Original Research *Featured Article*

Characterization of cultured multipotent zebrafish neural crest cells

Beste Kinikoglu[1,2], Yawei Kong[1,2] and Eric C Liao[1,2,3]

[1]Center for Regenerative Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA; [2]Division of Plastic and Reconstructive Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA; [3]Harvard Stem Cell Institute, Boston, MA 02114, USA

Corresponding author: Eric C Liao. Email: cliao@partners.org

Abstract

The neural crest is a unique cell population associated with vertebrate evolution. Neural crest cells (NCCs) are characterized by their multipotent and migratory potentials. While zebrafish is a powerful genetic model organism, the isolation and culture of zebrafish NCCs would provide a useful adjunct to fully interrogate the genetic networks that regulate NCC development. Here we report for the first time the isolation, *in vitro* culture, and characterization of NCCs from zebrafish embryos. NCCs were isolated from transgenic *sox10:egfp* embryos using fluorescence activated cell sorting and cultured in complex culture medium without feeder layers. NCC multilineage differentiation was determined by immunocytochemistry and real-time qPCR, cell migration was assessed by wound healing assay, and the proliferation index was calculated by immunostaining against the mitosis marker phospho-histone H3. Cultured NCCs expressed major neural crest lineage markers such as *sox10, sox9a, hnk1, p75, dlx2a*, and *pax3*, and the pluripotency markers *c-myc* and *klf4*. We showed that the cultured NCCs can be differentiated into multiple neural crest lineages, contributing to neurons, glial cells, smooth muscle cells, melanocytes, and chondrocytes. We applied the NCC *in vitro* model to study the effect of retinoic acid on NCC development. We showed that retinoic acid had a profound effect on NCC morphology and differentiation, significantly inhibited proliferation and enhanced cell migration. The availability of high numbers of NCCs and reproducible functional assays offers new opportunities for mechanistic studies of neural crest development, in genetic and chemical biology applications.

Keywords: Neural crest cells, *in vitro* culture, retinoic acid, zebrafish, multipotency

***Experimental Biology and Medicine* 2014; 239: 159–168. DOI: 10.1177/1535370213513997**

Introduction

The neural crest is a unique cell population induced at the lateral border of the neural plate during embryogenesis. During vertebrate embryogenesis, neural crest cells (NCCs) contribute to neural, skeletal, dermal, and mesenchymal structures,[1] and commonly referred to as the fourth germ layer due to this multipotency.[2] Many fundamental questions in developmental biology can be addressed by the study of this unique group of cells.[3] A systematic understanding of neural crest specification and differentiation requires the isolation of NCCs, which allows one to develop reproducible functional assays.[4] Further *in vitro* cell culture systems facilitate chemical genetics approaches, where small molecule screens can be carried out with robotic assistance, enabling these experiments to be scaled and automated.[5]

Although avian and murine NCCs have been identified *in vitro*, isolation and culture of zebrafish NCCs has not been reported. Zebrafish has long been used to study early development due to its advantages such as rapid development, optical clarity, and external development of large number of embryos. It has emerged as a powerful animal model for the study of basic development as well as human disease.[6] *In vivo* approaches to study neural crest development in zebrafish have been productive and *in vitro* culture systems of avian, murine, and human NCCs were successfully applied to study the effects of growth factors on NCC growth, differentiation, and migration, the effects of teratogenic compounds on NCC gene expression, the effects of inhibition of certain signaling pathways on NCC fate, and recently to model NCC-related rare genetic diseases.[5,7–21] Development of *in vitro* culture of NCCs and reproducible functional assays will provide a valuable and complementary approach to the *in vivo* experiments in zebrafish. Here we report the isolation, characterization, and culture of NCCs from zebrafish embryos. This study would provide new opportunities for studies of neural crest development

and disease, and for cell-based high-throughput drug screening applications.

Materials and methods

Zebrafish maintenance and the transgenic sox10:eGFP line

All fish were raised and cared for using established protocols as per Subcommittee on Research Animal Care, Massachusetts General Hospital. They were maintained and bred at 28°C on a 14-h light, 10-h dark cycle. Embryos were generated and collected by natural spawning and also kept at 28°C. Tubingen fish were used as wild type in our experiments. The *sox10:eGFP* transgene was generated as described.[22,23]

Isolation of gfp-positive NCCs

In 0.5% bleach-E3 solution, 10-somite stage embryos were decontaminated for 2 min. Then, they were rinsed several times with sterile E3 and dechorionated in 0.5 mg/ml pronase (Roche, Branchburg, NJ, USA)-E3 solution at 28°C for 10 min. The action of pronase was stopped by rinsing the embryos several times with sterile calcium-magnesium-free phosphate buffered saline (PBS) (Invitrogen, Grand Island, NY, USA). Next, the embryos were dissociated briefly on ice in 1 ml of 4% fetal bovine serum (Invitrogen)-PBS using a mechanical homogenizer and by pipetting. The homogenate was filtered through a 35-μm cell strainer (BD Biosciences, San Jose, California, USA) and the cells were sorted using an FACS Aria (BD Biosciences) into 1.5 ml Eppendorf tubes containing 500 μl of culture medium (described in section ''Culture of neural crest cells''). Same stage wild-type embryos were used as controls to set the FACS gateways. After collection, the cells were plated immediately on plastic tissue culture plates precoated with extracellular matrix proteins as described in section ''Culture of neural crest cells''.

Culture of NCCs

FACS-sorted gfp-positive cells were cultured in 12- or 24-well plates coated with collagen or a mixture of poly-ornithine hydrobromide (PO)/laminin/fibronectin or laminin alone. For collagen coating, the plates were incubated with a 500 μg/ml rat tail collagen (BD Biosciences) solution (dissolved in 0.02 N acetic acid) for 1 h at 28°C. Then, they were rinsed twice with sterile PBS before cell seeding. For the PO/laminin/fibronectin coating, the dishes were incubated with the PO solution (15 μg/ml in PBS) (Sigma, St. Louis, MO, USA) overnight at room temperature (RT). On the next day, they were rinsed three times with sterile PBS and then incubated with a mixture of fibronectin (1 μg/ml in PBS) (BD Biosciences) and laminin (10 μg/ml in PBS) (BD Biosciences) overnight at RT. The cells were seeded immediately after aspirating the solution, without letting the coating dry. For laminin coating, the plates were incubated with a 10 μg/ml laminin-PBS solution for 45 min at RT and then rinsed with sterile PBS thoroughly. The cells were seeded at a density of 12,500 cells / cm^2 and incubated in a humidified incubator at 28°C with 5% CO_2. The complex culture medium (50 ml) consisted of: 39.375 ml DMEM/F12 (Invitrogen), 5% zebrafish embryo extract, 12% heat inactivated embryonic stem cell qualified fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 500 μl N2 supplement (×100) (Invitrogen), 0.02 mg/ml insulin (Gemini Bio-Products, West Sacramento, CA, USA), 5000 UI Penicillin and 5000 μg Streptomycin (Invitrogen), 1 μg/ml amphotericin B (Sigma), 20 ng/ml FGF (R&D Systems, Minneapolis, MN, USA), and 20 ng/ml EGF (R&D Systems). Complex medium containing neither serum nor embryo extract was named as defined medium. All media were sterilized using 0.22 μm filtration and used within 1 week. For the preparation of the zebrafish embryo extract, 200 3-day-old embryos were decontaminated in 0.5% bleach-E3 for 2 min. Then, they were rinsed with calcium-magnesium-free PBS three times for 2 min. They were homogenized on ice using a mechanical homogenizer and by pipetting; 1 ml of DMEM/F12 was added to the homogenate and the mixture was centrifuged at 900 r/min at 4°C and for 6 min. The supernatant was filter-sterilized through a 0.2 μm filter, aliquoted, and stored at −20°C. The cells were passaged every 3 days by incubation with 0.01% trypsin-EDTA (Invitrogen) for 3 min at RT and could be maintained in culture for more than 21 days. For every new passage, the cells were subcultured in a mixture of filter-sterilized preconditioned medium collected from previous passage and fresh culture medium (mixed at a ratio of 1:1). Chemicals were added to the culture medium with no serum to induce differentiation into multilineages as follows. Neuronal: NGF (10 ng/ml) (R&D Systems), BDNF (10 ng/ml) (R&D Systems), glial cell line-derived neurotrophic factor (10 ng/ml) (R&D Systems), neurotrophin-3 (10 ng/ml) (R&D Systems), cyclic AMP (0.5 mM) (Sigma); glial: ciliary neurotrophic factor (10 ng/ml) (R&D Systems), neuregulin (20 ng/ml) (R&D Systems), cyclic AMP (0.5 mM) (Sigma); melanocytic lineage: phorbol esters (5 ng/ml) (Sigma), cholera toxin (20 nM) (Sigma), endothelin (100 nM) (Sigma). For chondrogenic differentiation the NCCs were cultured in the standard culture medium with no serum for 3 weeks.

Immunocytochemistry and Alcian blue staining

For immunocytochemistry, the cells were cultured on 12- or 15-mm diameter glass coverslips (ChemGlass, Vineland, NJ, USA) sterilized in an autoclave and coated subsequently with collagen or PO/laminin/FN solutions (as described in section ''Culture of neural crest cells''). At 70% confluence, they were fixed in 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.25% Triton X for 10 min, blocked in 2% bovine serum albumin (BSA)-PBS solution for 30 min, and incubated with the primary antibody overnight at 4°C or for 90 min at RT. The primary antibodies used were anti-HNK-1 (Sigma) (1:20), antinerve growth factor (p75) receptor (Advanced Targeting Systems, San Diego, CA, USA) (1:20), antiglial fibrillary acidic protein (GFAP) (Calbiochem, Billerica, MA, USA) (1:1000), anti-neuronal class III β-tubulin (TUJ1) (Covance, Princeton, NJ, USA) (1:500), anti-actin α smooth muscle (Sigma) (1:400), anti-mel-a (Thermo Scientific, Waltham, MA, USA)

(1:150), and antiphospho-Histone H3 (pH3) (Millipore, Billerica, MA, USA) (1:1500). Then, the cells were incubated with the secondary antibody Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) (1:200) for 1 h at RT and the cell nuclei were counterstained with DAPI (Molecular Probes). Images were captured utilizing an Olympus IX81 fluorescence microscope. For Alcian blue staining, cells were fixed in 4% PFA for 15 min and subsequently incubated in 50% ethanol for 10 min and in a 0.02% Alcian blue staining solution overnight at RT. Next day, they were rinsed with distilled water for 10 min and postfixed in 4% PFA. Images were captured utilizing a Nikon Eclipse TE 2000-U microscope.

Standard and real-time quantitative RT-PCR

For RT-PCR, RNA was isolated from cells using Trizol (Invitrogen) or RLT buffer (Qiagen, Valencia, CA, USA). cDNA was synthesized using the iScript cDNA synthesis kit (Biorad, Berkeley, CA, USA) with 1 μg total RNA in a total volume of 20 μl. Reaction mixtures were heated at 42°C for 90 min, then at 85°C for 5 min. For the standard PCR, from 1 μl of cDNAs, sequences of interest were amplified in a thermocycler in a total volume of 50 μl of mixture with Tag polymerase, using the Expand High Fidelity PCR System (Roche). The primers were obtained from Invitrogen and their sequences are presented in Table 1. Genes were amplified by denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min for 30 cycles. PCR products were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Concentrations and purity of total RNA and cDNA were measured using the Nanodrop 2000 software. For real-time quantitative PCR, sequences of interest were amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The data were analyzed using the delta CT method and β-actin served as the endogenous control gene.

Proliferation assay

All trans-retinoic acid (RA) (Sigma), dissolved in dimethyl sulfoxide (DMSO) (Sigma), was added to the adhered cells at a concentration of 1 μM. The same quantity of DMSO (1 μl/ml) was added to the controls. The proliferative indexes of the RA-treated cells and the control cells (treated with DMSO alone) were measured as reported previously.[24] Briefly, the cells were immunostained against the proliferation marker phospho histone H3 (pH3) at 72 h after RA and DMSO exposure and the number of pH3-positive cells were counted. The results represent the mean of three experimental replicates (10 fields of view) ± standard error of the mean (error bars). *p* Value was calculated by two-tailed Student's *t* test. Fluorescence images were captured utilizing an Olympus IX81 microscope.

Migration assay

The migration / motility of RA- and DMSO-treated NC cells was assessed by wound healing (''scratch'') assay following published protocols.[6,25] In brief, a total of 200,000 cells were seeded in each well of a collagen-coated 24-well plate and cultured with either RA (1 μM) or DMSO (both 1 μl/ml). At 24 h of plating, each well was scratched using a 100-μl pipette tip, washed, and image was taken (0 h) as reference point. At 48 h after scratching, the wells were washed and images of the identical fields marked at time 0 h were captured at × 40 magnification utilizing a Nikon Eclipse TE 2000-U microscope.

Statistical analysis

The statistical analysis of the data was done by using the two-tailed paired t-test with 5% significance level, $\alpha = 0.05$. The results were expressed as mean ± standard error of the mean.

Results

Isolation, characterization, and in vitro culture of NCCs

The *sox10:gfp*-positive cells were isolated from 10-somite stage embryos using fluorescence activated cell sorting (FACS) (Figure 1(a)–(e)). The isolated cells exhibited the typical stellate multipolar shape of normal NCCs (Figure 1(e)). After FACS isolation, the NCC expressed early NCC markers *hnk-1* and *p75*, the neural crest genes *sox10* and *sox9a*, and the migrating NCC marker *pax3* (Figure 2(a)–(c)). These gfp-positive cells constituted 8% of the whole cell population of the zebrafish embryo at the 10-somite stage. Along with NCC markers, NCC from 10-somites also expressed the pluripotency markers *c-myc* and *klf4* (Figure 2(c)). Quantitative PCR revealed that the major NCC markers *sox10* and *sox9a* were upregulated 24 and 5 folds, respectively, in gfp-positive cells compared with gfp-negative cells after FACS purification (Figure 2(d)). RNA *in situ* hybridization on isolated NCCs with *sox10* riboprobe demonstrated that the transgene expression mirrors that of endogenous *sox10* (suppl. Figure 1). The isolated cells did not adhere to uncoated tissue culture plastic. As a result, several proteins were tested as coating materials to enhance cell adhesion. Among these materials, the cells adhered best to collagen type I and a mixture of fibronectin/poly-ornithine bromide/laminin (Figure 3(a)–(f)). They did not adhere to laminin alone (Figure 3(b) and (e)) and feeder layers were not necessary. Using a suitable coating and a complex culture medium containing zebrafish embryo extract and fetal calf serum, along with other chemicals and growth factors, an optimum growth of the cells was achieved (Figure 3(d) and (f) and Figure 4(a)–(g)). Defined medium was prepared without serum or embryo extract, and the cells did not survive in this medium for more than 24 h (Figure 3(a)–(c)). The zebrafish embryo extract and not serum was essential for their survival. On the other hand, the presence of serum increased cell viability. An increase in the concentrations of embryo extract and serum in the complex medium resulted in an increase in survival up to 5% for embryo extract and 12% for serum. Above those concentrations, both serum and the embryo extract actually had a negative impact on cell viability. Gfp-negative cells did not survive under the conditions

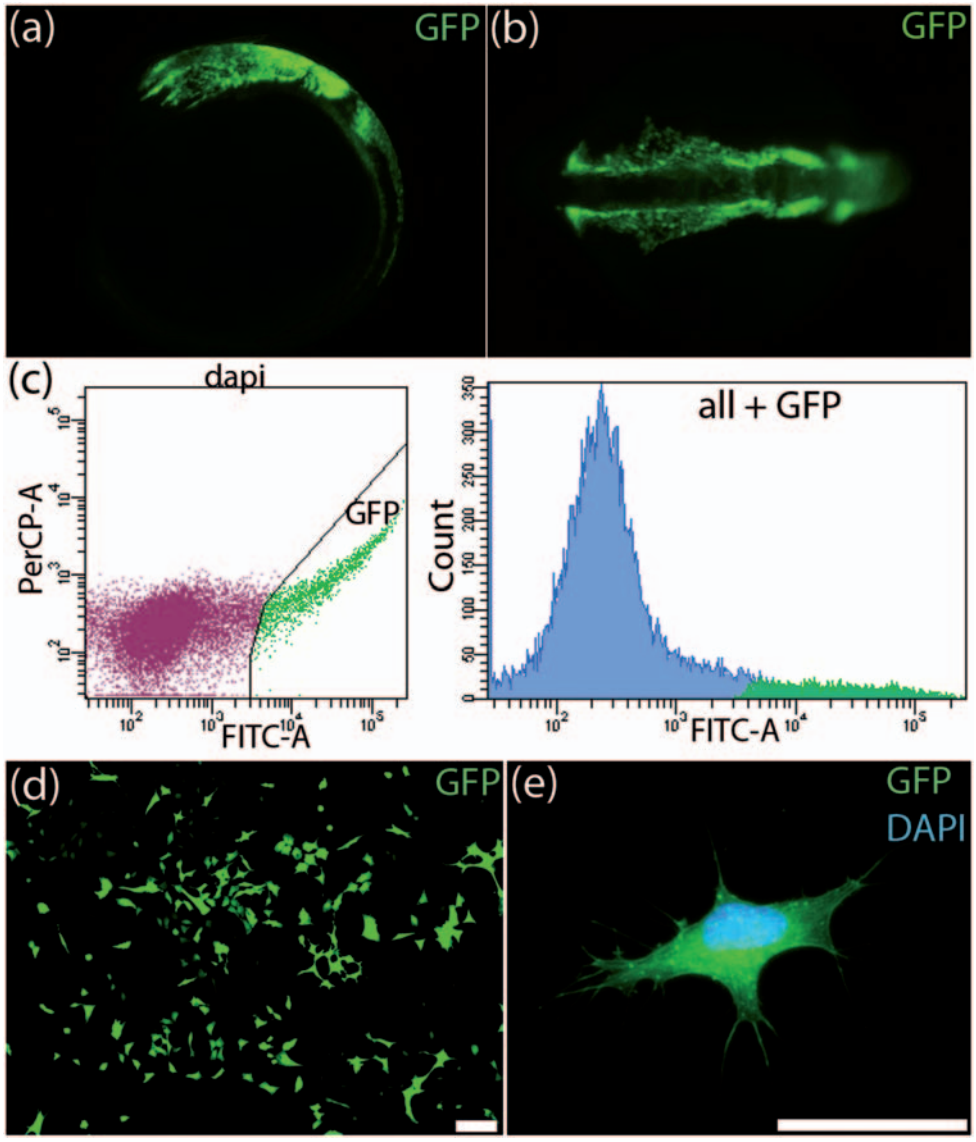

Figure 1 Isolation of neural crest cells from zebrafish embryos. (a) Lateral (anterior to the left) and (b) dorsal views of transgenic zebrafish embryos where neural crest cells are labeled with gfp. (c) FACS isolation of gfp-positive neural crest cells constituting 8% of the total cell population. (d) Gfp-positive neural crest cells in culture. (e) A neural crest cell at higher magnification exhibiting typical multipolar, stellate morphology of a normal neural crest cell. Bars = 50 μm. (A color version of this figure is available in the online journal)

optimized for NCCs indicating that these conditions were selective for *sox10:gfp*-positive NCCs. For the culture of amphibian cells, Leibovitz's L15 medium is generally the base medium of choice. However, in our hands, DMEM-F12, which is richer and mostly used for mammalian cells, turned out to support the growth of zebrafish NCCs much better when comparing to L15 or a mixture of L15 and DMEM-F12 (1:1) (data not shown).

Multilineage differentiation of NCCs

RT-PCR and immunocytological analyses showed that by day 3 in culture, the cells started to express the late neural crest marker *dlx2a*, and by day 15, the major differentiation markers *peripherin* (neuronal lineage), *tuj1* (neuron-specific class III beta-tubulin), *gfap* (glial lineage), *sm22* (smooth muscle lineage), and *dopachrome tautomerase (dct)* (melanocytic lineage) were expressed (Figure 5(a)–(f)). By day 21, cultured NCCs were positive for *collagen 2a* and stained for Alcian blue, which detects sulfated proteoglycans deposits that are indicative of functional chondrocytes (Figure 5(e) and (f)). Expression of *sox10* was decreased at days 15 and 21 compared to day 0.

Influence of RA on NCC morphology, differentiation, migration, and proliferation

In order to test the responsiveness of our neural crest culture system to exogenous signals and to validate reproducible functional assays, we investigated the effect of all trans-RA, a signaling molecule with morphogenetic effects during vertebrate embryogenesis, affecting NCC morphology, differentiation, migration, and proliferation *in vitro*. In our system, RA caused significant changes in all aspects

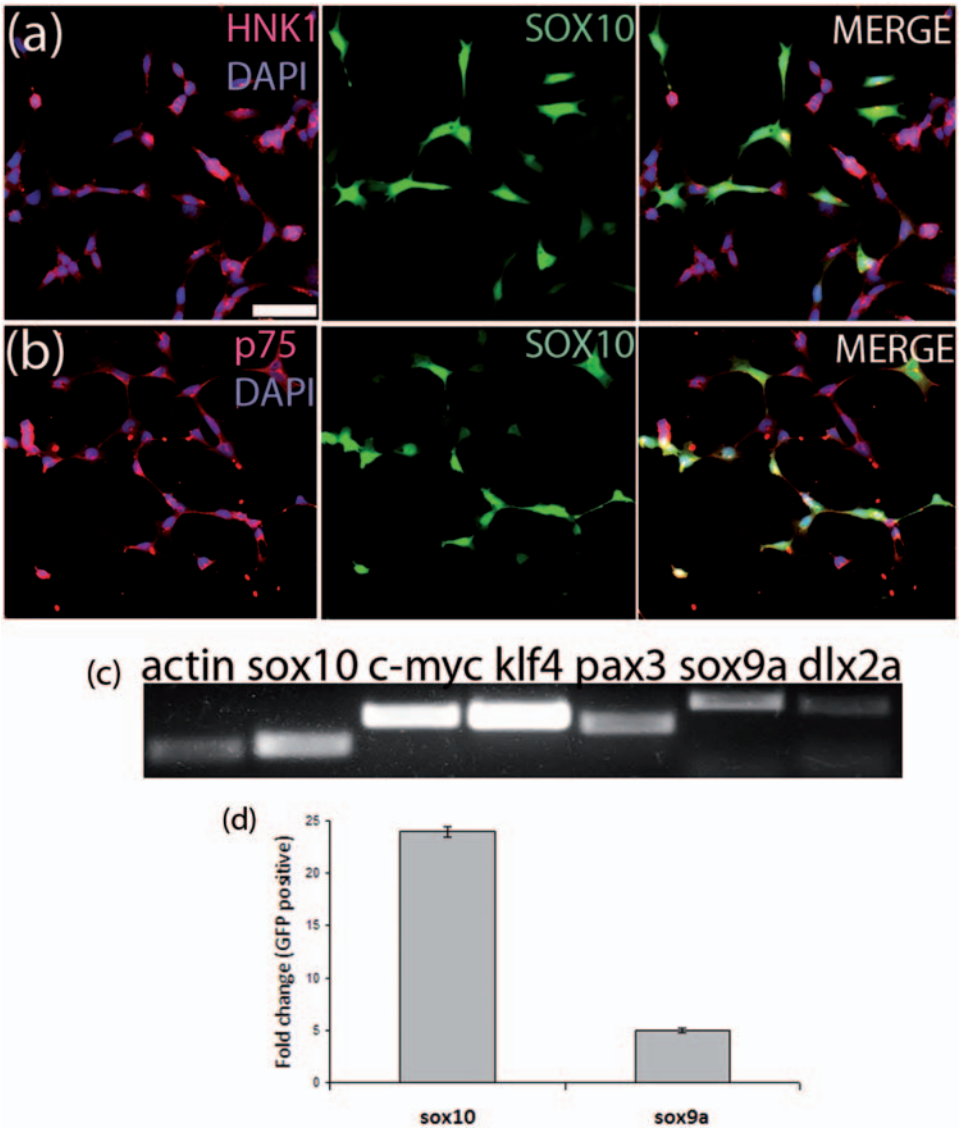

Figure 2 Confirmation of neural crest cell identity by gene expression analysis of isolated and cultured neural crest cells. Immunolabeling of (a) *hnk1* and (b) *p75*, early markers of neural crest cells. (c) RT-PCR analysis showed that the cells also expressed *sox10*, *sox9a*, *pax3*, and *dlx2a*, markers of neural crest cells, and the pluripotency markers *klf4* and *c-myc*. (d) Quantitative PCR showed that in the gfp-positive group of cells, the expressions of the major NCC markers *sox10* and *sox9a* were upregulated 24 and 5 folds, respectively, when compared with the gfp-negative group. Bars = 40 μm. (A color version of this figure is available in the online journal)

of NCC behavior, including differentiation, proliferation, and migration (Figure 6(a)–(i)). The cells treated with RA were scattered, flattened, spread with spindle-shaped appearance and outgrowing projections (Figure 6(b)), unlike DMSO-treated control cells, which were arranged as tightly packed polygonal cells (Figure 6(a)). The RT quantitative PCR data showed that in the group of cells treated with RA, neuronal marker *peripherin* and melonocytic marker *dct* were downregulated 6.5 and 3.6 folds, respectively, whereas the smooth muscle cell marker *sm22* and the neural crest marker *sox10* were upregulated 4.7 and 2.1 folds, respectively (Figure 6(c)). The expression levels of *collagen2a*, and *the gfap*, markers of chondrogenic and glial lineages, respectively, were unchanged (Figure 6(c)). Using a wound healing (''scratch'') assay, we observed a significant increase in the migration/motility of RA-treated versus control NCCs ($p < 0.001$) (Figure 6(d)–(f)). By 48 h after the scratch, the wound was completely closed by the RA-treated NCCs (Figure 6(e)). On the other hand, proliferation of the NCCs was severely inhibited in our system upon RA exposure (three-fold, $p < 0.001$) (Figure 6(g)–(i)).

Discussion

Access to a high number of NCCs at different stages of development offers a novel and unique *in vitro* model system that should contribute to a more detailed understanding of neural crest development. Traditionally, investigators have used tissue explants, such as the neural tube, to obtain primary NCCs for their studies. However, this

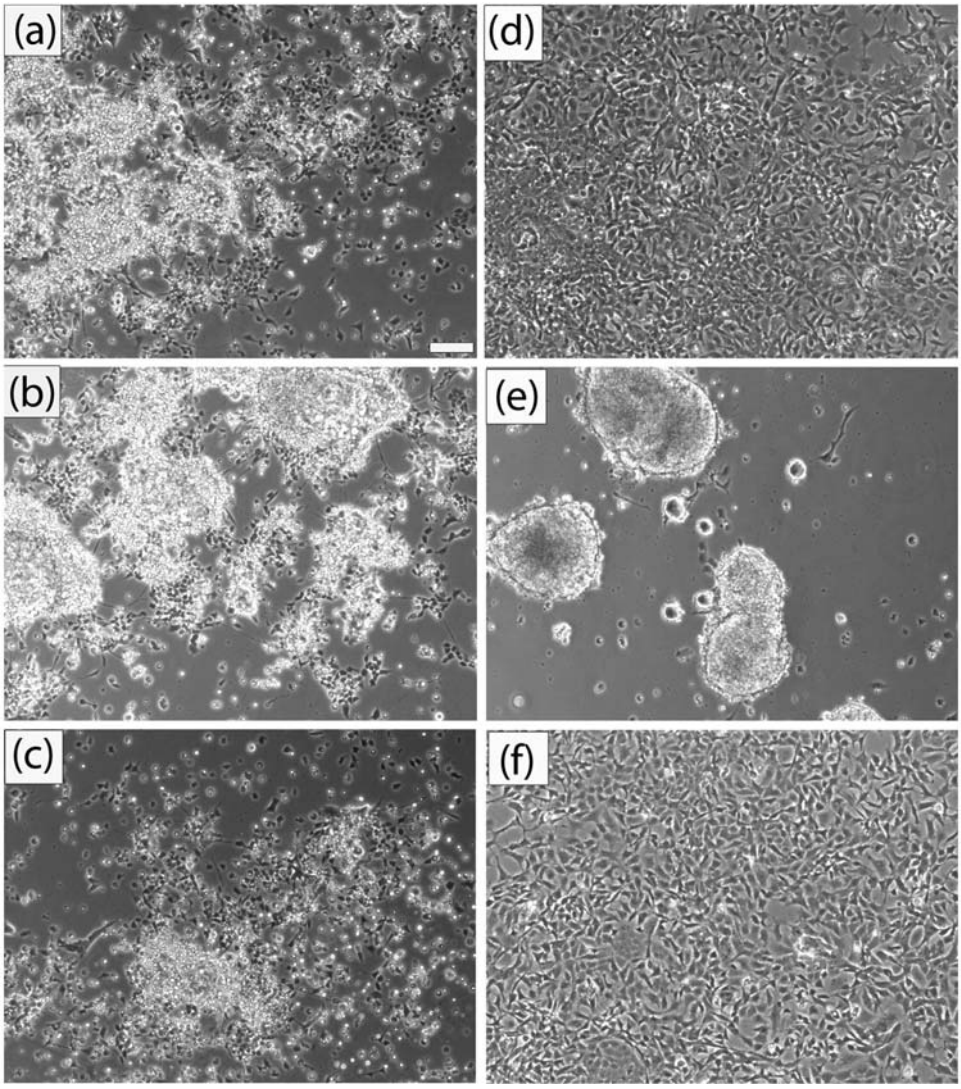

Figure 3 Neural crest cells were cultured under various conditions including cell surface coating proteins such as collagen (a and d), laminin (b and e) or PO/laminin/fibronectin (c and f) and cell culture media such as defined medium (a–c) or complex medium (d–f). Optimum neural cell growth was achieved using the complex medium and either collagen or PO/laminin/fibronectin as the surface-coating material (d and f). Bars = 40 μm

approach has inevitably resulted in simultaneous isolation of neural and non-NCCs as both of these cells migrate away from tissue explants.[26] Using the *sox10:egfp* zebrafish model, we have obtained a pure population of NCCs as indicated by the upregulation of the major NCC markers *sox10* and *sox9a* by 24 and 5 folds, respectively, in the gfp-positive group compared to the gfp negative group. *Sox10* expression is initiated in NCCs as they dissociate from the neural tube, and expression is maintained during NCC migration.[27] In addition to *sox10*, the gfp-positive cells that we isolated from our transgenic model expressed early NCC markers *hnk-1* and *p75*, neural crest specifier *sox9a*, and the migrating NCC marker *pax3*. They also expressed the pluripotency markers *c-myc* and *klf4*. It has been reported that both *c-myc* and *klf4* play an important role in the proliferation and maintenance of pluripotency of embryonic stem cells.[28,29] By day 3 in culture, the cells started to express RNA transcripts for the late neural crest marker *dlx2a*. By day 15, they were immunostained positive for the major differentiation markers *peripherin, tuj1, gfap, sm22, dct*, and they were *collagen 2a* and Alcian blue-positive by day 21. Differentiation into chondrocytes took longer than other lineages, as shown previously with NCCs derived from induced pluripotent stem cells.[30] This might be due to the fact that NCCs must go through the myofibroblastic stage before giving rise to mesenchymal cells such as chondrocytes or osteoblasts.[30] In our culture, differentiated neurons, smooth muscle cells, and chondrocytes stopped expressing *sox10*, whereas Schwann cells belonging to the glial lineage continued to do so. Indeed, it is reported that expression of *sox10* continues in the glial lineage, but turned off in many other neural crest derivatives.[31–33] For the optimization of *in vitro* culture conditions, we tested both complex and defined media, and several coating proteins such as collagen, poly-ornithine bromide, fibronectin, and laminin. The cells did

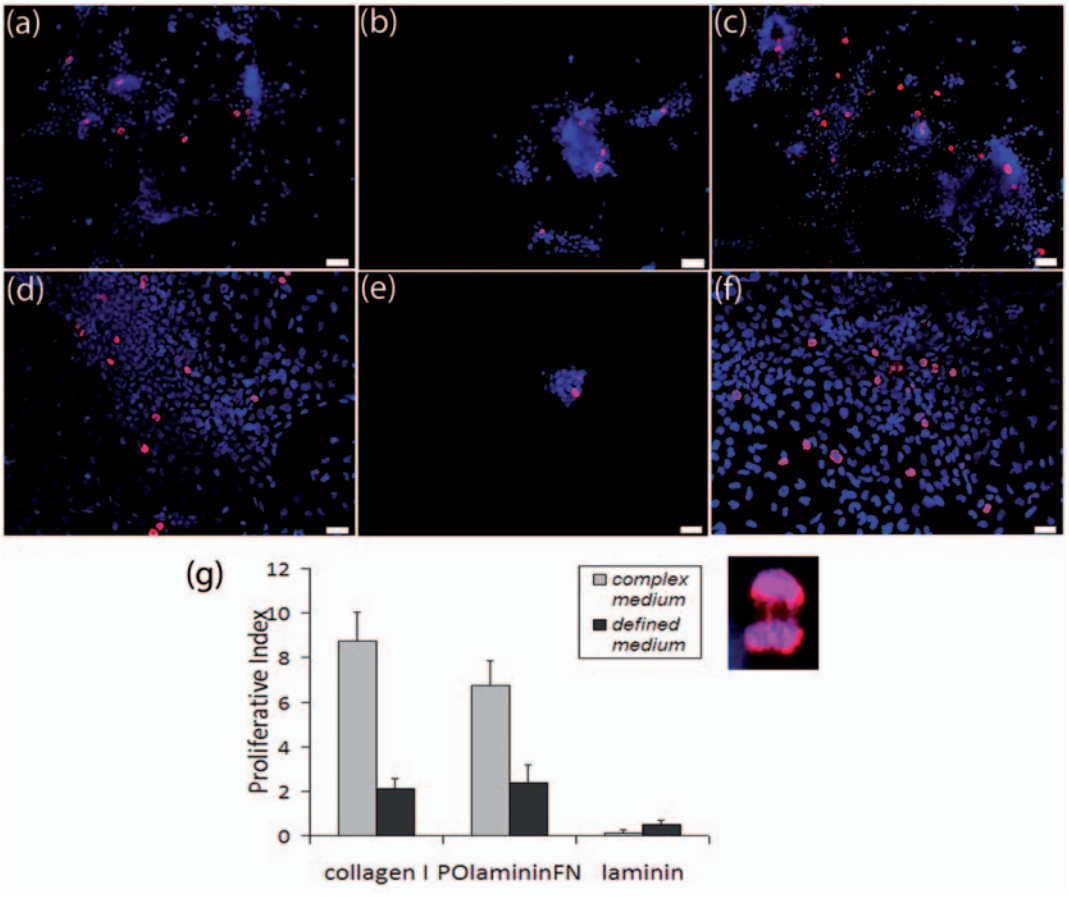

Figure 4 Proliferation of neural crest cells cultured under various conditions was compared by immunostaining against the mitosis marker pH3 (a–f). Neural crest cells cultured using complex medium and on collagen or PO/laminin/fibronectin coatings had the highest proliferative index (g). Bars = 20 μm. (A color version of this figure is available in the online journal)

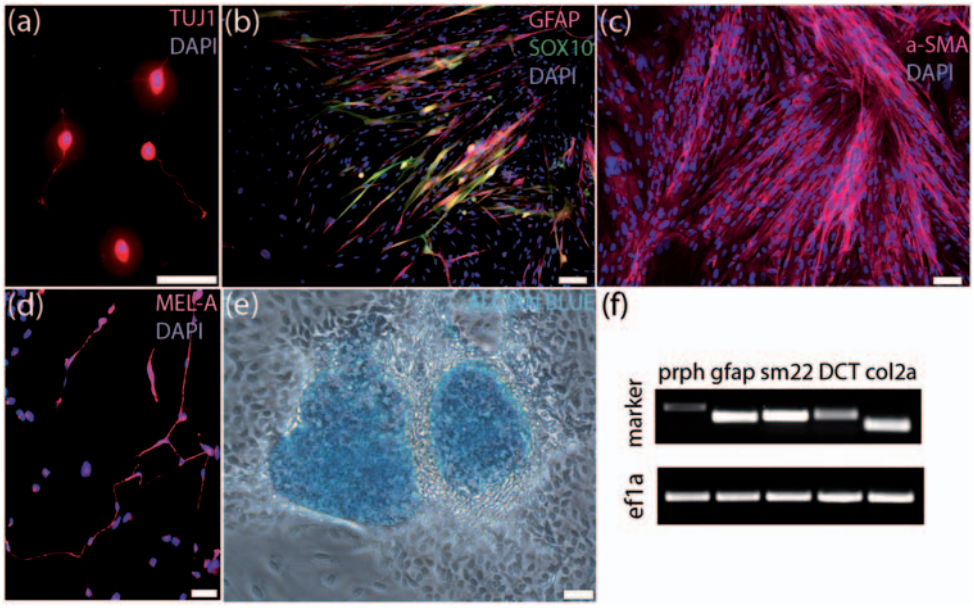

Figure 5 Multilineage differentiation of neural crest cells into neurons expressing *tuj1* (a), glial cells co-expressing *gfap* and *sox10* (b), smooth muscle cells expressing *α-sma* (c), melanocytes expressing *mel-a* (d), and chondrocytes positive for Alcian blue staining (e). RT-PCR confirmed the results of the immunohistological stainings (f). Bars = 50 μm. (A color version of this figure is available in the online journal)

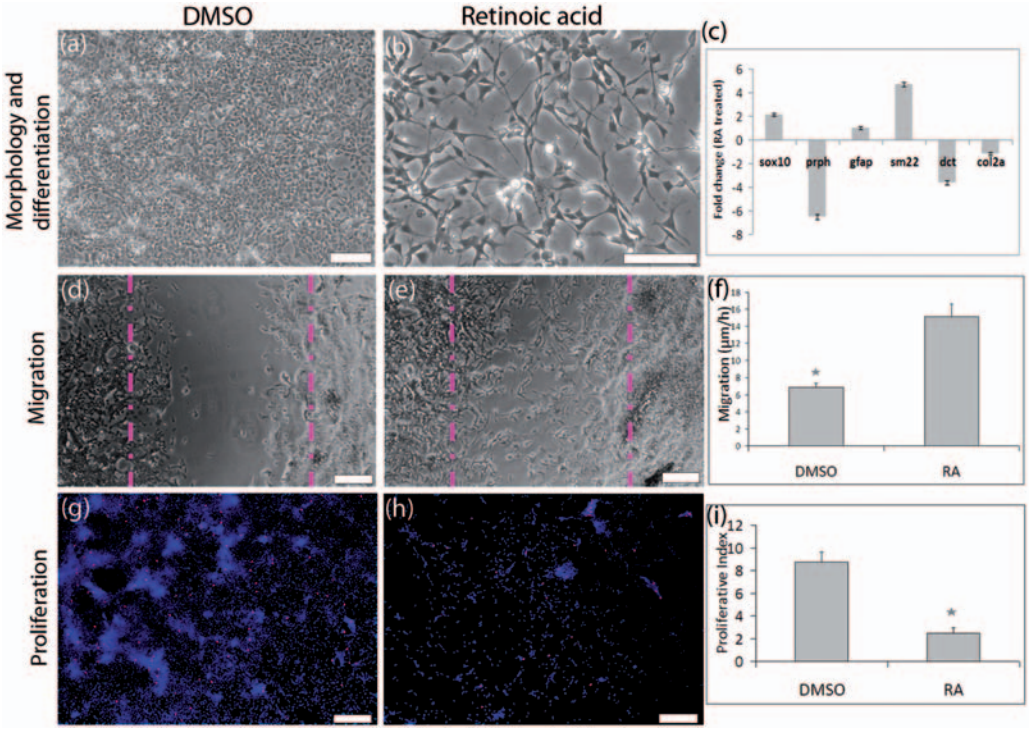

Figure 6 Effect of retinoic acid on neural crest cell behavior. Compared to DMSO-treated controls (a, d, g), the cells treated with retinoic acid had spindle-like morphology and grew projections (b). The results of the real-time quantitative PCR showed that upon retinoic acid exposure the expression of neural crest cell marker *sox10* and that of smooth muscle cell marker *sm22* were upregulated; whereas, the expression of *peripherin*, marker of neurons, and that of *dopachrome tautomerase*, marker of melanocytes, were downregulated (c). Retinoic acid significantly increased the motility of neural crest cells, as shown by the wound-healing assay (d–f), and inhibited their proliferation, as revealed by the *phospho histone3* proliferation assay (g–i). Bars = 100 μm. (A color version of this figure is available in the online journal)

Table 1 Primers used in standard and real time PCR experiments

Gene	Forward Primer	Reverse Primer
Sox10	CATTCAGAGCGCGAGCGAGGG	CGGTCCACTAGTTCTGCGGCC
C-myc	CAAGCGAAAAGACTTTCCAAG	CGCAGCCCATGTTTTTCTTAT
Klf4	CCTATTTTGGGGACAGCAGTT	CATGAAAAATACTCCCTGACTTGA
Dlx2a	CCTGCAGATGCTGAAAAGGAAGAA	TGGAGACTCACCGGAGGCCA
Sox9a	TCCACAACGCGGAGCTCAGC	CCCCTGGGACTGGCCTGAGT
Pax3	CTTTGCTATTTGGGCTCATTG	TGTCCAAAATGGAAAAAGGAA
Peripherin	CCTGGAGCTGGAGAAGAAGAT	AATTTGCCTCCTCAGGTCATT
Gfap	GCAAGTCTGGAGCTGGGTAGTTTTT	ACCTGGGTCCAAGAGATGAGTTAGC
Sm22	GCAGGTGCAGGATAAGATCG	GAGACCTGCTCCATCTGCTT
Dct	CTGCTCCTGCTGTTGATCGTGTTG	TAATGGCATATAATAATCAGTTTTATTACATTT
Col2a	GTTTCCCTCGTCCTGGTTCTG	CGTCTGGCTGTGATGCTCTTC
Actin	CATCCATCGTTCACAGGAAGTG	TGGTCGTTCGTTTGAATCTCAT

not adhere to bare tissue culture plastic or to dishes coated with laminin alone.

To apply the NCC functional assays we developed to examine the effect of exogenous signals, we investigated the effect of all trans-RA. RA is a vitamin A derivative, is endogenously synthesized in vertebrates, and is essential for normal embryonic development and the maintenance of differentiation in some adult tissues.[34] It is well-known that the excess or deficiency of retinoids induces abnormalities in mammalian embryos.[35] RA signals are involved in the induction of the neural crest and more specifically in the correct anteroposterior specification of the NCCs.[36] Treatment of zebrafish embryos with RA has been reported to result in abnormal cartilage, caudal midbrain, rostral hindbrain, retina, and fin morphogenesis.[37–40]

In our system RA caused significant changes in NCC morphology, differentiation, proliferation, and migration. The cells treated with RA were scattered, flattened, spread

with spindle-shaped appearance, and grew projections. The real-time quantitative PCR data showed that in the group of cells treated with RA, the neuronal marker *peripherin* and melanocytic marker *dct* were downregulated, whereas the smooth muscle cell marker *sm22* was upregulated. The change in morphology might indeed be an indicator of differentiation towards smooth muscle lineage, considering that *α-actin*-positive smooth muscle cell precursors have elongated shape with filamentous projections.[41] All trans-RA was also reported to promote a more differentiated smooth muscle cell phenotype when applied to embryonic stem cells,[42] and smooth muscle cells of the vessel walls were shown to exhibit retinoid receptor activity *in vitro*.[43] Retinoids were suggested to differentially affect emerging subsets of NCCs, inhibiting differentiation of some while promoting differentiation of others.[44] Our data confirm previous work where RA was reported to specifically upregulate *sm22*[45–47] and downregulate *peripherin*[48] and *dct*[49,50] in other cell types. Previously, it was also noted that RA treatment of embryoid bodies in the presence of fetal calf serum led to rapid activation of *sox10* mRNA expression.[51] We observed a similar activation of *sox10* RNA expression in our RA-treated neural crest system by 2.1 fold. Our results support the hypothesis that the teratogenic effects of RA might at least in part derive from reprogramming expression of genes which play critical roles during embryonic development regulating pathways that determine subsequent differentiation of NCCs.[44] Using a wound-healing (''scratch'') assay, we observed a significant increase in the migration of RA-treated versus control NCCs. *In vitro*, NCCs were able to migrate even better than the controls, but *in vivo* it is possible that they might have altered migration pathways due to RA exposure. Indeed, it was hypothesized that RA treatment does not inhibit migration but instead induces ectopic pathways in embryos.[34] On the other hand, proliferation of the NCCs was severely inhibited in our system upon RA exposure. RA was reported to induce growth arrest specific (Gas) genes in NCCs[52] and in chick embryos it was shown to block DNA synthesis,[53] which might explain the decreased proliferative activity observed in our system. Our data implicate NCCs as a target cell population for RA and suggest that it plays multiple critical roles in zebrafish NCC development.

Despite advances in understanding these unique cells, NCCs are not fully characterized and many questions regarding their properties are still debated.[54] Here, we present the first *in vitro* model of early neural crest development in zebrafish. We recapitulated the unique plasticity of the neural crest lineage *in vitro* by demonstrating multilineage differentiation into neuronal, glial, smooth muscle, melanocytic, and chondrogenic lineages and established a novel *in vitro* neural crest system with functional assays of cell behavior, which will be useful to gain mechanistic understanding of NCC development, and for cell-based high-throughput drug screening applications.

Author contributions: The project was conceived by ECL. BK developed and optimized the culture conditions. All authors participated in the design, interpretation of the studies and analysis of the data. BK and YK conducted the experiments, BK and ECL wrote the manuscript.

ACKNOWLEDGEMENTS

The authors would like to thank Christie Ciarlo, Charles Kaufman, and Leonard Zon for valuable discussions and the Center for Regenerative Medicine FACS Facility for excellent technical assistance. This work was supported by the Harvard Stem Cell Institute Seed Grant, Shriners Hospital for Children, and the March of Dimes Basil O'Connor Fellowship to ECL.

REFERENCES

1. Cordero DR, Brugmann S, Chu Y, Bajpai R, Jame M, Helms JA. Cranial neural crest cells on the move: their roles in craniofacial development. *Am J Med Genet A* 2011;**155A**:270–9
2. Hall BK. The neural crest as a fourth germ layer and vertebrates as quadroblastic not triploblastic. *Evol Dev* 2000;**2**:3–5
3. Etchevers H. Primary culture of chick, mouse or human neural crest cells. *Nat Protoc* 2011;**6**:1568–77
4. 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**:1468–75
5. Lee G, Papapetrou EP, Kim H, Chambers SM, Tomishima MJ, Fasano CA, Ganat YM, Menon J, Shimizu F, Viale A, Tabar V, Sadelain M, Studer L. Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 2009;**461**:402–6
6. Zhu H, Zon LI. Use of zebrafish models for the analysis of human disease. *Curr Protoc Hum Genet* 2002;**Chapter 15**: Unit 15 3
7. Ju C, Zhang K, Wu X. Derivation of corneal endothelial cell-like cells from rat neural crest cells in vitro. *PLoS One* 2012;**7**:e42378
8. Rovasio RA, Faas L, Battiato NL. Insights into stem cell factor chemotactic guidance of neural crest cells revealed by a real-time directionality-based assay. *Eur J Cell Biol* 2012;**91**:375–90
9. Jaurena MB, Carri NG, Battiato NL, Rovasio RA. Trophic and proliferative perturbations of in vivo/in vitro cephalic neural crest cells after ethanol exposure are prevented by neurotrophin 3. *Neurotoxicol Teratol* 2011;**33**:422–30
10. Garcez RC, Teixeira BL, Schmitt Sdos S, Alvarez-Silva M, Trentin AG. Epidermal growth factor (EGF) promotes the in vitro differentiation of neural crest cells to neurons and melanocytes. *Cell Mol Neurobiol* 2009;**29**:1087–91
11. Jiang HB, Tian WD, Liu LK, Xu Y. In vitro odontoblast-like cell differentiation of cranial neural crest cells induced by fibroblast growth factor 8 and dentin non-collagen proteins. *Cell Biol Int* 2008;**32**:671–8
12. Li F, Luo Z, Huang W, Lu Q, Wilcox CS, Jose PA, Chen S. Response gene to complement 32, a novel regulator for transforming growth factor-beta-induced smooth muscle differentiation of neural crest cells. *J Biol Chem* 2007;**282**:10133–7
13. Evrard YA, Mohammad-Zadeh L, Holton B. Alterations in Ca2+-dependent and cAMP-dependent signaling pathways affect neurogenesis and melanogenesis of quail neural crest cells in vitro. *Dev Genes Evol* 2004;**214**:193–9
14. Prince S, Wiggins T, Hulley PA, Kidson SH. Stimulation of melanogenesis by tetradecanoylphorbol 13-acetate (TPA) in mouse melanocytes and neural crest cells. *Pigment Cell Res* 2003;**16**:26–34
15. Kawakami T, Soma Y, Kawa Y, Ito M, Yamasaki E, Watabe H, Hosaka E, Yajima K, Ohsumi K, Mizoguchi M. Transforming growth factor beta1 regulates melanocyte proliferation and differentiation in mouse neural crest cells via stem cell factor/KIT signaling. *J Invest Dermatol* 2002;**118**:471–8
16. Sarkar S, Petiot A, Copp A, Ferretti P, Thorogood P. FGF2 promotes skeletogenic differentiation of cranial neural crest cells. *Development* 2001;**128**:2143–52

17. Wentzel P, Eriksson UJ. Altered gene expression in rat cranial neural crest cells exposed to a teratogenic glucose concentration in vitro: paradoxical downregulation of antioxidative defense genes. *Birth Defects Res B Dev Reprod Toxicol* 2011;**92**:487–97
18. Li J, Huang X, Xu X, Mayo J, Bringas P Jr, Jiang R, Wang S, Chai Y. SMAD4-mediated WNT signaling controls the fate of cranial neural crest cells during tooth morphogenesis. *Development* 2011;**138**:1977–89
19. Nakanishi K, Chan YS, Ito K. Notch signaling is required for the chondrogenic specification of mouse mesencephalic neural crest cells. *Mech Dev* 2007;**124**:190–203
20. Jia L, Cheng L, Raper J. Slit/Robo signaling is necessary to confine early neural crest cells to the ventral migratory pathway in the trunk. *Dev Biol* 2005;**282**:411–21
21. Testaz S, Jarov A, Williams KP, Ling LE, Koteliansky VE, Fournier-Thibault C, Duband JL. Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched- Smoothened-Gli signaling pathway. *Proc Natl Acad Sci U S A* 2001;**98**:12521–6
22. Curtin E, Hickey G, Kamel G, Davidson AJ, Liao EC. Zebrafish wnt9a is expressed in pharyngeal ectoderm and is required for palate and lower jaw development. *Mech Dev* 2011;**128**:104–15
23. Dougherty M, Kamel G, Shubinets V, Hickey G, Grimaldi M, Liao EC. Embryonic fate map of first pharyngeal arch structures in the sox10: kaede zebrafish transgenic model. *J Craniofac Surg* 2012;**23**:1333–7
24. Schlegelmilch K, Mohseni M, Kirak O, Pruszak J, Rodriguez JR, Zhou D, Kreger BT, Vasioukhin V, Avruch J, Brummelkamp TR, Camargo FD. Yap1 acts downstream of alpha-catenin to control epidermal proliferation. *Cell* 2011;**144**:782–95
25. Rodriguez LG, Wu X, Guan JL. Wound-healing assay. *Methods Mol Biol* 2005;**294**:23–9
26. Zhao H, Bringas P Jr, Chai Y. An in vitro model for characterizing the post-migratory cranial neural crest cells of the first branchial arch. *Dev Dyn* 2006;**235**:1433–40
27. 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
28. Zhang P, Andrianakos R, Yang Y, Liu C, Lu W. Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. *J Biol Chem* 2010;**285**:9180–9
29. Sato T, Okumura F, Ariga T, Hatakeyama S. TRIM6 interacts with Myc and maintains the pluripotency of mouse embryonic stem cells. *J Cell Sci* 2012;**125**:1544–55
30. Lee G, Chambers SM, Tomishima MJ, Studer L. Derivation of neural crest cells from human pluripotent stem cells. *Nat Protoc* 2010;**5**:688–701
31. Herbarth B, Pingault V, Bondurand N, Kuhlbrodt K, Hermans-Borgmeyer I, Puliti A, Lemort N, Goossens M, Wegner M. Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. *Proc Natl Acad Sci U S A* 1998;**95**:5161–5
32. Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M. Sox10, a novel transcriptional modulator in glial cells. *J Neurosci* 1998;**18**:237–50
33. Pusch C, Hustert E, Pfeifer D, Sudbeck P, Kist R, Roe B, Wang Z, Balling R, Blin N, Scherer G. The SOX10/Sox10 gene from human and mouse: sequence, expression, and transactivation by the encoded HMG domain transcription factor. *Hum Genet* 1998;**103**:115–23
34. Menegola E, Broccia ML, Di Renzo F, Massa V, Giavini E. Relationship between hindbrain segmentation, neural crest cell migration and branchial arch abnormalities in rat embryos exposed to fluconazole and retinoic acid in vitro. *Reprod Toxicol* 2004;**18**:121–30
35. Morriss-Kay G. Retinoic acid and development. *Pathobiology* 1992;**60**:264–70
36. Villanueva S, Glavic A, Ruiz P, Mayor R. Posteriorization by FGF, Wnt, and retinoic acid is required for neural crest induction. *Dev Biol* 2002;**241**:289–301
37. Ellies DL, Langille RM, Martin CC, Akimenko MA, Ekker M. Specific craniofacial cartilage dysmorphogenesis coincides with a loss of dlx gene expression in retinoic acid-treated zebrafish embryos. *Mech Dev* 1997;**61**:23–36
38. Zhang Z, Balmer JE, Lovlie A, Fromm SH, Blomhoff R. Specific teratogenic effects of different retinoic acid isomers and analogs in the developing anterior central nervous system of zebrafish. *Dev Dyn* 1996;**206**:73–86
39. Hyatt GA, Schmitt EA, Marsh-Armstrong N, McCaffery P, Drager UC, Dowling JE. Retinoic acid establishes ventral retinal characteristics. *Development* 1996;**122**:195–204
40. Ferretti P, Geraudie J. Retinoic acid-induced cell death in the wound epidermis of regenerating zebrafish fins. *Dev Dyn* 1995;**202**:271–83
41. Tollet J, Everett AW, Sparrow MP. Development of neural tissue and airway smooth muscle in fetal mouse lung explants: a role for glial-derived neurotrophic factor in lung innervation. *Am J Respir Cell Mol Biol* 2002;**26**:420–9
42. Huang H, Zhao X, Chen L, Xu C, Yao X, Lu Y, Dai L, Zhang M. Differentiation of human embryonic stem cells into smooth muscle cells in adherent monolayer culture. *Biochem Biophys Res Commun* 2006;**351**:321–7
43. Miano JM, Topouzis S, Majesky MW, Olson EN. Retinoid receptor expression and all-trans retinoic acid-mediated growth inhibition in vascular smooth muscle cells. *Circulation* 1996;**93**:1886–95
44. Williams SS, Mear JP, Liang HC, Potter SS, Aronow BJ, Colbert MC. Large-scale reprogramming of cranial neural crest gene expression by retinoic acid exposure. *Physiol Genomics* 2004;**19**:184–97
45. Kawai-Kowase K, Kumar MS, Hoofnagle MH, Yoshida T, Owens GK. PIAS1 activates the expression of smooth muscle cell differentiation marker genes by interacting with serum response factor and class I basic helix-loop-helix proteins. *Mol Cell Biol* 2005;**25**:8009–23
46. Jin L, Gan Q, Zieba BJ, Goicoechea SM, Owens GK, Otey CA, Somlyo AV. The actin associated protein palladin is important for the early smooth muscle cell differentiation. *PLoS One* 2010;**5**:e12823
47. Gollasch M, Haase H, Ried C, Lindschau C, Morano I, Luft FC, Haller H. L-type calcium channel expression depends on the differentiated state of vascular smooth muscle cells. *FASEB J* 1998;**12**:593–601
48. Kim KH. A role of retinoic acid in the regulation of the morphology and the levels of intermediate filament proteins and mRNAs in PC12 cells. *Exp Cell Res* 1992;**203**:374–82
49. Martinez-Liarte JH, Solano F, Garcia-Borron JC, Jara JR, Lozano JA. Alpha-MSH and other melanogenic activators mediate opposite effects on tyrosinase and dopachrome tautomerase in B16/F10 mouse melanoma cells. *J Invest Dermatol* 1992;**99**:435–9
50. Romero C, Aberdam E, Larnier C, Ortonne JP. Retinoic acid as modulator of UVB-induced melanocyte differentiation. Involvement of the melanogenic enzymes expression. *J Cell Sci* 1994;**107**:1095–103
51. Kawaguchi J, Mee PJ, Smith AG. Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. *Bone* 2005;**36**:758–69
52. Wang L, Mear JP, Kuan CY, Colbert MC. Retinoic acid induces CDK inhibitors and growth arrest specific (Gas) genes in neural crest cells. *Dev Growth Differ* 2005;**47**:119–30
53. Salvarezza SB, Rovasio RA. Exogenous retinoic acid decreases in vivo and in vitro proliferative activity during the early migratory stage of neural crest cells. *Cell Prolif* 1997;**30**:71–80
54. Crane JF, Trainor PA. Neural crest stem and progenitor cells. *Annu Rev Cell Dev Biol* 2006;**22**:267–86

(Received July 10, 2013, Accepted September 30, 2013)