assay for β-catenin activity in human embryonic stem cells (hESCs). (A) (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO) treatment of WA09 hESCs activates β-catenin signaling as shown by activation of the Top-Flash luciferase reporter. Data are expressed as the fold-increase in luciferase activity over untreated cells after normalization to the Fop-Flash control. All assays were performed in triplicate and represented as ±SD. (B) Top-Flash activity in hESCs grown in defined media treated with Wnt3a, Dkk1, Wnt3a, Dkk1, or BIO. Experiments were performed in triplicate and represented as ±SD.

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Fig. S3. WA09 human embryonic stem cells (hESCs) treated with SB 431542, Noggin, and (2Z,3E)-6-bromoindirubin-3′-oxime (BIO) down-regulate the pluripotency markers Oct4 and Nanog. hESCs were treated with the three factors for 12 d and then fixed and stained with antibodies for Oct4 and Nanog. DAPI was used to visualize DNA. (Scale bar, 100 μm.)

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Fig. S4. (A) WA09 human embryonic stem cells (hESCs) were treated with (2Z,3E)-6-bromoindirubin-3′-oxime (BIO) (0.5 μM) and SB 431542 for 15 d and then isolated by FACS for p75bright and p75dim populations. Pax6, Ap2, Sox2, p75, and Slug transcript levels in each population were then determined by quantitative RT-PCR and expressed as the fold ratio after normalization to Gapdh (assays performed in triplicate). (B) Immunocytochemistry of hESCs treated with BIO (0.1–2 μM) and SB 431542 for 10 d. Cells were probed with antibodies as indicated: Oct4, Nanog, Sox2, and DAPI (DNA). (Scale bar, 100 μM.)
Fig. S5. Western blot analysis for detection of pSmad1,5,8. Human embryonic stem cells (hESCs) (WA09) were grown for 3–6 d in the presence of (2’Z,3’E)-6-bromoindirubin-3’-oxime (BIO) and SB 431542 with Noggin alone and with BMP4 alone (5–50 ng/mL). Whole-cell lysates were then probed with antibodies for Smad1, Cdk2 (load control), or pSmad1,5,8.
Fig. S6. Human induced pluripotent stem cell (hiPSC) differentiation to neural crest cells. Flow cytometry analysis of hiPSC4 (A and B) and hiPSC5 (E and F) treated with (2Z,3E)-6-bromoindirubin-3′-oxime and SB431542 in defined media lacking Activin A. Flow cytometry analysis was performed using isotype antibody controls (A and E) and p75/Hnk1 antibodies (Right). The percentage of positive cells in bottom left and top right quadrants of A, B, E, and F is indicated. (C and G) Bright-field picture of neural crest-like cells. (Scale bar, 100 μm.) (D and H) Neural crest-like cells were analyzed by immunocytochemistry by probing with antibodies for Ap2, Sox2, p75, Pax6, and Hnk1. DNA was visualized by staining with DAPI. (Scale bar, 200 μm.)
Fig. S7. RUES1 and RUES2 hESC differentiation to neural crest cells. Flow cytometry analysis of RUES2 (A and B) and RUES1 (E and F) treated with (2′Z,3′E)-6-bromoindirubin-3′-oxime and SB431542 in defined media lacking Activin A. Flow cytometry analysis was performed using isotype antibody controls (A and E) and p75/Hnk1 antibodies (B and F). The percentage of positive cells is indicated in the bottom left and top right quadrants of A, B, E, and F. (C and G) Bright-field picture of neural crest-like cells. (Scale bar, 100 μm.) (D and H) Neural crest-like cells were analyzed by immunocytochemistry by probing with antibodies for Ap2, Sox2, p75, Pax6, and Hnk1. DNA was visualized by staining with DAPI. (Scale bar, 200 μm.)
**Fig. S8.** Differentiation of human induced pluripotent stem cell-derived (hiPSC4) neural crest-like cells to peripheral neurons. Generation of peripheral neurons is shown by staining with antibodies for peripherin and β-tubulin. DNA was visualized by staining with DAPI. (Scale bar, 200 μm.)

**Fig. S9.** Mesenchymal stem cells and derivatives generated from neural crest-like cells. Flow cytometry analysis of mesenchymal stem cells generated from RUES1, RUES2, and human induced pluripotent stem cell 5 (hiPSC5) neural crest shows loss of p75 and HNK1 (A) and increase of CD73, CD13 (B), CD105, and CD44 (C). (A–C) The percentage of positive cells for each antigen is shown at the bottom left and the top right quadrants. Mesenchymal stem cells can further differentiate into chondrocytes and osteocytes, as shown for hiPSC4 by staining with Alcian Blue, Alizarin Red, and alkaline phosphatase (AP) (D). (Scale bar, 200 μm.)
Table S1. Sources and other details relating to antibodies used in the study

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