Stem Cell Reports

Supplemental Information

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

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

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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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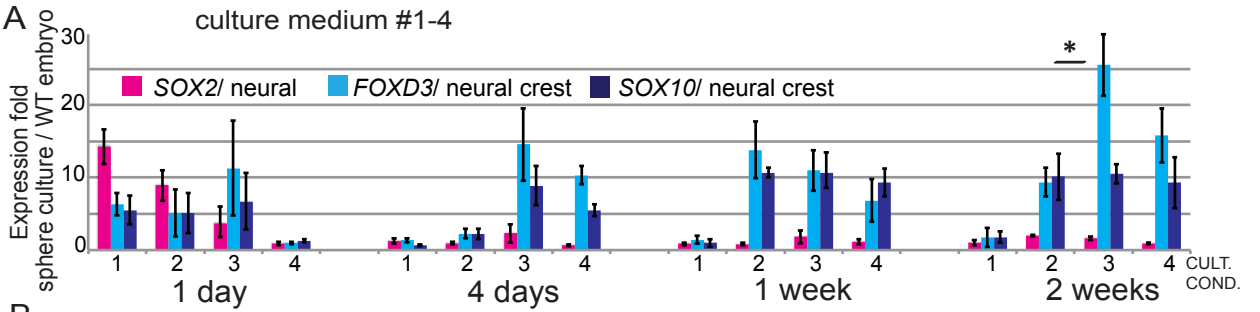
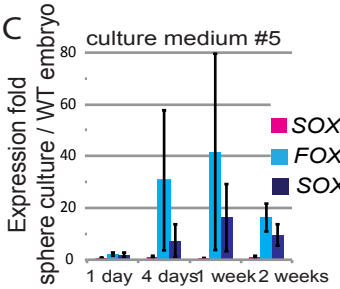
Supplemental figure S1

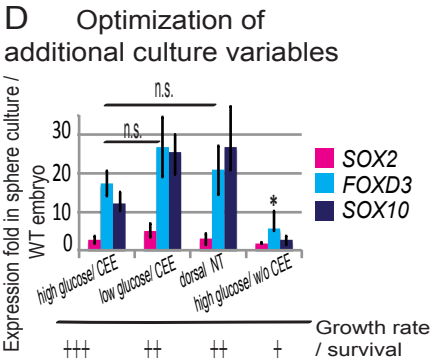


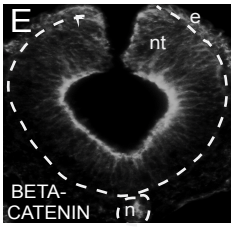
B

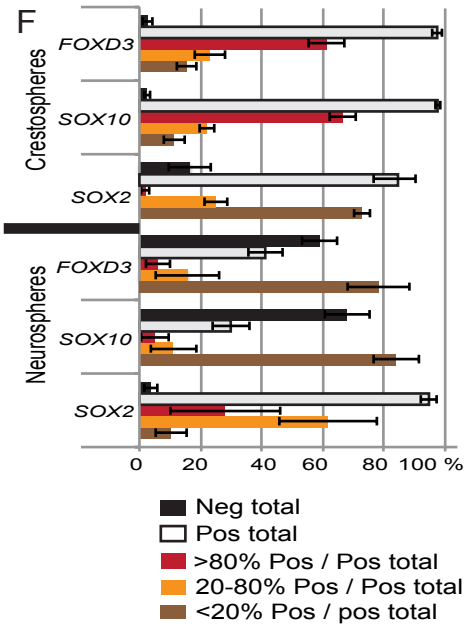
List of culture mediums:

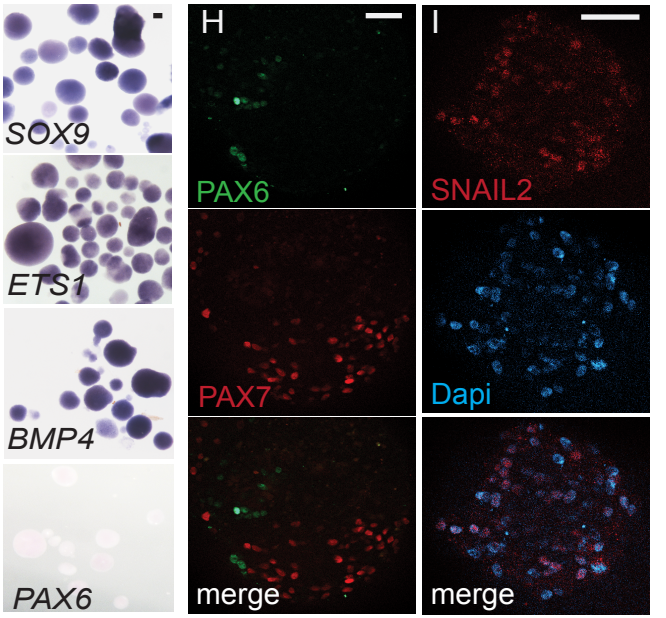
1. FGF 40 ng/ml / EGF40 ng/ml (neurospheres)
2. FGF 40 ng/ml / IGF 20ng/ml / RA 120nM
3. FGF 20ng/ml / IGF20 ng/ml / RA 60nM
4. FGF 20ng/ml / IGF 20ng/ml / RA 120nM
5. FGF 20ng/ml / IGF 20ng/ml / RA 240nM





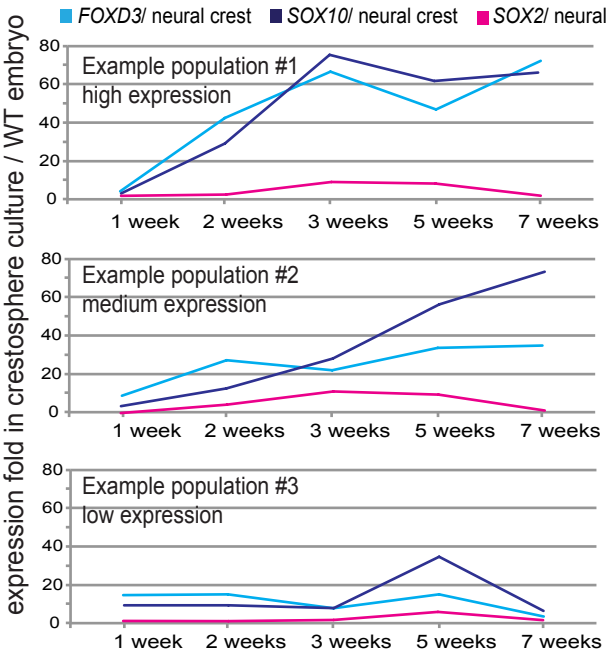






Supplemental figure S2

A



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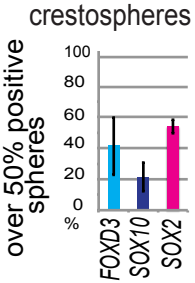

Supplemental figure S3