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Crestospheres: Long-Term Maintenance of Multipotent, Premigratory Neural Crest Stem Cells

Laura Kerosuo,[1] Shuyi Nie,[1] Ruchi Bajpai,[2] and Marianne E. Bronner[1,*]
[1]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125, USA
[2]Center for Craniofacial Molecular Biology and Department of Biochemistry, University of Southern California, Los Angeles, CA 90089, USA
*Correspondence: mbronner@caltech.edu
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SUMMARY

Premigratory neural crest cells comprise a transient, embryonic population that arises within the CNS, but subsequently migrates away and differentiates into many derivatives. Previously, premigratory neural crest could not be maintained in a multipotent, adhesive state without spontaneous differentiation. Here, we report conditions that enable maintenance of neuroepithelial "crestospheres" that self-renew and retain multipotency for weeks. Moreover, under differentiation conditions, these cells can form multiple derivatives in vitro and in vivo after transplantation into chick embryos. Similarly, human embryonic stem cells directed to a neural crest fate can be maintained as crestospheres and subsequently differentiated into several derivatives. By devising conditions that maintain the premigratory state in vitro, these results demonstrate that neuroepithelial neural crest precursors are capable of long-term self-renewal. This approach will help uncover mechanisms underlying their developmental potential, differentiation and, together with the induced pluripotent stem cell techniques, the pathology of human neurocristopathies.

INTRODUCTION

The neural crest is a uniquely vertebrate cell population characterized by its remarkable ability to form numerous differentiated derivatives, as diverse as facial skeleton and peripheral nervous system. From a stem cell biology perspective, the neural crest is an ideal embryonic source of multipotent stem cells for the purposes of regenerative medicine. Numerous studies have successfully isolated cell lines that differentiate into neural crest derivatives either in vitro or after transplantation into the embryo (Curchoe et al., 2010; Lee et al., 2007; Trentin et al., 2004). However, it remains controversial whether neural crest cells are indeed true stem cells or simply a transient multipotent progenitor population.

A cardinal feature of stem cells is their ability to give rise to multiple lineages and to self-renew. In contrast to true stem cells, primary cultures of embryonic neural crest cells examined to date have only limited capacity to self-renew in vitro (Stemple and Anderson, 1992; Trentin et al., 2004). Likewise, neural crest cells derived from peripheral nerve or facial skin have self-renewal capacity but tend to form a limited set of cell types reflecting their tissue of origin (Adameyko et al., 2009; Fernandes et al., 2004; Johnston et al., 2013; Li et al., 2007; Morrison et al., 1999). In general, adult neural-crest-derived "stem cells" have limited ability for long-term self-renewal or differentiation into a large repertoire of derivatives (Dupin and Coelho-Aguiar, 2013; Kruger et al., 2002). This raises the important question of whether a true neural crest stem cell that is multipotent and capable of self-renewal can be identified in either the embryo or adult.

Embryonic neural crest cells arise from the dorsal portion of the developing CNS around the time of neural tube closure. At this premigratory phase, neural crest precursors are characterized by combined expression of transcription factors such as *FOXD3*, *SOXE*, *SNAIL*, and *AP2α* (Khudyakov and Bronner-Fraser, 2009). They subsequently undergo an epithelial to mesenchymal transition (EMT), enabling them to leave the neural tube, and migrate extensively throughout the embryo. Single cell lineage analyses in vivo (Baggiolini et al., 2015; Bronner-Fraser and Fraser, 1988) and clonal analyses in vitro (Calloni et al., 2009) have shown that the majority of embryonic neural crest cells are multipotent. Indeed, premigratory precursors contribute not only to neural crest, but also to dorsal neural tube lineages (Baggiolini et al., 2015; Bronner-Fraser and Fraser, 1989). After emigration, however, their developmental potential is thought to become restricted by cues present in the environment and final site of localization. Finally, neural crest cells differentiate into many cell types that far exceed the repertoire traditionally considered as "ectodermal," including sensory and autonomic neurons and glia, bone and cartilage of the face, smooth muscle cells, adipocytes, melanocytes, and various endocrine cells (Dupin and Coelho-Aguiar, 2013).

Development of new methods for generating neural crest cells with self-renewal capacity is complicated by the transience of the embryonic premigratory crest stage. Our goal was to maintain, for extended time periods in vitro, primary neural crest stem cells derived from the embryo or human embryonic stem cells (hESCs) in a self-renewing state that reflects their premigratory character; under appropriate

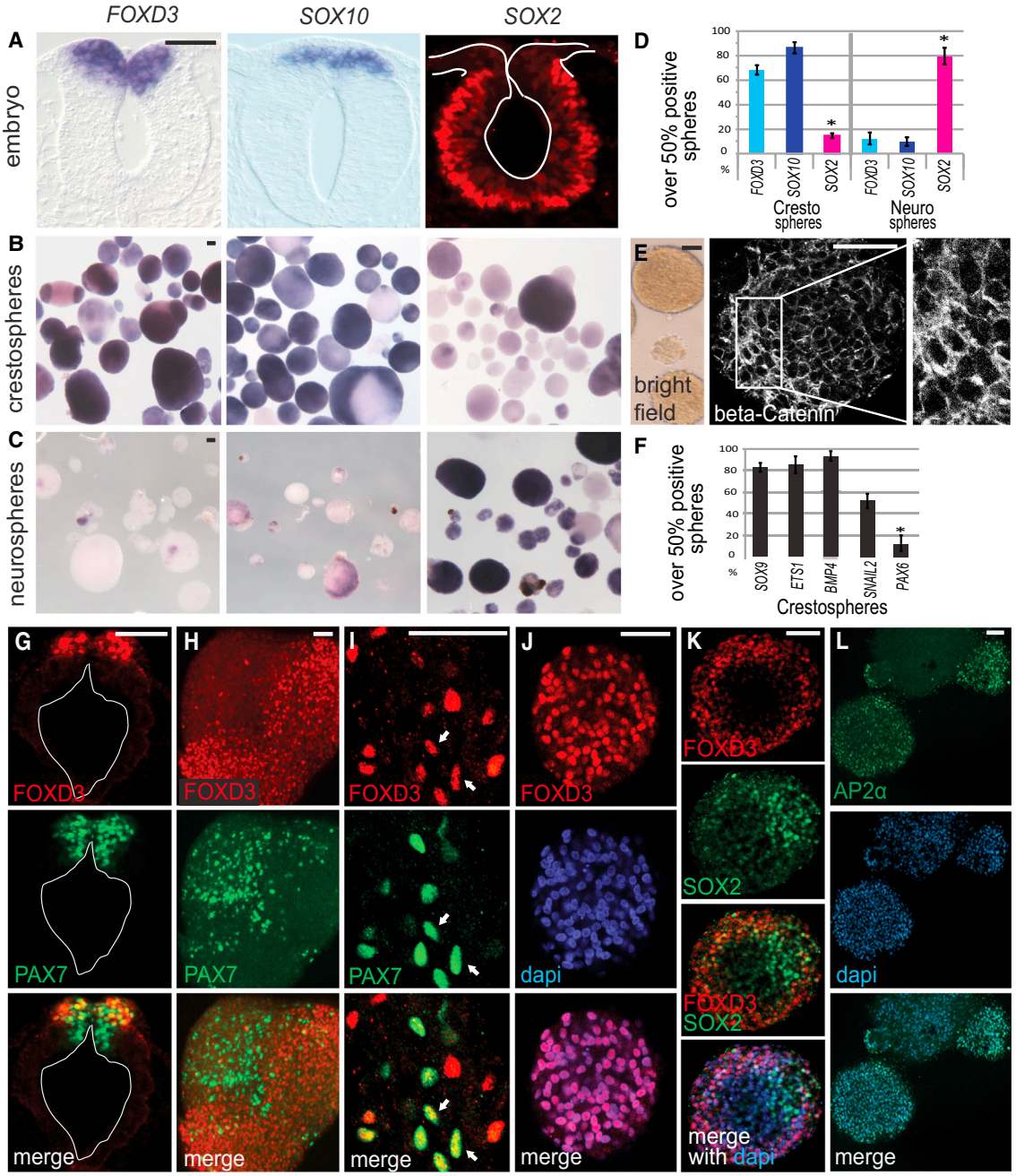

Figure 1. Chick Crestospheres Mimic Premigratory Neural Crest Cells

(A) Expression of the neural crest markers *FOXD3* and *SOX10* mRNA and SOX2 protein, highest in the ventral neural tube, in HH9 chick embryos.

(B and C) Highly enriched RNA expression of premigratory neural crest markers in 2-week-old crestospheres (B) compared with neurospheres (C) derived from equivalently staged neural tubes that express high mRNA levels of the neural marker *SOX2*.

(D) Quantification of spheres that consist of over 50% positive cells for each marker shows a significant difference between crestospheres (n = 5) and neurospheres (n = 6).

(E) The epithelial characteristics of floating crestospheres revealed by a brightfield image and β-Catenin staining showing adherens junctions typical of epithelial cells (magnified in inset).

(F) High expression of additional premigratory neural crest markers quantified from in situ hybridization (n = 3). Low *PAX6* levels reflect neural cells.

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conditions, these can then be differentiated into multiple derivatives. Such long-term maintenance of neural crest stem cells is useful not only for regenerative medicine, but also for understanding neural-crest-related birth defects.

RESULTS

Crestosphere Culture Conditions

Reasoning that neural crest precursors residing within the dorsal neural tube are most likely to have stem cell properties and given elegant classical studies on avian neural crest development, we used cranial to vagal chick embryonic neural tubes from five- to eight-somite stage embryos as starting material. We sought to find appropriate culture conditions that would maintain a molecular profile similar to premigratory crest cells and support maintenance of the self-renewing and multipotent neural crest state, thus mimicking the time point when they are premigratory and reside within an adhesive neuroepithelium.

To determine optimal conditions for enabling culture of these embryonic cells as epithelial "crestospheres," we tested the effects of different concentrations of growth factors and signaling molecules on expression of *SOX10*, *FOXD3*, and *SOX2*. *SOX10* and *FOXD3* are strongly expressed by premigratory neural crest (Figure 1A) and associated with maintenance of multipotency (Kim et al., 2003, 2014; Nitzan et al., 2013; Teng et al., 2008). In contrast, neural stem cell marker SOX2 is downregulated in dorsal relative to ventral neural tube regions (Figure 1A), albeit required at low levels for neural crest EMT (Cimadamore et al., 2011). Transcript levels were compared with those in whole embryo lysates using qPCR (see the Supplemental Results; Figure S1A). Because traditional neural stem cell medium, containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Molofsky et al., 2003) failed to sustain expression of *FOXD3* and *SOX10* (Figures 1C, 1D, and S1A–S1C), we turned to modified growth medium containing bFGF, retinoic acid (RA), and insulin-like growth factor1 (IGF1) previously used to support self-renewal of stem cells derived from embryonic sciatic nerve (Morrison et al., 1999), gut (Molofsky et al., 2003), or migratory neural crest (Mundell and Labosky, 2011) and implicated in aspects of neural crest development (Kerosuo and Bronner-Fraser, 2012; Martínez-Morales et al., 2011).

Neural crest marker expression was significantly increased when cells were grown in the presence of bFGF, IGF1, and RA. Of the concentrations tested, 20 ng/ml of bFGF and IGF1 plus 60 nM (19 mg/ml) RA were optimal to maintain high levels of *FOXD3* and *SOX10* as assessed by qPCR and in situ hybridization. Concomitantly, these conditions yielded low *SOX2* levels (Figures 1B, 1D, S1A–S1C, and S1F; Supplemental Results). In contrast, similar cells grown under neurosphere-promoting conditions contained high *SOX2* but low neural crest marker expression (Figures 1C and 1D). Crestospheres are epithelial in character, as evidenced by their morphology and β-Catenin expression, which outlines adherens junctions on their membranes (Figure 1E) similar to that seen in neuroepithelial cells prior to neural crest emigration (Figure S1E). Cultures prepared from either dorsal or entire neural tubes had equivalent ability to form crestospheres, suggesting that the culture conditions support neural crest at the expense of neural fate (Figure S1D).

Neural Crest Marker Expression in Crestospheres Reflects Endogenous Expression In Vivo

We next examined expression of additional premigratory neural crest (*SOX9*, *BMP4*, *ETS-1*, *SNAIL2*) and neural (*PAX6*) markers in crestopheres by in situ hybridization. The results reveal heterogeneous but high transcript levels for neural crest markers but only low levels of neural markers (Figures 1F and S1G). For cellular resolution, we performed immunostaining followed by confocal microscopy of crestospheres and compared expression patterns to those in the embryo. Neural crest marker expression in vivo initiates in the dorsal neural tube in a characteristic sequence. Expression of neural plate border gene PAX7 initiates at gastrula stages and is maintained in premigratory (Figure 1G) and early migrating neural crest cells (Basch et al., 2006). FOXD3 is expressed by the dorsal-most subset of PAX7$^+$ cells (Figure 1G). Similarly within crestospheres, PAX7$^+$ and FOXD3$^+$ domains are intermixed, with some double-labeled but other single-labeled cells (Figures 1H and 1I). Some crestospheres consist entirely of FOXD3-expressing cells (Figure 1J). SOX2 expression is very low in dorsal compared with more ventral regions of the neural tube (Figure 1A) similar to crestospheres where FOXD3$^+$ cells clearly segregate from high SOX2-expressing regions (Figure 1K), similar to segregation of PAX7$^+$ and PAX6$^+$ cells

(G) FOXD3 and PAX7 show only partially overlapping protein expression in the chick cranial neural tube.
(H) A large crestosphere with both FOXD3 and PAX7 protein-expressing domains.
(I) High-magnification image showing partially overlapping expression of FOXD3 and PAX7 protein in a sphere (white arrows mark characteristic examples).
(J) Confocal image of a small crestosphere exclusively expressing FOXD3.
(K) Confocal image showing largely nonoverlapping protein expression of high SOX2 and FOXD3 in a sphere.
(L) Heterogenous protein expression of AP2α in crestospheres. Scale bar represents 50 μm.

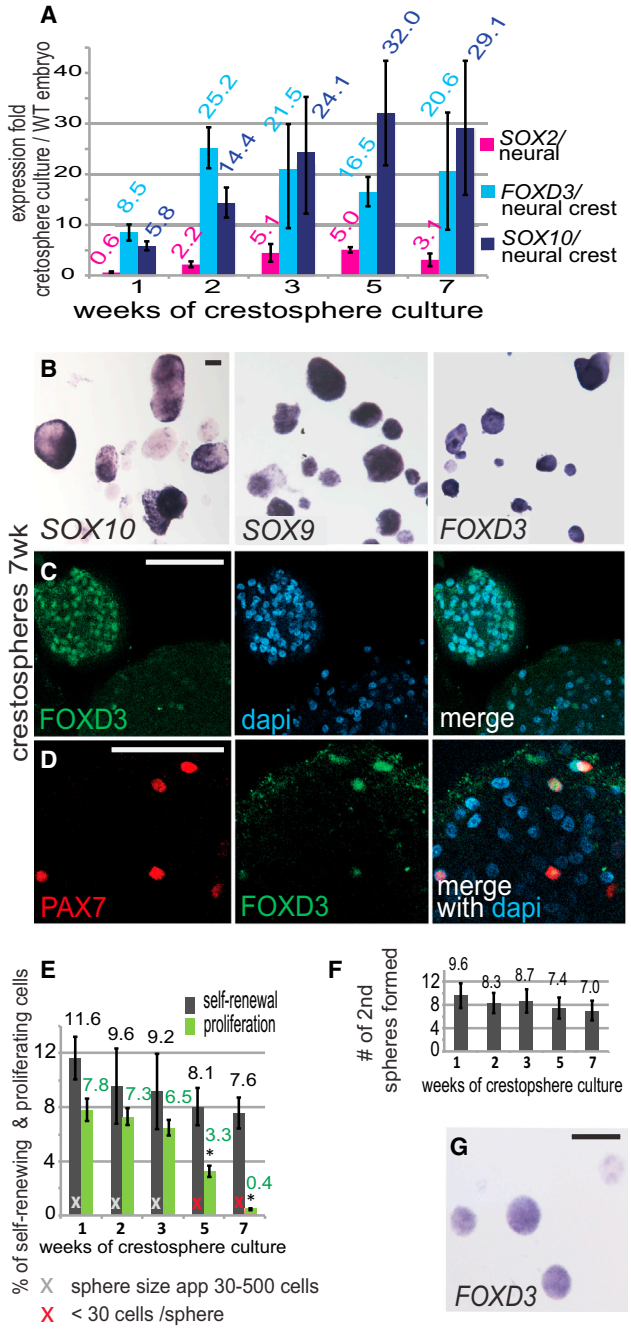

Figure 2. Long-Term Maintenance of Crestospheres

(A) *FOXD3* and *SOX10* mRNA expression was maintained throughout the 7-week crestosphere culture, initiated from pooled neural folds (n = 6; shown as average values).

(B–D) In situ hybridization (B) and immunostaining (C, D) reveal high and heterogeneous expression of premigratory neural crest markers in 7-week-old crestospheres.

(E) Sphere forming assay from single cells shows maintenance of self-renewal capacity through 7 weeks of crestosphere culture (week 1, n = 10; weeks 2, 3, and 7, n = 6; week 5, n = 12), with a drop in the proliferation rate after 5 weeks (n = 3).

(F) Secondary sphere formation is maintained throughout 7 weeks of crestosphere culture (n = 6).

(G) *FOXD3* mRNA-expressing crestospheres formed from single cells during the primary sphere-forming assay.

(Figure S1H). Other neural crest genes such as *AP2α* and *SNAIL* also exhibit high and heterogeneous expression (Figures 1L and S1I). Thus, whereas crestospheres contain a heterogeneous cell population, they are highly enriched in neural crest cells.

Cells Can Be Maintained as Self-Renewing Crestospheres for Several Weeks

In the embryo, neural crest cells are transiently premigratory for ~5 hr prior to initiating emigration. To test whether crestospheres maintained self-renewal capacity in the long term, we evaluated expression of neural crest markers and sphere-forming ability as a function of time. *FOXD3* and *SOX10* RNA expression persisted for up to 7 weeks in culture, the last time point examined (Figure 2A). Figure S2A shows examples of high, medium, and low expression by qPCR. Similarly, *FOXD3*, *SOX10*, *SOX9*, and PAX7 were expressed after 7 weeks of culture, although the frequency of high expressing spheres decreased by week 7 (Figures 2B–2D and S2B). Moreover, serial replating of pooled cultures revealed that ~10% of the cells in crestosphere cultures have the ability to self-renew and form new spheres from single cells (Figure 2E). Within 1 week, each primary sphere on average formed eight new secondary spheres (Figure 2F). Importantly, crestospheres formed from single cells retained expression of *FOXD3* (Figure 2G). Self-renewal capacity, as measured by ability of single cells to form new crestospheres, remained constant over the 7-week culture period. However, the cell proliferation rate, as assayed by phosphohistone3 staining, declined dramatically after 3 weeks, dropping from ~7% to 4% by week 5 and <1% by week 7. Consequently, crestospheres became progressively smaller during the course of the self-renewal assay (Figure 2E), possibly reflecting a slowdown of the proliferation rate in the transit amplifying cell population.

Crestospheres Form Migratory Cells and Neural Crest Derivatives In Vitro and In Vivo

Migration of crestospheres was triggered by plating onto an adhesion-promoting surface after withdrawal of stem cell medium. We compared the distances migrated by cells emanating from crestospheres with those from explanted neural tubes. The results show that both cell populations are able to undergo EMT and migrate in a similar fashion as a function of time (Figures S3A–S3C; Supplemental Results).

After transfer to differentiation conditions, expression of *FOXD3* and *SOX10* decreased in crestospheres derived

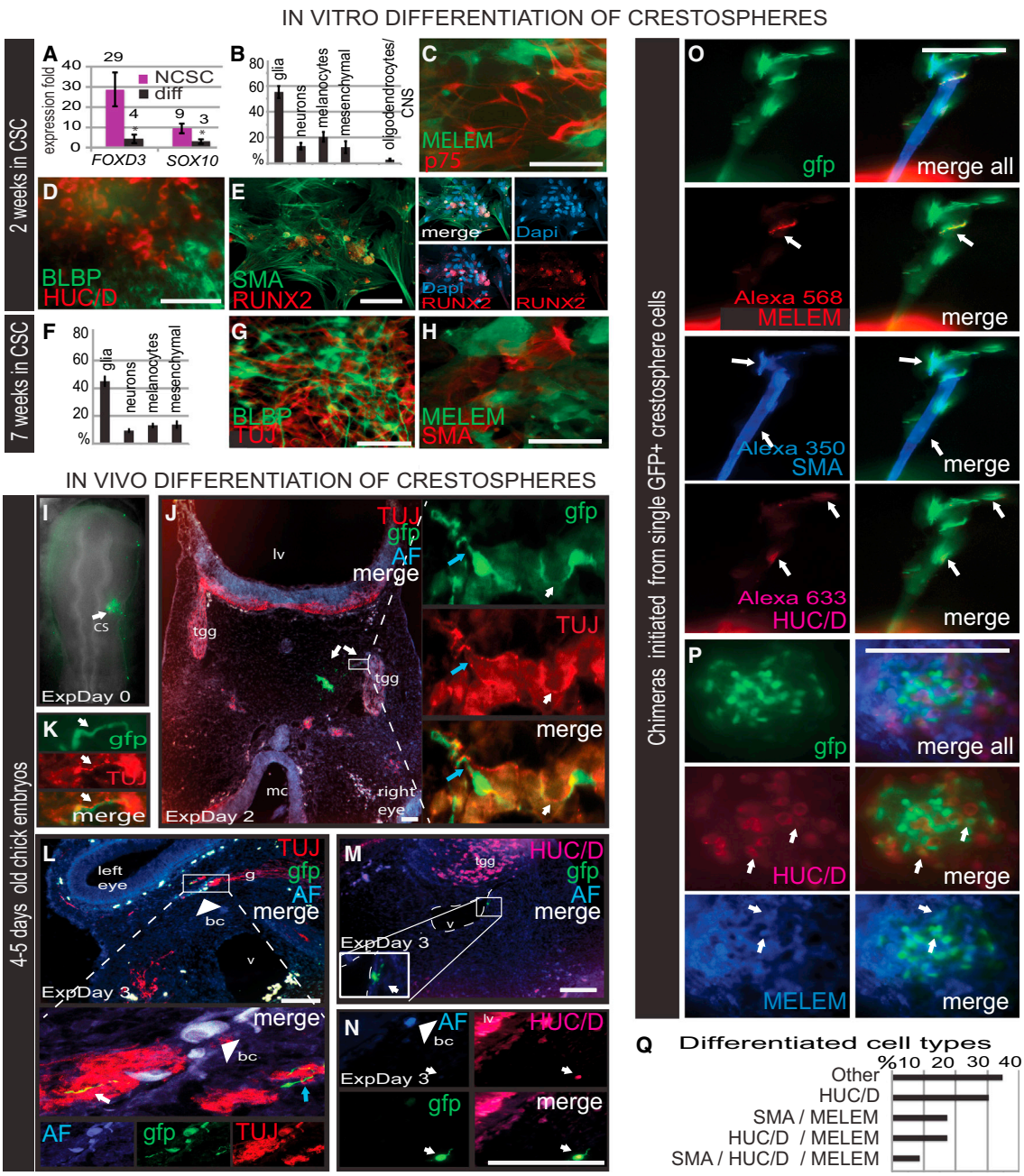

Figure 3. Multipotent Chick Crestospheres Differentiate into Multiple Neural Crest Derivatives

(A) qPCR shows a dramatic decrease in *FOXD3* and *SOX10* expression levels after 7 days of in vitro differentiation (n = 3).

(B–H) Seven day in vitro differentiation of either 2-week (B–E) or 7-week (F–H) cultured crestospheres results in production of melanoblasts (MELEM), neurons (TUJ-1, HUC/D), cells expressing P75, a common marker of many neural crest derived cells, glial cells (BCLP) as well as smooth muscle cells (SMA) and cartilage (RUNX2). Quantification of different derivatives shows similar results for 2- (B) and 7- (F) week spheres (n ranges: week 2, from 7 to 13; week 7, from 3 to 7). In contrast, few CNS-derived cells were observed as shown by oligodendrocyte marker 04.

(I) In vivo transplantation of 2-week cultured GFP crestospheres (CS) into the head mesenchyme of HH10 chick embryo (Day 0).

(J–L) GFP-positive cells were observed in the trigeminal ganglion (TGG) (J and K) and in a nerve bundle next to the left eye (L) 2 and 3 days after the transplantation, respectively. GFP^+ cells express neuronal marker TUJ-1 in cell body and overlapping axons (white arrow); TUJ-negative presumptive Schwann cells are intertwined with the neuronal axons (blue arrow).

(M) GFP^+ cells lining a blood vessel.

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from pooled cultures of 2-week-old spheres (Figure 3A); numerous neural crest derivatives were observed by immunostaining. Glial cells (BLBP) were predominant; roughly 10%–20% of cells positive for markers for neurons (HUC/D, TUJ1, ISLET), melanocytes (MELEM), mesenchymal smooth muscle (SMA), and cartilage (RUNX2), and ~30% of the cells were HNK1$^+$. By increasing the FBS from 1% to 10%, the percentage of mesenchymal cells increased significantly from 12% to 42% (SEM = 7.7, p = 0.004; data not shown). In contrast, very few oligodendrocytes, depicted with the O4 marker, were observed in crestospheres compared with neurospheres, suggesting a low presence of CNS cells in the former (see Figures 3B–3E and S3D–S3I and Supplemental Results), likely reflecting the shared lineage of premigratory neural crest and dorsal neural tube cells in vivo (Bronner-Fraser and Fraser, 1989; Baggiolini et al., 2015). The differentiation ability of crestospheres was maintained throughout the 7-week culture period (Figures 3F–3H and S3G).

To examine differentiation in vivo, we transplanted 2- to 3-week-old crestospheres derived from GFP transgenic chick embryos onto cranial crest migration pathways of HH9-10 chick embryos. After 2 or 3 days in ovo, we noted GFP-labeled cells within normal neural crest derivatives, forming trigeminal neurons, putative Schwann cells, as well as smooth muscle surrounding blood vessels (Figures 3I–3N).

Clonal Chimeric Cultures Verify Multipotency of the Cultured Crestosphere Cells

To examine crestosphere developmental potential at the individual cell level, we examined the differentiative potential of single GFP$^+$ cells, cocultured with unlabeled crestosphere cells. These clonal GFP cells contributed to multiple cell types, differentiating into neurons, melanocytes, and smooth muscle cells, demonstrating that crestosphere culture conditions are conducive to maintenance of multipotentiality of individual neural crest stem cells (Figures 3O–3Q; Supplemental Results).

Multipotency of hESC-Derived Neural Crest Cells

hESCs can be directed toward a neural crest cell fate from neural rosettes (Bajpai et al., 2010; Lee et al., 2007). However, these cells do not remain in a stem-like state but rather spontaneously detach from the rosettes, migrate and differentiate into neural crest derivatives, similar to neural crest behavior in the embryo.

To test whether hESCs can form crestospheres, we exposed the hESC lines H7 (Figure 4) and H9, directed toward a neural crest fate, to crestosphere conditions. The results show that these spheres express premigratory human neural crest markers AP2α, FOXD3, PAX7, and SNAIL when cultured for 1–2 weeks. Whereas expression of AP2α and FOXD3 partially overlap, they segregate from SOX2 (Figures 4A–4D). Like chick cells, ~30% of human crestospheres were HNK$^+$/P75$^+$ after 1 week in differentiation conditions and formed neural, glial, melanocytic, and mesenchymal neural crest derivatives after 2 weeks (Figures 4E–4K and S3J).

DISCUSSION

Here, we present culture conditions for maintaining premigratory neural crest cells in a self-renewing, multipotent state. For decades, investigators have been culturing migratory neural crest cells derived from embryonic dorsal neural tubes or folds. However, it was not previously possible to maintain these cells in an adhesive, premigratory state without spontaneous differentiation.

Our simple culture conditions allow maintenance of neural tube or human ES-derived cells as adhesive, neuroepithelial cells, as evidenced by their morphology and presence of β-Catenin in adherens junctions. These culture conditions appear to stop time and maintain the self-renewing state of the crestospheres for several weeks, although their proliferation rate begins to exhaust by 7 weeks. Switching into differentiation-promoting conditions shows that crestospheres are multipotent, forming neurons, glia, melanocytes, cartilage, and smooth muscle. Crestosphere cells can also contribute to migratory neural crest streams in vivo and subsequently differentiate into derivatives like peripheral neurons, smooth muscle, and other cell types. Expression of early neural crest markers *FOXD3*, *SOX10*, *SOX9*, *BMP4*, *ETS-1*, PAX7, SNAIL2, and AP2α was used to assay the neural crest cell state. Multiple loss-of-function studies and lineage analyses have suggested that these transcription factors are critical in early steps of neural crest development and specification (Barembaum and Bronner, 2013; Honoré et al., 2003; Kim et al., 2003; Luo et al., 2003; Mundell and Labosky, 2011; Murdoch et al., 2012; Nitzan et al., 2013; Teng et al., 2008; Tien et al., 2015).

There has been controversy regarding whether subsets of premigratory neural crest cells are predetermined to form

(N) Perinuclear HUC/D staining shows a GFP$^+$ neuron in the mesenchyme below the lateral ventricle (lv). Arrowhead marks autofluorescent (AF) blood cells (bc) visible in all three channels. g, ganglia; mc, mouth cavity.
(O and P) Clonal GFP$^+$ cells derived from individual premigratory neural crest cells grown as crestopheres give rise to neurons, melanocytes, and smooth muscle.
(Q) Summary of different derivatives formed. CSC, crestosphere culture. Scale bar represents 100 μm.

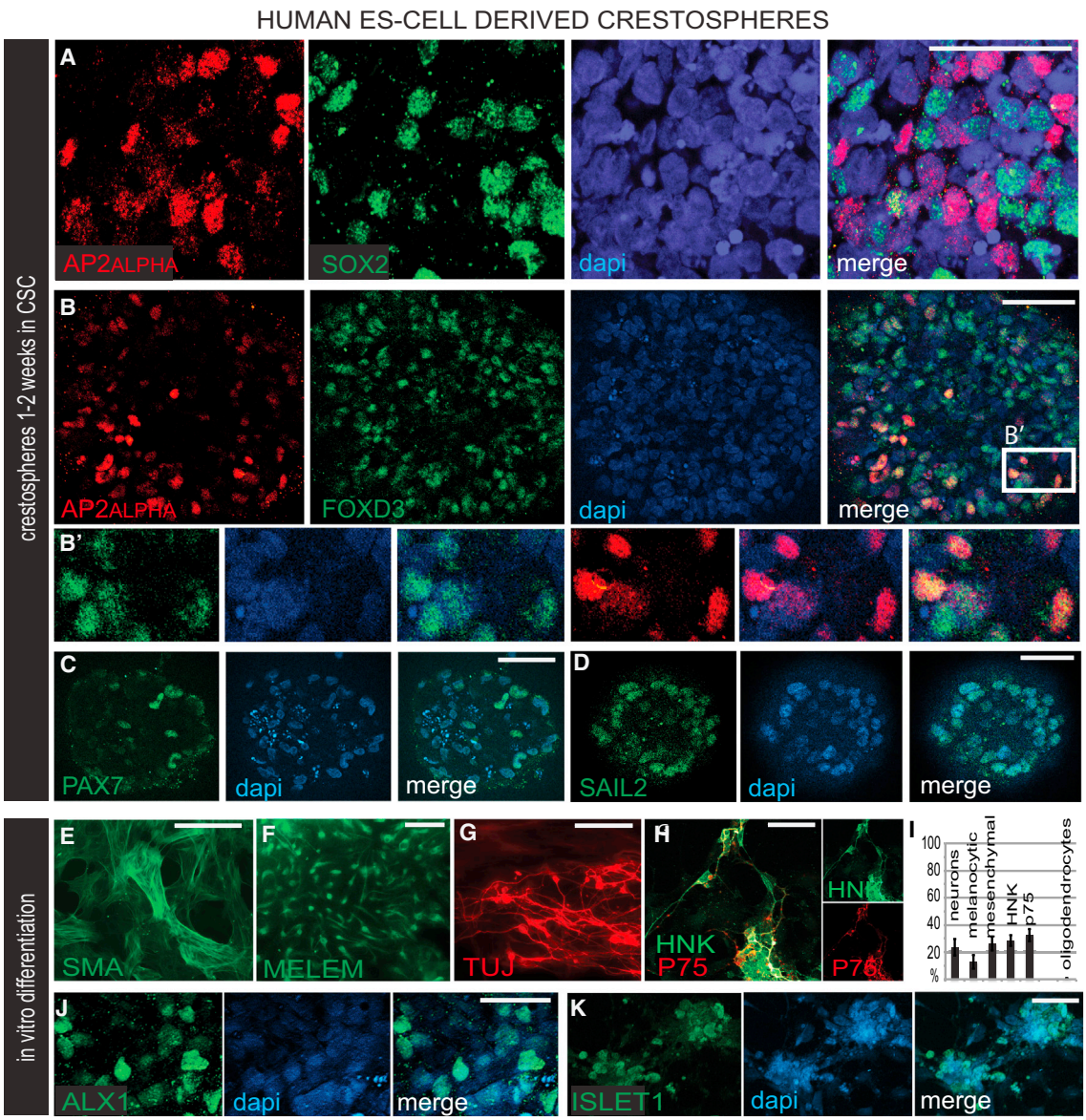

Figure 4. hESC-Derived Premigratory Neural Crest Cells Maintained in Crestosphere Cultures

(A) Confocal images showing AP2α^+ neural crest and SOX2$^+$ neural cells are largely nonoverlapping in a human crestosphere after 2 weeks of crestosphere stem cell culture (CSC).

(B) Double immunostaining with AP2α and FOXD3 reveals only partial overlap of the markers in a human crestosphere shown clearly in magnification box (B′).

(C and D) Expression of PAX7 and SNAIL in human crestospheres.

(E–H, J, and K) Human crestospheres differentiated for 1 week into mesenchymal smooth muscle cells (SMA), melanoblasts (MELEM), neurons (TUJ-1), P75$^+$ and HNK$^+$ neural crest derivatives, cartilage (ALX1), and sensory neurons (ISLET1) after 10 days of crestosphere culture.

(I) The various derivatives formed are similar to those observed in chick crestospheres (n ranges from 7 to 14). Scale bar represents 50 μm.

distinct cell types or multipotent and capable of forming numerous derivatives. Recently, this has been elegantly put to rest by single cell lineage analysis using Confetti transgenic mice. By labeling individual premigratory or migrating neural crest cells, the authors conclusively demonstrate that the majority of both are multipotent (Baggiolini et al., 2015), consistent with previous intracellular dye injections experiments in chick embryos (Bronner-Fraser and Fraser, 1988, 1989). Similarly, in vitro clonal analysis has demonstrated that many individual

clones formed from early migrating neural crest cells can form numerous derivatives (Baroffio et al., 1988; Calloni et al., 2009; Sieber-Blum et al., 1993). This suggests that at least some cell fate decisions take place during neural crest migration. Our results support this, as crestospheres initiated from single cells retained expression of *FOXD3*, reflecting their self-renewal ability as multipotent stem-like cells. Moreover, our clonal analysis shows that individual crestosphere clones can differentiate into neural, melanocytic, and mesenchymal cell types, reflecting their multipotency. Interestingly, our results also show that the expression of neural crest markers is heterogeneous and dynamic in crestospheres, suggesting that perhaps only a subpopulation of crestosphere cells (~10%) are true stem cells with the ability to self-renew.

Taken together, these results demonstrate that premigratory neuroepithelial cells maintained as crestospheres: (1) have high self-renewal capacity, (2) can be maintained for long periods in a stem-like state, (3) exhibit a profile characteristic of premigratory neural crest cells prior to EMT, and (4) can form multiple derivatives under differentiation conditions. These culture conditions promise to be useful for exploring the mechanism underlying maintenance of multipotency and the drivers of specific lineage specification. Furthermore, combined with the induced pluripotent stem cell techniques aimed at obtaining neural crest cells from individual patients, these conditions promise to provide an important tool for understanding pathology of human neurocristopathies that affect early stages of neural crest development.

EXPERIMENTAL PROCEDURES

Crestosphere Cultures

Dorsal or whole neural tubes from four- to eight-somite stage chick embryos were dissected and pooled from four to six embryos for each experiment. Human neural crest cells were obtained from neural rosettes as previously described (Bajpai et al., 2010) from two ESC lines (H7; H9) at a stage mimicking premigratory dorsal neural tube on day 8 of the neural crest induction protocol. The cells were then transferred to the crestosphere culture (CSC) medium (see the Supplemental Experimental Procedures for details). The hESC culture and differentiation experiments were done in accordance with USC-SCRO approved protocols.

qPCR

qPCR was performed by comparing relative expression of *FOXD3*, *SOX10*, and *SOX2* in the crestopsheres to whole chick embryo lysates from 5-8 somite stage (see the Supplemental Experimental Procedures for details).

In Vitro Differentiation

Pooled 2- or 7-week-old crestosphere cultures or GFP chimeric cultures were placed in differentiation-promoting medium (1% FBS) on poly-L-lysine coated glass coverslips and incubated at 37°C for 1 week and immunostained (see the Supplemental Experimental Procedures for details).

In Vivo Transplantation

Crestospheres derived from GFP chicken embryos were cultured in CSC medium for 2–4 weeks, and transplanted in ovo into the head mesenchyme of 10–13 somite stage chick embryos. Eggs were sealed and fixed 3 days later in 4% PFA on or near 4°C and cryosectioned before immunostaining (see the Supplemental Experimental Procedures for details).

Statistics

Averages were calculated from independent biological replicates (n), except for in vitro differentiation quantifications where n equals individual counted spots on the slides (see the Supplemental Results). Error bars represent SEM values and p values were calculated by using Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.08.017.

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Stem Cell Reports

Supplemental Information

Crestospheres: Long-Term Maintenance of Multipotent, Premigratory Neural Crest Stem Cells

Laura Kerosuo, Shuyi Nie, Ruchi Bajpai, and Marianne E. Bronner

Crestospheres: Long-term maintenance of multipotent, premigratory neural crest stem cells

Laura Kerosuo[1], Shuyi Nie[1], Ruchi Bajpai[2] and Marianne E. Bronner[1]
1. Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA 91125
2. Center for Craniofacial Molecular Biology, and Department of Biochemistry, University of Southern California, Los Angeles, CA 90089

SUPPLEMENTAL RESULTS

Optimization of crestosphere culture (CSC) medium and conditions

Relative RNA expression of *FOXD3*, *SOX10* and *SOX2* was monitored in culture conditions containing variable amounts of fibroblast growth factor (bFGF) and retinoic acid (RA) as well as epidermal growth factor (EGF) and insulin-like growth factor (IGF1). The expression levels were compared to those in the whole embryo of Hamburger and Hamilton (HH) stage 8-12 wild type embryos by using QPCR (Fig. S1, related to Fig 1).

Our results show that culture in the traditional neural stem cell medium with EGF and bFGF (Molofsky et al., 2003) did not promote enhanced expression of the neural crest markers *FOXD3* and *SOX10* (**medium #1**, n=3, **Day1** *SOX2* average 14.3, SEM 2.4; *FOXD3* average 6.3, SEM 1.5; *SOX10* 1.5, SEM 2.0, **Day4** *SOX2* average 1.3, SEM 0.4; *FOXD3* average 1.3, SEM 0.3; *SOX10* 0.7, SEM 0.2, **1 week** *SOX2* average 0.9, SEM 0.2; *FOXD3* average 1.4, SEM 0.6; *SOX10* 1.0, SEM 0.5, **2 weeks** *SOX2* average 1.0, SEM 0.4; *FOXD3* average 1.7, SEM1.3; *SOX10* 1.8, SEM 0.8, Fig S1A).

Neural crest marker expression was significantly increased when cultured in conditions #2-5 (listed in fig S1B) containing signaling molecules relevant for premigratory neural crest cells (bFGF, IGF1 and RA) in conditions modified and simplified from previously published neural crest cell studies (Molofsky et al., 2003; Morrison et al., 1999; Mundell and Labosky, 2011; McCabe et al., 2007). Among other signaling events, a rostrocaudal gradient of FGF and RA secreted by the paraxial mesoderm is formed in the dorsal neural tube: first high FGF promotes neural crest induction followed by increasing amounts of RA, which eventually leads to EMT (Kerosuo and Bronner-Fraser, 2012; Martinez-Morales et al., 2011). As a consequence, we tried different combinations of FGF and RA. Our results reveal similar neural crest marker expression in conditions #2 and 3, containing either a higher range of bFGF (40ng/ml) combined with medium level (120 nM) RA (#2) or a lower range of bFGF (20 ng/ml) combined with lower level (60 nM) of RA (#3). However, by 2 weeks of culture, conditions #3 with lower bFGF and lower RA had significantly more *FOXD3* expression than conditions #2 (p= 0.020640335, **medium #2**: n=3, **Day1** *SOX2* average 8.9, SEM 2.1; *FOXD3* average 5.1, SEM 3.2; *SOX10* average 5.1, SEM 2.8; **Day4** *SOX2* average 1.0, SEM 0.2; *FOXD3* average 2.2, SEM 0.7;

SOX10 average 2.2, SEM 0.7; **1 week** *SOX2* average 0.7, SEM 0.1; *FOXD3* average 13.8, SEM 3.9; *SOX10* average10.7, SEM 0.7;**2 weeks** *SOX2* average 2.0, SEM 0.1; *FOXD3* average 9.3, SEM 2.0; *SOX10* average 10.1, SEM 3.2; **medium #3:** n=6, **Day1** *SOX2* average 3.7, SEM 2.1; *FOXD3* average 11.3, SEM 6.5; *SOX10* average 6.7, SEM 3.9; **Day4** *SOX2* average 2.3, SEM 1.3; *FOXD3* average 14.6, SEM 5.0; *SOX10* average 8.9, SEM 2.7; **1 week** *SOX2* average 1.8, SEM 0.9; *FOXD3* average 10.9, SEM 2.8; *SOX10* average 10.6, SEM 2.4; **2 weeks** *SOX2* average 1.6, SEM 0.3; *FOXD3* average 25.5, SEM 4.3; *SOX10* average 10.5, SEM 1.3, Fig S1A).

Finally, we tested whether additional RA would further increase the proportional expression of neural crest markers. FGF was kept at the lower level (20ng/ml) and RA was increased to 120nM (medium #4) or 240nM (medium #5). We noticed that the adhesive integrity of the spheres was compromised with higher RA, perhaps due to an increased neuronal differentiation or onset of EMT. High RA resulted in a "looser" configuration of the spheres as evaluated by eye, an increase in single cells floating in the medium and cell death. This was particularly evident with 240nM RA, where the majority of the cells did not form spheres or had already detached from spheres and just a few spheres were visible surrounded by lots of debris. However, the few spheres left in the medium #5 expressed high levels of *FOXD3* although the presence of dying and detached cells caused overall high variation in the population (**medium #4**: n=4, **Day1** *SOX2* average 0.9, SEM 0.3; *FOXD3* average 1.0, SEM 0.2; *SOX10* average 1.3, SEM 0.2; **Day4** *SOX2* average 0.7, SEM 0.2; *FOXD3* average 10.3, SEM 1.3; *SOX10* average 5.5, SEM 0.8; **1 week** *SOX2* average 1.1, SEM 0.3; *FOXD3* average 6.8, SEM 3.0; *SOX10* average 9.3, SEM 1.9; **2 weeks** *SOX2* average 0.9, SEM 0.1; *FOXD3* average 15.8, SEM 3.8; *SOX10* average 9.3, SEM 3.5; (Figure S1A) and **medium #5**: n=3, **Day1** *SOX2* average 0.7, SEM 0.05; *FOXD3* average 2.1, SEM 0.7; *SOX10* average 1.9, SEM 0.9; **Day4** *SOX2* average 1.1, SEM 0.4; *FOXD3* average 30.6, SEM 27.1; *SOX10* average 7.3, SEM 6.2; **1 week** *SOX2* average 0.5, SEM 0.1; *FOXD3* average 41.6, SEM 37.9; *SOX10* average 16.2, SEM 13.0; **2 weeks** *SOX2* average 0.9, SEM 0.5; *FOXD3* average 16.4, SEM 5.5; *SOX10* average 9.5, SEM 4.1, Fig. S1C). Based on these results, we chose medium #3 for future experiments**, which we named "crestosphere culture medium" CSC** (Figures S1A-C).

In addition to culture medium composition, we found that different substrates influenced cell behavior, and that non-adhesive substrates were optimal for maintaining multipotency and producing crestospheres. The results showed that cultures of dissociated embryonic chick neural tube plated on regular cell culture plates resulted in attachment of the cells to the bottom of the wells (not shown) but when plated onto nonadherent plates, they instead formed floating spheres within 24h of culture (Fig 1E).

Next we optimized other variables of the culture conditions and technique. Our results combined from cultures after 4 or 7 days show that neural crest markers are similarly expressed (*FOXD3* p=0.35) with high (4.5 g/l) or low glucose (0.88 g/l) but growth rate, as estimated by the number of spheres in the culture wells, was clearly higher with the higher glucose concentration (Fig. S1D). We also tested whether

dissecting only the neural crest containing dorsal neural tubes instead of the entire neural tubes would increase neural crest marker expression but, surprisingly, we saw no significant difference (*FOXD3* p=0.56) between the two starting populations (Fig S1D). Finally, our results clearly demonstrate that the presence of chicken embryo extract (CEE) was crucial for the survival and neural crest marker expression (*FOXD3* p= 0.0063) of the crestospheres (Fig S1D, **high glucose**: n=12, *SOX2* average 2.7, SEM 1.1; *FOXD3* average 17.3, SEM 3.2; *SOX10* average 12.2, SEM 2.1; **low glucose**: n=3, *SOX2* average 5.0, SEM 1.8; *FOXD3* average 27.0, SEM 7.9; *SOX10* average 25.5, SEM 4.9; **dorsal NT**: n= 4, *SOX2* average 2.9, SEM 1.5; *FOXD3* average 20.8, SEM 6.4; *SOX10* average 26.7, SEM 8.3; **w/o CEE**: n=6, *SOX2* average 1.8, SEM 0.2; *FOXD3* average 5.6, SEM 2.5; *SOX10* average 2.5, SEM 1.1).

In situ hybridization based quantification of neural crest marker expression and PAX6 immunostain

We used *in situ* hybridization to quantify the intensity of the RNA expression of various neural crest markers as well as the neural stem cell markers *SOX2* and *PAX6.* After 1-2 weeks of crestosphere culture in the CSC medium, we counted the percentage of spheres that expressed the gene of interest by more than 50% of the crestospheres or neurospheres, respectively. Both sphere types were derived from equally staged neural tubes and prepared in the same way except for the difference in the crestosphere CSC versus traditional neurosphere medium. On average 68% (n=4, SEM= 4.0) of crestospheres expressed *FOXD3* and 86% (n=5, SEM=4.6) expressed *SOX10* in an over 50% positive manner, whereas the percentage for the neural stem cell marker *SOX2* was only 15% (n=5, SEM=2.2) and significantly lower than for the crest markers (sttest $p<0.01$, Fig 1B,D). Neurospheres, on the contrary, contained much more high *SOX2* expressing and less of neural crest marker expressing spheres (*FOXD3* average 12%, n=6, SEM=5.0; *SOX10* average 10%, n=6, SEM=3.5; *SOX2* average 79%, n=6, SEM=6.9, Fig 1C-D). A more detailed analysis of the same results with spheres divided into multiple subcategories (negative, positive, over 80% positive, 20-80% positive, under 20% positive) is presented in figure S1F.

A great majority of crestospheres were also intensively positive for additional neural crest markers tested as shown by average numbers of spheres with over 50% of positive cells: *SOX9* 83%, n=4, SEM=4; *ETS-11* 85%, n=3, SEM=7.7; *BMP4* 93%, n=3, SEM=4.4; *SNAIL2* 54%, n=4, SEM=7.1; and the expression of the neural tube gene *PAX6* was significantly lower as compared to the crest genes (13%, n=3, SEM=7.4, $p<0.01$, Fig 1F and S1F). Due to the weak staining of *PAX6*, we verified the expression by immunostaining: 15.7% (SEM 7.9, n=3) of the spheres expressed PAX6 positive cells and similarly to the *in situ* hybrisization results, in all of the positive spheres PAX6 was expressed by less than 20% of the total amount of cells in each sphere (Fig S1H).

Analysis of 7 weeks maintenance of FOXD3, SOX10 and SOX2 RNA expression

In the chick embryo, specified neural crest cells are maintained in a premigratory state for a period of approximately five hours. We addressed the self-renewal capacity of neural crest stem cells by testing how long we could maintain the crestospheres in a "premigratory" state by testing for co-expression of markers *FOXD3, SOX10, SOX9* and PAX7. Our QPCR results show maintenance of *FOXD3* and *SOX10* RNA expression for 7 weeks (when the experiment was ended) of crestosphere cultures representing the average values of 6 individual pools of crestospheres (Fig 2A, n=6, **week 1**: *SOX2* average 0.6, SEM 0.2; *FOXD3* average 8.5, SEM 1.6; *SOX10* average 5.8, SEM 0.9; **week 2**: *SOX2* average 2.2, SEM 0.6; *FOXD3* average 25.2, SEM 4.0; *SOX10* average 14.4, SEM 3.0; **week 3**: *SOX2* average 5.1, SEM 1.6; *FOXD3* average 21.5, SEM 10.3; *SOX10* average 24.1, SEM 10.9; **week 5**: *SOX2* average 5.0, SEM 0.6; *FOXD3* average 16.5, SEM 2.9; *SOX10* average 32.0, SEM 10.3; **week 7**: *SOX2* average 3.1, SEM 1.3; *FOXD3* average 20.6, SEM 11.6; *SOX10* average 29.1, SEM 13.2). Variation between the six individual populations was somewhat high and the changes of expression values between different time points are not statistically significant for any of the 3 markers ($p>0.05$). Examples of high, medium and low expression populations are shown in Figure S2A (**example #1**, *FOXD3* expression fold 1-7weeks: 4.2x; 42.2x; 66.7x; 46.9x; 72.2x, *SOX10* expression fold 1-7 weeks: 3.0x; 28.8x; 75.2x; 61.5x; 66.1x, *SOX2* expression fold 1-7 weeks: 1.8x; 2.4x; 8.4x; 7.7x; 1.8x, **example #2**, *FOXD3* expression fold 1-7weeks: 8.5x; 27.1x; 22.0x; 33.7x; 35.0x, *SOX10* expression fold 1-7 weeks: 4.1x; 13.6x; 29.0x; 57.4x; 74.4x , *SOX2* expression fold 1-7 weeks: 0.6x; 4.6x; 10.9x; 9.4x; 1.8x , **example #3**, *FOXD3* expression fold 1-7weeks: 14.8x; 14.8x; 31.5x; 15.2x; 3.5x , *SOX10* expression fold 1-7 weeks: 9.1x; 9.2x; 19.4x; 34.5x; 6.0x , *SOX2* expression fold 1-7 weeks: 0.5x; 0.4x; 4.9x; 4.8x; 0.8x).

Finally, *in situ* hybridization quantification of 3 individual pools of 7 week old crestospheres shows a change by time in the expression profile of the intensively positive spheres that express neural crest markers in an over 50% manner (*FOXD3* 44%, n=3, SEM=13.4; *SOX10* 24%, n=3, SEM=7.0) and a rise in *SOX2* expression was also detected (SOX2 49%, n=3, SEM=5.4; Fig S2B).

Crestospheres migrate in a similar fashion than neural crest cells from neural tube explants

To test the ability of crestosphere cells to migrate as compared to neural crest cells from the embryo we prepared neural tube explants and compared the time and length of migration to that of cells emerging from crestospheres. Perhaps due to the premigratory nature of crestospheres and thus a lack of cues for triggering EMT, the crestosphere cells started migration only a couple of hours after placement on the fibronectin coated surface whereas the migration of the neural crest cells in the explants started immediately (3h explants 51µm, n=8, SEM= 11.2; crestospheres 8µm, SEM=13.2). However, in 24 hours the crestosphere cells migrated on average a 294µm (n=8, SEM=5.2) distance out from the main sphere whereas the explant cells

migrated on average 355µm (n=8, SEM=11.2) from the explant. Even though the crestospheres didn't reach as far as the explant cells, taking into consideration the much bigger size and amount of cells of the explants and thus possible flattening of the dissected neural tube on the culture dish that may add to the "migration length", we conclude that the migration ability of crestospheres was similar to the *ex vivo* neural crest cells (Fig. S3A-C).

In vitro quantification shows similar differentiation pattern after 2 and 7 weeks of crestosphere culture

Crestospheres were differentiated for a week on poly-L-lysine coated glass cover slips in DMEM with 1% FBS and immunostained with markers for differentiated neural crest derivatives and the nuclei were stained with dapi. The percentage of differentiated cells was counted by choosing random spots (n) from each slide (total number of nuclei per cell type ranged from 3000 to 7000, total number of slides was minimum of 3/antibody). Each slide had cells differentiated from different batches of crestosphere cells.

2 week cultured chick crestospheres differentiated into glia (BLBP 55%, SEM=4.6, n=15) neurons (TuJ1 and HuC/D 13.2%, SEM=2.5, n=7), melanocytes (MELEM 20%, SEM=4.6, n=13) and smooth muscle cells (SMA 12.6%, SEM=4.5, n=13). There was no major contamination of neural cells in the cultures, only 2.8% (SEM=0.8, n=13) of the cells were positive for the CNS derived oligodendrocyte marker O4 (Fig 3B). After 7 weeks of crestosphere culture in CSC medium, the percentage of different derivatives was similar to the 2 week results (glia 45%, SEM=3.5, n=4; neurons 9%, SEM=1.0, n=7; melanocytes 13%, SEM=1.2, n=3; smooth muscle 14%, SEM=2.4, n=3; Fig 3F). Both after 2 weeks and 7 weeks of crestosphere culture, respectively, HNK, the marker for early migrating neural crest cells as well as some peripheral ganglion cells was expressed similarly (2wk 33.7%, SEM=3.3, n=7; 7wk 31%, SEM=4.4, n=5 Fig. S3G). As an additional control for excluding contamination of CNS cells within the crestosphere population we compared the percentage of oligodendrocytes from crestospheres with those derived from neurospheres. 19% (SEM 1.7; n=5) of neurospheres and 2.8% (SEM 0.8; n=13) of crestospheres became O4-positive oligodendrocytes (ttest p= 6.8 E-19, Fig S3H-I).

In vivo transplantations

GFP expressing chick crestospheres were transplanted into the head mesenchyme of 10-12 somite stage host chick embryos to study whether they were able to incorporate into the neural crest stream. Embryos were analyzed either 2 (n=5) or 3 (n=5) days after the transplantation (Fig 3I-N). At 2 days after *in ovo* transplantation, we detect GFP positive cells in the mesenchyme (14 times), around blood vessels (5 times), in ganglia (17 times) and in the branchial arches (1 time). At 3 days after the transplantation, we detected eGFP positive cells in the mesenchyme (12 times), around blood vessels (6 times), in gaglia (23 times) and in the brachial arches (3 times).

In vitro differentiation of GFP chimeras shows multipotentiality of crestospheres
Of the 38 clones of chimeras that had GFP positive cells originally derived from a single GFP+ cell in each well, we could detect various neural crest derivatives ranging from all three different types of derivatives tested (HUC/D+ neurons, MELEM+ melanoblasts and mesenchymal SMA+ smooth muscle) to only one or two of the derivative types (Figs. 3O-Q), thus verifying that the crestosphere culture conditions are sufficient to maintain neural crest cells in a multipotent state. Additionally, all clones contained a few and some clones consisted inclusively of differentiated GFP positive cells that were not positive for any of the markers used, perhaps indicating the presence of glial cells, precursors or other neural crest derivatives (Fig 3P). The most commonly detected differentiated cell type was neurons, perhaps reflecting a bias of the culture conditions as summarized in Fig 3Q. Many clones (15/38) were GFP negative, perhaps reflecting cell death during the 6 week culture period.

Human ES cell derived crestospheres reflect premigratory neural crest cells
The human ES cell derived crestospheres were similar to chick crestospheres (Fig. 4A-D). To exclude contamination of CNS derived neural stem cells we also immunostained the spheres with CD133 and were not able to detect any specific expression (Fig S3K-N).

The human ES cell derived crestospheres (cultured for 1-2 weeks in CSC medium) were differentiated like the chick spheres. The percentages of neurons (HUC/D and TUJ+ 23%, SEM=6.0, n=7), melanocytes (MELEM+ 12.7%, SEM= 4.9, n=7) smooth muscle (SMA+26%, SEM=5.2, n=8) and oligodendrocytes (O4+ 0.7%, SEM= 0.4, n=13), the neurotrophin receptor P75 expressed by early migrating neural crest cells and ganglia (32%, SEM=4.7, n=14) and the general migratory neural crest marker HNK1 (29%, SEM=4.0, n=7) was measured (Figs 4I, S3J).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Crestosphere cultures

The chick crestospheres were generated by pooling 4-6 entire neural tubes from 4-8 somite stage of either wild type (McIntyre Poultry, CA, USA) or GFP embryos (Clemson Public Services Activities, Clemson University, SC, USA). Each pool represents one "n" in the results. For isolation, neural tubes were carefully dissected from neighboring tissue using microscissors (FST 15003-08). The very anterior tip was excluded and the neural tube was collected up to the second somite level covering the cranial and part of the vagal neural tube. In some cases only the dorsal portions that contain the premigratory neural crest cells were collected. The neural tubes were mechanically dissociated in 50-100µl of Ringers balanced salt solution 30 times by using a p200 tip in an eppendorf tube. Dissociated tissue pieces were placed on ultra-low attachment 6-well plates (Corning, 3471) in 1ml of the **crestosphere culture medium (CSC, see below)** in 37*C (5% CO_2) that was

modified and simplified from previous NCC culture studies performed for self-renewing neural crest cells isolated from the sciatic nerve (Morrison et al., 1999) the gut (Molofsky et al., 2003) or the migratory neural crest (Mundell and Labosky, 2011).

The CSC medium always consisted of a basic component of DMEM with 4.5g/l glucose (Corning 10-013-CV), or with low glucose for testing the conditions (1g/l, 10567-014 Gibco), 1X penicillin/streptomycin (15140-122 Gibco), 1X B27 supplement (17564-044 Gibco), 7.5% Chicken embryo extract (CEE, see below) supplemented with the growth factors 20ng/ml IGF (IGF1 Recombinant Human Protein, PHG0078 Invitrogen), 20ng/ml FGF (FGF-Basic AA 10-155 Recombinant Human Protein, PHG0024 Invitrogen) and 60nM RA (190269 MP Biomedicals), which thus are the conditions for medium #3. Alternatively, when conditions were tested (mediums #1-5 listed in Fig S1B), combinations of different concentrations of the same growth factors were used in the same DMEM/B27/CEE/antibiotics base with the exception of medium #1 that also contained epidermal growth factor EGF (PHG0311L Gibco). New medium was added and the spheres were mechanically triturated by pipetting 10-20 times every two to three days. Because RA rapidly degrades, fresh RA acid was added every 3 days.

The human ES cells culture and differentiation experiments were done in accordance with USC-SCRO approved protocols. H7 and H9 lines were obtained from USC stem cell core and amplified in mTESR medium (Stemcell Technologies Inc). Cells were harvested with collagenase IV treatment and differentiated as clusters in suspension in medium containing 1: 1 mix of DMEM-F12 (Cellgro), neurobasal (Life Technologies) supplemented with 0.5x GEM21 (100x stock, Gemini Bio products), 0.5x N2 supplement (100x stock, Gemini), 1x Glutamax supplement (100x stock, Invitrogen), 0.5x antibiotic, 20ng/mL of EGF, 20ng/mL bFGF, 5ug/mL bovine insulin (Sigma-Aldrich) for eight days before transferring the neural crest induced rosettes into the chick neural crest stem cell medium.

Chick Embryo Extract

In sterile conditions, headless 11 days old chick embryos were rinsed with cold DMEM on a double layer of Gauze on a 500ml beaker until blood was removed, transferred into a 10ml syringe and pushed through into a 50ml falcon tube. The minced embryos were weighed and diluted with DMEM (1g/ml) and stirred at +4*C over night. Ice chilled hyaluronidase ($4x10^{-5}$ g/1g of minced embryos, LS002592 Worthington Biochemical Corporation) was added and stirred for 1h at +4*C. Then the lysates were ultracentrifugated (30min 46 000g) and the clear supernatant was filter sterilized (0.45µm filter, 430768 Corning), aliquoted and stored in -80*C.

In vitro differentiation cultures

Crestospheres were lightly dispersed mechanically into smaller clumps and changed onto poly-L-lysine (Sigma P5899 100µg/ml H_2O 15min RT*C) or fibronectin (Sigma F1141 5µg/ml PBS 10min RT*C) coated glass coverslips (12mm) on 24-well

(nunclon surface, Nunc) culture plates (both surfaces produced all derivatives in an equivalent manner). They were cultured in differentiation medium (1% FBS, 1X B27 in DMEM) for 7 days and immunostained.

QPCR

The RNA from individually originated pools of neural crest spheres was isolated by using the Ambion® RNAqueous-Micro Kit and cDNA was reverse transcribed by using Superscript II (Invitrogen 18064-014). QPCR was performed by using iTaq SYBR®Green supermix (BioRad 172-5125) and Abiprism 7000 Sequence detection system. The results were analyzed by using the ΔΔCT method (Livak and Schmittgen, 2001). The following primers were used: *GaphdH*fwd ATCACTATCTTCCACCACCGT; *GapdH*rev: AGCACCACCCTTCAGATGAG; *SOX10*fwd AGCCAGCAATTGAGAAGAAGG; *SOX10*Rev GAGGTGCGAAGAGTTGTCC; *FOXD3*fwd TCTGCGAGTTCATCAGCAAC; *FOXD3*rev TTCACGAAGCAGTCGTTGAG; *SOX2*fwd TATCTACCAGGTGCTGAAGTA *SOX2*Rev AGAGGGAGTGTGCCATTA

Immunostaining

For the immunostaining, crestospheres or differentiated neural crest cells were fixed with 4% paraformaldehyde in PBS for 15min RT*C, and the 3-4 days old embryos were fixed over night at +4*C, washed twice with PBS and blocked with 5% donkey serum and the Abs were diluted in the same blocking solution. The chick embryos were embedded in gelatin. Immunostaining was performed on 12 μm cryosections or on whole crestospheres using the following antibodies: PAX7, MELEM, HNK clone 3H5, SNAIL2/SLUG clone 62.1E6, AP2α clone 5E4, ISLET1 clone 39.3F7, RUNX2 clone 1B9 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at 1:5-1:10 dilution, SOX2 (Santa Cruz sc17320 1:2000), FOXD3 (Rb polyclonal, a gift from Patricia Labosky, 1:500), PAX6 (Covance PRB 2184 1:2000), TUJ-1 (Covance MMS-435P 1:400), HuC/D (Invitrogen / molecular probes 16A11 1:300), GFAP (SMI22; Sternberger Monoclonals, Covance 1:800) BLBP (Millipore ABN14 1:200, antigen retrieval by brief boiling in 10mM trisodium Citrate pH6 prior to staining), SMA (Sigma A5228 1:1000,) P75 (Promega, G323A; 1:350), O4 (MBS604817 MyBioSource.com, 1:15), β-CATENIN (Abcam ab6301 clone 15B8, 1:1000). Antibodies that specifically recognize human neural precursors and neural crest were SOX2 (Santa Cruz Sc17380; 1:500); CD133 (orb10288 biorbyt, 1:100), ALX1 (Sigma hpa 001598, 1:100) and TFAP2-α (Santa Cruz, SC12726; 1:1000); respectively. Secondary Alexa Abs (Molecular Probes) were used 1:1000. The cells were imaged using fluorescence microscopy (Zeiss Axioscope 2 and Zeiss ApoTome.2) or confocal imaging (Zeiss LSM 5 Exciter).

In situ hybridization assay for crestospheres

Crestospheres were fixed with 4% paraformaldehyde over night +4°C, washed with phosphate-buffered saline/0.1% Tween, dehydrated in MeOH, and stored at −20°C. The avian probes for *SOX10*, *SOX9*, *FOXD3*, *BMP4*, and *SOX2* were made by cloning respective genes to DNA vectors from reverse transcription (RT) PCR products made by using chicken whole embryo cDNA as template. *In situ* hybridization was

performed as described for whole mount embryos (Acloque *et al.*, 2008). The digoxigenin-conjugated RNA probes were visualized using anti–dig-AP antibody (1:2000; 11093274910; Roche Diagnostics, Mannheim, Germany) and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine (11383213001 and 11383221001; Roche Diagnostics).

Self-renewal and proliferation assays

For the primary self-renewal assay, crestospheres from pooled bulk cultures were dissociated into single cells using 0.125% trypsin – EDTA (T4049 Sigma, diluted 1:2 in sterile PBS) for 15-30 min at 37^{o}C accompanied by mechanical trituration until complete dissociation. The separation into single cells was verified by microscopic visualization from 5 parallel samples, each dissociated into crestosphere cell pools. Cells were counted using a hematocytometer. Single cells were plated in a concentration of 15 cells/150µl/well on ultra low adherence 96 well plates (Corning Costar 3474) in CSC medium for 7 days after which the newly formed spheres were counted. RA was added once on day 3. The self-renewal percentage was measured as the number of spheres / the number of cells plated. The results are shown as average numbers from different crestosphere pools and the error bars represent standard error of mean (SEM) values. Secondary sphere formation was analyzed by mechanically dissociating individual spheres into small pieces and culturing the cells of 1 sphere in 6 wells (with 100 µl CSC medium in each well of the 96/well plate) for 7 days, when the number of newly formed secondary spheres was counted. RA was added once on day 3. The results represent average numbers of new spheres formed from 6 individual crestospheres and the error bars represent SEM values. Proliferation was measured using immunostaining for phosphohistone H3 (06-570 Upstate, Millipore 1:500) and the nuclei were stained with dapi. The numbers represent the percentage of proliferating cells at the time of crestosphere fixation, for each individual value (n) 2000 – 5000 nuclei from 6-9 crestospheres were counted and the results represent averages of 3 individually started crestosphere populations. The error bars represent SEM values.

Clonal chimera assays

Crestospheres derived from wild type as well as GFP chicken embryos that had been cultured for 10 days, respectively, were dissociated into single cells (as described above) and plated at a ratio of 3 GFP cells with 2x10^{4} WT cells in 100 µl CSC medium in each well of the 96/well plate (5 plates total). After 3 days of culture, the wells with only single GFP cells or small clusters tightly attached to each other (indicating they descend from the same original GFP positive cell) were selected for further studies. Most GFP clusters fused with the nonlabeled WT crestospheres. Finally after 2 weeks of culture, the clones were transferred into 24-well plates together with additional 3x10^{4} WT cells in each well, cultured for additional 2 weeks while lightly dissociating them mechanically once a week. After 4.5 weeks, chimeric cultures with individual GFP clones were lightly dissociated using 0.125% trypsin – EDTA (5min, 37^{o}C) together with mechanical dissociation and plated on poly-L-lysine coated wells and cultured in differentiation promoting medium (as above) for 8 days, fixed with 4% PFA 20min at room temperature and each well was immunostained by

using antibodies against neurons (HUC/D), melanoblasts (MELEM) and smooth muscle (SMA) and visualized by using Alexa 633, 568 and 350 secondary antibodies, respectively.

Migration assay
Premigratory (4-5som) cranial neural tubes were dissected out and placed on fibronectin coated (5µg/ml in PBS 2h RT) culture wells and cultured in a DMEM with 1% FBS. Similarly, crestospheres were removed from CSC medium and placed in equivalent culture conditions. The distance of the migration of each sphere / explant was measured from five furthest migration points and averaged at two time points 3h and 24h. The averages of 8 explants / crestospheres, respectively, were counted. At 24h the cultures were fixed (4%PFA 1h RT), stained with phalloidin (Molecular Probes A12380) and imaged.

Supplemental figure legends

Figure S1
QPCR results of optimization of culture conditions that support long term maintenance of crestospheres. S1A. RNA expression levels of *FOXD3, SOX10* and *SOX2* by QPCR in neural crest spheres cultured in four different culture conditions for two weeks (mediums #1-4; mediums 1-2: n=3; medium 3: n=6, medium 4: n=4) **S1B.** A list of the variables in the crestosphere mediums tested. **S1C.** RNA expression levels of *FOXD3, SOX10* and *SOX2* by Q-PCR in neural crest spheres cultured in culture medium #5 for two weeks (note the different scale, n=3) **S1D.** Further optimization of the crestosphere culture conditions (in the chosen medium #3) by variations in glucose concentration (high n=12, low n=3), Chicken Embryo Extract (w/o CEE n=6) supplement as well as using only the dorsal neural tube (NT, n=4) as compared to the entire neural tube as starting material for the cultures. In addition to expression of the neural crest and neural markers, growth rate and survival was also monitored. **S1E** Immunostaining with β-CATENIN in the chick embryo at the stage (HH9) when neural crest cells are still premigratory and reside within the neural epithelium. Adherence junctions typical for epithelial cells are clearly seen in the neural tube (NT), ectoderm (e) and the notochord (n). **S1F** A more detailed quantification of the expression levels of *FOXD3*, *SOX10* and *SOX2* (compare to Fig 1D) in crestospheres (n=5) versus neurospheres (n=6) by *in situ* hybridization after 2 weeks of stem cell culture in CSC. The positive cells are further charachterized as subgroups of high expression >80%, medium expression 20-80%, and low expression <20% of positively stained cells in the sphere shown as a percentage of the total amount of spheres with positive expression. **S1G**. *In situ* hybridization of crestospheres after 1-2 weeks of stem cell culture in the CSC medium shows high RNA levels of neural crest markers *SOX9, ETS-1* and *BMP4* and very low expression levels of the neural marker *PAX6*. **S1H** Double immunostaining with the neural crest marker PAX7 and neural marker PAX6 show no overlap. **S1I**. Immunostaining of a crestosphere showing SNAIL2 positive cells. Scale bar 50µm.

Figure S2

Long-term maintenance of heterogenous neural crest marker expression. S2A Examples of high, medium and low expression of *SOX10* and *FOXD3*. **S2B** *In situ* hybridization quantification of 3 individual pools of 7 week old crestospheres shows a change by time in the expression profile of the intensively positive spheres that express neural crest markers in an over 50% manner and a rise in *SOX2* expression was also detected (n=4).

Figure S3

Crestosphere migration ability is comparable to neural tube explants. S3A-C The distance migrated by neural crest cells from crestospheres is similar to that exhibited by primary neural crest emigrating out from the neural tube explants. **S3D-F** Crestosphere cells express ISLET1 as an indication of peripheral neurons, glial marker GFAP, neural marker TUJ1 as well as the migratory neural crest cell marker HNK1 followed by 2 weeks of stem cell crestosphere culture and 1 week of differentiation in 1% FBS. **S3G** Roughly 30% of the differentiated cells expressed HNK1 in a similar fashion following 2 (n=7) or 7 weeks (n=5) of crestosphere culture, respectively. **S3H** Roughly 20% of neurosphere cells (n=22) and 2.5% of crestosphere cells (n=13), respectively, differentiate into O4+ oligodendrocytes. **S3I** An image of an oligodendrocyte differentiated from chick and (**S3J**) human ES cell derived crestospheres, respectively. **S3K-N** Human ES cell derived crestospheres are not positive for the neural stem cell marker CD133. An example of negative cells with some background staining not matching the membrane staining pattern of CD133 can be seen in spheres double stained with AP2α. Scale bar 50 μm.

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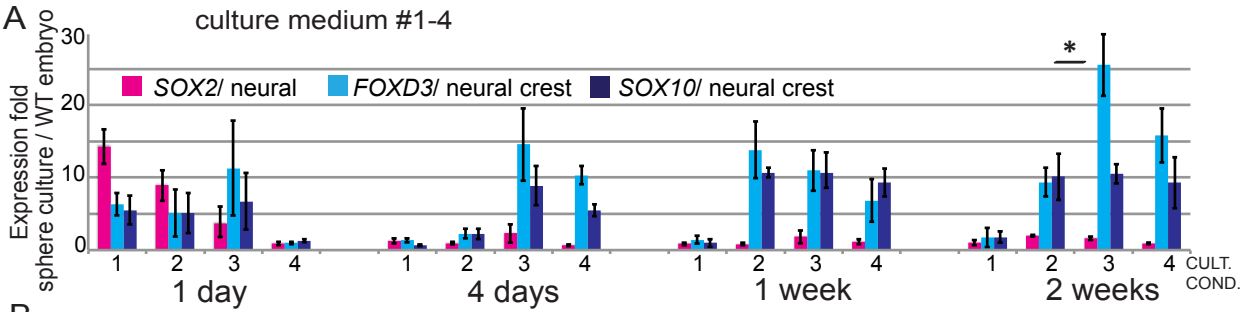
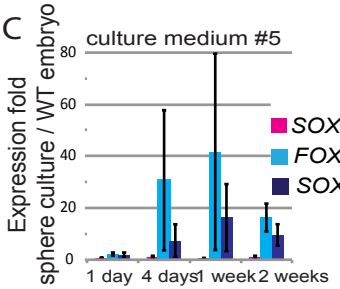
Supplemental figure S1

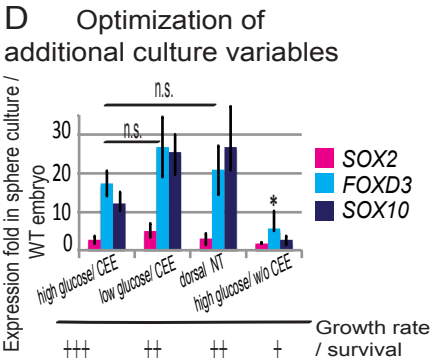


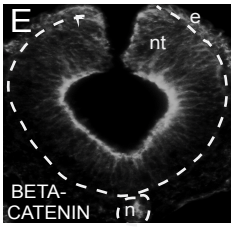
B

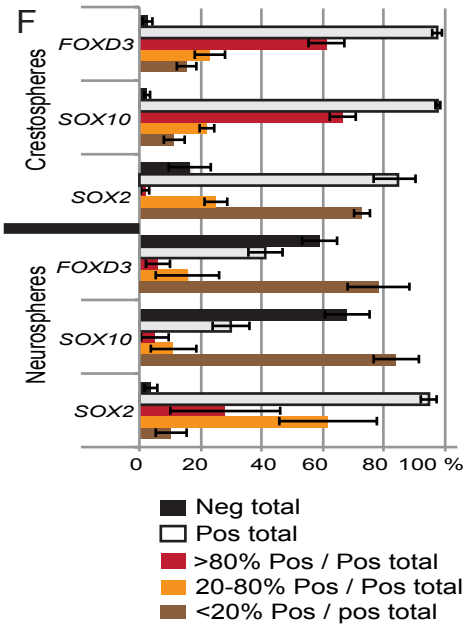
List of culture mediums:

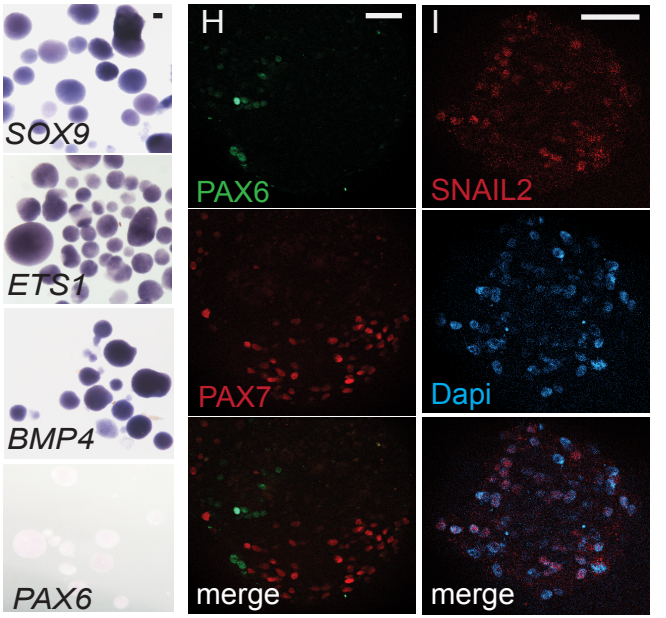
1. FGF 40 ng/ml / EGF40 ng/ml (neurospheres)
2. FGF 40 ng/ml / IGF 20ng/ml / RA 120nM
3. FGF 20ng/ml / IGF20 ng/ml / RA 60nM
4. FGF 20ng/ml / IGF 20ng/ml / RA 120nM
5. FGF 20ng/ml / IGF 20ng/ml / RA 240nM





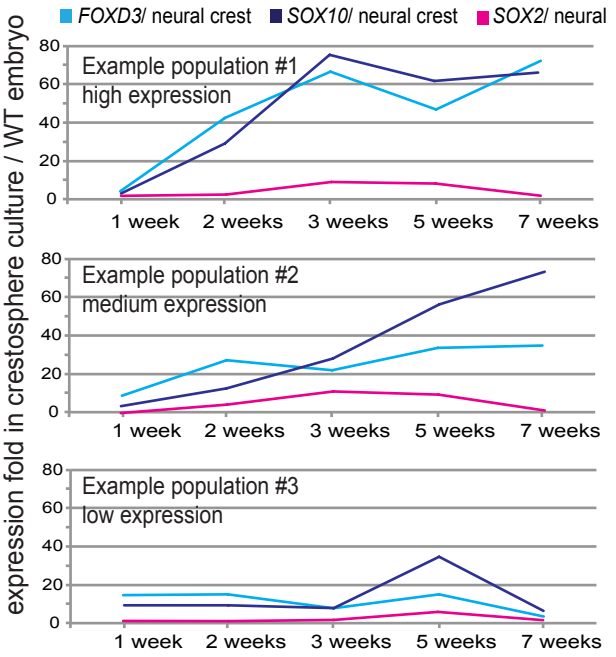






Supplemental figure S2

A



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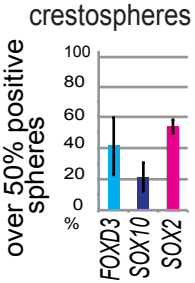

Supplemental figure S3