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Derivation of Cranial Neural Crest-like Cells from Human Embryonic Stem Cells

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Abstract

The neural crest is a transient population of multipotent progenitors contributing to a diverse array of tissues throughout the vertebrate embryo. Embryonic stem (ES) cells are able to form embryoid body and spontaneously differentiate to various lineages, following a reproducible temporal pattern of development that recapitulates early embryogenesis. Embryoid bodies were triturated and the dissociated cells were processed for fluorescence-activated cell sorting (FACS), and more than 1% of cells were identified as frizzled-3⁺/cadherin-11⁺. Expression of marker genes associated with various terminal fates was detected for chondrocytes, glia, neurons, osteoblasts and smooth muscles, indicating that the FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were multipotent progenitor cells capable of differentiating to fates associated with cranial neural crest. Moreover, the sorted cells were able to self-renew and maintain multipotent differentiation potential. The derivation of cranial neural crest-like multipotent progenitor cells from ES cells provides a new tool for cell lineage analysis of neural crest in vitro.

Keywords

embryonic stem cells; neural crest; multipotent; differentiation; osteogenesis

Introduction

Human embryonic stem (ES) cells are pluripotent lines derived from the inner cell mass of developing blastocysts [1–3]. Upon removal of antidifferentiation agents, human ES cells are able to spontaneously differentiate to various lineages, following a reproducible temporal pattern of development that recapitulates early embryogenesis [4–6]. When human ES cells differentiate in suspension culture, they form a three-dimensional aggregate of cells known as an embryoid body (EB) [7]. EBs can be assayed at various stages of development for the presence of specific lineage-determined populations.

The neural crest is a transient population of multipotent progenitors arising at the lateral edge of the neural plate in vertebrate embryos. After delamination and migration from the neuroepithelium, these cells contribute to a diverse array of tissues throughout the embryo

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[8]. Recent studies report the presence of neural crest derivatives among human ES cell progeny [9,10] and the derivation of neural crest stem cells from human ES cells [11].

Wnt-frizzled signaling plays an important role in the induction and differentiation of neural crest [12]. It has been demonstrated that frizzled proteins are functional Wnt receptors [13, 14]. Wnt1 has been implicated in the regulation of neural crest development [15–17]. Significantly, skeletogenic neural crest cells in the branchial arches are derived from Wnt1 expressing precursor cells in CNS [18]. Endogenous Wnt1 and frizzled-3 have been shown to function as a ligand-receptor pair in the induction of neural crest [19]. Cadherin-11 is the major mesenchymal cadherin excluded from all epithelial tissues [20,21]. In *Xenopus*, cranial neural crest cells express cadherin-11 as they begin to emigrate from the neural fold [22,23]. Murine cadherin-11 is strongly expressed in the mesoderm during gastrulation and in migrating neural crest cells, especially in mesenchymal cells of the head region originated from cranial neural crest [20,24,25].

The temporal and spatial expression patterns of frizzled-3 and cadherin-11 during embryogenesis make these two molecules ideal surface markers to enrich cranial neural crest cells by fluorescence activated cell sorting (FACS). In this study, we derived from human ES cells frizzled-3⁺/cadherin-11⁺ cranial neural crest-like multipotent progenitor (CNCLMP) cells.

Materials and Methods

Human ES Cell Culture and EB Formation

H9 cells were obtained from WiCell Research Institute (Madison, WI) [1]. HUES-1 and HUES-3 were obtained from HUES Facility, Harvard University [26]. Human ES (hES) cells were maintained on feeders prepared from mouse embryonic fibroblasts (MEF). To induce EB formation, hES cells were trypsinized and resuspended on a plastic Petri dish to allow their aggregation and prevent adhesion. About 10⁶ hES cells were seeded in each 60mm Petri dish (Fisher). The EBs were grown in EB medium which was the same as ES medium except that it lacked hbFGF.

Immunofluorescence Staining and FACS of Human Embryoid Body Cells

Human EBs cultured for 10 days were mechanically triturated after exposure to Ca²/Mg²-free PBS solution for 20 min at 25°C, and processed for double (frizzled-3-FITC and cadherin-11-rhodamine) staining along with appropriate negative controls and single color positive controls to establish compensation settings for FACS. The stained cells were resuspended in culture medium at 2–3 × 10⁶/ml, sorted by flow cytometry (Moflo, Cytomation, Inc.) and collected into culture medium. The viability of the cells after sorting was determined by vital trypan blue exclusion.

Complete medium—L-15CO₂ supplemented with 100 µg/ml transferrin (Calbiochem), 5 µg/ml insulin (Sigma), 16 µg/ml putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM selenious acid (Sigma) 1 mg/ml bovine serum albumin, (crystallized, Gibco/BRL), 39 pg/ml dexamethasone (Sigma), 5 µg/ml α -d-1-tocopherol (Sigma), 63 µg/ml β -hydroxybutyrate (Sigma), 25 ng/ml cobalt chloride (Sigma), 1 µg/ml biotin (Sigma), 10 ng/ml oleic acid (Sigma), 3.6 mg/ml glycerol, 100 ng/ml α -melanocyte-stimulating-hormone (Sigma), 10 ng/ml prostaglandin E1 (Sigma), 67.5 rig/ml triiodothyronine (Sigma), 100 ng/ml epidermal growth factor (Upstate Biotechnology), 4 ng/ml basic fibroblast growth factor (Upstate Biotechnology), 20 ng/ml 2.5S nerve growth factor (Upstate Biotechnology), and 10% chick embryo extract.

Neuronal differentiation—Cells are cultured for 7 days in complete medium supplemented with 20 ng/ml IGF-1 (R&D Systems) and 50 ng/ml BMP2 (R&D Systems).

Glial differentiation—Cells are cultured for 7 days in complete medium supplemented with 20 ng/ml IGF-1 (R&D Systems) and 1 nM NRG1 (R&D Systems).

Smooth muscle differentiation—Cells are cultured for 7 days in complete medium supplemented with 20 ng/ml IGF-1 (R&D Systems) and 0.1 ng/ml TGF β 1 (R&D Systems).

Osteogenic Differentiation

Cells are transferred to gelatin-coated tissue culture plates in α DMEM media supplemented with 10% fetal bovine serum (Hyclone), 2 mM glutamine, 100 U/ml of penicillin, 100 μ g/ml of streptomycin, ascorbic acid phosphate (50 μ g/mL, Sigma), β -glycerophosphate (10 mM, Sigma) and dexamethasone (10^{-7} M, Sigma) (mineralization medium). The cells are incubated under these conditions for up to 15 days with a media change every 2 days.

Chondrogenic Differentiation—The chondrogenic medium contained the following components: DMEM/F12 (1:1), 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin, 10 ng/ml transforming growth factor- β 1 (TGF- β 1) (R&D Systems), 100 nM dexamethasone (Sigma), 6.25 μ g/ml insulin (Sigma), 50 nM ascorbate-2-phosphate (Sigma), 110 mg/L sodium pyruvate (Sigma). Cells are cultured in the media for up to 15 days with media changes twice per week.

RT-PCR Analysis

Total RNA is extracted from cells using RNAqueous4PCR (Ambion) following the instructions of the manufacturer. Reverse transcription is performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR reactions are set up using Platinum PCR SuperMix High Fidelity (Invitrogen) following the instructions of the manufacturer.

Fate Mapping of CNCLMP Cells

Cell-type specific antibodies were used to assess the differentiation of CNCLMP cells as follows: osteoblast, Runx2 (Active Motif); chondrocyte, type II collagen (Chemicon); neuron, NFM (neurofilament M, Chemicon); glia, GFAP (glial fibrillary acidic protein, Sigma); smooth muscle, SMA (smooth muscle actin) (monoclonal anti smooth muscle actin clone 1A4 Cy3 conjugate, Sigma).

Immunocytochemistry

All stainings are performed on cells after two PBS (calcium- and magnesium-free) washes, followed by fixation in 4% paraformaldehyde for 10 min at room temperature.

Analysis of mineral deposition

Culture wells were washed once in phosphate-buffered saline (PBS) and fixed for one hour at 4°C in 70% ethyl alcohol. The Alizarin Red solution (40 mM, pH 4.2) was filtered through Whatman paper, then applied to the fixed wells for 10 minutes at room temperature. Non-specific staining was removed by several washes in water. Quantification of calcium concentration was measured by the intensity of absorption at 612 nm (QuantiChrom™ Calcium Assay kit; BioAssay Systems). The total amount of protein in each sample was used as a standard to normalize the calcium data.

Results

Frizzled-3 and Cadherin-11 are co-expressed in EBs

Murine frizzled-3 is expressed in the dorsal neural tube where the neural crest is derived [27]. Murine cadherin-11 is strongly expressed in migrating cranial neural crest [20,24]. Therefore, cranial neural crest cells that begin to emigrate from neural fold are hypothesized to be frizzled-3⁺/cadherin-11⁺. Using indirect immunofluorescent staining, we showed that frizzled-3 and cadherin-11 were co-expressed in a subpopulation of cells within EBs (Fig.1), suggesting that these cells express cell markers associated with cranial neural crest in vivo.

Isolation and characterization of frizzled-3⁺/cadherin-11⁺ cells from EBs

Human EBs cultured for 10 days were mechanically triturated and the dissociated cells were processed for double staining along with appropriate negative controls and single color positive controls to establish compensation settings for FACS. More than 1% of cells were frizzled-3⁺/cadherin-11⁺ (Fig. 2A, Region R3). It should be noted that populations of either frizzled-3⁺ (Region R2) or cadherin-11⁺ (Region R5) cells also existed, indicating that the expression of either of these two molecules are tightly regulated and that ES cells differentiate along distinct cell lineages in EBs. FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were cultured for 28 days and collected for gene expression analysis by RT-PCR (Fig. 2B). Expression of marker genes associated with various terminal fates was detected for chondrocytes, glia, neurons, osteoblasts and smooth muscles, indicating the FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were multipotent progenitor cells capable of differentiating to cell lineages associated with cranial neural crest (Fig. 2B). However, the odontoblast marker (dentin sialophosphoprotein, DSPP, 353 bp) was absent, an outcome likely due to the lack of induction from a competent dental epithelium since odontoblast differentiation requires such induction [28]. Furthermore, epithelia were absent in FACS-sorted frizzled-3⁺/cadherin-11⁺ cells, evidenced by the lack of expression of epithelial markers keratin 7 and 14 (Fig. 2C). Immunofluorescence staining further confirmed that FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were capable of differentiating to cranial neural crest lineages upon exposed to inducing signals (Fig. 2D).

Osteogenic differentiation of frizzled-3⁺/cadherin-11⁺ cells

To further examine their osteogenic differentiation potential, FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were induced for 4 weeks. Mineral deposition was analyzed by Alizarin Red staining. Dexamethasone (10⁻⁷M) induced osteogenic differentiation of FACS-sorted frizzled-3⁺/cadherin-11⁺ cells in mineralization media, as evidenced by the presence of Alizarin Red-positive mineral modules (Fig. 3A). Calcium concentration was also measured to quantify mineral deposition. FACS-sorted frizzled-3⁺/cadherin-11⁺ cells cultured in the presence of dexamethasone gave rise to significantly increased level of mineral deposition, which is consistent with the Alizarin Red staining results (Fig. 3B). Taken together, these data demonstrated that FACS-sorted frizzled-3⁺/cadherin-11⁺ cells possess strong osteogenic differentiation potential.

Self-renewal of FACS-sorted frizzled-3⁺/cadherin-11⁺ cells

To examine whether FACS-sorted frizzled-3⁺/cadherin-11⁺ cells express neural crest stem cell (NCSC) markers Sox10 and p75 [29–31], sorted cells were plated and subject to immunostaining analysis. More than 90% of the cells were Sox10⁺/p75⁺ (Fig. 4A). To determine whether FACS-sorted cells were able to self-renew, cells were cultured for 10 days (3rd passage) and then subject to different induction signals to assess their multi-lineage differentiation potentials. Using immunofluorescence staining to identify lineage-specific markers, we demonstrated that the 3rd passage cells maintained their multipotency in

differentiating to various cranial neural crest lineages (Fig. 4B). However, cells cultured for longer periods (more than three passages) underwent spontaneous differentiation. These data indicated that FACS-sorted frizzled-3⁺/cadherin-11⁺ cells have limited ability to self-renew and maintain their capacity for multi-lineage differentiation (multipotency) into neurons, glial cells, myofibroblasts, chondrocytes and osteoblasts.

Discussion

Human ES cells can reproducibly differentiate in vitro into EBs with three embryonic germ layers. Given the fact that this process recapitulates early embryogenesis, we hypothesize that neural crest-like cell population are present in EBs. In order to isolate neural crest-like cells from EBs, we identified two cell surface markers, frizzled-3 and cadherin-11 that are expressed in neural crest cells during embryogenesis. We then detected robust expression of frizzled-3 and cadherin-11 in a subpopulation within EBs (Fig. 1). This enabled us to tightly gate the FACS sorting to obtain a homogenous frizzled-3⁺/cadherin-11⁺ population. As a result, contamination of cells with alternative lineages was significantly limited (Fig. 2). With appropriate inducing signals, FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were able to differentiate to various neural crest lineages, such as neurons, glial cells, smooth muscle cells, chondrocytes, and osteoblasts (Fig. 2 and Fig. 3). Moreover, these FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were capable of self-renewing and maintaining their differentiation potential in short-term cultures (Fig. 4). A recent study reported the derivation of neural crest stem cells from human ES cells at the neural rosette stage. It is surprising that large number of cells with a neural crest profile is present in rosette cultures considering that similar conditions are routinely used for the generation of hES cell-derived central nervous system progeny [11].

Neural crest cells are multipotent stem cells that contribute to a diverse array of tissues throughout the embryo. During craniofacial development, cranial neural crest contributes extensively to the formation of mesenchymal structures in the head and neck. The major difference between the cranial and trunk neural crest is the ability of cranial neural crest to generate skeletal derivatives. Fate maps of the cranial neural crest suggest that the majority of the head skeleton is neural crest derived [32,33], with the exception of parietal bones having a mesoderm origin [34].

Given its osteogenic differentiation potential, frizzled-3⁺/cadherin-11⁺ cells behave more like cranial neural crest than trunk neural crest. Therefore, bone tissues generated from frizzled-3⁺/cadherin-11⁺ CNCLMP cells share similar developmental origin with craniofacial bones, thereby representing a superior therapeutic tissue source for craniofacial bone repair.

A common theme in vertebrate organogenesis is the reciprocal interaction between epithelium and mesenchyme. During dental development a specialized region of the oral epithelium known as the dental placode signals to an underlying cranial neural crest derived population of cells to alter their cell fate and to differentiate as dental mesenchyme including a terminal phenotype of odontoblasts. It will be interesting to utilize the unique properties of reciprocal tissue interactions occurring during odontogenesis to test the competency of the ES cell-derived CNCLMP cells to respond to signals from a dental epithelium. Previous studies has demonstrated that premigratory neural crest cells and other non-odontogenic CNC cells, when recombined with a competent epithelium, can receive signals causing them to differentiate to odontogenic lineages [28,35]. The emergence of induced genes consistent with an odontogenic phenotype would argue that the CNCLMP cells are as plastic in their ability to respond to inductive signals as are authentic populations of cranial neural crest cells that reside in the first brachial arch.

At least some neural crest cells are multipotent, as shown by clonal analysis in vitro [31,36] and dye labeling in vivo [37–39]. It is possible that FACS-sorted CNCLMP cells are a mixed population in which a given cell does not have the potential to differentiate to all the cranial neural crest lineages. To address this question, further investigation is necessary to establish single-colony derived strains from CNCLMP cells and subsequently to carry out fate-mapping analyses. It has been shown that bone morphogenetic protein (BMP) and Wnt synergistically suppress differentiation and maintain multipotency of neural crest stem cells (NCSCs) [40]. Neural crest stem cells generated from hES cell-derived neural rosette are capable of forming proliferating neurosphere-like structures in response to FGF2/EGF exposure [11]. We observed limited self-renewal of FACS-sorted CNCLMP cells (Fig. 4). It will be interesting to examine whether CNCLMP cells respond in a similar way to these signaling molecules, and consequently maintain long-term self-renewal activity.

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References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145–1147. [PubMed: 9804556]
2. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 2000;18:399–404. [PubMed: 10748519]
3. Watt FM, Hogan BL. Out of Eden: stem cells and their niches. *Science* 2000;287:1427–1430. [PubMed: 10688781]
4. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001;108:407–414. [PubMed: 11489934]
5. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 2002;99:4391–4396. [PubMed: 11917100]
6. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. *Diabetes* 2001;50:1691–1697. [PubMed: 11473026]
7. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 2000;6:88–95. [PubMed: 10859025]
8. LaBonne C, Bronner-Fraser M. Molecular mechanisms of neural crest formation. *Annu Rev Cell Dev Biol* 1999;15:81–112. [PubMed: 10611958]
9. Fang D, Leishear K, Nguyen TK, Finko R, Cai K, Fukunaga M, Li L, Brafford PA, Kulp AN, Xu X, Smalley KS, Herlyn M. Defining the conditions for the generation of melanocytes from human embryonic stem cells. *Stem Cells* 2006;24:1668–1677. [PubMed: 16574754]
10. Pomp O, Brokman I, Ben-Dor I, Reubinoff B, Goldstein RS. Generation of peripheral sensory and sympathetic neurons and neural crest cells from human embryonic stem cells. *Stem Cells* 2005;23:923–930. [PubMed: 15883233]
11. Lee G, Kim H, Elkabetz Y, Al Shamy G, Panagiotakos G, Barberi T, Tabar V, Studer L. Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells. *Nat Biotechnol* 2007;25:1468–1475. [PubMed: 18037878]
12. Yanfeng W, Saint-Jeannet JP, Klein PS. Wnt-frizzled signaling in the induction and differentiation of the neural crest. *Bioessays* 2003;25:317–325. [PubMed: 12655639]
13. Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* 1996;382:225–230. [PubMed: 8717036]

14. Yang-Snyder J, Miller JR, Brown JD, Lai CJ, Moon RT. A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr Biol* 1996;6:1302–1306. [PubMed: 8939578]
15. Dorsky RI, Moon RT, Raible DW. Control of neural crest cell fate by the Wnt signalling pathway. *Nature* 1998;396:370–373. [PubMed: 9845073]
16. Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S. Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* 1997;389:966–970. [PubMed: 9353119]
17. Saint-Jeannet JP, He X, Varmus HE, Dawid IB. Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc Natl Acad Sci U S A* 1997;94:13713–13718. [PubMed: 9391091]
18. Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 2000;127:1671–1679. [PubMed: 10725243]
19. Deardorff MA, Tan C, Saint-Jeannet JP, Klein PS. A role for frizzled 3 in neural crest development. *Development* 2001;128:3655–3663. [PubMed: 11585792]
20. Hoffmann I, Balling R. Cloning and expression analysis of a novel mesodermally expressed cadherin. *Dev Biol* 1995;169:337–346. [PubMed: 7750649]
21. Simonneau L, Kitagawa M, Suzuki S, Thiery JP. Cadherin 11 expression marks the mesenchymal phenotype: towards new functions for cadherins? *Cell Adhes Commun* 1995;3:115–130. [PubMed: 7583005]
22. Hadeball B, Borchers A, Wedlich D. Xenopus cadherin-11 (Xcadherin-11) expression requires the Wg/Wnt signal. *Mech Dev* 1998;72:101–113. [PubMed: 9533956]
23. Vallin J, Girault JM, Thiery JP, Broders F. Xenopus cadherin-11 is expressed in different populations of migrating neural crest cells. *Mech Dev* 1998;75:171–174. [PubMed: 9739138]
24. Kimura Y, Matsunami H, Inoue T, Shimamura K, Uchida N, Ueno T, Miyazaki T, Takeichi M. Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos. *Dev Biol* 1995;169:347–358. [PubMed: 7750650]
25. Kimura Y, Matsunami H, Takeichi M. Expression of cadherin-11 delineates boundaries, neuromeres, and nuclei in the developing mouse brain. *Dev Dyn* 1996;206:455–462. [PubMed: 8853994]
26. Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 2004;350:1353–1356. [PubMed: 14999088]
27. Borello U, Buffa V, Sonnino C, Melchionna R, Vivarelli E, Cossu G. Differential expression of the Wnt putative receptors Frizzled during mouse somitogenesis. *Mech Dev* 1999;89:173–177. [PubMed: 10559494]
28. Lumsden AG. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* 1988;103:155–169. [PubMed: 3250849]
29. Britsch S, Goerich DE, Riethmacher D, Peirano RI, Rossner M, Nave KA, Birchmeier C, Wegner M. The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev* 2001;15:66–78. [PubMed: 11156606]
30. Southard-Smith EM, Kos L, Pavan WJ. Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat Genet* 1998;18:60–64. [PubMed: 9425902]
31. Stemple DL, Anderson DJ. Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* 1992;71:973–985. [PubMed: 1458542]
32. Noden DM. Interactions and fates of avian craniofacial mesenchyme. *Development* 1988;103:121–140. [PubMed: 3074905]
33. Couly GF, Coltey PM, Le Douarin NM. The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* 1993;117:409–429. [PubMed: 8330517]
34. Jiang X, Iseki S, Maxson RE, Sucov HM, Morriss-Kay GM. Tissue origins and interactions in the mammalian skull vault. *Dev Biol* 2002;241:106–116. [PubMed: 11784098]
35. Wang YH, Upholt WB, Sharpe PT, Kollar EJ, Mina M. Odontogenic epithelium induces similar molecular responses in chick and mouse mandibular mesenchyme. *Dev Dyn* 1998;213:386–397. [PubMed: 9853960]

36. Lee HY, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. *Science* 2004;303:1020–1023. [PubMed: 14716020]
37. Bronner-Fraser M, Fraser S. Developmental potential of avian trunk neural crest cells in situ. *Neuron* 1989;3:755–766. [PubMed: 2484346]
38. Bronner-Fraser M, Fraser SE. Cell lineage analysis reveals multipotency of some avian neural crest cells. *Nature* 1988;335:161–164. [PubMed: 2457813]
39. Fraser SE, Bronner-Fraser M. Migrating neural crest cells in the trunk of the avian embryo are multipotent. *Development* 1991;112:913–920. [PubMed: 1935700]
40. Kleber M, Lee HY, Wurdak H, Buchstaller J, Riccomagno MM, Ittner LM, Suter U, Epstein DJ, Sommer L. Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. *J Cell Biol* 2005;169:309–320. [PubMed: 15837799]

Figure 1. Human EBs express markers known to be expressed by cranial neural crest in vivo
Human ES cells were induced to form EBs. Whole-mount indirect immunofluorescent staining of EBs was performed using specific antibodies against frizzled-3 and cadherin-11. Panel A, cadherin-11 staining; Panel B, frizzled-3 staining; Panels C, merged view of A and B. Fluorescence-conjugated secondary antibodies alone produced no staining pattern. Panel D, FITC-conjugated rabbit anti-goat IgG; panel E, Alexa Fluor 594 conjugated chicken anti-rat IgG; panel F, merged view of D and E.

Figure 2. Isolation and characterization of FACS-sorted putative cranial neural crest cells

(A) Human EBs cultured for 10 days were tritured and the dissociated cells were processed for double staining along with appropriate negative controls and single color positive controls to establish compensation settings for the FACS. Region R2: frizzled-3⁺; R3: frizzled-3⁺/cadherin-11⁺; R4: frizzled-3⁺/cadherin-11⁻; R5: cadherin-11⁺. (B) RT-PCR analysis of marker genes for CNCLMP terminal cell types. **Lane 1**, Molecular Weight Marker; **Lane 2**, type II collagen (chondrocyte); **Lane 3**, dentin sialophosphoprotein (odontoblast); **Lane 4**, glial fibrillary acidic protein (glia); **Lane 5**, peripherin (neuron); **Lane 6**, Runx2 (osteoblast); **Lane 7**, smooth muscle actin (smooth muscle); **Lane 8**, GAPDH. (C) RT-PCR analysis of epithelial markers. **Lanes 1 and 8**, Molecular Weight Marker; **Lanes 2 and 5**, keratin 7; **Lanes 3 and 6**, keratin 14; **Lanes 4 and 7**, GAPDH. **Lanes 2–4**, frizzled-3⁺/cadherin-11⁺ cells; **Lanes 5–7**, positive controls. (D) Immunofluorescence staining of differentiated CNCLMP cells.

Figure 3. Osteogenic differentiation of FACS-sorted frizzled-3⁺/cadherin-11⁺ cells

FACS-sorted frizzled-3⁺/cadherin-11⁺ cells were induced to osteogenic differentiation for 4 weeks in three different media. **CTRL** medium, α DMEM medium with 15% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 0.1 mM β -mercaptoethanol, 1 $\times$ non-essential amino acid; **MINERAL** medium, **CTRL** medium supplemented with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate; **DEX** medium, **MINERAL** medium supplemented with 10⁻⁷M dexamethasone. (A) Mineral deposition was analyzed by Alizarin Red staining. (B) Quantification of calcium concentration of FACS-sorted frizzled-3⁺/cadherin-11⁺ cells that were induced to osteogenic differentiation for 4 weeks. Relative calcium concentration was calculated by normalization of the absolute calcium concentration to total protein concentration in each sample.

Figure 4. Self-renewal of FACS-sorted frizzled-3⁺/cadherin-11⁺ cells

(A) Detection of neural crest stem cell (NCSC) markers Sox10 and p75 in FACS-sorted frizzled-3⁺/cadherin-11⁺ cells. (B) Maintenance of multi-lineage differentiation potentials for FACS-sorted CNCLMP that have been passed three times in vitro.