Long-Term Expandable SOX9+ Chondrogenic Ectomesenchymal Cells from Human Pluripotent Stem Cells

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SUMMARY

Here we report the successful generation and long-term expansion of SOX9-expressing CD271+PDGFRα+CD73+ chondrogenic ectomesenchymal cells from the PAX3/SOX10/FOXD3-expressing MIXL1-CD271hiPDGFRαhiCD73hi neural crest-like progeny of human pluripotent stem cells in a chemically defined medium supplemented with Nodal/Activin/transforming growth factor β (TGFβ) inhibitor and fibroblast growth factor (FGF). When “primed” with TGFβ, such cells efficiently formed translucent cartilage particles, which were completely mineralized in 12 weeks in immunocompromized mice. The ectomesenchymal cells were expandable without loss of chondrogenic potential for at least 16 passages. They maintained normal karyotype for at least 10 passages and expressed genes representing embryonic progenitors (SOX4/12, LIN28A/B), cranial mesenchyme (ALX1/3/4), and chondroprogenitors (SOX9, COL2A1) of neural crest origin (SOX8/9, NGFR, NES). Ectomesenchyme is a source of many craniofacial bone and cartilage structures. The method we describe for obtaining a large quantity of human ectomesenchymal cells will help to model craniofacial disorders in vitro and potentially provide cells for the repair of craniofacial damage.

INTRODUCTION

Adult mesenchymal stromal cells (MSCs), defined in vitro by their potential to contribute to bone-cartilage-fat cell lineages, are currently used for cell-based bone and cartilage therapies because of their ready accessibility. However, as for other adult stem cells, it is difficult to obtain sufficient MSCs for treatment. Expansion culture is therefore necessary before transplantation; however, it tends to cause the loss of long-term viability of the MSCs and their capacity to differentiate, especially into chondrocytes (Somoza et al., 2014). Different types of bone and cartilage are formed most actively during embryonic skeletogenesis from one of three precursor cell types: paraxial mesoderm, lateral plate mesoderm, and cranial neural crest. Such embryonic cells and their osteochondrogenic progeny may be as effective as or more effective than MSCs for the regeneration of adult bone and cartilage.

The early processes of in vitro differentiation of pluripotent embryonic stem cells (ESCs) mimic those of in vivo embryogenesis (Nishikawa et al., 2007). Therefore, ESCs and induced pluripotent stem cells (iPSCs) (collectively designated pluripotent stem cells or PSCs) would appear to be the practical source of embryonic precursor cells in humans. In fact, in vitro induction of osteogenesis and chondrogenesis from human PSCs (hPSCs) and mouse (mPSCs) has been demonstrated by many groups (Nakayama and Umeda, 2011). With the exception of recent reports, including ours (Craft et al., 2013; Diekman et al., 2012; Nakayama et al., 2003; Toh et al., 2009; Umeda et al., 2012; Zhao et al., 2014), many of the earlier reports described spontaneous differentiation of hPSCs followed by enrichment of mesenchymal cells by further culturing the progeny in MSC medium. As adult human tissue-derived MSCs or chondroprogenitors (Koelling et al., 2009; Pitterer et al., 1999), mesenchymal cells derived from mESCs/iPSCs were able to be expanded extensively; however, expansion occurred with the loss of their chondrogenic activity (Bakre et al., 2007; Diekman et al., 2012). Thus far, the potential benefits of bone and cartilage repair of hPSC-derived osteochondroprogenitors over those of adult MSCs, whether in quantity or in quality, have not been demonstrated, even in vitro (Nakayama and Umeda, 2011). In theory, the wealth of information on the signaling mechanisms involved in mouse skeletogenesis should be of great help in improving the expansion culture methods. However, the unclear embryonic origins of the chondrogenic activity developed from PSCs and the undefined conditions used for expansion have hampered full use of the information and thereby hindered progress.

A large portion of craniofacial bone and cartilage arises from osteochondrogenic progeny (i.e., ectomesenchyme) from cranial neural crest (Santagati and Rijli, 2003), generated from the junction between anterior neuroectoderm...
and surface ectoderm (Milet and Monsoro-Burq, 2012). Neural crest cells have been developed from hESCs in 12–28 days of differentiation culture either through neuroepithelial intermediates induced by suppression of Nodal/Activin/transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) signaling in a defined medium (Chambers et al., 2009; Smith et al., 2008) or directly by activation of WNT signaling with suppression of Nodal/Activin/TGFβ signaling (Menendez et al., 2011). Further differentiation and expansion for 2–3 weeks of such neural crest cells or earlier neural cells in a serum-containing medium generate MSC-like cells with variable chondrogenic activity, but never sufficient to reproducibly form cartilage particles that accumulate proteoglycan-rich, mature matrices uniformly (hereafter designated “full-cartilage”). Our group has focused on generating and characterizing paraxial mesodermal progeny from mPSCs/hPSCs, which are highly chondrogenic (Tanaka et al., 2009; Umeda et al., 2012; Zhao et al., 2014). In the current study, we report simple, effective methods for the specification of neural crest-like progeny from hPSCs and subsequent generation and expansion of chondrogenically committed ectomesenchymal cells without loss of their chondrogenic activity over 7–8 weeks in chemically defined media (CDM). The outcomes were achieved by the control of fibroblast growth factor (FGF) signaling and Nodal/Activin/TGFβ signaling. We have also defined the cellular developmental pathway from hPSCs to such ectomesenchymal cells using the neural crest markers the low-affinity nerve growth factor receptor (CD271) (Lee et al., 2007; Stemple and Anderson, 1992), and the platelet-derived growth factor receptor a (PDGFRα) (Morrison-Graham et al., 1992; Weston et al., 2004), and the MSC markers, CD73 and CD13 (Olivier et al., 2006; Pittenger et al., 1999).

RESULTS

Signaling Requirement for Early Development of Neural Crest-like Progeny from hPSCs

Generation of osteochondrogenic ectomesenchyme from hPSCs first requires specification of cranial neural crest-like progeny. In order to reproducibly generate strong chondrogenic activity from hPSCs, we first optimized the method for specification of neural crest using CD271 as the readout. Fluorescence-activated cell sorting (FACS) analyses revealed that when H9 hESCs and BJ5 hiPSCs were differentiated in CDM using the conventional embryoid body (EB)-forming culture or 2D differentiation culture in the presence of SB431542 or other Nodal/Activin/TGFβ signaling inhibitor (e.g., A83-01 or SJN2511), they generated progeny that expressed CD271 (Figures 1A and S1). The CD271hi cell population, but not the CD271lo progeny, also expressed PDGFRα at low levels. Both populations lacked CD73 and CD13. The effect of SB431542 was dose dependent at least up to 10 μM (Figure S1D).

The CD271hiPDGFRαlo cell development reached a peak around day 6 of differentiation (Figure S1C), in parallel with the highest expression of the “neural crest specifier” genes PAX3 and PAX7 (Nelms and Labosky, 2010) (Figure 1B). In contrast, the neuroectoderm marker SOX1 was never induced, and the ESC marker NANOG was downregulated. Furthermore, the isolated CD271hi(PDGFRαlo)/CD73− progeny had higher levels of SOX10 and FOXD3 transcripts than the CD271lo−(PDGFRα+)CD73− cells, indicating enrichment of the fraction with neural crest-like progeny (Figure 1D). SOX9 is also implicated in the specification of cranial neural crest (Nelms and Labosky, 2010) and is expressed in premigratory human neural crest (Betters et al., 2010). The SOX9 reporter, GFP (Figures 1F and S1I) and SOX9 transcripts (Figure S1G) were expressed in the CD271hi−CD73− fraction. The PAX3 protein (Figure 1E) and PAX3 transcript (Figure S1G) were also detected in the CD271hi−CD73− fraction.

Furthermore, in sharp contrast to the results obtained from the hPSC differentiation to paraxial mesoderm (Umeda et al., 2012), when MIXL1 (an early mesendoderm gene)-green fluorescence protein (GFP) knockin hESCs (MIXL1-GFP) (Davis et al., 2008) were differentiated under similar conditions, no MIXL1-GFP+ progeny developed (Figure 1A). There was also negligible induction of a second mesendoderm transcript, T(Figure S1B) (Umeda et al., 2012). Therefore, neither CD271hi(PDGFRα+)CD73− nor CD271lo(PDGFRα+)CD73− cells were likely to be mesendodermal derivatives.

BMP and WNT are implicated in the neural crest specification (Milet and Monsoro-Burq, 2012). As expected, the BMP inhibitor Noggin suppressed the SB431542-induced development of the CD271hi(PDGFRαhi)(CD73+CD13−) neural crest-like progeny from H9 hESCs (Figure S1E). The WNT inhibitor FZD also showed an inhibitory effect, consistent with the findings of Menendez et al. (2011) (Figure S1D). Interestingly, BMP4 at 10 ng/ml, a concentration sufficient to induce mesoderm (Wang and Nakayama, 2009), was as inhibitory as Noggin, and the GSK3 inhibitor that mimics canonical WNT signaling showed weakly inhibitory effects (Figure S1E). However, when SOX9-GFP iPSCs were used, the GSK3 inhibitor was found to enhance the genesis of CD271hi−CD73− cells (Figure S1I).

Thus, inhibition of Nodal/Activin/TGFβ signaling with appropriate levels of BMP and WNT signaling is required for the effective development of CD271hi(PDGFRαhi)CD73−CD13− neural crest-like progeny from hPSCs (hereafter called CD271hiCD73− progeny) more quickly than previously attained (Lee et al., 2010; Menendez et al., 2011), potentially reflecting the specification of cranial instead of trunk neural crest cells.
Mesenchymal Cells Derived from the Nonmesendodermal hESC Progeny by Conventional Methods Show Weak, Transient Chondrogenic Activity

The neural crest-like progeny were then directed to commit to chondrogenic ectomesenchyme. First, using a conventional EB-outgrowth method (Hwang et al., 2006) (Figure S2A), we generated mesenchymal cells from the SB431542-treated H9 and MIXL1-GFP hESCs. In knockout serum replacement-based SR medium or serum-containing D10 medium, expansion of the outgrowth cells led to enhanced expression of CD73 and later CD13, but loss of the expression of CD271 (Figures S2D and S2E). As we reported previously (Umeda et al., 2012), MIXL1-GFP+ mesendodermal progeny were never detected during such studies (data not shown).

In 3D-pellet culture, the generated mesenchymal cells gave rise to a particle containing an area that weakly stained metachromatically (pink to purple) with Toluidine Blue and immunostained with anti-type II collagen (COL2) antibody at passage 1 (p1) (Figure S2F) and p2, but not from p3 to p5. The lack of chondrogenic activity in the primary

Figure 1. Directed Specification of Neural Crest from hPSCs
(A) FACS analysis demonstrating the SB431542-dependent development of CD271\(^{\text{hi}}\)PDGFR\(^{\text{a}}\)lo progeny during 2D differentiation of H9 and MIXL1-GFP hESCs in CDM. SB: 10 \(\mu\)M SB431542.
(B) Time-dependent changes in the gene expression profile during differentiation of H9 hESCs in the presence of SB (n = 3 technical repeats, mean ± SD).
(C) Isolation of H9-derived CD271\(^{\text{hi}}\)PDGFR\(^{\text{a}}\)CD73\(^{\text{lo}}\) and CD271\(^{\text{lo}}\)PDGFR\(^{\text{a}}\)CD73\(^{\text{hi}}\) progeny by FACS. (Left) Isotype control.
(D) Real-time RT-PCR analysis with cells from (C) demonstrating enrichment of neural crest-related transcripts in the CD271\(^{\text{hi}}\)CD73\(^{\text{lo}}\) cell fraction. Pre, presort cells (n = 3 technical repeats, mean ± SD).
(E) Intracellular FACS staining by anti-PAX3 antibody demonstrating presence of PAX3 protein in the H9-derived CD271\(^{\text{hi}}\)CD73\(^{\text{lo}}\) cell fraction. (Upper) Isotype control.
(F) FACS demonstration of SOX9-GFP expression in the CD271\(^{\text{hi}}\)CD73\(^{\text{lo}}\) progeny in SOX9-GFP hiPSCs differentiated for 6 days.
outgrowth cells (p0), suggests that a short-term expansion of the outgrowth cells is required for its development and/or accumulation. However, as reported by others (Nakayama and Umeda, 2011), we did not observe robust chondrogenic activity leading to a full-cartilage particle, as found for paraxial mesoderm derived from mPSCs and hPSCs (Nakayama et al., 2003; Umeda et al., 2012). Thus, conventional culture methods failed to generate and maintain strong chondrogenic activity from hPSC-derived neural crest-like progeny.

**Generation and Selective Expansion of CD271+PDGFRα+CD73+ Mesenchymal Cells in CDM in the Presence of FGF2 and SB431542**

Either in a FACS-purified form or in an unpurified mixture with other nonmesendodermal (i.e., MIXL1+) cells, the CD271hiCD73+ neural crest-like progeny failed to adhere to the culture dish in the absence of fibronectin and grew poorly in the medium in which they were specified, i.e., CDM plus SB431542 (SB; Figures 2B and S3A). Therefore, we tested the effects of growth factors, such as FGF2 that have been used for maintaining neural crest cells (Stemple and Anderson, 1992) and generating chondrogenic activity (Abzhanov et al., 2003) in culture, and of other factors, such as endothelin1, PDGF, and Sonic hedgehog that have been linked to cranial skeletogenesis and ectomesenchymal specification in vivo (Le Douarin and Creuzet, 2009). FGF2 alone significantly increased viability and supported growth of the neural crest-like progeny on fibronectin but was often associated with significant slowdown in an early stage of expansion culture. The addition of both FGF2 and SB431542 maintained the growth rate at least until p16 and supported maintenance of the normal karyotype at least to p10 (Figure S3B).

Direct expansion of the nonmesendodermal progeny mix in CDM in the presence of FGF2 and SB431542

**Figure 2. FGF2+SB431542 Generates and Expands SOX9-Expressing CD271+PDGFRα+CD73+ Ectomesenchymal Cells from Unsorted Nonmesendodermal Progeny**

(A) Graphical representation of the experimental procedure. SB, SB431542; F, FGF2; FSB, FGF2+SB431542.
(B) Growth curve of H9 hESC-derived ectomesenchymal cells expanded under F, FSB, and SB conditions (n = 3 independent cultures, mean ± SD).
(C) FACS analysis demonstrating the effect of FSB on the selective accumulation of CD271+CD73+ mesenchymal. H9 hESCs and BJ5 hiPSCs were differentiated (Pre) and expanded under the conditions indicated: D10, F, FSB, and FLDN (FGF2+LDN193189). p, passage; isotype control, Figure S3C.
(D) Micrographs of mesenchymal cells (p4) expanded under F and FSB.
(E) Real-time RT-PCR analysis demonstrating changes in the neural crest gene expression during expansion under F and FSB (n = 3 technical repeats, mean ± SD).
resulted in accumulation of CD271+/PDGFRα+/CD73+ mesenchymal cells (Figures 2C, S3, and S4). The cells were positive for the MSC markers CD105, CD166, CD29, CD44, and CD56, but negative for MSC markers CD13 and STRO1 (Figures S3D and S3E). In contrast, CD271 expression decreased in the presence of FGF2 alone, causing the CD271+/PDGFRα+/CD73+ cells to become the predominant cell population by p3 (Figure 2C). Similar results were obtained from expansion in D10 medium. The BMP receptor inhibitor LDN193189 was unable to demonstrate that they were chondrogenic using 2D microscopy. Under the condition in which PDGF-BB was added to the chondrogenesis medium on day 0 and then replaced with TGFβ3 on day 6, which was supplemented with BMP4 on day 10 (hereafter designated as PDGF/TGFβ/BMP condition) (Umeda et al., 2012), CD271+/CD73+ cells gave rise to acid Alcian Blue-positive cartilage nodules, the capacity of which lasted at least until p10 (i.e., 5 weeks) of expansion culture (Figures 3F and S4G). Since the CD271+/CD73+ ectomesenchymal cells maintained under FGF2+SB431542 conditions showed no chondrogenic activity and ceased to grow at p2. In contrast, the CD271+/CD73+ cells generated and maintained under FGF2 conditions showed weak chondrogenic activity as early as p3. The SB431542-cultured cells showed no chondrogenic activity and ceased to grow at p2.

**Brief Treatment with TGFβ Facilitates Formation of Macroscopic Cartilage Particle Formation from the CD271+/CD73+ Cells**

The ectomesenchymal cells expanded in CDM in the presence of FGF2 or FGF2+SB431542 were subjected to 3D-pellet culture under the PDGF/TGFβ/BMP condition. The early (p2) FGF2-expanded cells formed a large cartilage particle that stained metachromatically (pink to purple) with Toluidine Blue, but then lost such chondrogenic activity by
p4–p5 (Figures 4A and 4C), in keeping with the loss of the CD271+ cell population (Figures 2C and S3D).

On the other hand, the (FGF2+SB431542)-expanded cells did not form a particle when subjected directly to pellet culture (no pellet, Figure 4B), suggesting that (FGF2+SB431542)-expanded cells are defective in the induction of mesenchymal condensation, a prerequisite for cartilage formation. Supporting this suggestion, 2D-micromass culture demonstrated that inclusion of SB431542 until the point of induction of chondrogenesis using TGFβ3 and BMP4 severely inhibited or delayed the formation of cartilage nodules (Figures S4A and S4B). Thus, suppression of Nodal/Activin/TGFβ signaling allows the CD271+CD73+ cells to grow but not to initiate chondrogenesis under the action of TGFβ and BMP.

A brief treatment (“priming”) with TGFβ is known to commit mouse neural crest stem cells to mesenchymal lineages (John et al., 2011). Therefore, we reasoned that stimulation with TGFβ before pellet culture might be required for efficient cartilage particle formation. When the (FGF2+SB431542)-expanded cells were passaged once into CDM containing FGF2+TGFβ3 (FT), cultured for 3 to 4 days to confluence, and then subjected to 3D-pellet culture, large translucent full-cartilage particles filled with

Figure 3. The SOX9-Expressing CD271+CD73- Ectomesenchymal Cells Are Chondrogenic and Derived from the CD271hiCD73-C0 Neural Crest-like Cell Fraction
(A) Graphical representation of the experimental procedure.
(B) FACS analysis of the sorted CD271hiCD73-C0 and CD271loCD73-C0 progeny, expanded under F, FSB, FSJN (FGF2+SJN2511), and FA83 (FGF2+A83-01) for three passages.
(C) Real-time RT-PCR with cells in (B) demonstrating SOX9 expression specifically maintained in the FSB-expanded CD271hi cells. sort, pre-expanded cells (n = 3 technical repeats, mean ± SD).
(D) Immunofluorescence detection of SOX9 protein in the FSB-expanded CD271hi cells. Pink, SOX9; blue (nucleus), DAPI; graph, the value of % SOX9+ nuclei was the average of scoring from four areas (n = 4 technical repeats, mean ± SD); ++, strong signal; +, weaker signal; isotype control, Figure S4J.
(E) SOX9-GFP expression in the FSB-expanded CD271hi cells. SOX9-GFP hiPSCs were differentiated, and the CD271hiCD73-C0 progeny were isolated and expanded under FSB for five passages as in (A) and then FACS analyzed (Figure S3H). H9 hESC-derived, FSB-expanded cells in (B) were used as the GFP-negative control.
(F) Chondrogenic activity of the CD271hi or CD271lo progeny maintained under FSB. Cells were grown under FSB and F conditions for five passages and subjected to 2D-micromass cultures, followed by Alcian Blue staining and quantification. *p < 0.05 (n = 3 independent cultures, two masses/culture, mean ± SD).
matrices that stained metachromatically with Toluidine Blue and immunostained with the COL2 antibody were generated effectively (Figures 4Bi, 4Bv, 4Bvi, S4F, and S4H). The SB431542-dependent maintenance of chondrogenic activity during expansion culture was confirmed by the quantitative comparison of sulfated glycosaminoglycan (sGAG) levels in 24 days of pellet culture using ectomesenchymal cells maintained under FGF2+SB431542 (FSBp11/FT) and under FGF2 alone (Fp4) (Figure 4C).

When FGF2+TGFβ3 pretreatment was extended for a few more passages (FTp2), the cells maintained their pellet-forming capability but gradually lost their chondrogenic potential, resulting in particles with a small cartilaginous area (Figure 4Bii). Pretreatment with D10 medium also induced pellet-forming activity in the (FGF2+SB431542)-expanded cells, which led to a full-cartilage particle (Figure 4Biii), but further maintenance in D10 completely suppressed chondrogenic activity (Figure 4Biv). Neither pretreatment was able to recover lost chondrogenic activity.

Figure 4. Pretreatment with TGFβ Facilitates Cartilage Particle Formation from the FSB-Expanded Ectomesenchymal Cells (A and B) Cartilage particle formation from the H9 hESC-derived ectomesenchymal cells maintained under F (A) or FSB (B) by 3D-pellet culture. Toluidine Blue staining (purple) of sections of the cartilage particles, except for (Bvi), which is COL2 immunostained (brown). Some cultures were passaged once or twice (p2) to FT (CDM plus FGF2+TGFβ3) (Aii, Bi, Bii, Bv, Bvi), or D10 (Aiv, Biii, Biv), prior to pellet culture.

(C) Capacity of sGAG production in cartilage particles. The ectomesenchymal cells cultured as indicated (→FT, passage to FT) were subjected to pellet culture and total DNA and sGAGs were quantified. Negative control: undifferentiated ESCs. *p < 0.05 (n = 3 independent cultures, one pellet/culture mean ± SD).

(D) Upregulation of N-cadherin surface expression on the FSB-expanded ectomesenchymal cells during FT treatment. The ectomesenchymal cells maintained under F (Fp2) and FSB (FSBp5) were either passaged to FSB (→FSB), FT (→FT, FTp2), or D10 (→D10) or not passaged (Fp2), and then FACS analyzed.

(E) Effect of FT treatment on SOX9 protein expression. FSB-expanded p7 ectomesenchymal cells followed by FT culture (i.e., p8) were treated and analyzed as in Figure 3D. Graph, n = 4 technical repeats, mean ± SD. Isotype control, Figure S4J.

(F) Real-time RT-PCR analysis for investigating the effect of FT treatment on SOX9 gene expression (n = 3 technical repeats, mean ± SD).
in FGF2-expanded cells (Figures 4Aiii and 4Aiv). A brief TGFβ pretreatment thus seemed to enhance the condensing capacity but not the chondrocyte-forming ability of the expanded ectomesenchymal cells. In support of this observation, the proportion of cells expressing SOX9 protein increased only slightly from 76%–83% (Figure 3D) to 97% (Figure 4E), and the transcription levels of SOX9 (Figure 4F), SOX5, and SOX6 (data not shown) were unaltered by the 4-day FGF2+TGFβ3 treatment.

In contrast, FGF2+TGFβ3 treatment (→FT, Figure 4D) led to dramatic downregulation of the expression of CD271 in 3 to 4 days and complete loss of expression during the subsequent culture for two passages (→Fp2) and then subjected to FT treatment and 3D-pellet culture. The capacity for sGAG production is displayed as in (B). *p < 0.05 (n ≥ 3 independent cultures, one pellet/culture, mean ± SD).

(E) Real-time RT-PCR analysis for investigating the effect of FSB on maintaining SOX9 expression (n = 3 technical repeats, mean ± SD).

(Figure 5. The Long-Term Expanded Ectomesenchymal Cells Are Still Dependent on FSB)

(A) SB431542-dependent maintenance of CD271 expression. Ectomesenchymal cells maintained under FSB (p10) were either passaged one to three times to FSB again (p11, p14), or to FGF2 (→F, Fp3) and then FACS analyzed.

(B) Maintenance of sGAG production capacity. The ectomesenchymal cells maintained under FSB for 11–16 passages followed by FT treatment, and the freshly isolated chondrogenic paraxial mesoderm (PM) (Umeda et al., 2012) were subjected to pellet culture. The capacity for sGAG production is displayed as in Figure 4C. Negative control, hESCs; positive control, bovine articular cartilage (n = 3 technical repeats, mean ± SD).

(C) "Full-cartilage"-forming capacity remained in the FSB-expanded p13 ectomesenchymal cells derived from CD271+CD73−H9 progeny. Toluidine Blue staining.

(D) SB431542-dependent maintenance of chondrogenic activity. The p10 ectomesenchymal cells were passaged to either FSB for four passages (FSBp14) or F for two passages (→Fp2) and then subjected to FT treatment and 3D-pellet culture. The capacity for sGAG production is displayed as in (B). *p < 0.05 (n = 3 independent cultures, one pellet/culture, mean ± SD).
Furthermore, when 3D-pellet culture was performed, the (FGF2+SB431542)-expanded ectomesenchymal cells maintained their chondrogenic activity during further expansion (p11–16), as judged by the accumulation of sGAGs in the developed cartilage particles (Figure 5B), and the “full cartilage”-forming activity was preserved at least to p13 (Figure 5C). Interestingly, such chondrogenic activity was indistinguishable from that of the KDR ‘PDGFRα’ chondrogenic paraxial mesodermal cells (PM) we previously reported (Umeda et al., 2012). However, even two passages without SB431542 caused the p10 cells to begin showing signs of decline in their capacity to generate similar chondrocytes in quantity and quality (→p2, Figures 5D and 5E). These results indicate that expansion under FGF2-conditions does not select a permanently differentiated cell type and would likely be involved in “cell adhesion,” “angiogenesis,” and/or “skeletal system development.” Furthermore, as for the predicted developmental potentials, (FGF2+SB431542)-expanded cells still showed relevance to “peripheral nervous system development,” “melanocyte differentiation,” and/or “eye development” (Figure 6D), although essential genes such as SOX10, ASCL1-4, NEUROD1/2/4/6, NEUROG1-3, OLIG1-3, MITF, and PAX6 (Nelms and Labosky, 2010) were either not (yet) expressed or expressed at very low levels (FPKM ≤1; data not shown). In contrast, (FGF2+SB431542→FGF2)-cultured cells seemed to be associated more with “ossification,” “odontogenensis,” and “embryonic vicerocoranium morphogenesis.” In addition, consistent with the removal of SB431542, enhancement of “TGFβ receptor signaling” was suggested.

**The Presence of SB431542 Maintains Expression of Genes Representing “Proliferative” and “Primitive” Stages of Differentiating Neural Crest**

We performed genome-wide, comparative transcriptome analyses to elucidate the molecular basis of the effect of Nodal/Activin/TGFβ signaling-suppression on the maintenance of the proliferative and chondrogenic properties of the CD271+CD73+ ectomesenchymal cells. The CD271+CD73+ neural crest-like progeny of H9 hESCs were isolated and cultured under both conditions, the presence of SB431542 being necessary for preventing the loss of chondrogenic activity and CD271 expression for an extended period.

The overall mRNA profiles of the three resultant cell populations demonstrated that (FGF2+SB431542)-expanded neural crest cells are distinct from both FGF2- and (FGF2+SB431542→FGF2)-expanded cells (Figures 6A and 6B), whereas little distinction was found between the last two. However, the possibility remained that the FGF2-cultured neural crest cells are different types of cells from (FGF2+SB431542)-expanded cells. Therefore, to investigate the effect of TGFβ suppression, further comparative analyses focused on (FGF2+SB431542)- and (FGF2+SB431542→FGF2)-cultured cells.

First, gene ontology (GO) analysis suggested that (FGF2+SB431542)-expanded cells would likely be involved in “DNA replication,” “mitotic cell cycle,” and “cell division” (Figure 6D), while (FGF2+SB431542→FGF2)-cultured cells seemed to have the property of differentiated cells and would likely be involved in “cell adhesion,” “angiogenesis,” and/or “skeletal system development.” In Vivo Stability of the Cartilage Particle Developed In Vitro with the CD271+CD73+ Cells: A Comparative Analysis

Cartilage particles generated under standard “PDGF/TGFβ/ BMP” conditions (Umeda et al., 2012) with the CD271+CD73+ ectomesenchymal cells, derived either from a mixture of the 6-day differentiated H9 hESCs or from the FACS-isolated CD271+CD73+ neural crest-like progeny (Figures 7C and 7D), were all mineralized after subcutaneous transplantation into immunocompromized mice for 8–12 weeks (black area with von Kossa staining),
implying the capacity of the CD271⁺CD73⁺ cell to induce endochondral ossification. Consistently, the detection of RUNX2 transcript (2D-micromass culture, Figure 7E) and COL10A1 transcript (3D-pellet culture, Figure 7F) during chondrogenesis suggested that the ectomesenchymal cell-derived chondrocytes matured into hypertrophic chondrocytes.

Such a property of the ectomesenchymal cells might reflect a more general property of hPSC-derived chondrogenic cells. Therefore, we also examined the cartilage particles produced by the hPSC-derived KDR PDGFRα⁺ chondrogenic paraxial mesodermal progeny (Umeda et al., 2012). First, we significantly improved the yield of KDR PDGFRα⁺ progeny expressing higher levels of MEOX1, TCF15, as well as UNC1 (Figure S5) by replacing BIO with CHIR99021 and inhibiting FGF signaling with PD173074 at a later stage of differentiation. Next, cartilage particles were generated and transplanted subcutaneously. The transplanted particles were mineralized at the periphery, but four of six particles stably maintained internal unmineralized cartilaginous areas even after 12 weeks (Figure 7A). In some case, the whole cartilaginous particles remained unmineralized (Figure 7B). These results indicate that the strong tendency of terminal

Figure 6. Comparative Transcriptome Analysis of Ectomesenchymal Cells Using the RNA-seq Technology
(A) Heat map of the top 250 genes, which are differentially expressed among the three groups. Lanes 1–3: "FSB"; lanes 4, 5, 8: "F"; lanes 6, 7, 9: "FSB→F."
(B) Principal component analysis of the expression pattern of protein-coding genes among the three groups. Blue, "FSB"; red, "F"; green, "FSB→F."
(C) Selected list of culture condition-specific genes and common genes. Genes that gave a p < 0.05 were picked from Tables S3 and S4 except for few with * that gave slightly more variations (0.05 < p < 0.1). ND, not determined.
(D) GO analysis. Selected GO categories from Table S1 (upper) and a selected list of development-related GO terms from Table S2 (lower).
(E) RT-PCR confirmation of the relative gene expression levels predicted by the FPKM values (n = 3 independent cultures, mean ± SD).
maturation in vivo is not a general property of the hPSC-derived chondrogenic progeny. The neural crest-derived ectomesenchymal cells may innately possess such a property or may have been primed for it during expansion.

Endochondral ossification would replace the transplanted cartilage (consisting of human chondrocytes) with bone tissue made by osteoblasts and osteocytes of murine origin. We therefore addressed the question of whether the donor human chondrocytes completely disappeared from the bony particles generated in 12 weeks. As expected, immunohistochemical analyses revealed that the large hypertrophic chondrocytes showed human-specific signals (Figure 7G). Interestingly, human cells were also detected in a marrow-like space and around the COL1-positive bone pieces (Figure 7H), suggesting that the transplanted cartilage particles may contain cells with osteoblastic/osteocytic potential.

**DISCUSSION**

We have established a novel serum-free culture method for generating and expanding CD271⁺CD73⁺ SOX9⁺ chondrogenic ectomesenchymal cells from hPSC-derived, CD271⁺CD73⁻ neural crest-like progeny. The differentiation pathways from hPSCs to chondrocytes via chondrogenic ectomesenchymal cells postulated from this study are summarized in the Graphical Abstract. Under optimal conditions, the generation of neural crest-like progeny
from hPSCs in CDM was achieved more quickly than previously reported, and the resultant ectomesenchymal cells displayed long-term chondrogenic activity.

Quantitative data on the efficiency of genesis of SOX9-expressing chondroprogenitors from hESCs were first shown by Oldershaw et al. (2010). Under their conditions in which hESC differentiation is directed initially to mesoderm then toward chondrocytes, approximately 8.5 chondroprogenitors of 75%–97% SOX9+ are produced per hESC by day 14 of differentiation. In contrast, while only about 0.6 CD271hiCD73− neural crest-like progeny were generated per hPSC by day 6 of differentiation in CDM plus SB431542 (data not shown), our method for neural crest-like cell culture in CDM in the presence of FGF2+SB431542 led to 19 to 38 CD271−CD73+ chondroprogenitors per hPSC by day 14 (Figure S3A). Approximately 76%–85% of these cells continued to express SOX9 protein on days 27–33 (p8–p10), a proportion that increased slightly to about 97% after 3 days of treatment with FGF2+TGFβ3. Further expansion yielded approximately 1 × 107 chondrogenic ectomesenchymal cells per hPSC by day 51 (p16, 24 population doublings). Thus, the method described here allows human chondrogenic cells to expand for an extended period without the loss of purity and chondrogenic activity, resulting in a much larger yield of chondroprogenitor cells (although not homogenous) from hPSCs than previously attained.

The effect of SB431542 on expansion of endothelial progenitors derived from mouse and human ESCs has also been reported (James et al., 2010; Watabe et al., 2003). A lack of Nodal/Activin/TGFβ signaling may generally promote proliferation and prevent terminal differentiation of embryonic stem/progenitor cells. In this respect, the ways in which FGF2+SB431542 helps to maintain SOX9 and CD271 while suppressing N-cadherin expression during expansion of the chondrogenic CD271−CD73+ cells and in which CD271hiCD73− neural crest-like cells are directed to give rise to such cells are interesting topics for future study. In this respect, comparative transcriptome analysis has revealed that the CD271−CD73+ cells accumulated under FGF2+SB431542 conditions possess a similar mRNA profile to primitive neural crest/ectomesenchymal cells, although they lacked SOX10 expression, which is critical for neural and melanocytic lineage commitment. Thus, suppression of Nodal/Activin/TGFβ signaling does not seem to freeze the developmental stage of the hPSC-derived neural crest during expansion. Such suppression may instead simply support the high proliferative potential of the cells as well as the expression of SOX9 (and COL2A1), and thereby maintain chondrogenic activity. SOX9 expression initiated at the specification and premigratory stages is transient in trunk neural crest but persists in cranial neural crest (Cheung and Briscoe, 2003; Cheung et al., 2005; McKeown et al., 2005).

The chondrogenic CD271−CD73+ ectomesenchymal cells that maintain SOX9 transcription and translation (SOX9-GFP+/lo) may therefore represent proliferating cranial neural crest, with a slight commitment to non-neural lineages.

Genesis of MSC-like cells from hESCs directly or via neuroectoderm specification has been demonstrated (Chambers et al., 2009; Mahmood et al., 2010). As shown in Figure S4D, the colonogenic (CFU-F) activity, representing self-renewal activity of MSCs, emerged as early as p2 of the expansion culture under FGF2 alone (i.e., in CD271−CD73 cells) or FGF2+SB431542 (i.e., in CD271−CD73+ cells). However, we were unable to demonstrate significant adipogenic or osteogenic activity in either cell type (data not shown), although the GO analysis predicted osteogenic activity in cells generated under FGF2 (data not shown) and FGF2+SB431542→FGF2 conditions. This discrepancy may be due to some degree of heterogeneity in the cell types generated under these conditions or to the assay method employed, which is widely used for MSCs (Pittenger et al., 1999), but is less well validated for mesenchymal cells derived from human neural crest. Furthermore, the FGF2+SB431542 conditions applied to the expansion culture of mouse bone marrow MSCs resulted in inhibition of proliferation and promotion of adipocytic differentiation (data not shown). The CD271−CD73+ ectomesenchymal cells are therefore very likely not to be MSCs, although the possibility that they are derived from an MSC-like precursor has not been excluded.

Thus, hPSC-derived chondrogenic ectomesenchymal cells are amenable to large-scale production in CDM without loss of activity. Although some hESC-derived neural crest stem cells are known to self-renew in culture for an extended period, during which they maintain the capacity to produce multipotential MSC-like activity (Menendez et al., 2011), no previous reports have described a method as simple and effective as ours for generating large quantities of (osteochondrogenically committed mesenchymal cells under clinically applicable, defined conditions. Therefore, the chondrogenic ectomesenchymal cells produced by the culture technology described are set to become a competitive alternative to adult MSCs, especially for craniofacial regenerative therapy. Moreover, taking advantage of high yields of chondroprogenitor cells, we have successfully modeled neonatal-onset multisystem inflammatory disease by applying our method to patient-derived iPSCs (Yokoyama et al., 2015).

EXPERIMENTAL PROCEDURES

Cells

H9 (WA09), HES3 (ES03), MIXL1-GFP hESCs, and the human fibroblast-derived iPSC line, BJ5, were maintained on feeder cells as described (Umeda et al., 2012). The SOX9-GFP hiPSC
(CY2-SOX9-2A-ZsGreen-2A-Puro) line from NIH was maintained feeder free in E8 medium.

**Neural Crest Cell Differentiation from hPSCs**

For generating neural crest-like progeny, H9 hESCs and BJ5 hiPSCs were transferred to gelatin-coated dishes and cultured feeder free in the differentiation medium: CDM supplemented with SB431542. The SOX9-GFP hiPSCs were directly induced to differentiate by changing the medium to differentiation medium.

**Paraxial Mesoderm Generation from hESCs**

The hPSCs were differentiated as described (Umeda et al., 2012) (“2i” condition) or using improvements involving CHIR99021, Noggin, PD173074, and PDGF with or without SB431542 (“3i+P” or “4i+P,” Figure S5).

**Flow Cytometry**

FACS analysis and cell sorting were performed as described (Umeda et al., 2012). Intracellular staining for FACS analysis using mouse anti-PAX3 monoclonal antibody was performed according to the manufacturer’s recommendations.

**Ectomesenchymal Cell Genesis and Expansion**

The differentiated hPSCs before and after sorting for CD271hiCD73- were plated onto fibronectin-coated dishes and cultured in CDM supplemented with FGF2 and/or SB431542 (or other small molecules) or in the serum-containing D10 medium.

**Immunofluorescence Detection of SOX9**

The CD271hiCD73- neural crest-like progeny were cultured in an eight-well chamber slide for 3 days in CDM containing either FGF2-SB431542 or FGF2-TGFβ3 and immunostained with the anti-hSOX9 polyclonal antibody.

**Chondrogenesis Assays**

2D-micromass culture and 3D-pellet culture were performed and analyzed as described (Nakayama et al., 2003; Tanaka et al., 2009; Umeda et al., 2012).

**Quantification of Sulfated Glycosaminoglycans**

DNAs and sulfated glycosaminoglycans (sGAGs) from micromasses and cartilage particles were isolated, quantified, and analyzed as described (Zhao et al., 2014).

**Subcutaneous Transplantation and Immunohistorical Analysis**

Cartilaginous particles were transplanted under the dorsal skin of NODScid or NODScid Il2rg<sup>-/-</sup> (NSG) mice for 8–12 weeks and analyzed as described (Zhao et al., 2014). To detect remaining human cells, the fixed particles were first decalcified and then paraffin embedded, sectioned, and stained with the mouse antihuman mitochondria monoclonal antibody.

**Gene-Expression Profiling**

Total RNAs were isolated and reverse transcribed (RT), and real-time PCR was performed using the Taqman Gene Expression Assay. The results were displayed as described (Zhao et al., 2014). RNA sequencing was performed on an Illumina HiSeq 1500. Sequenced reads were deposited to Gene Expression Omnibus (GSE64752) and mapped against the human reference genome (GRCh37). Expression levels were calculated as fragments per kilobase of exon per million mapped fragments (FPKMs) (Tables S1, S2, S3, and S4).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at [http://dx.doi.org/10.1016/j.stemcr.2015.02.012](http://dx.doi.org/10.1016/j.stemcr.2015.02.012).

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Long-Term Expandable SOX9⁺
Chondrogenic Ectomesenchymal Cells
from Human Pluripotent Stem Cells
Katsutsugu Umeda, Hirotsugu Oda, Qing Yan, Nadine Matthias, Jiangang Zhao, Brian R. Davis, and Naoki Nakayama
Fig. S1

**a**
CDM + 10 µM SB431542 (SB)

**b**
FACS and RNA analyzes

**c**
PDGFRα

**d**
IsoSB 1 SB 3 SB 10 FZD

**e**
None BMP4 Nog BIO

**f**
CDM + SB (+CHIR d0-4)

**g**
H9 hESCs sorted

**h**
BD5 hiPSCs

**i**
SOX9-GFP hiPSCs

**j**
CD271

**k**
CD73

**l**
SOX9-GFP
Fig. S2

- **a**
  - Time points: d0, d6, d14, d17, d20, d23, d26, d29
  - EB in CDM+SB
  - Outgrowth in KSR p0
  - Expansion in KSR / D10 p1-5
  - FACS and RNA analysis (b, c)
  - FACS analysis and pellet culture (d-f)

- **b**
  - PDGFRα vs. CD271

- **c**
  - PAX3 expression over time

- **d**
  - Graph showing % positive cells at EB, P0, P1, P2, P3, P5

- **e**
  - Counts of CD73, PDGFRα, CD13, CD271 for SR and D10

- **f**
  - Images of COL2, CD73, PDGFRα, CD13, CD271 for SR and D10
  - Scale bar: 200 µm
a

b

Fig. S3
Fig. S4

**a** FSB p3  FSB p3 (F/TB)  FSB p3 (FSB/TB)

FSB p10  F p3  SB p2

**b**

OD600

|         | F/TB | FSB/TB | P/TB |
|---------|------|--------|------|
| p5      | 0.4  | 0.3    | 0.2  |
| p6      | 0.3  | 0.2    | 0.1  |
| p7      | 0.2  | 0.1    | 0.0  |
| p8      | 0.1  | 0.0    | 0.0  |
| p9      | 0.0  | 0.0    | 0.0  |
| p10     | 0.0  | 0.0    | 0.0  |

**c**

Relative Expression

|         | SOX9 | NKX3.2 | COMP |
|---------|------|--------|------|
| p4      | 0.5  | 0.3    | 0.2  |
| p5      | 0.4  | 0.2    | 0.1  |
| p6      | 0.3  | 0.1    | 0.0  |
| p7      | 0.2  | 0.0    | 0.0  |
| p8      | 0.1  | 0.0    | 0.0  |
| p9      | 0.0  | 0.0    | 0.0  |
| p10     | 0.0  | 0.0    | 0.0  |

**d**

CFU-F frequency

|         | SB | FSB | F |
|---------|----|-----|---|
| p5      | 0.2| 0.1 | 0.0|
| p6      | 0.1| 0.0 | 0.0|
| p7      | 0.0| 0.0 | 0.0|
| p8      | 0.0| 0.0 | 0.0|
| p9      | 0.0| 0.0 | 0.0|
| p10     | 0.0| 0.0 | 0.0|

**e**

BJ5 unsorted FSBp5

CD271 vs CD73

**f**

Mix progeny

FT

BJ5 unsorted progeny

500 µm

**g**

BJ5-CD271hi

FSB p1  FSBp3

BJ5-CD271lo

FSB p2  FSBp4

**h**

BJ5 FACS-purified progeny

CD271hi

FSB p5  p9

FT

500 µm

**i**

CD271 vs CD73

**j**

FSB p8  FSB p7→FT

IgG  DAPI

50 µm

**k**

IgG  DAPI

50 µm

**l**

IgG  COL1  DAPI

50 µm

100 µm
Fig. S5
a

b

Neuronal rosette (200x)  Pigmented cells (200x)  White adipose tissue (200x)

Cartilage (200x)  Bone (200x)  Gland (100x)
2. Legends to Supplemental Figures

Fig. S1 Specification of neural crest-like progeny from hPSCs.

(a) Graphical representation of the standard differentiation protocol. (b) Time-dependent changes in the T transcript during H9 hESC differentiation as in (a). (n=3 technical repeats, mean ± SD) Note that the weak expression of T transcript on day 2 was approximately 1/50 to 1/100 of the level achieved under conditions for induction of paraxial mesoderm (Umeda et al., 2012). (c) Time-dependent changes in the CD271\(^{hi}\)PDGFR\(\alpha^{lo}\) progeny profile during 2D differentiation of H9 hESCs. (d) Nodal/Activin/TGF\(\beta\) receptor inhibitor-dependent development of CD271\(^{hi}\)PDGFR\(\alpha^{lo}\) progeny from hESCs. The H9 hESCs were differentiated for 6 days in 2D in the presence of SB431542 (1-10 \(\mu\)M, SB1-SB10), A83-01 (A83), SJN2511 (SJN), LDN193189 (LDN), or FZD, either alone (FZD) or in the presence of 10 \(\mu\)M SB431542 (FZD+SB), and analyzed by FACS. (e) Factor-dependent development of CD271\(^{hi}\)PDGFR\(\alpha^{lo}\) progeny from hESCs. The H9 hESCs were differentiated for 6 days in 2D in the presence of 10 ng/ml BMP4 (BMP), 100 ng/ml Noggin (Nog) or 5 \(\mu\)M BIO in the presence or absence of 10 \(\mu\)M SB431542 (SB), and subjected to FACS analysis. (f) Graphical representation of the standard differentiation protocol. CDM + SB: CDM in the presence of 10 \(\mu\)M SB431542 (g) Enrichment of neural crest cell-related transcripts in the CD271\(^{hi}\)(PDGFR\(\alpha^{lo}\))CD73\(^{-}\) progeny. The fractionated cells in Fig. 1c were subjected to real-time RT-PCR analysis using the primers indicated. (n=3 technical repeats, mean ± SD) (h) Development of CD271\(^{hi}\)CD73\(^{-}\) (CD271\(^{hi}\)) and CD271\(^{lo}\)CD73\(^{-}\) (CD271\(^{lo}\)) progeny from BJ5 hiPSCs. BJ5 cells were differentiated as in (f) without CHIR99021 (CHIR) and analyzed by FACS for CD271 and CD73 expression. (i) Enhancement of the genesis of CD271\(^{hi}\)CD73\(^{-}\) neural crest-like cells from SOX9-GFP hiPSCs. The SOX9-GFP hiPSCs underwent direct (2D) differentiation to neural crest under CDM+SB in the presence of 2 \(\mu\)M CHIR99021 from day 0 to day4, and were analyzed by FACS on day 6 (f). Note that the
SOX9-GFP hiPSC differentiation was not very efficient under the standard CDM+SB condition (Fig. 1f). It has been demonstrated that activation of canonical WNT signaling and suppression of BMP signaling leads to neural crest formation from hPSCs (Menendez et al., 2011). Although BMP inhibition was not included, CHIR99021 treatment for the initial 4 days of differentiation was found to enhance the CD271⁺GFP⁺/⁻ cell genesis by day 6.

**Fig. S2 EB-outgrowth and enrichment of mesenchymal progeny by conventional expansion culture results in transient chondrogenic activity.**

(a) Graphical representation of the experimental procedure for EB outgrowth. (b) FACS analysis for CD271 and PDGFRα expression on the day-6 EB cells generated from H9 hESCs as in (a). (c) Time-dependent changes in PAX3 expression during H9 hESC differentiation as in (a). (n=3 technical repeats, mean ± SD) (d) Surface marker expression profiles of EB outgrowth-derived mesenchymal cells expanded in SR medium (passage [p]0: primary outgrowth cells, p1-p5: expanded outgrowth cells). (e) Representative CD73, PDGFRα, CD13, and CD271 expression on expanded outgrowth cells at p5 in D10 and SR medium. Note that CD271 disappeared during expansion in D10 and SR media, but CD13 appeared preferentially in D10. (f) Cartilage formation from the outgrowth-derived mesenchymal cells. The primary (p0) and expanded (p1-p5) outgrowth cells were subjected to 3D pellet culture. The resulting particles were sectioned and stained with Toluidine Blue and anti-COL2 antibody (COL2). Arrows indicate small cartilage nodules. Green is tissue dye.

**Fig. S3 Characterization of the ectomesenchymal cells.**

(a) Display of the Fig. 2b results according to time. (n=3 independent cultures, mean ± SD) (b) Karyotype analysis of the (FGF2+SB431542)-expanded CD271⁺CD73⁺ ectomesenchymal cells (p10). Karyotype analysis was performed at the Clinical and Research Cytogenetic Laboratory,
Texas Children’s Hospital. Cytogenetic evaluation of harvested cells revealed a normal female karyotype in all metaphase cells analyzed. (c) SB431542 dose-dependent generation and maintenance of CD271⁺CD73⁺ ectomesenchymal cells from the CD271⁺CD73⁻ neural crest-like progeny. F: CDM plus FGF2, FSB 1-10 μM: CDM plus FGF2 + 1-10 μM SB431542. (d) Time-course of the SB431542 (FSB) effect on the expression of CD13 and PDGFRα on ectomesenchymal cells during expansion in CDM plus FGF2 (F). Note: CD13 expression was induced under F (but not FSB) culture at p6, while slight up-regulation of PDGFRα expression was observed during expansion under both conditions. (e) MSC marker analysis on (FGF2+SB431542)-expanded CD271⁺CD73⁺ ectomesenchymal cells (p6). Note: The cells expressed all MSC markers tested except STRO1. (f) SOX9-GFP hiPSCs were differentiated for 6 days as in Fig. 1f. The resultant progeny were then expanded under FGF2+SB431542 conditions for 2 passages and fractionated by FACS for the isolation of SOX9-GFP⁺ and SOX9-GFP⁻ mesenchymal cells. (g) RNAs were isolated from the sorted GFP⁺ and GFP⁻ cells, pre-sorted progeny (diff presort) and the undifferentiated hiPSCs, and subjected to real-time RT-PCR. (n=3 technical repeats, mean ± SD) Note that the SOX9 transcript is enriched in the GFP⁺ cells. (h) SOX9-GFP hiPSC-derived ectomesenchymal cells expanded (p5) under FGF2+SB431542 were CD271⁺CD73⁺. Supplemental to Fig. 3e.

**Fig. S4 Biological activities of the FSB-expanded ectomesenchymal cells.**

(a) Cartilage nodule formation by the H9 hESC-derived, FGF2+SB431542 (FSB)-, FGF2 (F)-, and SB431542 (SB)-expanded mesenchymal cells (without FACS purification of CD271⁺³ progeny). The FSB-, F-, and SB-expanded cells at p2, p3, and p10 were subjected to 2D-micromass culture under PDGF/TGFβ/BMP, FGF/TGFβ/BMP (F/TB), or FGF+SB/TGFβ/BMP (FSB/TB) conditions, and stained with acid Alcian Blue. (b) A culture step in the absence of SB is necessary for induction of chondrogenesis. The FSB-expanded p10 cells (a) were subjected
to 2D-micromass cultures under FGF/TGFβ/BMP (F/TB), FGF+SB/TGFβ/BMP (FSB/TB) and PDGF/TGFβ/BMP (P/TB) conditions. The micromass cultures were fixed and stained with acid Alcian Blue, and the bound Alcian Blue was extracted from each micromass, and quantified by OD600. (n=8 technical repeats, mean ± SD) Note: Interestingly, efficient cartilage nodule formation was also observed under F/TB (i.e. FGF2 replacing PDGF during the initial 6 days of micromass culture). (c) Expression of cartilage-specific genes during 2D-micromass cultures. The kinetics of chondrogenesis from the (FGF2+SB431542)-expanded cells was also monitored by the expression during micromass culture of the chondroprogenitor genes NKX3.2, SOX9 and RUNX2 (Fig. 7e) and the chondrocyte/cartilage matrix genes COL2A1 (Fig. 7e), COL10A1 and COMP. The FSB-expanded p7 ectomesenchymal cells were subjected to differentiation under PDGF/TGFβ/BMP, and the resulting micromasses were harvested on days 0, 6, 10, and 14 and subjected to real-time RT-PCR analysis with primers for the genes indicated. (n=3 technical repeats, mean ± SD) Note: except for COL10A1 expression, which was first detected after day 14, the expression of other genes was upregulated between days 6 and 10 and the level increased by day 14. In contrast to human paraxial mesoderm (Umeda et al., 2012), RUNX2 expression was enhanced in a time dependent manner even before day 14. (d) CFU-F generation during the expansion of ectomesenchymal cells under SB431542 (SB), FGF2+SB431542 (FSB), and FGF2 (F) conditions. Three factors suggest that the long-term expandable CD271⁺CD73⁺ chondrogenic ectomesenchymal cells might consist of, or contain, MSCs: 1) the CD271⁺CD73⁺ chondrogenic ectomesenchymal cells are also CD56⁺ (Fig. S3e), 2) the CD271⁺CD56⁺TNAP (tissue-nonspecific alkaline phosphate)⁺ uncultured hMSCs are highly enriched in chondrogenic activity that is difficult to maintain during conventional culture, as is the expression of CD271, CD56 and TNAP (Aicher et al., 2011), and 3) one of the developmental origins of bone marrow MSCs is neural crest (Morikawa et al., 2009; Takashima et al., 2007). Therefore, ectomesenchymal cells were plated at clonal densities at each passage, and the
cultures were maintained for 14 days in αMEM 20% (v/v) FBS at 37°C under 5% O₂/5% CO₂. Cells were fixed in Z-Fix and stained with Toluidine Blue. The CFU-F frequency was calculated as follows: the number of colonies was divided by the plating cell number, and displayed as mean ± SD (thin vertical bars): n=3 technical repeats. The STRO1⁺ human bone marrow MSCs expanded to p5-p6 gave a CFU-F frequency of approximately 0.1. (e) Selective growth of CD271⁺CD73⁺ ectomesenchymal cells from an unsorted mixture of non-mesendodermal progeny of BJ5 hiPSCs, containing neural crest-like cells, under FGF2+SB431542 (FSB) conditions (p5). Supplemental to Fig. 2c. (f) Cartilage particle formation with the FSB-expanded ectomesenchymal cells. The 3-day preculture in CDM plus FGF2+TGFβ3 (FT) allowed p4 and p8 ectomesenchymal cells to form full-cartilage particles during 3D pellet culture. (g) FACS analysis of the sorted CD271⁺hiCD73⁻ (CD271⁺hi) and CD271⁺loCD73⁻ (CD271⁺lo) progeny of BJ5 hiPSCs (Fig. S1h) expanded under FSB. Supplemental to Fig. 3b. (h) Cartilage particle formation with the FSB-expanded ectomesenchymal cells derived from the CD271⁺hi progeny of BJ5 hiPSCs. The 3-day preculture under FT allowed p5 and p9 ectomesenchymal cells to form full-cartilage particles. (i) H9 hESCs were differentiated and subjected to FACS analysis as described in Fig. 1c. (j) Isotype controls for Figs. 3d (FSBp8) and 4e (FSBp7→FT) are shown. In both cases, the anti-SOX9 antibody was replaced with goat IgG. (k,l) Isotype controls for Fig. 7g (k) and for Fig. 7h (l). In both cases, the anti-human mitochondria antibody was replaced with mouse IgG.

**Fig. S5 Improving the method of paraxial mesoderm specification.**

(a, b) Requirement for Noggin and determination of proper timing. H9 hESCs were differentiated for 7 days toward paraxial mesoderm as described in “Supplemental Experimental Procedures”. Briefly, differentiation was initiated under modified 3i (a) and 3i+ P (b) conditions, in which Noggin (N) was added on days 0, 1 or 2 (Nd0-Nd2) (a, b), and removed from day 3 (Nd3-) (b).
Transcripts for *UNCX* and *MEOX1* were quantified by real-time RT-PCR. Note that the presence of Noggin from day 1 or day 2 till the end of culture gave the best results. (c) Effect of FGFR inhibitor PD173074 and determination of optimal concentrations. H9 hESCs were differentiated for 8 days under modified 3i+P conditions (CHIRN) and 2i conditions (Umeda et al., 2012) (BION) in which PD173074 was added on day 3 at the indicated concentrations. Transcripts for *UNCX*, *MEOX1*, *TBX18* and *TCF15* were quantified by real-time RT-PCR. Note that approximately 0.4 µM PD173074 enhanced *MEOX1*, *UNCX* and *TBX18* expression. (d) Effect of SB431542 and determination of proper concentrations. H9 hESCs were differentiated for 8 days under modified 4i+P conditions, in which SB431542 was added on day 3 at the indicated concentration. The transcripts for *MEOX1* and *TCF15* were quantified by real-time RT-PCR. Note that >2 µM SB431542 enhanced *MEOX1* and *TCF15* expression. (e) The APJ−KDR−PDGFRα+(K-P+) fraction and a minor APJ−KDR−PDGFRα+(A+P+) fraction are concentrated in the paraxial mesodermal progeny. H9 hESCs were differentiated for 7 days under the 4i+P condition. Differentiated progeny were fractionated by FACS based on the expression of KDR, PDGFRα and APJ, and transcripts for *UNCX*, *MEOX1* and *TCF15* were quantified by real-time RT-PCR in each sorted fraction. Note that as for differentiation under the 2i condition we reported previously (Umeda et al., 2012), the KDR−PDGFRα+ fraction is enriched in paraxial mesodermal progeny. At this stage, the APJ+ cells represented a very minor population (data not shown) and contained paraxial mesodermal cells at a similar concentration to the KDR−PDGFRα+ fraction. (a-e, n=3 technical repeats, mean ± SD)

**Fig. S6 Characterization of the BJ5 hiPSC line.**

(a) Karyotype analysis of the BJ5 hiPSC line was performed at the Clinical and Research Cytogenetic Laboratory, Texas Children’s Hospital. Cytogenetic evaluation of harvested cells revealed a normal male karyotype in all metaphase cells analyzed (46,XY). (b) Teratoma
formation of the BJ5 hiPSC line (p15) was performed by Applied StemCell (Menlo Park, CA). The BJ5 hiPSCs (2-3x10⁶) were injected in 30% (v/v) Matrigel (BD) into kidney and testis of 6-week-old Fox Chase SCID beige mice (Charles River, Wilmington MA). On days 50 and 65 post injection, tumors were fixed with 10% (v/v) formalin, paraffin embedded, sectioned, and histologically examined by hematoxylin and eosin staining. Cell types originating from all three embryonic germ layers were found. Undifferentiated neoplastic cells were also observed.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cells, factors, and antibodies

H9 (WA09) and HES3 (ES03) hESCs from WiCell (Madison WI) were maintained on mouse embryonic fibroblast feeder cells in SR medium: Dulbecco’s modified Eagle’s medium (DMEM): Ham’s F12 (1:1), 20% (v/v) KnockOut Serum Replacement (KSR), 2 mM GlutaMAX, 0.1 mM nonessential amino acids (all from Invitrogen, Carlsbad, CA) and 90 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis MO), supplemented with 4 ng/ml FGF2 (R&D Systems, Minneapolis, MN) as described (Umeda et al., 2012). MIXL1-GFP hESCs were maintained in the same manner except that the FGF2 concentration was 10 ng/ml (Davis et al., 2008). The BJ5 hiPSC line was established by the retroviral transduction of the four Yamanaka-factors plus NANOG to the human normal foreskin fibroblast line BJ, purchased from American Type Culture Collection (Manassas, VA) (Takahashi and Yamanaka, 2006). BJ5 cells were maintained in the same manner as hESCs except that the concentration of FGF2 was higher (40 ng/ml). The BJ5 cells were karyotypically normal and formed teratomas in immunocompromised mice (Fig. S6). The SOX9-GFP hiPSC (CY2-SOX9-2A-ZsGreen-2A-Puro, in which the ZsGreen open reading frame [ORF] is inserted with the 2A peptide in frame at the C-terminal end of hSOX9 ORF) line was kindly provided by Dr. M. Rao at the NIH Center for Regenerative Medicine and maintained on a vitronectin-coated plate in E8 medium (Invitrogen). Antibodies, cytokines, media and supplements, buffers and specialized tissue culture plates were generally sourced as described (Wang and Nakayama, 2009; Wang et al., 2010).

Embryoid body (EB)-outgrowth culture

Mesenchymal cells were generated from hESCs as described (Hwang et al., 2006) with modifications. H9 and MIXL1-GFP hESCs were separated into small clumps with collagenase I (Worthington BioChemical, Lakewood, NJ) and resuspended in chemically defined medium (CDM): Iscove modified Dulbecco’s Medium: Ham’s F12 (1:1), 5 mg/ml fatty acid-free bovine
serum albumin (Sigma), 2% (v/v) chemically defined lipid concentrate (Invitrogen), 2 mM GlutaMax (Invitrogen), 100 µg/ml holo-transferrin (Sigma), 20 µg/ml insulin (Sigma), 0.17 mM ascorbic acid-2-phosphate (Sigma), and 0.45 mM monothioglycerol (Sigma) for 6 days. The EBs generated were transferred to a gelatine-coated dish and cultured in SR medium for 8-10 days to allow attachment of EBs to the plate and outgrowth of mesenchymal cells. The mesenchymal cells were enriched further by expansion culture (3-5 passages) either in D10 medium: DMEM (Invitrogen), 10% (v/v) fetal bovine serum (FBS, HyClone, Logan UT) and 2 mM GlutaMax, or in SR medium. The medium was changed every 3-4 days, and the cells were passaged on reaching 80-90% confluence.

**Neural crest cell differentiation from hPSCs.**

H9 hESCs and BJ5 hiPSCs were treated with collagenase I to generate clumps, which were plated onto gelatin-coated dishes without feeder cells. The clumps were cultured for 6 days in Differentiation medium: CDM supplemented with 10 µM SB431542 (Tocris, Ellisville MO) and/or other protein factors and small molecules at 37°C under 5% CO₂/5% O₂. Media was changed once on day 3. The SOX9-GFP hiPSCs maintained feeder-free in the E8 medium were directly induced to differentiate by changing the medium to Differentiation medium and culturing for 6 days. When specified, CHIR99021 (BioVision, Milpitas CA) was added from day 0 to day 4. Factors used: mNoggin-Fc chimera (Noggin, R&D) at 100 ng/ml, mFrizzled8-Fc chimera (FZD, R&D) at 2 µg/ml, hBMP4 (R&D) at 10 ng/ml, BIO (Calbiochem, San Diego CA) or CHIR99021 at 2 µM, SB431542 at 1-10 µM, A83-01 (Tocris) at 0.1 µM, SJN2511 (Tocris) at 1 µM, and LDN193189 (Stemgent, Cambridge MA) at 0.5 µM. On the day of harvest, a suspension of single cells was prepared by treatment of the culture with 0.05% (w/v) trypsin-EDTA or TrypLE Select (diluted 2.5 fold with 0.5 mM EDTA in PBS) (Invitrogen).

**Paraxial mesoderm generation from hPSCs**

The HES3 and H9 hESCs were differentiated using the original method based on BIO and Noggin as described (here designated ‘2i condition’) (Umeda et al., 2012). Briefly, hESCs were
differentiated as aggregates in the CDM (without ascorbic acid, -AA) with 2 µM BIO and 100 ng/ml Noggin at 37°C under 5% CO₂/5% O₂. The developed EBs were then transferred on day 3 into a semi-solid CDM(-AA) (i.e. including 0.9% [w/v] methylcellulose [Methocel A4M, Dow Chemical, Midland MI]), supplemented with 5 µM BIO, and 100 ng/ml Noggin. The improved method used for hiPSCs and H9 hESCs was as follows: briefly, hPSCs were differentiated as aggregates in CDM(-AA) with 5 µM CHIR99021, and Noggin was added at 100 ng/ml on day 1 or 2. The developed EBs were transferred onto a semi-solid CDM(-AA), supplemented with 0.1 µM CHIR99021, 0.2-0.4 µM PD173074 (FGFR inhibitor, Tocris), 100 ng/ml Noggin and 5 ng/ml hPDGF-BB (R&D) on day 3 (the condition designated ‘3i+P’, Fig. S5). When needed, SB431542 was also added at 5 µM on day 3 (i.e. 4i+P). The EBs were collected on day 7 or 8.

Flow cytometry

The single-cell suspension from differentiated hPSCs was stained with anti-human KDR (#101M20, RELIATech, Germany), CD13 (#0778, Immunotech/BeckmanColter, Indianapolis IN), PDGFRα (#556001 BD Biosciences, San Jose CA), CD29 (#561794, BD), CD44, (#555478 and 560890, BD), CD271 (#560326 and 557195, BD), CD56 (#318303, BioLegend, San Diego CA, and #12-0569-71, eBioscience, San Diego CA), CD73 (#344004, BioLegend), CD105 (#323203, BioLegend), CD166 (#343903, BioLegend) and STRO1 (kindly provided by Dr. P. Simmons) monoclonal antibodies, as described (Umeda et al., 2012). Intracellular staining for FACS analysis using mouse anti-PAX3 monoclonal antibody (#1.C2457A, R&D) was performed according to the manufacturer’s recommendations, except that it was preceded by treatment with LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen) and anti-human CD271 antibody (#557195, BD) staining. For staining with anti-N-cadherin antibody (#17-3259-41, eBioscience), the ectomesenchymal cells were harvested with Non-Enzymatic Cell Dissociation Buffer (Invitrogen). Negative controls to determine FACS gates were fluorochrome-conjugated or unconjugated isotype immunoglobulins purchased from eBioscience and BioLegend (Figs. 1c-left, 1e-upper, S1h-left and S3c-rightmost). The GFP⁺ FACS gates for the SOX9-GFP hiPSC-
derived progeny (Figs. 1f and S1h) were determined by the corresponding cell population derived from H9 hESCs (data not shown). FACS analysis was performed on LSRII (BD); cell sorting was done with Aria (BD). Viable single cells were gated using 4′,6-diamidino-2-phenyindole (DAPI, Sigma). Sorted cells ranged in purity from 92 to 96%.

**Ectomesenchymal cell genesis and expansion**

The single-cell suspension from differentiated hPSCs before or after sorting with antibodies to CD271 and CD73 were plated onto fibronectin-coated dishes (10 µg/ml human fibronectin [Sigma], overnight at 4°C) and cultured in CDM supplemented with various factors or small molecules, or in D10 medium at 37°C under 5% CO₂/5% O₂. Factors used: FGF2 at 5-10 ng/ml, hPDGF-BB at 10 ng/ml, SB431542 at 1-10 µM, A83-01 at 0.1 µM, SJN2511 at 1 µM, and LDN193189 at 0.5 µM. Generated cells were passaged every 3-4 days by 0.05% Trypsin-EDTA or diluted TrypLE Select. For 3D-pellet culture, cells were passaged into CDM containing 5 ng/ml FGF2 + 10 ng/ml TGFβ3 (R&D) or D10 medium, and maintained for 3-4 days (without further passage) or for 9-11 days (with 2 passages) at 37°C under 5% CO₂, prior to forming a pellet.

**Growth curves and karyotype analysis**

Mesenchymal cells were maintained by passaging every 3 days in CDM containing 5-10 ng/ml FGF2 and 3-10 µM SB431542. The total number of cells in each passage was calculated as a ratio of harvested cell number to seeded cell number, multiplied by the calculated cell number of the previous passage. The p10 cells were karyotyped with a standard G-banding technique.

**Immunofluorescence detection of SOX9**

The CD271<sup>hi</sup>CD73<sup>-</sup> neural crest-like progeny cultured in CDM containing FGF2+SB431542 were transferred to an 8-well chamber slide (Nunc, Rochester, NY) coated with fibronectin and cultured for 3 days in either the same medium or CDM with FGF2+TGFβ. Cultures were stopped and fixed with 10% (v/v) buffered Zn-Formalin (Z-fix, Anatech, Battle Creek, MI) for 15
min, then immunostained initially with the goat anti-hSOX9 polyclonal antibody (AF3075, R&D), then with biotinylated donkey anti-goat IgG (705-065-147, Jackson ImmunoResearch, West Grove, PA), and finally with AlexaFluor (AF) 594-conjugated streptavidin (S32356, Invitrogen). The stained slides were inspected with a BX61 fluorescence microscope (Olympus, Center Valley, PA) one day after being mounted with a DAPI-containing solution (Invitrogen).

**Chondrogenesis assays**

TGFβ signaling is essential for normal chondrogenesis from cranial neural crest (Chai et al., 2003; Cheah et al., 2010), FGF and TGFβ signaling are implicated in mesenchymal condensation during limb, vertebral and craniofacial skeletogenesis (Kronenberg et al., 2009). Therefore, 2D-micromass culture was performed with expanded ectomesenchymal cells (1.5 x10^5 cells) spotted in 5 µl onto a fibronectin-coated 24-well plate (BD), in serum-free chondrogenic medium supplemented with 40 ng/ml PDGF-BB and 1% (v/v) FBS (Nakayama et al., 2003; Tanaka et al., 2009; Umeda et al., 2012). On day 6, 10 ng/ml TGFβ3 either supplemented or replaced PDGF, and on day 10, 50 ng/ml BMP4 was added (PDGF/TGFβ/BMP, or PTB) (Umeda et al., 2012). In some experiments, FGF2 or FGF2+SB431542 was added instead of PDGF on day 0, followed by replacement with TGFβ3 on day 6 and addition of BMP4 on day 10 (FGF/TGFβ/BMP or FGF+SB/TGFβ/BMP, respectively). The 2D cultures were maintained at 37°C under 5% CO₂/5% O₂ for 14-16 days, and either fixed with Z-Fix, and stained with 1% (w/v) acid Alcian Blue (Sigma) pH 1, as described (Nakayama et al., 2003; Tanaka et al., 2009; Umeda et al., 2012), or subjected to analyses of DNA, RNA and sulfated glycosaminoglycans (sGAGs). The stained micromasses were then treated with 400 µl/well of 4 M guanidine hydrochloride (Sigma) in Tris-HCl-EDTA pH 7.5 (Hoemann, 2004). The absorbance of the extract was measured at 600 nm (OD600) using SpectraMax M2 (Molecular Devices, Sunnyvale CA) to determine the relative amount of bound
sGAGs per micromass, and displayed as mean OD600 with the standard deviation (SD) as a thin vertical line.

For 3D pellet culture, aliquots of $2.0 \times 10^5$ cells were centrifuged to form pellets, and cultured in 0.5 ml of serum-free chondrogenic media supplemented with defined factors (without 1% FBS). The pellet cultures were maintained under PDGF/TGFβ1/BMP conditions at 37°C under 5% CO₂ for 19-24 days, and either fixed with Gendre’s Fluid overnight, paraffin-embedded, sectioned (4 µm), and stained with 0.1% (w/v) Toluidine Blue (Sigma) or immunostained with anti-COL2 antibody as described (Nakayama et al., 2003; Umeda et al., 2012), or subjected to analyses of DNA, RNA and sGAGs (Zhao et al., 2014). In the case of paraxial mesoderm, cartilage particles were generated with $3.0 \times 10^5$ freshly sorted (uncultured) EB cells as described (Umeda et al., 2012).

**Quantification of sulfated glycosaminoglycans**

The micromass was directly lysed using guanidine isothiocyanate buffer (RLT, Qiagen, Valencia CA) (Zhao et al., 2014). The cartilage particle was first homogenized in liquid N₂, and then lysed with RLT. Insoluble materials were removed, and DNA, RNA and proteins were individually isolated using AllPrep DNA/RNA/Protein mini kit (Qiagen). The isolated proteins and the insoluble materials were both subjected to papain digestion for 18-20 h at 60°C (125 µg/ml papain, 10 mM cysteine in sodium phosphate-EDTA pH 6.5 [all from Sigma]), and released sGAGs were quantified with 1,9-dimethyl methylene blue (DMMB [Sigma], 16 µg/ml in 41 mM glycine·HCl, 85 mM NaCl pH 3.0), as described (Hoemann, 2004; Zhao et al., 2014), using bovine tracheal chondroitin-4-sulfate (Biocolor, UK) as standard. The OD590-530 was measured with SpectraMax M2. The DNA isolated with the AllPrep kit and DNA present in the papain-digest of insoluble materials were both quantified using Hoechst33258 (Sigma, 0.2 µg/ml in 10 mM Tris·HCl, 1 mM EDTA, 100 mM NaCl pH 7.5), as described (Hoemann, 2004; Zhao et al., 2014). The fluorescence (emission 460 nm, excitation 360 nm) was measured with
a SpectraMax M2 or Infinite M1000 (Tecan, Melbourne, Australia). The sGAGs and DNA from a soluble extract and the corresponding insoluble material were combined to obtain total sGAGs and DNA per micromass or particle. The amount of sGAGs was then normalized to the amount of corresponding DNA and displayed as mean µg sGAG/µg DNA with SD as a thin vertical line.

Subcutaneous transplantation and immunohistological analysis
One to three cartilaginous particles (approximately 2-3 mm “wet” diameter), formed by 3D-pellet culture for 19-24 days using either freshly sorted KDR\(^+\)PDGFR\(\alpha^+\) paraxial mesoderm cells derived from HES3 or H9 hESCs (Umeda et al., 2012) or the expanded CD271\(^+\)CD73\(^+\) ectomesenchymal cells derived from H9 hESCs, were wrapped in one 0.5 cm x 1 cm Gelfoam (Pharmacia Upjohn, Kalamazoo MI) and transplanted at 1 or 2 sites under the dorsal skin of NODScid or NODScid Il2rg\(^-\)/\(^-\) (NSG) mice for 8-12 weeks. The cartilage/bone particles were harvested, fixed with Z-fix for 4 days, embedded in plastic, sectioned, and stained with von Kossa, and counterstained with van Gieson, or with Toluidine Blue as described (Zhao et al., 2014). The transplantation experiments were performed under the regulation of the IACUC for the University of Texas Health Science Center at Houston. To detect remaining human cells, the fixed particles from NSG mice were first decalcified in 10% (w/v) EDTA (pH 8.0) for 3-7 days at 4\(^\circ\)C, then paraffin embedded, sectioned, rehydrated, treated with antigen retrieval solution (DACO, Denmark), blocked, and stained with the mouse anti-human mitochondria monoclonal antibody (MAB1273, Millipore, Temecula CA) and rabbit anti-human COL1 antibody (#NB600-408, Novus Biologicals, Littleton CO). The former staining was visualized using biotin-conjugated donkey anti-mouse IgG (715-065-151, Jackson) followed by AF594-conjugated streptavidin (Invitrogen). The latter was visualized with AF555-conjugated goat anti-rabbit IgG antibody (Invitrogen).

Gene-expression profiling
The isolated total RNAs were reverse transcribed (RT), and real-time polymerase chain reaction (PCR) was performed using the Taqman Gene Expression Assay and ABI7900 (Applied Biosystems, Foster City CA) as described (Umeda et al., 2012; Zhao et al., 2014). The expression levels of individual genes from triplicate reactions (n=3) were normalized against EEF1A1 transcript. The mean relative expression is presented in a bar or line graph with SD shown as a thin vertical line.

For RNA Sequencing analysis (RNA-seq), three independent sets of (FGF2+SB431542)-expanded cells (p6-p7), FGF2-expanded cells (p6), and (FGF2+SB431542 [p4]→FGF2 [p3])-expanded cells were prepared from three batches of FACS-isolated CD271hiCD73- neural crest-like progeny of H9 hESCs. Total RNA was extracted with an RNeasy mini kit (Qiagen). RNA-sequencing libraries were prepared using a SureSelect Strand Specific RNA Library Preparation kit (Agilent technologies, Santa Clara CA). Sequencing was performed on an Illumina HiSeq 1500 using a TruSeq Rapid SBS kit (Illumina, San Diego CA) in a 50-base single-end mode. Sequenced reads were mapped against the human reference genome (GRCh37), using TopHat v2.0.12 (http://ccb.jhu.edu/software/tophat/index.shtml). Expression levels were calculated as fragments per kilobase of exon per million mapped fragments (FPKMs) using Cufflinks v2.1.1 (http://cole-trapnell-lab.github.io/cufflinks). Principal component analysis of protein coding genes was performed using R (version 3.0.1). Gene ontology analysis was performed using an in-house program. The raw data have been deposited to GEO (#GSE64752), and analyzed data are summarized in Tables S1-4.

**Statistical Analysis**

The P-value was determined by the Student-t test using Excel (Microsoft, Redmond WA) or KaleidaGraph (Synergy, Reading PA) software. P values <0.05 were considered significant.
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