FIGURE LEGENDS

Figure 1

Differentiation of NC cells from HESC

A) Expression of neuroectoderm (NE), neural crest (NC) and epithelial to mesenchymal transition (EMT) specific genes by qRT-PCR during H9s HESC differentiation in chemically defined medium (CDM), supplemented with FGF2, SB-431542 (FSB) and with (red line) or without WNT3A (black line). Cells were studied as undifferentiated HESC, neuroectoderm day 7 (D7) and with serial passages (P1-P5). mRNA levels were normalized to the housekeeping gene *PBGD* and then presented relative to HESC expression which was set to 1. The results presented are representative of 3 independent experiments.

B) Flow cytometry analysis of neuroectoderm marker SOX1 and NC marker P75 in the differentiation of H9s HESC to neuroectoderm. FSB media was used to differentiate H9s HESC to neuroectoderm over four or seven days. SOX1 and P75 were analyzed before (D4, D7) and after (D4 P1 and D7 P1) splitting the neuroectoderm at indicated time points. Red contour plots represent SOX1+P75 double stained populations, blue contour plots represent IgG control staining.

C) Histogram of flow cytometric analysis of the NC marker TFAP2A (AP2) in H9s HESC differentiation with FSB to neuroectoderm over four or seven days. TFAP2A expression was measured before (D4, D7) and after (D4 P1, D7 P1) splitting the neuroectoderm at indicated time points. Red histograms represent TFAP2A stained populations, blue histograms represent IgG control staining.

D) Immunocytochemistry for the NC marker HNK1 and the neuroectoderm marker SOX1 with DAPI counterstain in H9s HESC differentiation to neuroectoderm. Upper panels, differentiation of HESC colonies over four days (D4) and after passage (D4 P1). Bottom panels, differentiation over seven days. Colonies passaged at day 7 showed a remaining SOX1+ population (D7 P1, white square) shown in D7 P1 ZOOM. The SOX1+ population was not detected in colonies passaged on day 4. White scale bar: 100 µm, blue scale bar: 50 µm, red scale bar: 20 µm.

E) Expression of the NC marker *TFAP2A* and neuroectoderm marker *GBX2* by qRT-PCR. H9s HESC colonies were differentiated with FSB media over four or seven days to neuroectoderm. RNA samples were collected at day 4 and day 7 before and after passage (D4, D7, D4 P1 and D7 P1, respectively). The relative mRNA level was normalized to the housekeeping gene *PBGD*. The results are presented as mean ± s.d. of 3 independent experiments. *$P < 0.05$; **$P < 0.01$, two-sided Student's T-test.

Figure 2

Increased purity and maturation of HESC derived NC cells with serial passages

A) Immunostaining of the NC markers SOX9, P75, HNK1, and the neuroectoderm marker SOX1 with DAPI counterstain, in H9s HESC-derived NC after 2 passages (NC P2) in FSB media. Blue scale bar: 50 µm, white scale bar: 100 µm.

B) Flow cytometric analysis of P75 and HNK1 in H9s HESC after four days of differentiation in FSB media before passage (H9s 4D IN FSB) and H9s-derived NC after 3 passages maintained in FSB as single cells (H9s NC P3). Red contour plots represent HNK1+P75 double stained populations, blue contour plots represent IgG controls.

C) Flow cytometric analysis of TFAP2A and P75 in the differentiation of undifferentiated H9s and H9s-derived NC after 3 passages maintained in FSB as single cells (H9s NC P3). Colonies were passaged at day 4 of differentiation. Red contour plots represent TFAP2A+P75 double stained populations, blue contour plots represent IgG controls.

D) Flow cytometric analysis of SOX17 and SOX10 in H9s HESC, endoderm cells differentiated from H9s (H9s END), and H9s HESC at day 4 of differentiation in FSB without splitting (H9s 4D IN FSB). SOX10 induction was observed following 4 days of FSB treatment, whereas the population was negative for expression of the endoderm marker SOX17. Red contour plots represent SOX17+SOX10 double stained populations, blue contour plots represent IgG controls.

E) Top panel: qRT-PCR expression analysis of NC markers *PAX3*, *ZIC1* and *SOX9*.
Bottom panel: qRT-PCR expression analysis of NC markers *P75, TFAP2A, TWIST1*.
mRNA of HESC-derived NC cells at different passages were used to perform the qRT-PCR. The relative mRNA levels were relative to NC passage 1 levels (NC P1). The relative mRNA level was normalized to the housekeeping gene *PBGD*. P1, P3, P5, P7, P8, and P13: number of passages of the neural crest cells as single cells. Results presented as mean ± s.d. of 3 independent experiments. *P < 0.05; **P < 0.01, ***P < 0.001. Two-sided Student's T-test.

F) Representative images of in vitro scratch wound assay performed in H9s HESC derived NC cells (NC P7). Pictures were obtained from 0 to 24 hours post-scratch. White scale bar: 100 µm.

G) Flow cytometric analysis of TFAP2A and P75 in H9s HESC and H9s HESC-derived NC passage 12 in FSB (NC P12). Red contour plots represent TFAP2A+P75 double stained populations, blue contour plots represent IgG controls.

H) Chemotaxis potential and migration in representative H9s-derived NC P7 was assessed using a CytoSelect™ transwell cell migration assay kit. The NC chemo-attractant FGF8B was added to FSB media to assess migration. DMEM/F12 + 10% fetal bovine serum (SERUM) was used as a positive control of migration. FSB migration without any chemo-attractant was set as 1 and data is expressed relative to this. Equal cell numbers were used in each condition and results are presented as mean ± s.d. of 3 independent experiments. *P < 0.05; **P < 0.01.

Figure 3

NC P2 cells are a mix of pre-migratory NC and neural progenitor cells that are purified into migratory NC cells with serial passages (P7)

A) Heat map showing the Euclidean distance metric among HESC, NC P2, NC P7 and neuroectoderm D7 populations. The global gene expression patterns were sorted based on similarity by hierarchical clustering. Red (upregulation) and blue (downregulation) depict differential gene expression from the mean across all samples.
NC P2, NC P7: H9s HESC treated four days with FSB then split as single cells for two or seven passages, respectively. NE D7: neuroectoderm cells obtained from H9s HESC after 7 days in FSB.

B) Principal component analysis showed the NC P2 population as an intermediate population between neuroectoderm and NC P7.

C) Fold change gene expression of NC and neural markers between NC P2 and NC P7 populations. Cluster 14 depicts the NC genes upregulated in NC P7 compared with NC P2. Cluster 33 illustrates the NC genes upregulated in NC P2 compared with NC P7. Benjamini Hochberg FDR 0.05 was used as a cutoff for ANOVA analysis of differentially expressed genes.

D) Gene interaction pathway for NC cell differentiation (WikiPathway WP2064 revision 47071). Differentially expressed genes are coloured in red for upregulated gene expression in NC P2 or blue for upregulated gene expression in NC P7. Pathvisio software was used to depict the neural crest pathway. Legend: Arrows represent stimulatory interaction while T lines represent inhibition. The colouration intensity of the box is dependent on the level of differential expression between these two populations. Those which are highest in NC P7 are blue, and those highest in NC P2 are red.

Figure 4

HESC-derived NC cells engraft, migrate and differentiate into specific NC developmental location in the chicken embryo

A) Fluorescent images of HESC-derived NC GFP+ cells showing survival into the chicken embryo, 20 hours after engraftment at Hamburger and Hamilton stage 16 (HH16). Clumps of 100-150 HESC-derived NC GFP+ cells at P2 were embedded into Matrigel and engrafted into the chicken embryo at HH9-10. The clumps were inserted in-between the otic vesicle and the third somite, near to the neural tube (white arrow, left panel). Blue scale bar: 100 µm. White scale bar: 50 µm.

B) Fluorescent images showing HESC-derived NC GFP+ cells migrating from the Matrigel inside the embryo (HH17) 42 hours after engraftment. White scale bar: 50 µm.

C) Top row: Confocal images from the chicken embryo aorta 4.5 days after HESC-derived NC GFP+ cells engraftment (HH29). The GFP+ cells migrated to the aortic arch (GFP+ picture) and differentiated into SMC (ACTA2 picture) contributing to the aortic wall.
Middle row: Confocal images from chicken aorta 4.5 days after HESC-derived endoderm GFP+ cells insertion (HH29). No GFP+ cells were visible in the aortic arch of the chicken embryo when HESC-derived endoderm GFP+ cells were injected instead of the HESC-derived NC GFP+ cells.
Bottom row: Confocal images from chicken myocardium and epicardium 4.5 days after HESC-derived NC GFP+ insertion (HH29). No GFP+ cells were identified in the epicardium or the myocardium of the chicken embryo. GFP+ cells were restricted to the aorta. White scale bar: 50 µm.

Figure 5

Differentiation of HESC-derived NC to their derivatives

A) HESC-derived NC cells were plated on polyornithine-laminin-coated culture dishes to induce neuronal differentiation. (i, ii), beta III Tubulin immunocytochemistry showing differentiation to peripheral neurons. (iii), NC cells stained with beta III Tubulin as a negative control. (iv), magnification of the white square in (ii). White Scale bar: 100 µm, blue scale bar: 50 µm. Representative images of 3 independent biological replicates are shown.

B) Immunocytochemistry of the smooth muscle cell (SMC) proteins CNN1, ACTA2, and SM22A following HESC-derived NC differentiation to SMCs with PDGF-BB (10ng/ml) and TGF-β1 (2ng/ml) for twelve days (12D PT). White Scale bar: 100 µm, red scale bar: 20 µm. Representative images of 3 independent biological replicates.

C) Quantitative RT-PCR showing the upregulation of the specific melanocyte genes *MITF* and *KIT* and the downregulation of specific NC genes *FOXD3* and *P75* in NC P7 derived melanocytes (NC-MEL). The relative mRNA level was normalized to the housekeeping gene *PBGD*. Results are presented as mean ± s.d. of 3 independent experiments. *P < 0.05; **P < 0.01, ***P < 0.001.

D) Flow cytometry analysis of CD44 and P75 following differentiation of HESC-derived NC to mesenchymal stem cells (MSC). Green contour plots represent CD44+P75 double staining in HESC-derived NCs, whereas red contour plots represent the double staining in MSCs derived from this population. IgG isotype control staining for P75 and CD44 in both HESC-derived NC and MSC populations are shown as blue and orange contour plots, respectively. MSC derived from NC showed robust expression of CD44 and the absence of P75 expression. Representative results of 3 independent biological replicates are shown.

E) Alcian blue staining, (F) Oil Red O staining and (G) Alizarin Red staining, demonstrating chondrocyte, adipocyte, and osteocyte differentiation from HESC-derived NC, respectively. Representative images of 3 independent biological replicates are shown. White scale bar: 100 µm, red scale bar: 20 µm.

Figure 6

NC cells transfected with TCOF1 siRNA impair regular migration of NC and MSC

A) H9s-derived NC cells were transiently transfected with a siRNA to TCOF1. Left panel: Flow cytometry was performed for CD44 and P75 following 5 days of differentiation from NC to MSC, demonstrating that TCOF1 Knockdown (KD) does not impair MSC differentiation.
Right panel: Flow cytometric analysis of CD44 and P75 in NC transiently transfected with a siRNA to TCOF1. Red contour plots represent CD44 + P75 double stained populations, blue contour plots represent isotype control staining.

B) MTT cell proliferation assay was performed using TCOF1 knockdown NC cells (NC siRNA TCOF1) and siRNA scramble NC cells (NC siRNA SCRAMBLE) during 4 days. Results are presented as mean ± s.d. of 3 independent experiments. *P < 0.05; **P < 0.01. Two-sided student's T-test.

C) Representative images of scratch wound assays of HESC-derived NC cells transiently transfected with Scramble siRNA or TCOF1 siRNA. Images were collected 4 days following transfection, at the indicated timepoints. Scale bar: 100 µm

D) Box plot depicting the quantification of chemotaxis potential and migration of NC cells transfected with TCOF1 siRNA or Scramble siRNA during a 6 hour CytoSelect™ Cell Migration Assay. FGF8B was used as a NC chemo-attractant. DMEM/F12 + 10% fetal bovine serum (SERUM) was used as a positive control for cell migration. Data is expressed relative to the NC cells transfected with TCOF1 siRNA, maintained in FSB medium. *P < 0.05; **P < 0.01. Two-sided student's T-test.

E) Representative images of scratch wound assays of NC-derived MSC transiently transfected with Scramble siRNA or TCOF1 siRNA. Images were collected after 5 days of differentiation, at the indicated timepoints. Scale bar: 100 μm

Figure 7

Generation of TCOF1 heterozygous knockout HIPSC using CRISPR/Cas9

A) Schematic of CRISPR/Cas9-mediated non-homologous end joining strategy to generate insertions or deletions (INDELs) leading to HIPSC TCOF1 knock out clones.

B) Representative genomic sequencing of HIPSC transfected with specific CRISPR TCOF1 gRNA. Heterozygous knock out clones showed the same deletion leading to a change in codon reading frame and the introduction of a premature STOP codon in *TCOF1* Exon 1.

C) Immunoblot for Treacle protein demonstrating that NC derived from HIPSC TCOF1$^{+/-}$ clones (C12 and C24) showed a reduction in Treacle compared with NC derived from HIPSC TCOF1$^{+/+}$ (C8) and NC derived from H9s (WT).

D) Time course MTT proliferation assay of TCOF1$^{+/+}$ NC derived from H9s (WT), HIPSC (C8) and NC derived from TCOF1$^{+/-}$ HIPSC (C12 and C24), over a 4-day period. Proliferation rate decreased significantly in mutated cells compared with wild-type. Results are presented as mean ± s.d. of 3 independent experiments. **P < 0.01, ***P < 0.001, two-sided Student's T-test

E) Flow cytometric analysis of Annexin V staining depicting the apoptotic rate in TCOF1$^{+/+}$ neuroectoderm-derived cells from H9s (WT), HIPSC (C8) and neuroectoderm derived from TCOF1$^{+/-}$ HIPSC (C12 and C24). Red histograms represent Annexin V staining, blue histograms represent the unstained population.

F) Single cell analysis of cell migration in NC derived from TCOF1$^{+/+}$ HIPSC (C8) and TCOF1$^{+/-}$ HIPSC (C24). TCOF1$^{+/-}$ NC demonstrate impaired migration and reduced directionality of movement. Each dot and tail represents a single cell analyzed, with 30 cells analyzed from both conditions, over a 12-hour period.

Supplementary Figure 1

Optimization of NC cells differentiation by splitting neuroectoderm-derived HPSC at day 4 into single cells

A) Expression levels of *P75*, *HNK1,* and *PAX6* by qRT-PCR during seven days of HESC differentiation to neuroectoderm. The mRNA levels were normalized to the housekeeping gene *PBGD*. Black line: differentiation to neuroectoderm with chemically defined medium (CDM) supplemented with FGF2 and SB-431542 (FSB). Red line: FSB media supplemented with 25ng/ml WNT3A.

B) Schematic of the differentiation protocols used to derive neuroectoderm (NE) and neural crest (NC) cells from HPSC.

Supplementary Figure 2

Characterization of NC cells derived from HIPSC

A) Histogram of the percentage of P75+ cells in HIPSC (left panel), HIPSC-derived NC P7 (middle panel) and HIPSC-derived NC P14 (right panel) determined by flow cytometry. Red histograms represent P75 stained populations, blue histograms represent IgG controls.

B) Flow cytometry analysis of TFAP2A and P75 expression in wild-type HIPSC and their NC-derivatives (NC P12). Red contour plots represent TFAP2A + P75 double stained populations, blue contour plots represent IgG controls.

C) Cell counts performed over a 4-day period from HIPSC-derived NC at P2, P7, and P14. The doubling time of the NC cells was 17.0 ± 0.71 h.

D) MTT proliferation assay performed on HIPSC-derived NC over a 4-day period in culture. Cells were seeded at various densities as depicted in the figure. Results are presented as mean ± s.d. of 3 independent experiments.

E) Quantitative RT-PCR analysis of endoderm (*EOMES* and *SOX17*), mesoderm (*NKX2.5* and *KDR*) and neuronal (*TUBB3* and *MAP2*) gene expression in NC P2 and NC P7 populations. The relative mRNA level was normalized to the housekeeping gene *PBGD*, and expression levels are relative to those at NC P7. The results are presented as mean ± s.d. of 3 independent experiments. ***P < 0.001, two-sided Student's T-test. ENDO: Endoderm cells differentiated from HPSC [98]; MESO: Mesoderm cells differentiated from HPSC [34]; NEURO: Neurons differentiated from HPSC [99].

Supplementary Figure 3

Transplanted NC cells localize specifically to NC locations in the chicken embryo

(A-F) Cross section of the chicken ascending aorta where fluorescent human NC cells were detected (arrowheads); (B, E) Human cells identified by their bright and distinct Hoechst 33342 staining (arrowheads); (C, F) The human NC cells also expressed ACTA2 (arrowheads); (G) Fluorescent human NC cells were seen in the cerebral cortex (parietal lobe) of chicken at HH stage 32 (arrowheads); (H) Human cells identified by distinct Hoechst 33342 staining; (I) GFP+ human neural crest cells identified in perivascular locations within a network of blood vessels (lectin stained) and within the cerebrum of chicken embryos; (J) GFP+ human neural crest cells deposited in the cortex (frontal lobe) by blood vessels in the brain (identified by lectin); (K) myocardial and overlying epicardial (arrow) regions of the chicken embryo heart with no evidence of GFP+ human NC cells. Myo; Myocardium, Epi; Epicardium. White scale bar: 100 µm, blue scale bar: 50 µm.

Supplementary Figure 4

NC cells successfully differentiate into their derivatives

A) Bright field image of representative HESC-derived NC cells. Scale bar 50 µm.

B) Bright field image of neurons-derived from NC cells. Scale bar 20 µm.

C) Bright field image of representative melanocytes derived from NC cells. Scale bar 50 µm.

D) Bright field image of NC derived mesenchymal stem cells (MSC). Scale bar 50 µm. Bottom, qRT-PCR showing expression levels of the specific MSC marker Endoglin (*ENG*). The relative mRNA level was normalized to the housekeeping gene *PBGD.* Results are presented as mean ± s.d. of 3 independent experiments. **P < 0.01, two-sided Student's T-test

E) Bright field picture of representative NC P7 derived chondrocytes (NC-CHOND). Scale bar 100 µm. Bottom, qRT-PCR showing expression levels of specific chondrocyte marker Aggrecan (*ACAN*). The relative mRNA level was normalized to the housekeeping gene *PBGD.* Results are presented as mean ± s.d. of 3 independent experiments. **P < 0.01, two-sided Student's T-test

F) Bright field picture of representative NC P7 derived adipocytes (NC-ADIPO). Scale bar 50 µm. Bottom, qRT-PCR showing expression levels of specific adipocyte marker *PPARG*. The relative mRNA level was normalized to the housekeeping gene *PBGD*. Results are presented as mean ± s.d. of 3 independent experiments. ***P < 0.001, two-sided Student's T-test

G) Top panel: Immunocytochemistry for Osteocalcin and COL1A1 in osteocytes derived from NC P7 (NC-OSTEO). Scale bar 20 µm. Bottom panel: qRT-PCR showing expression levels of the specific osteocyte genes Sclerostin (*SOST*) and Collagen1A1 (*COL1A1*). The relative mRNA level was normalized to the housekeeping gene *PBGD*. Results are presented as mean ± s.d. of 3 independent experiments. ***P < 0.001, two-sided Student's T-test

H) Quantitative RT-PCR showing expression levels of the specific SMC genes *MYH11*, *MYOCD*, *TAGLN, SMTN-B* and *ACTA2*, following SMC differentiation from NC-P7. The relative mRNA level was normalized to the housekeeping gene *PBGD.* Results are presented as mean ± s.d. of 3 independent experiments. *P < 0.05; **P < 0.01, two-sided Student's T-test.

I) Quantitative RT-PCR expression analysis of NC and SMC markers at different passages. Top graph: Expression analysis of specific NC genes (*P75, TFAP2A* and *TWIST*) measured by qRT-PCR. The relative mRNA level was normalized to the housekeeping gene *PBGD*. The results are presented as mean ± s.d. of 3 independent experiments. *P < 0.05; **P < 0.01, ***P < 0.001. Two-sided Student's T-test. NC P1: NC differentiated from HPSC passage 1; NC P9: NC differentiated from HPSC passage 9; NC P7+2; NC differentiated from HPSC passage 7 when they were frozen. Those cells were thawed and passaged twice in FSB media before harvesting
Bottom graph: Expression level analysis of specific SMC genes (*ACTA2, CNN1, TAGLN, MYH11*) measured by qRT-PCR. The relative mRNA level was normalized to the housekeeping gene *PBGD.* The results are presented as mean ± s.d. of 3 independent experiments. **P < 0.01, ***P < 0.001. Two-sided Student's T-test. NC P9; NC differentiated from HPSC passage 9; NC P9-SMC: SMC derived from NC P9; NC P7+2-SMC: SMC derived from NC cells that were frozen down at P7, thawed and passaged twice in FSB media before commencing SMC differentiation.

J) Viability assay graph showing the percentage of viable NC P2 and P7 after thaw and culture for 3-hours in FSB media.

Supplementary Figure 5

TCOF1 siRNA transient transfection in NC and MSC downregulates Treacle protein

A) Quantitative RT-PCR showing the expression levels of TCOF1 in HESC-derived NC cells transfected with either TCOF1 siRNA or Scrambled siRNA (left). qRT-PCR showing the expression levels of TCOF1 in wild-type NC-derived mesenchymal stem cells (MSC) transfected with TCOF1 siRNA or scramble siRNA (right). The relative mRNA level was normalized to the housekeeping

gene *PBGD.* The results are presented as mean ± s.d. of 3 independent experiments. **P < 0.01, two-sided Student's T-test.

B) Histogram of the percentage of Treacle positive cells determined by flow cytometry analysis in HESC-derived NC cells transfected with TCOF1 siRNA or scrambled siRNA. Red histograms represent Treacle staining, blue histograms represent IgG isotype controls.

C) Immunocytochemistry for Treacle protein (TCOF1) in HESC-derived NC transfected with either TCOF1 siRNA or Scramble siRNA. IgG is shown as a negative control. Scale bar 20 µm.

Supplementary Figure 6

$TCOF1^{+/-}$ HIPSC-derived MSC show a defect in cell migration

A) Representative images of scratch wound assay performed in $TCOF1^{+/+}$ NC derived from H9s (WT), HIPSC (C8) and NC derived from $TCOF1^{+/-}$ HIPSC (C24). Pictures were obtained at the indicated time points. Scale bar 100 µm.

B) Representative images of scratch assays carried out in MSCs derived from NC $TCOF1^{+/+}$ (WT, C8) and NC $TCOF1^{+/-}$ (C24). Pictures were obtained at the indicated time points. Scale bar 100 µm.

Supplementary Table 1

Quantitative RT-PCR Primers used to measure the expression levels of the genes listed in the table

Supplementary Table 2

Antibodies used in immunofluorescence, western blot and flow cytometry assays