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Report

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

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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
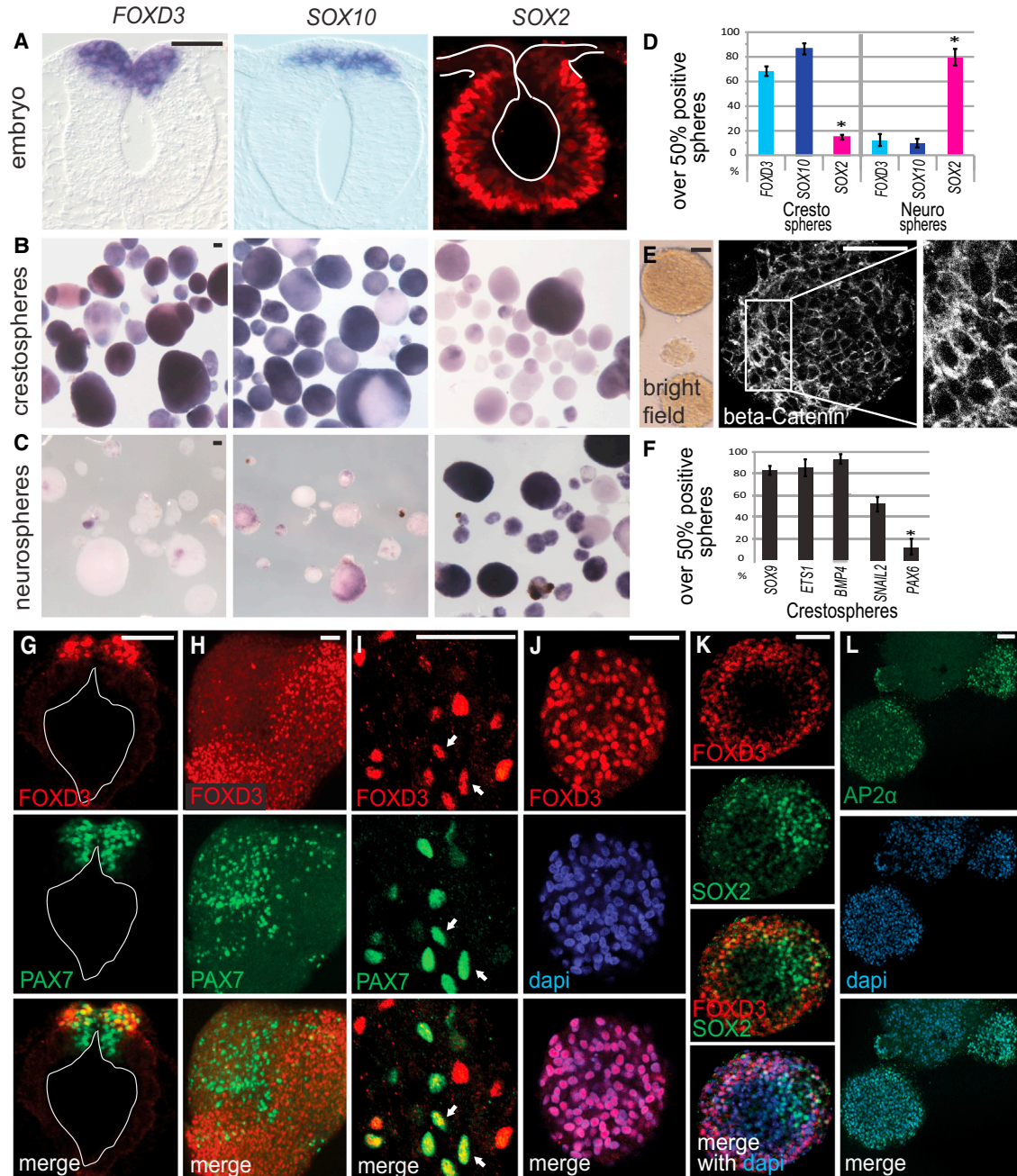
Figure 1. Chick Crestospheres Mimic Premigratory Neural Crest Cells

(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.
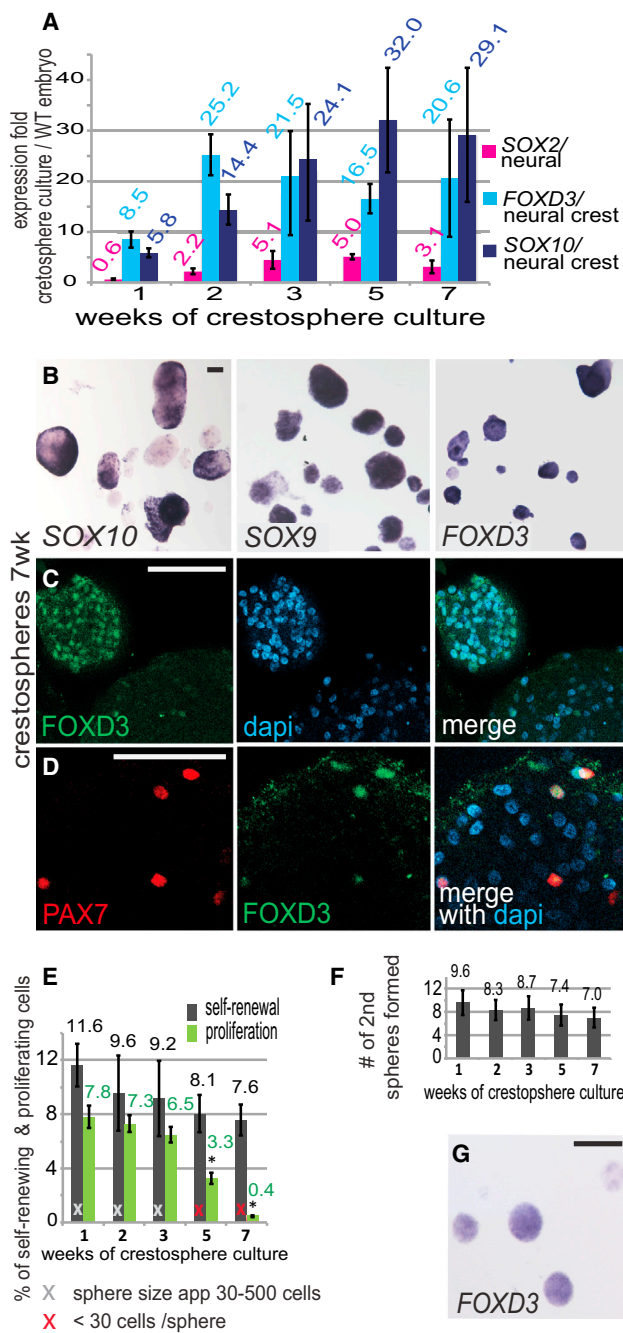
Figure 2. Long-Term Maintenance of Crestospheres

(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).

(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

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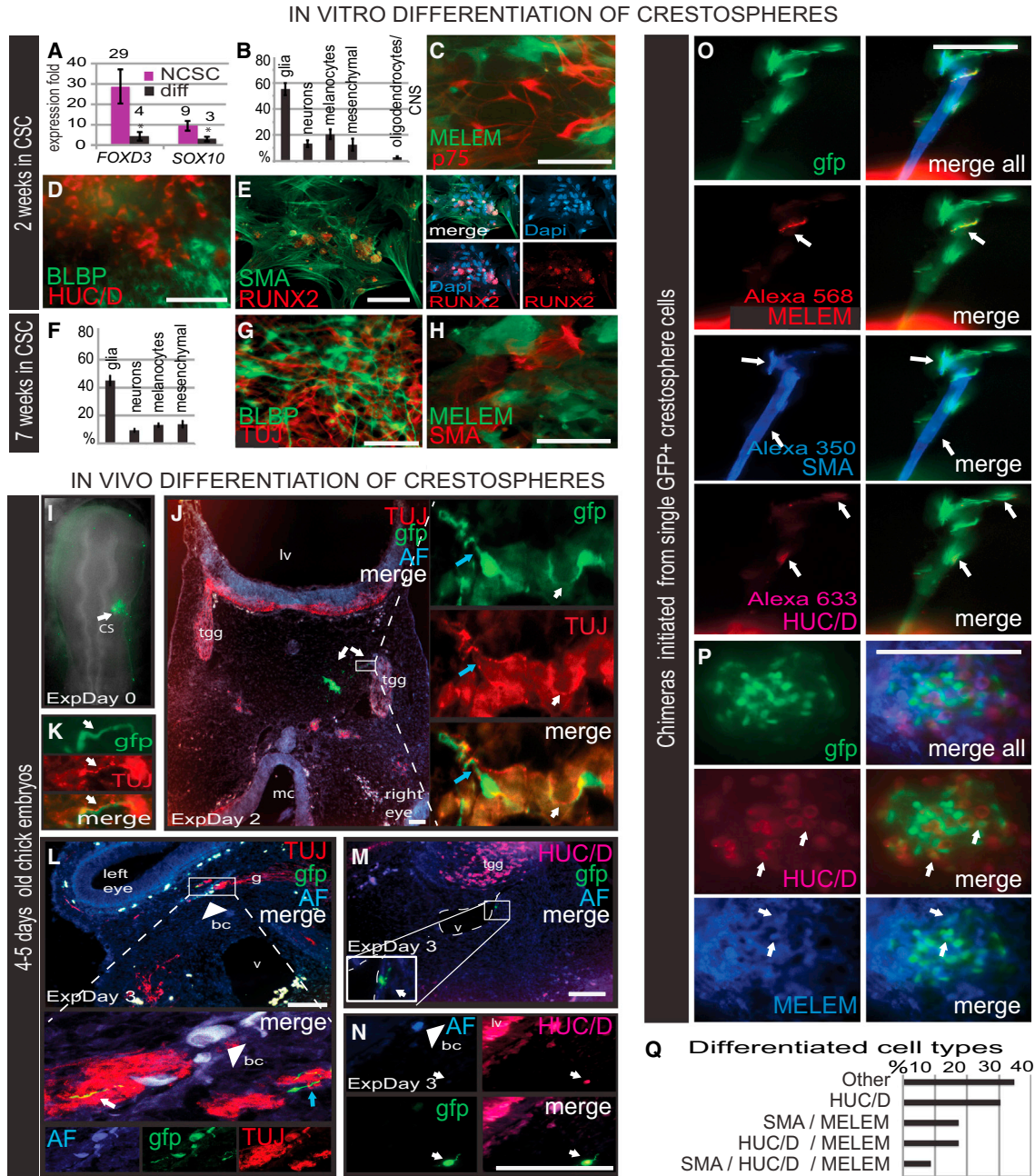
Figure 3. Multipotent Chick Crestospheres Differentiate into Multiple Neural Crest Derivatives

(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 mela-noblasts (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 O4.

(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 crestospheres give rise to neurons, melanocytes, and smooth muscle.

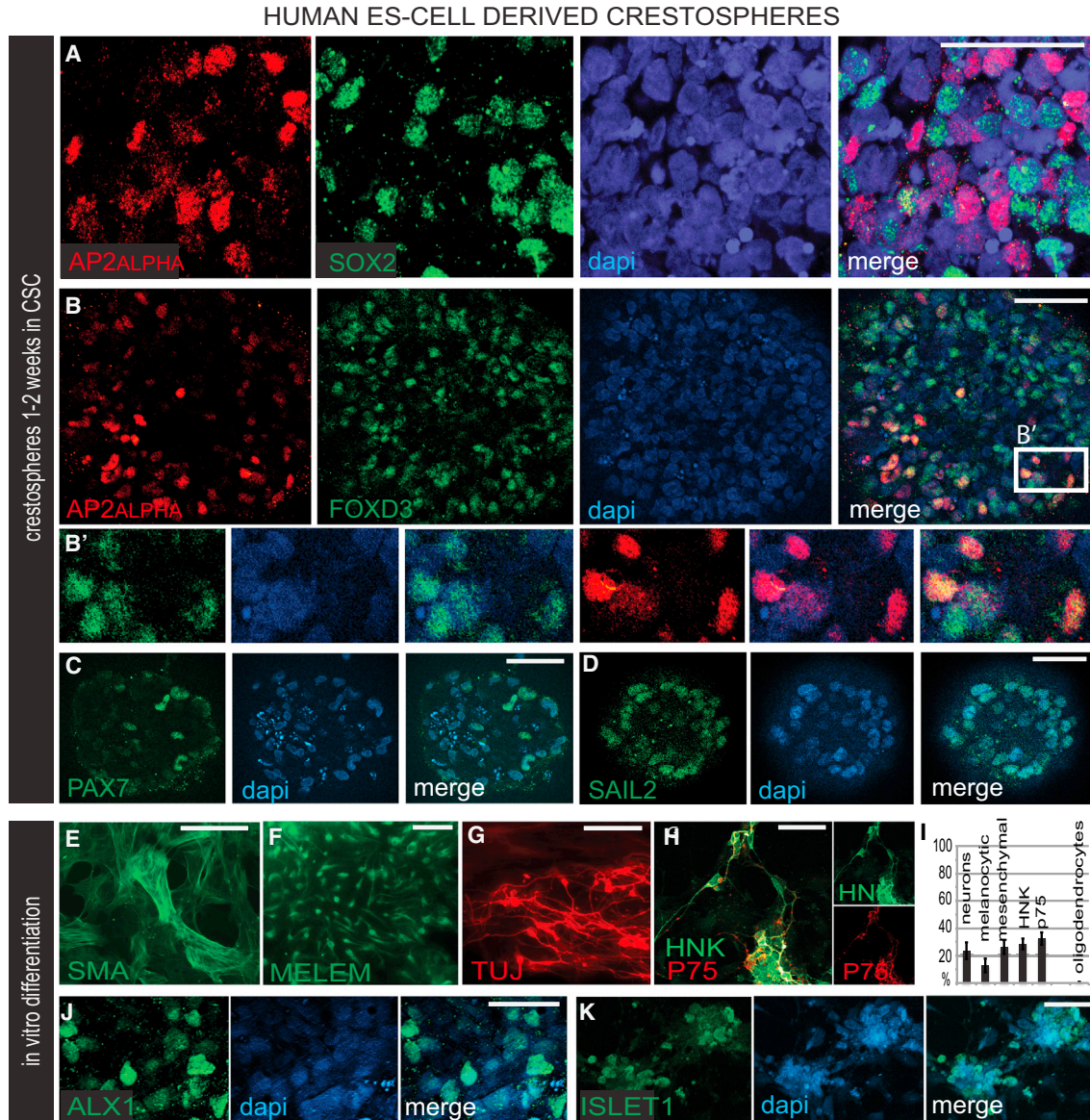(Q) Summary of different derivatives formed. CSC, crestosphere culture. Scale bar represents 100 μm.

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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