A Stable Cranial Neural Crest Cell Line from Mouse

Mamoru Ishii,¹ Athena C. Arias,¹ Liqiong Liu,¹ Yi-Bu Chen,² Marianne E. Bronner,³ and Robert E. Maxson¹

Cranial neural crest cells give rise to ectomesenchymal derivatives such as cranial bones, cartilage, smooth muscle, dentin, as well as melanocytes, corneal endothelial cells, and neurons and glial cells of the peripheral nervous system. Previous studies have suggested that although multipotent stem-like cells may exist during the course of cranial neural crest development, they are transient, undergoing lineage restriction early in embryonic development. We have developed culture conditions that allow cranial neural crest cells to be grown as multipotent stem-like cells. With these methods, we obtained 2 independent cell lines, O9-1 and i10-1, which were derived from mass cultures of *Wnt1-Cre; R26R-GFP*-expressing cells. These cell lines can be propagated and passaged indefinitely, and can differentiate into osteoblasts, chondrocytes, smooth muscle cells, and glial cells. Whole-genome expression profiling of O9-1 cells revealed that this line stably expresses stem cell markers (*CD44*, *Sca-1*, and *Bmi1*) and neural crest markers (*AP-2 α* , *Twist1*, *Sox9*, *Myc*, *Ets1*, *Dlx1*, *Dlx2*, *Crabp1*, *Epha2*, and *Itgb1*). O9-1 cells are capable of contributing to cranial mesenchymal (osteoblast and smooth muscle) neural crest fates when injected into E13.5 mouse cranial tissue explants and chicken embryos. These results suggest that O9-1 cells represent multipotent mesenchymal cranial neural crest cells. The O9-1 cell line should serve as a useful tool for investigating the molecular properties of differentiating cranial neural crest cells.

Introduction

NEURAL CREST CELLS, a multipotent population of migratory cells, are of central importance to vertebrate organogenesis [1–3]. Neural crest cells are specified at the border between neural and non-neural ectoderm during embryogenesis. Subsequently, they undergo a transition from epithelium to mesenchyme, and migrate, often, long distances. They ultimately differentiate into a variety of cell types and contribute to many organs. Anomalies in neural crest development underlie the pathophysiology of a variety of human diseases [4,5], highlighting the importance of understanding of the molecular and cellular mechanisms that govern neural crest development.

Depending on the site of origin along the anterior–posterior axis of the embryo, neural crest cells fall into 3 populations, cranial, cardiac, and trunk, each with a unique developmental potential. The cranial neural crest, which originates in the portion of the neural tube from the neural fold anterior to rhombomere 6, has the ability to produce a greater diversity of derivatives than other crest populations: Cranial neural crest cells give rise to derivatives of the ectoderm, including peripheral nerves, as well as mesenchymal cell types, including skeletal bone, cartilage, dentin, smooth muscle, and corneal endothelial cells [6]. Trunk neural crest cells form a more

limited set of cell types, including peripheral nerves, melanocytes, and the adrenal medulla [6]. Cardiac neural crest cells contribute to the smooth muscle lining of the outflow tract [7].

Clonal culture experiments have shown that trunk neural crest cells include a population of self-renewing multipotent stem cells [8]. Whether other neural crest populations also contain such populations is not clear. In the case of cranial neural crest cells, individual cells derived from quail embryos have been shown to be multipotent [9,10]. However, these cells have not been maintained as stable, long-term cell lines; consequently, the question of whether these cells have the ability to self-renew has not been addressed.

One of our main long-term goals is to test the hypothesis that the cranial neural crest contains a population of self-renewing stem cells. For this purpose, we have sought to develop clonal culture conditions that will allow us to evaluate the differentiation potential of individual cranial neural crest cell clones. As a first step toward this goal, we have identified conditions that enable murine cranial neural crest cells to be cultured en masse, and conditions in which such mass-cultured cranial neural crest cells to be maintained in vitro in a multipotent state. We have isolated 2 multipotent non-clonal cell lines, one of which we have characterized extensively. The cell line designated O9-1 has the capacity to differentiate in vitro into osteoblasts, a defining characteristic of cranial

¹Department of Biochemistry and Molecular Biology, USC/Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California.

²Norris Medical Library, University of Southern California, Los Angeles, California.

³Division of Biology, California Institute of Technology, Pasadena, California.

neural crest cells, as well as chondrocytes, smooth muscle, and glial cells. In vivo, O9-1 cells can contribute to cranial mesenchymal derivatives, including osteoblasts and smooth muscle cells. The O9-1 cell line can be passaged extensively, stably maintaining its multipotency. Whole-genome expression profiling suggests that the O9-1 cell line has characteristics of both cranial neural crest and stem cells. We believe that the O9-1 cell line will be a powerful asset in the investigation of the molecular properties of cranial neural crest cells.

Materials and Methods

Culture medium

The basal medium for neural crest culture was prepared as following. The Dulbecco's modified Eagle's medium (DMEM), 15% fetal bovine serum (FBS), 0.1 mM minimum essential medium (MEM) non-essential amino acids, 1 mM sodium pyruvate, 55 μ M β -mercaptoethanol, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine, was conditioned by STO feeder cells (ATCC) for an overnight. The medium was filtered (0.22- μ m pore size) and supplemented with 25 ng/mL basic fibroblast growth factor (bFGF; R&D Systems) and 1,000 U/mL leukemia inhibitory factor (LIF; Millipore).

Neural crest cell culture

Cranial neural crest cells labeled with *Wnt1-Cre; R26R-GFP* [7,11,12] were obtained from E8.5 mouse embryos. Cranial tissues above the level of the second pharyngeal arch were dissected in DMEM supplemented with 10% fetal calf serum (FCS), washed by phosphate-buffered saline (PBS), and then incubated in 0.025% trypsin, 1 mg/mL collagenase in 0.25 mM EDTA for 5 min at 37°C. Cells were dissociated by gentle pipetting and neutralized in DMEM with 10% FCS. Then, cells were initially expanded on a Matrigel- (Becton Dickinson) coated plate with the basal medium at 37°C, 5.5% CO₂. Confluent culture was subjected to fluorescence-activated cell sorting (FACS) by the MoFlo cytometer (cytometry), and GFP-positive cells were seeded on a Matrigel-coated plate with the basal medium. Cells were seeded at 10,000–15,000 cells/cm², and it took 3–4 days before they reach to the confluence. For passaging, cells were rinsed with PBS twice and treated with 0.05% trypsin in 0.5 mM EDTA at 37°C for 3 min. Cells were neutralized in 10% FCS in DMEM and gently pipetted. Cells were maintained in logarithmic growth, and as a standard procedure, culture was diluted 2 to 3 times with aimed seeding density $\sim 0.8\text{--}1.0 \times 10^5$ cells/cm². Under this condition, cranial neural crest cells can be passaged for an extended time.

Cell differentiation

To induce differentiation, cells were cultured in the following conditions: osteogenic differentiation medium [α -MEM, 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.1 μ M dexamethasone, 10 mM β -glycerophosphate, 50 μ g/mL ascorbic acid, and 100 ng/mL BMP2 (gift from B. Frenkel)], smooth muscle differentiation medium (DMEM, 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin), and glial differentiation medium [DMEM/F12, 1 $\times$ B27 (Invitrogen), 2 mM L-glutamine, 50 ng/mL BMP2, 50 ng/mL LIF (Millipore), 1% heat-inactivated FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin]. For chondrogenic differ-

entiation, monolayer culture was initially treated with the osteogenic medium for 3 days. Then, cells were trypsinized and cultured as a micromass format in a chondrogenic medium [α -MEM, 5% FCS, 1% ITS (BD Biosciences), 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 ng/mL TGF- β 3 (R&D systems), 50 μ g/mL ascorbic acid, 10 ng/mL BMP2, 0.1 μ M dexamethasone, and 1 mM sodium pyruvate] for additional 7 days.

Osteogenic and chondrogenic differentiation was assessed by Alizarin red, alkaline phosphatase (ALP), and Alcian blue staining [13]. To detect smooth muscle and glial cell differentiation, immunofluorescence was performed with anti- α -smooth muscle actin (α SMA; Sigma), anti-Fabp7, and anti-GFAP (Millipore) antibodies.

Primers

Primers used in this study are shown in Supplementary Data S1 (Supplementary Data are available online at www.liebertpub.com/scd).

FACS analysis

O9-1 and i10-1 cells were immunophenotyped by phycoerythrin-conjugated anti-CD44 and allophycocyanin-conjugated anti-Sca-1 antibody, as well as isotype controls purchased from eBioscience. The cells were incubated with 1/200 diluted antibodies at 4°C for 30 min and washed. Analysis was performed by MoFlo (Cytometry).

Microarray analysis

RNA samples were extracted from a triplicated culture of O9-1 cells grown in the basal medium (passage13) and purified using a PicoPure RNA Isolation Kit (Arcturus). Genome-wide RNA expression profile of O9-1 cells was conducted using Illumina MouseRef-8 V2.0 Expression BeadChip (Illumina). The raw intensity data were imported into Illumina GenomeStudio v3 and subsequently exported to Partek Genomic Suite v6 (PGS; Partek, Inc.) using a plug-in script provided by Partek without normalization and background correction. As controls, GEO data sets generated from E8.25 mouse ectoderm ([14], GSE13040) and E12.5 mouse dorsal root ganglia (DRG) ([15], GSE24730) were directly imported into PGS. All data sets were combined and then subjected to log transformation and quantile normalization [16]. To detect differentially expressed genes (DEG) in O9-1 cell line versus the control tissues, a one-way ANOVA was performed using the gene expression workflow in PGS. Lists of the most significant DEG were generated with stringent criteria: a false discovery rate corrected $P < 0.05$ using the Benjamini-Hochberg step-up procedure [17] to account for multiple testing and the difference in mean gene transcript level was at least 2-fold in either direction. We used the same approach to identify DEG of the human trunk neural crest cell lines ([18], GSE14340) versus human Schwann cells (GSE4030).

Analysis of developmental potency of cultured neural crest cells by microinjection into mouse and chick embryos

To assess the ability of cultured neural crest cells to undergo osteogenic and smooth muscle differentiation, we

cultured the upper half of E13.5 mouse heads as described [19] with some modifications. In typical experiments, $\sim 3.5 \times 10^6$ of O9-1 cells were resuspended in 100 μ L of 10% FBS in DMEM. O9-1 cells (P18) were injected with a mouth pipette into area adjacent to the frontal bone primordium. Explants were cultured in the BGJb medium for 3 days (see Fig. 4). In the case of the smooth muscle lineage, O9-1 cells were prepared as described above and injected into the frontonasal prominence of the HH stage17 chicken embryos by following to [20]. Embryos were cultured for 24 h and harvested. Both mouse organ explants and chicken embryos were fixed in 4% paraformaldehyde and cryosectioned. In the case of mouse explants, injected cells were visualized by means of immunohistochemistry with anti-GFP antibody (Invitrogen) or immunofluorescence with the TSA tyramide system (PerkinElmer) due to a high level of host autofluorescence. Osteogenic differentiation was assessed by ALP staining [13], and smooth muscle differentiation was assessed by immunofluorescence with anti- α SMA antibody, followed by anti-mouse IgG Rhodamine (Invitrogen) staining.

CD44 immunofluorescence

Cranial tissues of E8.5 through E10.5 *Wnt1-Cre; R26R-GFP* mouse embryo were fixed in 4% paraformaldehyde and cryosectioned. Tissues were stained with anti-CD44 antibody (eBioscience) and visualized by anti-Rat IgG Alexa568 (Invitrogen).

Results

Mass-cultured cranial neural crest cells sustain stem-like potency

We isolated cranial neural crest cells from E8.5 mouse embryos by means of FACS (Fig. 1). The cells were labeled with a *Wnt1-Cre; R26R-GFP* reporter. We found that by using a combination of Matrigel-coated culture dishes and a medium conditioned by STO feeder cells and supplemented with bFGF and LIF, we were able to obtain sustained growth of mouse cranial neural crest. bFGF is essential for adequate growth of these cells. We have thus far isolated 2 independent mass culture lines, O9-1 and i10-1. When cultured with various differentiation media, both of these cell lines were capable of differentiating into several different cell types, including osteoblasts, chondrocytes, smooth muscle cells, and glial cells (Fig. 1; data not shown). Neither cell line showed a neuronal phenotype when exposed to conditions that were capable of causing neuronal differentiation [21], and melanocyte conditions were not tested.

By performing reverse transcriptase polymerase chain reaction (RT-PCR) analysis at different passages, we found that both cell lines continually express the neural crest markers, *AP-2 α* , *Twist1*, and *Snail1*, and the stem cell markers, *nestin*, *CD44*, and *Sca-1* (Figs. 1 and 2; data not shown). Flow cytometry analysis showed that more than 84% of O9-1 cells are positive for both CD44 and Sca-1. In the case of i10-1, the CD44-Sca1 double-positive fraction was more than 97% (Fig. 1). O9-1 cells stably maintained their multipotency as well as the expression of key markers through passages P17 and P22 (see Fig. 2).

Findings of sustainable differentiation potential, multipotency, and expression of neural crest markers suggest that

these mass-cultured cells have key properties expected of cranial neural crest cells. Since O9-1 and i10-1 cell lines had equivalent marker gene expression profiles, proliferation ratios, and differentiation potential (Figs. 1 and 2; data not shown), we chose to focus on the O9-1 cell line in subsequent analyses.

Whole-genome expression profiling revealed that the O9-1 cell line exhibits characteristics of mesenchymal cranial neural crest

Initial marker analysis with RT-PCR suggested the O9-1 cell line has features of neural crest cells as well as mesenchymal and neural stem cells. To further test the hypothesis that the O9-1 cell line represents a native state of developing cranial neural crest cells, we carried out whole-genome expression analysis by means of Illumina microarrays (Illumina). To detect DEG, E8.25 mouse ectoderm ([14] GSE13040) and E12.5 mouse DRG ([15] GSE24730) were used as controls. E8.25 ectoderm should contain few or no neural crest cells, whereas E12.5 DRG is formed entirely from a trunk neural crest population differentiating into neuronal, glial, and smooth muscle cells. Thus, comparison of O9-1 cell line with E8.25 ectoderm should highlight the difference between neural crest and non-neural crest cells, whereas comparison with E12.5 DRG should illuminate differences of undifferentiated versus differentiated neural crest, as well as cranial versus trunk neural crest cells.

Markers of neural crest were increased significantly in the O9-1 cell line relative to both ectoderm and the DRG cell line (fold change >2 ; $P < 0.05$): these include *Tcfap2a* (*AP-2 α*), *Twist1*, *Sox9*, *Myc*, *Ets1*, *Dlx1*, *Dlx2*, *Crabp1*, *Epha2*, and *Itgb1*. Additional neural crest markers (a total of 25) also showed higher levels of expression (Table 1 and Supplementary Data S2). *Snail1* and *Msx1* were increased in the O9-1 cell line in comparison with E12.5 DRG cell line, but not in comparison with ectoderm.

We did not detect an increase in the expression of *Foxd3* or *Sox10* in O9-1 cells, as would be expected of cells capable of ectodermal fates. We noted a higher level of *Sox9* expression and a lower level of *Sox10* expression, characteristics of cranial neural crest cells that have migrated into the pharyngeal arches and become committed to mesenchymal fates [22–24]. These data suggest that the O9-1 cell line models the ectomesenchymal cells in the cranial region.

Expression profiling of O9-1 cells showed some similarity to neural crest stem cells found in the enteric nerve (enNCSCs) [25,26]. The O9-1 cell line expresses *Bmi1*, *Arpc1b*, *CD9*, and *Cyba*, which are highly expressed in enNCSCs. These results suggest that the O9-1 cell line may represent an undifferentiated subpopulation of cranial neural crest. In agreement with this, additional stem cell markers, including *CD44*, *CD47*, *Ly6a* (*Sca-1*), and *Klf4*, were highly expressed in O9-1 cells. Markers that represent differentiating neural crest showed a lower level of expression [*Tkrf* and *Tkrc* (neuronal cell differentiation); *Mbp*, *Plp* and *Gap43* (Schwann cell differentiation); and *Col2a1* (chondrogenic differentiation)]. A downregulation of differentiation markers was more obvious in comparison with DRG. Such markers include neuronal (*Neurod1*, *Neurod4*, *Nefin*, and *Prph1*), glial (*Fabp7* and *Slc1a3*), and smooth muscle (α SMA) lineage markers (see Table 1). These results suggest that the cell line O9-1 exhibits

FIG. 1. Sustainable stem-like potency of cranial neural crest cell lines. **(A)** Overall protocol. Cranial neural crest cells labeled with *Wnt1-Cre; R26R-GFP* were obtained from E8.5 mouse embryos. Dissociated cells were initially expanded in vitro for 3 days and sorted by fluorescence-activated cell sorting (FACS) (arrowhead). Sorted cells were cultured on Matrigel-coated plates with a basal medium. Under this condition, cranial neural crest cells can be passed for an extended time. Two independent mass culture lines were established (O9-1 and i10-1). The morphology **(B)** and growth ratio **(C)** of mass culture O9-1 cells. Doubling time is ~ 27 h. **(D–G)** Long-term cultured mass cranial neural crest differentiates into multiple cell lineages. Shown is the line O9-1, which is capable of differentiating into osteoblasts **(D)**, chondrocytes **(E)**, smooth muscle cells **(F)**, and glial cells **(G)**. **(H)** These cells express GFP as a transgenic reporter (shown is line O9-1). **(I)** Marker gene expression analysis by RT-PCR. Both lines (O9-1/passage11 and i10-1/passage10) express *AP-2 α* , *Twist1*, and *Snail1* (neural crest markers). They also express *nestin*, *CD44*, and *Sca-1* (stem cell markers). **(J)** Flow cytometry analysis of the O9-1 cell line for CD44 and Sca-1 expression. More than 84.17% of O9-1 cells are double positive for CD44 and Sca-1 (right). Isotype antibodies (negative control; left and middle) show no staining. **(K)** Cell growth of the O9-1 and i10-1 lines is highly dependent on the basic fibroblast growth factor (bFGF) supplement. Error bars are standard deviations derived from 3 independent experiments. Scale bars: in **(B, D–F, H)** 200 μ m; in **(G)** 100 μ m. RT-PCR, reverse transcriptase-polymerase chain reaction.

properties expected of cranial neural crest cells committed to a multipotent ectomesenchymal lineage.

Thomas and colleagues [18] established stable trunk neural crest cell lines from the human fetus (trunk hNCC). These cells can be passaged multiple times in vitro during which time they continuously express neural crest markers [18]. The

culture also contains cells that express smooth muscle, neuronal, or glial cell markers [18], suggesting that these cells differentiate into the derivatives of trunk neural crest. We first determined DEG in the human trunk neural crest cell lines (GSE14340) versus human Schwann cells (GSE4030), and then asked whether these results are relevant to the DEG

FIG. 2. O9-1 cells exhibit sustainable multipotency and expression of marker genes for neural crest and stem cells. (A–L) Differentiation ability of the O9-1 cell line in its earlier (P17) and later (P22) passage points. O9-1 cells have an identical morphology at those passage points (A, B) and are capable of differentiating into osteogenic (C, D), chondrogenic (E, F), smooth muscle (G, H), and glial lineages (I–L). To evaluate cell differentiation, Alizarin red, Alcian blue, immunofluorescence of α SMA, Fabp7, and GFAP were performed, respectively. (M) RT-PCR results show continuous expression of neural crest and stem cell markers. Scale bars: in (A, E, G) 200 μ m for (A, B, E–H); in (C) 2 mm for (C, D); in (L) 100 μ m for (I–L).

in the O9-1 and mouse DRG (see above). Intriguingly, these comparisons showed certain similarities in the expression of genes involved in neural crest development. Some neural crest markers were increased (*Twist1*, *Myc*, *Snail1*, *Sox9*, *Epha2*, *Rarg*, *Rac1*, and *Cald1*), while others were decreased or unchanged [*Sox10*, *Ngfr* (p75), and *Ednrb*] (Supplementary Data S3). In addition, neuronal (*Neurod4* and *Nefn*) as well as glial differentiation markers (*Mbp*, *GAP43*, and *Fabp7*) were both decreased (Supplementary Data S3). These results suggest that the O9-1 and trunk hNCC cell lines both retain certain properties of neural crest and negatively regulate neuronal lineage differentiation. Differences between the O9-1 and trunk hNCC cell lines were also evident. For example, some of the key markers expressed in the O9-1 cell line were not detected in the trunk hNCC lines (*Crabp1*, *Ets1*, *Dlx1*, *Dlx2*, *CD44*, *Bmi1*, *Barx1*, *Prrx1*, *Arpc1b*, *CD9*, and *Cyba*, Table 1, not shown). In addition, *Hox* cluster genes were significantly decreased in O9-1 cells (total of 21 *Hox* genes), whereas they were increased in the trunk hNCC lines (total of 19 *Hox* genes) (Supplementary Data S2 and S3, data not shown). The observed differences between O9-1 and trunk

hNCC lines in the expression of *Hox* genes were consistent with the expected differences between cranial versus trunk neural crest cells [6]. The trunk hNCC lines were reported to have a spontaneous expression of the smooth muscle marker, α SMA [18]. Our analysis also detected an increased level of α SMA expression, together with other smooth muscle markers *TAGLN* (*SM22*) and *CNN1* (*Calponin 1*). In contrast, O9-1 cells exhibit reduced expression of α SMA (see above) and no change in *Tagln* and *Cnn1* (not shown). Thus, it seems that spontaneous differentiation of smooth muscle is more efficiently repressed in the O9-1 cell line than in the human trunk neural crest cell line.

The osteogenic differentiation of O9-1 cells can be controlled precisely by extrinsic conditions

Osteogenic potential distinguishes cranial neural crest from other neural crest populations [6]. We therefore investigated the osteogenic differentiation of O9-1 cells in more detail. We placed cells in osteogenic conditions and monitored cellular morphology and the expression of various

TABLE 1. O9-1 ILLUMINA ANALYSIS

<i>Symbol</i>	<i>Fold-change</i>	<i>P value</i>	<i>Fold-change</i>	<i>P value</i>	<i>Annotation</i>
	<i>O9-1 up vs. DRG</i>		<i>O9-1 up vs. ectoderm</i>		
Crabp1	28.8	6.00E-07	5.03	4.37E-05	Neural crest
Dlx1	17.6	1.88E-05	25.6	9.27E-06	Neural crest
Dlx2	7.39	3.16E-06	7.09	3.58E-06	Neural crest
Epha2	4.89	2.74E-07	4.02	6.00E-07	Neural crest
Ets1	2.73	1.66E-03	4.26	2.35E-04	Neural crest
Itgb1	2.63	6.65E-05	6.03	1.82E-06	Neural crest
Myc	2.34	2.27E-08	2.49	1.50E-08	Neural crest
Sox9	6.48	7.18E-07	2.49	4.72E-05	Neural crest
Tcfap2a (AP-2 α)	3.66	1.04E-04	3.72	9.60E-05	Neural crest
Twist1	4.81	6.22E-06	6.80	1.93E-06	Neural crest
Cald1	2.09	2.01E-05	2.21	1.32E-05	Neural crest migration
Cfl1	2.74	2.06E-04	2.69	2.29E-04	Neural crest migration
Rac1	4.28	2.88E-06	3.03	1.41E-05	Neural crest migration, post-migratory cell proliferation
Barx1	6.23	1.27E-08	5.70	1.71E-08	Pharyngeal arch mesenchyme
Prrx1 (Prx1)	18.2	9.19E-08	32.6	3.08E-08	Facial mesenchyme
Rarg	5.31	2.01E-07	4.81	2.88E-07	Pharyngeal arches, periocular mesenchyme, cranial ganglia
Gli3	2.71	2.84E-06	2.22	1.04E-05	Palatogenesis
Runx2	2.54	2.97E-07	2.33	5.30E-07	Osteogenic differentiation, multipotent stem-like cell
Spp1	53.7	1.60E-06	106	6.25E-07	Osteogenic differentiation
Col1a1	6.25	7.36E-08	17.5	5.10E-09	Osteogenic differentiation
Arpc1b	12.2	1.97E-09	17.8	8.41E-10	Enteric nerve neural crest stem cell
Cd9	6.64	7.65E-06	12.9	1.31E-06	Enteric nerve neural crest stem cell
Cyba	5.44	3.99E-05	4.49	7.93E-05	Enteric nerve neural crest stem cell
Bmi1	4.59	1.10E-06	5.79	4.77E-07	Enteric nerve neural crest stem cell
Cd44	19.2	3.28E-08	14.2	6.17E-08	Stem cell
Cd47	2.08	1.10E-03	4.45	2.13E-05	Stem cell
Klf4	6.19	3.09E-08	7.47	1.72E-08	Stem cell
Ly6a (Sca-1)	7.99	5.35E-08	7.18	7.31E-08	Stem cell
	<i>O9-1 down vs. DRG</i>		<i>O9-1 down vs. ectoderm</i>		
Cxcr4	-2.75	4.54E-05	-4.39	5.05E-06	Neural crest
Id2	-5.70	4.43E-06	-9.67	9.27E-07	Neural crest, neuronal differentiation
Meis1	-3.11	1.71E-04	-6.56	9.52E-06	Neural crest
Ngfr (p75)	-29.2	2.74E-07	-2.26	9.47E-04	Neural crest, neuronal differentiation, trunk neural crest stem cell
Robo1	-7.02	2.48E-06	-7.85	1.79E-06	Neural crest, axon guidance
Tcfap2b	-5.37	7.71E-03	-4.98	9.47E-03	Neural crest
Zic1	-15.5	4.02E-05	-2.43	1.37E-02	Neural crest, neuronal differentiation
Zic2	-2.27	6.85E-03	-2.99	1.66E-03	Neural crest, neuronal differentiation
Reln (reelin)	-3.92	1.25E-07	-6.12	2.32E-08	Neuronal development
Ntrk2 (Tkrb)	-4.11	2.82E-05	-2.52	3.12E-04	Neuronal differentiation
Ntrk3 (Tkrc)	-3.88	4.77E-05	-2.92	1.79E-04	Neuronal differentiation
Mbp	-2.49	3.83E-04	-3.01	1.35E-04	Schwann cell differentiation
Plp	-30.7	4.47E-07	-5.95	2.08E-05	Proteolipid protein (myelin) 1
Gap43	-61.3	6.11E-09	-4.26	3.06E-06	Neural crest migration, Schwann cell differentiation, neuroblastoma
Vldlr	-3.92	3.54E-06	-6.34	5.95E-07	Neuronal differentiation
Col2a1	-4.88	1.87E-04	-3.31	8.63E-04	Chondrogenic differentiation
Ckb	-41.4	2.30E-08	-4.24	6.32E-06	Enteric nerve neural crest stem cell
Ednrb	-68.2	2.82E-07	-2.87	8.15E-04	Enteric nerve neural crest stem cell
Rbp1	-6.93	1.86E-05	-6.60	2.15E-05	Enteric nerve neural crest stem cell
Bmp4	-3.72	4.52E-05	-5.07	1.33E-05	Osteogenic, chondrogenic, smooth muscle differentiation
Bmp7	-2.97	1.38E-05	-2.57	3.14E-05	Osteogenic differentiation
	<i>O9-1 up vs. DRG</i>		<i>O9-1 down vs. ectoderm</i>		
Hk2	3.00	1.36E-03	-3.73	5.22E-04	Neural crest
Msh6	2.06	2.40E-03	-2.11	2.05E-03	Neural crest
Msx1	4.63	1.22E-06	-2.03	1.13E-04	Neural crest

(continued)

TABLE 1. (CONTINUED)

Symbol	Fold-change	P value	Fold-change	P value	Annotation
<i>O9-1 up vs. DRG</i>					
Myo10	2.9	1.40E-05			Neural crest
Snail (Snail1)	3.03	2.28E-04			Neural crest
<i>O9-1 down vs. DRG</i>					
Acta1 (α SMA)	-2.41	3.70E-03			Smooth muscle differentiation
Ednra	-2.75	2.12E-03			Pharyngeal arch development
Nefm	-66.1	8.80E-08			Neuronal differentiation
Neurod1	-3.65	2.69E-05			Neuronal differentiation
Neurod4	-7.78	8.22E-06			Neuronal differentiation
Sostdc1	-5.19	1.47E-02			Tooth development
Fabp7	-5.92	1.53E-05			Glial differentiation
Myt1	-35.9	2.69E-08			Myelin transcription factor 1
Mapt	-46.2	1.00E-06			Neuron-specific protein
Prph1	-32.0	7.04E-06			Peripheral neurons
(Peripherin)					
Slc1a3	-5.96	4.30E-06			Glial differentiation
Smad1	-2.79	6.69E-06			TGF/BMP signal effector
Smad5	-2.32	8.20E-06			TGF/BMP signal effector
<i>O9-1 down vs. ectoderm</i>					
Sp7 (Osterix)			-2.85	8.54E-08	Osteogenic differentiation

osteogenic markers at different time points (day 0, 1, 3, 5, 7, and 10).

The morphology of O9-1 cells began changing on the second day (day 1). The cells lost their fibroblastic appearance and became more cuboidal (Fig. 3B). The cells formed aggregates by day 5 (Fig. 3D) and nodule-like structures by day 10 (Fig. 3F).

Initially, the expression of osteogenic markers [ALP and *osteocalcin* (OCL)], as well as the osteogenic regulator, *Msx2* [27], was undetectable. Within 1 day, the expression of these markers was readily apparent (Fig. 3). Also, *Runx2* and *osteopontin* (*Spp1*) expression rose from barely detectable to prominent during the course of the experiment. That O9-1 cells do not differentiate spontaneously along an osteogenic pathway, but instead require an osteogenic medium, for such differentiation shows that the induction of osteogenic differentiation of O9-1 cells is governed by extrinsic conditions, and occurs in a controlled manner.

O9-1 cells assume mesenchymal cranial neural crest fates when injected into embryos

We made use of a mouse organ culture system as well as chicken in ovo culture system to ask whether the O9-1 cell line can integrate into differentiating cranial structures derived from cranial neural crest. Undifferentiated cells propagated in the basal medium were injected into E13.5 mouse periorbital prominences, which were then cultured in the BGJb medium for 3 days. We also injected O9-1 cells into the frontonasal prominence of chicken embryos and cultured them for 24 h (Fig. 4). We examined mesenchymal neural crest derivatives for contributions of O9-1 cells. We found that injected O9-1 cells differentiated into osteogenic and smooth muscle lineage cells, which are mesenchymal derivatives (Fig. 4). Of 14 injected mouse explants, over 25% showed integration into the osteogenic lineage, and over 20% showed differentiation into smooth muscle. In surviving chicken embryos, 1 of 3 showed smooth muscle differentia-

tion. These results provide evidence that the O9-1 cell line closely approximates undifferentiated ectomesenchymal cells derived from the cranial neural crest.

CD44 marks migratory and postmigratory cranial neural crest cells in mouse

As noted above, O9-1 cells express the stem cell marker, CD44. This molecule is expressed in migratory and post-migratory cranial neural crest cells in *Xenopus* [28] and migratory cranial neural crest cells in chicken [29]. In *Xenopus*, downregulation of *CD44* by morpholino antisense resulted in delayed migration of cranial neural crest, suggesting that CD44 has an essential role in neural crest migration [28]. However, the expression pattern of CD44 in murine neural crest has not been described in detail. Thus, to verify the utility of CD44 as a marker of neural crest cells in the mouse, we assessed its expression in mouse embryos at E8.5–E10.5. We found that CD44 is expressed in cranial neural crest cells in both the migratory and post-migratory phases from E8.75 through E9.5 (Fig. 5, data not shown), supporting the hypothesis that the O9-1 cell line represents a population of cranial neural crest in the migratory through post-migratory stages. At E8.75, virtually no positive cells were found in the early phase of migration (not shown), whereas a majority of the late migratory or post-migratory cells were positive (Fig. 5). Expression of CD44 in cranial neural crest was markedly reduced by E10.5 (not shown).

Discussion

Here, we describe stable cell lines that exhibit properties of an undifferentiated mesenchymal population of cranial neural crest cells. One such cell line, O9-1, is capable of differentiating into 4 cell types, including osteoblasts, chondrocytes, smooth muscle cells, and glial cells. O9-1 cells express neural crest markers, as well as stem cell markers. They

FIG. 3. Osteogenic differentiation of O9-1 cells. (A–F) Treatment of O9-1 cells with an osteogenic differentiation medium resulted in a dramatic morphological change from a fibroblast-like appearance to a cuboidal shape (panel A–C), and tight adhesion between the cells. By day 5 (D), the cells had condensed. By day 10 (E, F), nodule-like structures had formed (E, arrowheads). Such structures are typical of osteogenic tissues. All of those images were taken under the bright field. (G) RT-PCR was performed to see the level of mRNA expression of various osteogenic markers. On day 0, nearly undetectable level of alkaline phosphatase (ALP), osteocalcin (OCL), and *Msx2* mRNA were seen. However, shortly after osteogenic medium treatment has begun (day 1), expression of these markers became significantly high. *Runx2* and *Spp1* (osteopontin) mRNA were expressed at low level in day 0. It was gradually increased on later time points. These data indicate that tightly controlled osteogenic differentiation takes place in the O9-1 cell line. nd, bone nodule. Scale bar: 200 μ m. Color images available online at www.liebertpub.com/scd

also express genes involved in craniofacial development, and are able to integrate into differentiating bone and smooth muscle in E13.5 mouse embryos, as well as smooth muscle in chicken embryos. We suggest that these cells will provide a tool for investigating the molecular properties of differentiating neural crest cells.

A major limitation in the investigation of the molecular properties of cranial neural crest cells is the difficulty of obtaining sufficient quantities of cells for biochemical studies. One approach that would overcome this limitation is a system for culturing cranial neural crest in such a way as to provide a large quantity of homogeneous cells that represent the native state of cranial neural crest cells. Although cell lines derived from trunk neural crest are available (trunk hNCC [N1–N5] [18], MONC-1 [30], and JoMa1.3 [31]), there is no generally accepted sustainable cell culture model for cranial neural crest.

There is evidence that early-stage cranial neural crest cells have stem-like properties. Calloni and colleagues [9] isolated single neural crest cells from quail embryos and showed that they can differentiate into 6 cell-types, in vitro. Because these cells were not a stable cell line and thus could not be maintained in culture, it was not possible to determine whether they had the ability to self-renew. To address this issue, Trentin and colleagues established stable cranial neural crest cell lines with the ability to self-renew [32]. These cells showed a limited ability to differentiate, functioning as bi-potent progenitors [32]. The question of whether a popula-

tion of self-renewing neural crest stem cells exists in vivo therefore remains open. Our work, by identifying conditions that can maintain cranial neural crest cells in a multipotent state, suggests an approach to the clonal culture of cranial neural crest, and thus a direct test of hypothesis that a population of multipotent cranial neural crest cells exists in vivo. We note that although we have sought conditions that maintain a normal karyotype during cell expansion, we were unsuccessful to date. Thus, a form of transformation may be required to maintain sustainable growth of cranial neural crest in vitro.

Several neural crest markers were either not detectable in O9-1 cells or were reduced in O9-1 cells relative to DRG and ectoderm; these include *Foxd3*, *Tcfap2b*, *Sox10*, *Ngfr* (p75), *Zic1*, *Zic2*, *Id2*, and *Robo1* (see Table 1). Some of these markers are known to be expressed in neural crest precursors in the neural plate as well as in differentiating neuronal and glial cells [33–37]. The reduced expression of these markers may indicate that O9-1 cells have a non-neural character. Indeed, our finding of increased expression of *Sox9* and no change in expression of *Sox10* relative to E8.25 ectoderm and E12.5 DRG is consistent with the view that O9-1 cells resemble undifferentiated mesenchymal cranial neural crest cells.

Our data suggest that there may be a difference in the potency of O9-1 cells in vitro versus in vivo. The O9-1 cell line can differentiate in vitro into both mesenchymal (osteogenic, chondrogenic, and smooth muscle) and non-

FIG. 4. Ectomesenchymal potential of the O9-1 cells in differentiating mouse organ explants and chicken embryo. To verify the differentiation ability of the O9-1 cell line in the cranial tissues, we performed microinjection experiments with an in vitro organ culture model. **(A)** Diagrams illustrate the strategy of O9-1 microinjection into E13.5 mouse upper cranial explants. We cultured these explants for 3 days in the BGjb medium. Injected cells were traced in histological sections by means of GFP labeling and staining with the osteogenic differentiation marker, ALP, and the smooth muscle differentiation marker, α -smooth muscle actin (α SMA). **(B–G)** In the upper cranial explants, O9-1 cells were found among differentiating osteogenic cells in the frontal bone primordium. Arrows indicate that the O9-1 cells positive for both GFP **(B, E)** and ALP **(C, F)**. **(E–G)** show the area demarcated in panels **(B–D)**. **(H–K)** A subpopulation of O9-1 cells injected into the upper cranial explants expresses α SMA. Arrows indicate the O9-1 cells positive for both GFP **(I)** and α SMA **(J)**. **(L)** We also microinjected O9-1 cells into the frontonasal prominence of HH stage17 chicken embryos. After injection, embryos were cultured in ovo for 24 h, and injected cells were traced histologically by GFP and staining with the smooth muscle differentiation marker, α SMA. **(M–P)** O9-1 cells can differentiate into the smooth muscle cells in chick. Arrows indicate the O9-1 cells positive for both GFP (green) **(N)** and α SMA (red; Rhodamine) **(O)**. In some area, smooth muscle differentiation of the host embryo (arrowhead in **O**) and the O9-1 cell line appears to be synchronized. These lineage-tracing experiments revealed that O9-1 cells are capable of differentiating into mesenchymal lineages. fbp, frontal bone primordium; bh, brain hemisphere; nt, neural tube. Scale bars: in **(D, G, P)** 100 μ m for **(B–D)**, **(E–G)**, and **(M–P)**; in **(K)**, 50 μ m for **(H–K)**.

FIG. 5. CD44 marks a subset of migratory and post-migratory cranial neural crest cells in E8.75 mouse embryos. (A–H) Sections of cranial region of E8.75 mouse embryo carrying the *Wnt1-Cre; R26R-GFP* reporter. Migratory as well as post-migratory neural crest cells are labeled by GFP in this system (green signal in B and F). (C, D, G, H) Immunostaining reveals that a subset of cranial neural crest expresses CD44 (yellow signal in D and H, arrows). Panels (B) and (F) show the boxed area in (A) and (E). Note that not all of the migratory neural crest cells are positive for CD44 (arrowhead in B). ne, neural ectoderm; pa, first pharyngeal arch. Scale bars: in (D), 50 μ m for (B–D); in (H), 50 μ m for (F–H); in (E), 100 μ m for (A, E).

mesenchymal (glial) lineages. In contrast, we have thus far only been able to demonstrate osteogenic and smooth muscle fates, *in vivo*. This difference may be technical and caused, for example, by our limited ability to inject O9-1 cells into embryos at the right time and place to observe non-mesenchymal fates. Alternatively, it may indicate that, as the marker studies suggest, O9-1 cells model the mesenchymal neural crest lineages more closely than the non-mesenchymal ones.

We found that CD44 is expressed in a subset of migratory cranial neural crest cells as well as postmigratory neural crest in E8.75 mouse embryos as well as in O9-1 cells (Figs. 1 and 5). Chung and colleagues have also found that CD44 is expressed in stem-like cells derived from the postmigratory cranial neural crest in mouse mandibular tissues [38]. These findings are consistent with the results of Menendez and colleagues [39], who showed that CD44 is exclusively expressed in neural crest cells restricted to a mesenchymal fate in human.

Microarray analysis showed that O9-1 cells express the osteogenic markers, *Runx2*, *Spp1* (*osteopontin*), and *Col1a1* (*type I collagen*). On the other hand, several additional osteogenic genes, including *Sp7* (*Osterix*), and elements of the Bmp signaling pathway were decreased relative to either DRG or ectoderm (see Table 1). Despite the expression of osteogenic markers, the O9-1 cell line does not differentiate spontaneously into osteogenic cells, but instead is highly dependent on the osteogenic medium for osteogenic differentiation (Fig. 3). It is interesting to note that *Runx2* expression is linked to multipotent stem-like characteristics of cranial neural crest in the quail [9]. Similarly, *osteopontin* has roles in settings outside osteogenic differentiation, controlling tumor progression and metastasis [40]. Thus, expression

of *Runx2* and *osteopontin* may be an indication of the stem cell character and migratory properties of O9-1 cells.

Migratory cranial neural crest cells are distinguished by the expression *Cald1* and *Cf11* [41,42]. Postmigratory neural crest cells express their own unique set of markers, including *Barx1*, *Prrx1* (*Prx1*), and *Rarg* [43–45]. *Rac1* functions in both the migratory and postmigratory neural crest [46–48]. Our array analysis revealed that O9-1 cells express genes typical of each category, including the migratory neural crest markers and the postmigratory markers. These results suggest that the O9-1 cell line has some properties of both phases of neural crest cell development.

Previous studies showed enhanced chondrogenic and glial differentiation of cultured mouse cranial neural crest by bFGF via activation of the Notch-signaling pathway [49,50]. In our culture conditions, bFGF functions together with LIF to promote the sustainable expansion of the culture while maintaining the cells in an undifferentiated state. bFGF controls the balance between proliferation and differentiation of mesenchymal cranial neural crest *in vitro* [51]. We found that, indeed, O9-1 cells have significantly reduced expression of Notch receptors (Supplementary Data S2, data not shown). Thus, bFGF may act differently depending on the context of the cell culture condition, including supplemented cofactors.

Finally, we found recently that osteogenic progenitors derived from O9-1 cells exhibit Bmp2 responsiveness similar to the frontal bone primordium of E12.5 mouse embryos (Jingjing Sun, M.Ishii, and R.Maxson, unpublished observations). These cells can be transfected efficiently, both with plasmid constructs as well as RNAi, and can be used for chromatin immunoprecipitation assays. We conclude that the O9-1 multipotent cranial neural crest cell line will serve

as a useful tool for the investigation of the biochemical mechanisms of cranial neural crest development.

Acknowledgments

We thank Dr. Baruch Frenkel for providing Bmp2, and we thank members of the Maxson lab for helpful discussions. This work was supported by grants from the National Institutes of Health R01DE016320 and R01DE019650 to R.M. and the CIRM training grant TB1-01192 to A.A.

Author Disclosure Statement

No competing financial interests exist in this study.

References

1. Sauka-Spengler T and M Bronner-Fraser. (2008). A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol* 9:557–568.
2. Nelms BL and PA Labosky. (2010). *Transcriptional Control of Neural Crest Development*. Morgan & Claypool Life Sciences, San Rafael, CA, pp 1–227.
3. Chai Y and RE Maxson, Jr. (2006). Recent advances in craniofacial morphogenesis. *Dev Dyn* 235:2353–2375.
4. Aclouque H, MS Adams, K Fishwick, M Bronner-Fraser and MA Nieto. (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 119:1438–1449.
5. Cordero DR, S Brugmann, Y Chu, R Bajpai, M Jame and JA Helms. (2011). Cranial neural crest cells on the move: their roles in craniofacial development. *Am J Med Genet A* 155A: 270–279.
6. Santagati F and FM Rijli. (2003). Cranial neural crest and the building of the vertebrate head. *Nat Rev Neurosci* 4:806–818.
7. Jiang X, DH Rowitch, P Soriano, AP McMahon and HM Sucov. (2000). Fate of the mammalian cardiac neural crest. *Development* 127:1607–1616.
8. Stemple DL and DJ Anderson. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* 71:973–985.
9. Calloni GW, NM Le Douarin and E Dupin. (2009). High frequency of cephalic neural crest cells shows coexistence of neurogenic, melanogenic, and osteogenic differentiation capacities. *Proc Natl Acad Sci U S A* 106:8947–8952.
10. Baroffio A, E Dupin and NM Le Douarin. (1988). Clonogenic ability and differentiation potential of migratory neural crest cells. *Proc Natl Acad Sci U S A* 85:5325–5329.
11. Chai Y, X Jiang, Y Ito, P Bringas, Jr., J Han, DH Rowitch, P Soriano, AP McMahon and HM Sucov. (2000). Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127:1671–1679.
12. Belteki G, J Haigh, N Kabacs, K Haigh, K Sison, F Costantini, J Whitsett, SE Quaggin and A Nagy. (2005). Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res* 33:e51.
13. Ishii M, J Han, HY Yen, HM Sucov, Y Chai and RE Maxson, Jr. (2005). Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. *Development* 132:4937–4950.
14. Sherwood RI, TY Chen and DA Melton. (2009). Transcriptional dynamics of endodermal organ formation. *Dev Dyn* 238:29–42.
15. Newbern JM, X Li, SE Shoemaker, J Zhou, J Zhong, Y Wu, D Bonder, S Hollenback, G Coppola, et al. (2011). Specific functions for ERK/MAPK signaling during PNS development. *Neuron* 69:91–105.
16. Bolstad BM, RA Irizarry, M Astrand and TP Speed. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19:185–193.
17. Benjamini Y and Y Hochberg. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc* 57:289–300.
18. Thomas S, M Thomas, P Wincker, C Babarit, P Xu, MC Speer, A Munnich, S Lyonnet, M Vekemans and HC Etchevers. (2008). Human neural crest cells display molecular and phenotypic hallmarks of stem cells. *Hum Mol Genet* 17:3411–3425.
19. Slavkin H, G Nuckolls and L Shum. (2000). Craniofacial development and patterning. *Methods Mol Biol* 136:45–54.
20. Bronner-Fraser M and M Garcia-Castro. (2008). Manipulations of neural crest cells or their migratory pathways. *Methods Cell Biol* 87:75–96.
21. Greenwood AL, EE Turner and DJ Anderson. (1999). Identification of dividing, determined sensory neuron precursors in the mammalian neural crest. *Development* 126:3545–3559.
22. John N, P Cinelli, M Wegner and L Sommer. (2011). Transforming growth factor beta-mediated Sox10 suppression controls mesenchymal progenitor generation in neural crest stem cells. *Stem Cells* 29:689–699.
23. O'Donnell M, CS Hong, X Huang, RJ Delnicki and JP Saint-Jeannet. (2006). Functional analysis of Sox8 during neural crest development in *Xenopus*. *Development* 133: 3817–3826.
24. Spokony RF, Y Aoki, N Saint-Germain, E Magner-Fink and JP Saint-Jeannet. (2002). The transcription factor Sox9 is required for cranial neural crest development in *Xenopus*. *Development* 129:421–432.
25. Iwashita T, GM Kruger, R Pardal, MJ Kiel and SJ Morrison. (2003). Hirschsprung disease is linked to defects in neural crest stem cell function. *Science* 301:972–976.
26. Molofsky AV, R Pardal, T Iwashita, IK Park, MF Clarke and SJ Morrison. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 425:962–967.
27. Ishii M, AE Merrill, YS Chan, I Gitelman, DP Rice, HM Sucov and RE Maxson, Jr. (2003). Msx2 and twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. *Development* 130:6131–6142.
28. Casini P, I Nardi and M Ori. (2012). Hyaluronan is required for cranial neural crest cells migration and craniofacial development. *Dev Dyn* 241:294–302.
29. Corbel C, A Lehmann and F Davison. (2000). Expression of CD44 during early development of the chick embryo. *Mech Dev* 96:111–114.
30. Rao MS and DJ Anderson. (1997). Immortalization and controlled in vitro differentiation of murine multipotent neural crest stem cells. *J Neurobiol* 32:722–746.
31. Maurer J, S Fuchs, R Jager, B Kurz, L Sommer and H Schorle. (2007). Establishment and controlled differentiation of neural crest stem cell lines using conditional transgenesis. *Differentiation* 75:580–591.
32. Trentin A, C Glavieux-Pardanaud, NM Le Douarin and E Dupin. (2004). Self-renewal capacity is a widespread

- property of various types of neural crest precursor cells. *Proc Natl Acad Sci U S A* 101:4495–4500.
33. Aruga J and K Mikoshiba. (2011). Role of BMP, FGF, calcium signaling, and Zic proteins in vertebrate neuroectodermal differentiation. *Neurochem Res* 36:1286–1292.
 34. Lee KF, E Li, LJ Huber, SC Landis, AH Sharpe, MV Chao and R Jaenisch. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* 69:737–749.
 35. Haldin CE and C LaBonne. (2010). SoxE factors as multi-functional neural crest regulatory factors. *Int J Biochem Cell Biol* 42:441–444.
 36. Brose K, KS Bland, KH Wang, D Arnott, W Henzel, CS Goodman, M Tessier-Lavigne and T Kidd. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96:795–806.
 37. Jen Y, K Manova and R Benezra. (1997). Each member of the Id gene family exhibits a unique expression pattern in mouse gastrulation and neurogenesis. *Dev Dyn* 208:92–106.
 38. Chung IH, T Yamaza, H Zhao, PH Choung, S Shi and Y Chai. (2009). Stem cell property of postmigratory cranial neural crest cells and their utility in alveolar bone regeneration and tooth development. *Stem Cells* 27:866–877.
 39. Menendez L, TA Yatskevych, PB Antin and S Dalton. (2011). Wnt signaling and a Smad pathway blockade direct the differentiation of human pluripotent stem cells to multipotent neural crest cells. *Proc Natl Acad Sci U S A* 108:19240–19245.
 40. Rangaswami H, A Bulbule and GC Kundu. (2006). Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol* 16:79–87.
 41. Nie S, Y Kee and M Bronner-Fraser. (2011). Caldesmon regulates actin dynamics to influence cranial neural crest migration in *Xenopus*. *Mol Biol Cell* 22:3355–3365.
 42. Gurniak CB, E Perlas and W Witke. (2005). The actin depolymerizing factor n-cofilin is essential for neural tube morphogenesis and neural crest cell migration. *Dev Biol* 278:231–241.
 43. Thomas T, H Kurihara, H Yamagishi, Y Kurihara, Y Yazaki, EN Olson and D Srivastava. (1998). A signaling cascade involving endothelin-1, dHAND and msx1 regulates development of neural-crest-derived branchial arch mesenchyme. *Development* 125:3005–3014.
 44. Trumpp A, MJ Depew, JL Rubenstein, JM Bishop and GR Martin. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev* 13:3136–3148.
 45. Ruberte E, P Dolle, P Chambon and G Morriss-Kay. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* 111:45–60.
 46. Thomas PS, J Kim, S Nunez, M Glogauer and V Kaartinen. (2010). Neural crest cell-specific deletion of Rac1 results in defective cell-matrix interactions and severe craniofacial and cardiovascular malformations. *Dev Biol* 340:613–625.
 47. Fuchs S, D Herzog, G Sumara, S Buchmann-Moller, G Civerni, X Wu, A Chrostek-Grashoff, U Suter, R Ricci, et al. (2009). Stage-specific control of neural crest stem cell proliferation by the small rho GTPases Cdc42 and Rac1. *Cell Stem Cell* 4:236–247.
 48. Kee Y, BJ Hwang, PW Sternberg and M Bronner-Fraser. (2007). Evolutionary conservation of cell migration genes: from nematode neurons to vertebrate neural crest. *Genes Dev* 21:391–396.
 49. Ijuin K, K Nakanishi and K Ito. (2008). Different downstream pathways for Notch signaling are required for gliogenic and chondrogenic specification of mouse mesencephalic neural crest cells. *Mech Dev* 125:462–474.
 50. Nakanishi K, YS Chan and K Ito. (2007). Notch signaling is required for the chondrogenic specification of mouse mesencephalic neural crest cells. *Mech Dev* 124:190–203.
 51. Sarkar S, A Petiot, A Copp, P Ferretti and P Thorogood. (2001). FGF2 promotes skeletogenic differentiation of cranial neural crest cells. *Development* 128:2143–2152.

Address correspondence to:

Prof. Robert E. Maxson

Department of Biochemistry and Molecular Biology

USC/Norris Comprehensive Cancer Center

Keck School of Medicine

University of Southern California

Los Angeles, CA 90033

E-mail: maxson@usc.edu

Received for publication March 26, 2012

Accepted after revision August 13, 2012

Prepublished on Liebert Instant Online August 13, 2012