A novel human pluripotent stem cell-derived neural crest model of Treacher Collins Syndrome shows defects in cell death and migration

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Running title: Neural Crest Derivation and TCS.

Footnotes and abbreviations:
HPSC: human pluripotent stem cells, HIPSC: human induced pluripotent stem cells, HESC: human embryonic stem cells, MSC: mesenchymal stem cells, NC: neural crest,
NC-SMC: neural crest smooth muscle cells, TCS: Treacher Collins Syndrome.

ABSTRACT

The neural crest (NC) is a transient, multipotent cell population present during embryonic development. The NC can give rise to multiple cell types and is involved in a number of different diseases. Therefore, the development of new strategies to model NC in vitro enables investigations into the mechanisms involved in NC development and disease. Here we report a simple and efficient protocol to differentiate human pluripotent stem cells (HPSC) into NC using a chemically defined media, with basic fibroblast growth factor (FGF2) and the transforming growth factor-β inhibitor SB-431542. The cell population generated expresses a range of NC markers including P75, TWIST1, SOX10 and TFAP2A. NC purification was achieved in vitro through serial passaging of the population, recreating the developmental stages of NC differentiation. The generated NC cells are highly proliferative, capable of differentiating to their derivatives in vitro and engraft in vivo to NC specific locations. In addition, these cells could be frozen for storage and thawed with no loss of NC properties, nor the ability to generate cellular derivatives. We assessed the potential of the derived NC population to model the neurocristopathy, Treacher Collins Syndrome (TCS), by using siRNA knockdown of TCOF1 and by creating different $TCOF1^{+/-}$ HPSC lines via CRISPR/Cas9 technology. The NC cells derived from $TCOF1^{+/-}$ HPSCs recapitulate the phenotype of the reported Treacher Collins Syndrome murine model. We also report for the first time an impairment of migration in $TCOF1^{+/-}$ NC and mesenchymal stem cells. In conclusion, the developed protocol permits the generation of the large number of NC cells required for developmental studies, disease modeling, and for drug discovery platforms in vitro.

INTRODUCTION

In mammalian development, neural crest (NC) cells are a transient multipotent population arising from the neural plate border, usually contemporaneously with neural tube closure [1]. These migratory cells invade adjacent tissues and differentiate into multiple cell types including mesenchymal stem cells (MSC), vascular smooth muscle cells (SMC), adipocytes, osteocytes, chondrocytes, melanocytes, glia, and Schwann cells [2]. NC cells are also implicated in a broad range of pathologies making the in vitro generation of these cells of high clinical relevance.

The development and function of the NC have been well characterized in animal models including the chick, zebrafish, and mouse [3-6]. NC specification and induction depends on signaling molecules and transcription factors, whose actions are coordinated during gastrulation and neurulation. The neural plate border is specified by the cross talk between BMP, WNT and FGF signaling [7]. Initially, intermediate levels of BMP signaling induce neural folds with anterior character over the whole neural plate border. Next, the posterior regions of the neural plate border are transformed into NC under the effects of FGF, WNT and retinoic acid [8]. After specification, NC cells undergo epithelial-mesenchymal transition (EMT) before delaminating from the border of the neuroepithelium [9]. Subsequently the arising NC cells migrate in response to specific cues and express specific proteins including Twist [10], Sox10 [3,11], and p75 [12]. These factors control cellular events such as delamination, cell proliferation, migration, and differentiation [13-15]. Defects in the complex processes that choreograph NC development are involved in congenital human diseases known as neurocristopathies.

Human embryonic stem cells (HESC) and human induced pluripotent stem cells (HiPSC) are collectively referred to as human pluripotent stem cells (HPSC). Some of the most relevant NC differentiation protocols from HPSC are based on initially producing in vitro neuroectoderm [16-18] and then purifying the nascent NC population using different approaches such as dual SMAD inhibition [18-21] and/or WNT pathway activation [22-24]. A common limitation of these approaches is the use of undefined basement membrane extracts as a coating for cell adhesion, while only a few protocols have so far been validated in vivo [21,24-26]. Further optimization and validation of NC in vitro differentiation protocols would be valuable for studies into human development and disease.

Treacher Collins Syndrome (TCS) [27,28] (OMIM: 154500) is a neurocristopathy resulting in a severe congenital craniofacial disorder which occurs one in every 50,000 births [29]. TCS is primarily associated with autosomal dominant haploinsufficiency-inducing mutations in the *TCOF1* gene located on chromosome 5 [30], which leads to deficient ribosome biogenesis [31]. *Tcof1* is expressed broadly throughout the mouse embryo, with high activity in the neuroepithelium where it plays an essential role in cell survival [32]. Extensive apoptosis of the neuroepithelial progenitor has been reported in TCS, resulting in impaired NC differentiation and subsequent defects in craniofacial development [32].

In this study, we report a complete differentiation protocol using simple conditions that permits the generation of the NC from HPSCs using a combination of FGF signaling and TGF-β inhibition. Derived NC cells are proliferative, can be maintained over multiple passages, can differentiate to a variety of cell types in vitro and have been validated in a developmental chick embryo model. Furthermore, we have utilized CRISPR/Cas9 technology to generate *TCOF1*$^{+/-}$ lines as a model of Treacher Collins Syndrome, and have revealed abnormalities in cell migration, which may play a role in the underlying pathology of the disease. The development of this protocol permits the generation of NC and its derivatives in a chemically-defined media for developmental studies, disease modeling, and drug discovery.

MATERIALS AND METHODS

HPSC culture

Human embryonic stem cells (HESC; H9s line, Wicell, Madison,WI) and human induced pluripotent stem cells (HIPSC; BBHX8 line [33], Cambridge Biomedical Research Centre HIPSC core facility) called together human pluripotent stem cells (HPSC) were maintained with chemically defined media (CDM), plus bovine serum albumin fraction A (CDM-BSA) as previously described [34]. CDM-BSA comprised of Iscove's modified Dulbecco's medium (ThermoFisher Scientific) plus Ham's F12 NUT-MIX (ThermoFisher Scientific) medium in a 1:1 ratio, supplemented with Glutamax-I (ThermoFisher Scientific), BSA (5mg/ml; Europa Bioproducts), chemically defined lipid concentrate (ThermoFisher Scientific), transferrin (15 µg/ml, Roche Diagnostics), insulin (7 µg/ml, Roche Diagnostics) and monothioglycerol (450µM, Sigma). For the maintenance of HPSCs, CDM-BSA was supplemented with Activin A (10 ng/ml, R&D Systems) and FGF2 (12 ng/ml, R&D Systems), and cells were maintained on tissue culture treated plastic coated with 0.1% gelatin (Sigma Aldrich).

HPSC were also cultured and maintained in TeSR™-E8™ media (STEMCELL Technologies) using Vitronectin XF (STEMCELL Technologies) as chemically defined xeno-free cell culture matrix.

HPSC Differentiation to Neural Crest (NC)

For NC differentiation, HPSC were detached from gelatin-coated plates using 1 mg/ml collagenase IV (Gibco). Clumps were triturated, counted and plated at a density of 300 colonies/well in 0.1% gelatin-coated 6 well plates in CDM-BSA supplemented with Activin A (10 ng/ml, R&D Systems) and FGF2 (12 ng/ml, R&D Systems), referred to herein as CDM-BSA+FA.

After 24 hours in CDM-BSA, the media was changed to CDM-PVA supplemented with FGF2 (12ng/ml R&D Systems) and SB-431542 (10µM, Tocris), referred to herein as FSB, for four days without splitting. CDM-PVA has the same composition as CDM-BSA, with polyvinyl alcohol (PVA, 1 mg/ml, Sigma) instead of BSA. On day 4 in FSB, the differentiating HPSC were dissociated using TrypLE Express™ (Gibco) and seeded as single cells at a 1:3 ratio on 0.1% gelatin-coated plates in FSB. The single cells were maintained in FSB (with daily media changes) on 0.1% gelatin-coated plates over several passages, the splitting of those single cells was performed every 3-4 days (80-90% confluence) at 1:3 ratio using TrypLE Express™ (Gibco).

For differentiating HPSC grown on Vitronectin XF and maintained with TeSR™-E8™, the HPSC colonies were detached using 0.5mM EDTA (Gibco). Clumps were counted and seeded at a density of 300 colonies/well in vitronectin XF-coated 6 well plates in TeSR™-E8™ media. After 24 hours in TeSR™-E8™, the media was changed to FSB and cells were maintained in FSB on vitronectin XF without splitting for 4-days. At day 4 in FSB, the differentiating HPSC were dissociated with TrypLE Express™, seeded as single cells on 0.1% gelatin-coated plates and split every 3-4 days as described above.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was synthesised from 250 ng total RNA using the Maxima First Strand cDNA Synthesis kit (ThermoFisher Scientific). qRT-PCR reaction mixtures were prepared with the FAST-SYBR Green Master Mix (ThermoFisher Scientific) and analysed on a 7500 Fast Real-time PCR system (ThermoFisher Scientific). C_T values were normalised to porphobilinogen deaminase (PBGD). Primer sequences are listed in Supplementary Table 1.

Flow cytometry

Cells were fixed with Cytofix/Cytoperm Fixation solution (BD Biosciences) for 20 min at 4^{o}C, then washed with Perm Wash Buffer/PBS (1X, BD Biosciences) and permeabilized with Perm Wash Buffer/PBS + 0.1% Triton-X100 for 30 minutes. Cells were blocked with 3% BSA in 1X Perm Wash Buffer at room temp for 30 minutes. After blocking, cells were incubated in primary antibody (Supplementary Table 2) diluted in 1X Perm Wash Buffer + 0.1% Triton-X100 4^{o}C for 45 minutes. Alexa Fluor®-tagged secondary antibody was added after primary incubation for 1 hour at room temperature. Samples were run on a Beckman Coulter CyAn-ADP flow cytometer, and subsequent datasets were analysed using FlowJo software.

Immunocytochemistry

Adherent cells were fixed using 4% PFA, permeabilised with 0.5% Triton-X100 in PBS (Sigma) and blocked with PBS + 3% BSA for 60 minutes at room temperature (RT). Primary antibody (Supplementary Table 2) incubations were performed at 4^{o}C overnight and Alexa Fluor® tagged secondary antibodies applied for 45 minutes at RT the following day. Nuclei were counterstained with DAPI (0.1 µg/ml, Sigma). Images were acquired on a Zeiss LSM 700 confocal microscope and analysed with ImageJ software.

Western Blotting

Cells were lysed in RIPA buffer containing phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (Sigma), on ice for 15 minutes and protein content was quantified using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific). 10µg of protein per sample was resolved by electrophoresis and transferred to PVDF membranes. Membranes were blocked for 1 hour at room temperature with 5% milk in Tris-Buffered Saline containing 0.1% Tween-20 (TBS-t, Sigma), and incubated overnight with either anti-TCOF1 (1:1,000 Abnova), or anti-beta actin (1:10,000 Sigma). Membranes were washed with TBS-t and incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Membranes were washed and developed using the Pierce ECL2 Western Blotting Substrate (ThermoFisher Scientific).

Migration assays

Neural crest and mesenchymal stem cells were plated onto six-well plates and allowed to form a confluent monolayer. The cell monolayer was then scratched in a straight line to make a 'scratch wound' with a 1-ml pipette tip and the cell debris was removed by washing the cells with phosphate-buffered saline. Cells were maintained in FSB (for NC cells) or DMEM + 10% FBS (for MSC), and images of the closure of the scratch were captured at different time points as indicated.

A chemotaxis assay was performed using the CytoSelect™ 24-Well Cell Migration and Invasion Assay Combo Kit (Cell Biolabs) following the manufacturer's instructions. Briefly, 5x10^{5} cells were plated in a pre-warmed 24-well migration plate in FSB media. The chemo-attractant FGF8B (30 ng/ml, R&D Biosystems) was added separately to FSB media. Cell media was added to the lower well of the migration plate. FSB media was used as a control. Cells were incubated for 8 hours at 37 degrees and quantification was performed following the manufacturer's instructions.

For single cell analysis of cell migration, cells were imaged real-time on an In-Cell Analyser 2200 (GE Healthcare Life Sciences) with images collected every 30 minutes for a 12-hour period. Cells were tracked using the Pointing Cell Tracking plugin for ImageJ (https://imagej.nih.gov/ij/plugins/pointing-cell-tracking/index.html) and migratory profiles were generated using the freely available Chemotaxis and Migration Tool from Ibidi (ibidi.com/chemotaxis-analysis/171-chemotaxis-and-migration-tool.html).

Microarray Hybridization and Analysis

RNAs isolated from H9s HESC, neuroectoderm [34] and NC passage 2 and 7 (NC P2, NC P7) were hybridized with Illumina Human HT-12 BeadChip (Illumina Inc., San Diego, http://www.illumina.com). All the data processing and analysis were performed using the algorithms included with the Bioconductor package beadarray and *Lumi* implemented in R software environment for statistical computing and graphics (R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org).

Microinjection of HPSC-derived NC cells and HPSC-derived endoderm cells in chicken embryos

For injections into the cardiac neural crest pre-migratory region, chicken (*Gallus domesticus*) eggs (Winter Egg Farm, Cambridge, UK) were incubated in a digital cabinet incubator (OVA Easy 380, Brinsea) for 32 hours until Hamburger-Hamilton stage 9-10 (HH9-10). Eggs were windowed and injected under the embryo with India Ink to improve contrast. Small cuts were made with a BD Microlance needle (size 3) through the vitelline membrane and ectoderm directly adjacent to the neural tube, at a level just posterior to the forming otic vesicle. Clumps of 50-100 cells in Matrigel were injected into the cut site used a pulled glass capillary tube. Eggs were re-sealed with tape and cultured a further 20, 42 and 108 hours after injection to visualise migrating neural crest cells at HH16, HH17, and HH26 respectively. HPSC-derived endoderm cells were used as a negative control and generated as previously reported [35].

For systemic injections into developing chicken embryo, eggs were incubated until HH24. A small window was made, and 500-1000 NC cells were administered into the extraembryonic vessels. Either GFP^+ HPSC-derived NC cells or GFP^+ HPSC-derived endoderm cells were administered as previously reported [16] [36]. The window was covered with parafilm (VWR), and eggs were placed horizontally in the incubator until HH34. Embryos were staining using whole mount immunocytochemistry as previously described [36].

Differentiation of the NC to various cell types

NC populations were differentiated to smooth muscle cells (SMCs) using a combination of PDGF-BB (10ng/ml, Peprotech) and TGF-β1 (2ng/ml, Peprotech), as previously reported [37]. NC differentiation to neuronal populations and mesenchymal stem cells (MSCs) was performed as previously reported [21]. NC differentiation to melanocytes was performed as previously described [38]. MSC populations were subsequently differentiated to chondrocytes, adipocytes, and osteocytes using the StemPro Chondrogenesis Differentiation Kit (ThermoFisher Scientific), StemPro Adipogenesis Differentiation kit (ThermoFisher Scientific), and StemPro Osteogenesis Differentiation Kit (ThermoFisher Scientific), respectively, following the manufacturer's instructions.

Cell Proliferation assay

To assess cell proliferation, the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT, Promega) kit was used, as per the manufacturer's instructions.

Freezing and thawing of neural crest cells

Confluent NC cells were dissociated with TrypLE Express™ (ThermoFisher Scientific). NC cells were pelleted and resuspended in CDM-PVA media. $2X10^6$ cells were added to a cryovial using 90% CDM-PVA media with 10% DMSO. NC cryovials were thawed at 37 degrees, and cells transferred to a 15 ml Falcon tube containing 8 ml of fresh CDM-PVA media. Cells were centrifuged for 3 minutes at 1200 rpm. Cell pellets were resuspended in 1 ml FSB media, and 3 $x10^5$ NC cells were plated per well of a 0.1% gelatin-coated well of a 6 well plate. Cells were incubated at 37 degrees overnight in a 5% CO_2 incubator. Cell viability was assessed as previously reported [39].

siRNA knockdown and transient transfections

TCOF1 mRNA was knocked down with siRNA (ThermoFisher Scientific Assay ID S13920). siRNA transfection (25nM) was performed using DharmaFECT-1 transfection reagent (Dharmacon), following the manufacturer's instructions.

Generation of a TCOF1-targeting CRISPR guide RNA/Cas9 Construct

A gRNA targeting the TCOF1 gene was designed to target Exon1 according to the rule of 5'-GN20NGG-3' (sequence 5'-TGGCTATGTGCGTGCGGCGC-3'). Oligonucleotides were synthesized and ligated into pSpCas9(BB)-2A-Puro (PX459) V2.0 as previously reported [40].

Gene Targeting

For gene targeting, $2.5X10^6$ HIPSC were electroporated with one μg of generated TCOF1 targeting Cas9 plasmid in 100ul of nucleofection mix from the P3 Primary Cell 4D-Nucleofector X Kit (Lonza) using a 4D-nucleofector system (Lonza). Transfected cells were plated onto DR4 strain feeders (Jackson Laboratory) and cultured in advanced DMEM/F12 (Gibco) + 20% KOSR supplemented with FGF2 (4ng/ml) and Rho Kinase inhibitor (Y-27632, 10μM). One day after transfection, cells were selected with puromycin (1μg/ml, Sigma) for 36 hours. Resistant colonies were picked, expanded, and mutation introduction was assessed by PCR and Sanger sequencing.

Statistics

One-way ANOVA (Tukey's multiple comparisons test) and two-sided Student's t-test were used to determine statistically significant differences between the groups. Results are presented as mean ± s.e.m. P values ≤ 0.05 were considered statistically significant. All experiments represent the results of at least three independent biological replicates (measurements of biologically distinct samples). *P< 0.05; **P < 0.01; ***P < 0.001.

RESULTS

Dissociation of Differentiating Neuroectoderm During Early Development Promotes NC Differentiation.

It has been demonstrated that neuroectoderm can be generated from HPSCs utilizing a combination of FGF2 and the TGF-β inhibitor SB-431542 (referred to herein as FSB) for seven days [16]. Our group

has previously reported the expression of some NC markers during this differentiation process, including *SNAI1*, *SNAI2*, and *PAX3*, suggesting that a mixed cell population may be generated at these stages [37]. We hypothesized that cell dissociation with trypsin, a serine protease, might facilitate EMT and the generation of the NC from this population [41-45]. We, therefore, differentiated the H9s HESC line to neuroectoderm over seven days with FSB media, with and without WNT3A (25ng/ml, R&D Systems). At day 7 of differentiation, the neuroectoderm was trypsinized and replated as single cells in CDM-PVA media supplemented with FGF2 and SB-431542 (FSB) +/- WNT3A for up to 5 passages. Quantitative RT-PCR over this period showed increased expression of NC differentiation-associated genes (*PAX3, ZIC1, CD49,* and *SOX9*) and the mesenchymal gene (*VIM*) (Fig.1A). Conversely, neuroectoderm genes (*OLIG3, PAX6*) and an epithelial gene (*CDH1*) were downregulated (Fig.1A), suggesting that cell dissociation promoted NC marker expression and EMT. Interestingly, the addition of WNT3A did not have any effect on the expression of these markers during the differentiation process (Fig.1A).

To further analyze the onset of NC markers during the differentiation of HESC to neuroectoderm over the first seven days, we assessed the expression of NC (*HNK1*, *P75*) and neuroectoderm (*PAX6*) genes on a daily basis. We observed the greatest expression of *HNK1* and P*75* on day 4 of the differentiation process (Supplementary Fig.1A). Therefore, we hypothesized that dissociation of the developing neuroectoderm at day 4 instead of day 7, may increase the efficiency of NC generation during the differentiation process. We analyzed SOX1 and P75 expression by flow cytometry in passaged and non-passaged neuroectoderm at day 4 or day 7 of differentiation in FSB. SOX1 is the earliest known specific marker of the neuroectoderm lineage and is activated during gastrulation [46]. Neuroectoderm passaged at day 4 (D4 P1) demonstrated a higher induction of P75 and lower expression of SOX1 when compared with non-passaged cells or cells split at day 7 (Fig.1B). Furthermore, the cells split at day 4 attached and survived better than the cells split at day 7 (data not shown). Consistent with these results, TFAP2A, an essential transcription factor for the development of the NC [47,48] was highly expressed in the D4 P1 population (Fig.1C). SOX1 was detected at the edges of the neuroectoderm at day 4 and day 7 of differentiation in FSB by immunocytochemistry (Fig.1D). Following passage at day 4, SOX1 expression was significantly reduced. Conversely, SOX1 positive cells remained if passaging was delayed until day 7 (Fig.1D), suggesting that the greatest reduction of SOX1 was obtained by passaging the differentiating neuroectoderm in FSB on day 4. Furthermore, HNK1 was highly expressed in passaged neuroectoderm at day 4 (Fig.1D) and qRT-PCR analyses confirmed these cells showed significantly higher levels of NC marker *TFAP2A* and lower levels of neuroectoderm marker *GBX2* [49] than cells from day 7 passaged neuroectoderm (Fig.1E).

Together, these findings suggest that differentiation of neuroectoderm from HPSCS can generate a mixed population containing both neuroectoderm and NC cells. The NC population can be enriched from the differentiating neuroectoderm by passaging at day 4 of the protocol.

Serial Passage Increases Purity of NC Cells

Following passaging at day 4, cells were maintained in FSB medium upon reaching confluence (NC P1). Every four days, the confluent NC cells were split at a 1:3 ratio and seeded for the next passage (Supplementary Fig.1B). After two passages cells expressed the NC proteins SOX9, HNK1, and P75, and did not express the neuroectoderm marker SOX1 (Fig.2A). We detected expression of the NC markers

P75 and TFAP2A by flow cytometry following subsequent passaging (Fig.2B and C) [21]. While over 80% of the early NC population expressed P75, only 50% of the population was double positive for both P75 and TFAP2A at this stage (Fig.2C). Furthermore, SOX10, a marker of migratory NC, was induced at D4 of (Fig.2D). These data confirmed that we were generating NC at this time point of the differentiation process, although the yield was suboptimal.

We questioned whether the differentiated NC would retain their NC-like identity following further expansion. Interestingly, the expression levels of the NC markers *PAX3* and *ZIC1*, markers of early NC induction [50,51], peaked at passage 3 (P3). In contrast, *SOX9* which is an essential factor for the further development of migratory NC [52] was observed to be highly expressed at passage 8 (P8) (Fig.2E top). The expression levels of the neural crest markers *P75, TFAP2A,* and *TWIST1* increased significantly upon serial passaging (P1, P7, and P13), as assessed by qRT-PCR (Fig.2E bottom), suggesting that this approach could purify the NC population.

To further confirm the NC cells retain their NC identity and show purification with passaging, we used flow cytometry to examine the expression of NC and non-NC markers. Furthermore, we repeated the flow analysis of P75 and TFAP2A in late passages of NC (P12) and observed that 99% of the cells expressed P75, and 69% showed both P75 and TFAP2A (Fig.2G). Similar results were also obtained using HIPSC to generate NC cells (Supplementary Fig.2A-C). To assess if the purified NC population contained additional cell populations, we measured the expression of endoderm (*SOX17* and *EOMES*), mesoderm, (*NKX2.5* and *KDR*), and neuronal (*TUBB3* and *MAP2*) genes in the populations (Supplementary Fig.2E). We observed negligible expression of these genes in the NC populations when compared to HPSC-derived endoderm, mesoderm, or neurons, demonstrating that the NC did not contain a mixture of these populations in culture. Taken together, these data suggest that NC purification may be achieved by serial passaging of NC cells in FSB media. Furthermore, we found we could expand the NC cells up to passage 15 without losing their NC identity [53].

NC Cells are proliferative and Migrate in Response to Specific Chemo-Attractants.

To assess whether passage number or cell density affects the proliferation rate of the differentiated NC, we performed sequential cell counting and an MTT assay at different NC passages (Supplementary Fig.2C, D). The MTT Cell Proliferation Assay is used to calculate the cell proliferation rate. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by active cells, to purple formazan that can be solubilized and quantified by spectrophotometric means.

We found that the proliferation rate of NC was independent of passage number (Supplementary Fig.2C) or cell density (Supplementary Fig.2D). In vivo, NC cells migrate over great distances in response to chemotactic cues to contribute to tissue development during embryogenesis [54]. To validate a similar migratory function in our cells, we performed an in vitro scratch assay. NC cells were able to migrate and cover the scratch within 24 hours (Fig.2F). FGF8 is chemotactic and chemokinetic for NC in vivo and in vitro [19,54]. We, therefore, examined whether the HESC-derived NC cells would respond to this cue using the CytoSelect 24-well Cell Migration Assay (8 µm). In response to FGF8B, NC migration was significantly greater than in FSB media alone (Fig.2H). Overall, these data demonstrate that the HESC-NC cells recapitulate critical NC functions.

Early Passages of HESC-NC Comprise a Mixed Population of Neural Progenitors and Pre-Migratory NC Cells that is purified with Serial Passage.

To uncover the differences between early and later passages of NC, we performed a microarray gene expression analysis using H9s HESC and three independent differentiations of H9s HESC to neuroectoderm [34], NC at passage 2 (P2) and NC at passage 7 (P7). Hierarchical clustering separated these populations (Fig.3A). Interestingly, PCA analysis distributed NC P2 in between neuroectoderm and NC P7 population (Fig.3B). Based on this, we hypothesized that the early NC population could be a mixture of neuroectoderm and NC cells.

The NC P2 population expressed markers of neural progenitors including *AXIN2* [55], *FGFR3* [56] and *NOTCH3* [57] (Fig.3C). These genes however, were markedly downregulated at later passages (Fig.3C). Furthermore, the NC P2 population expressed markers of neural plate border / pre-migratory NC cells including *CDH2* [58], *CDH6* [59], *FOXD3* [60,61], *BMP7,* and *RHOB* [9,62] (Fig.3C). These neural plate border genes are thought to promote conversion of pre-migratory NC P2 to migratory cells as they also expressed migratory markers such as *B3GAT1* (HNK1) [63,64] in this mixed population (Fig.3C).

As the WNT signaling pathway has been identified as playing important roles in NC development in vivo [23,65-67], we wondered why exogenous WNT3A was not required in our in vitro differentiation system. Interestingly, genes related to activation of WNT signaling, such as *WNT1, FZD3, DVL2 [68],* and *MSX1 [69]*, were significantly expressed in the early NC P2 population (Fig.3C). These data suggest there may be endogenous activation of WNT during the early stages of NC differentiation, as observed in the neural plate border intermediate cells in vivo [69] and in vitro [23]. Interestingly, it has been proposed that NC induction requires intermediate levels of BMP signaling [70], as part of a BMP gradient between epidermal ectoderm which expresses both BMP7 and BMP4 [71] and the neural plate. BMP7 induces the expression of the RHOB protein in the cells destined to become NC in the neural plate border [9,62]. Consistent with developmental NC induction, *BMP7* and *RHOB* were highly upregulated in the early NC P2 population compared with NC P7. In contrast, the NC P7 population expressed higher levels of migratory NC genes such as *SOX10, SOX9, TWIST1* and *P75* [52,58,72,73] when compared to the early NC P2 population (Fig.3C).

Finally, we plotted gene expression differences in the NC pathway using an open source pathway archive (WikiPathway WP2064 revision 47071). Most genes upregulated at NC P2 corresponded to genes expressed in premigratory NC cells (Fig.3D). This population also showed some migratory NC genes, suggesting a mixed population. However, genes upregulated in the NC P7 population corresponded for the most part with genes expressed in the migratory NC (Fig.3D). These data revealed evidence of endogenous WNT and BMP activity in the NC P2 population. In these early passages, a mixed NC population could be purified in vitro after several further passages to express a migratory NC transcriptional profile by passage 7.

HESC-Derived NC Cells Survive, Engraft, Migrate and Differentiate In Vivo at NC Specific Location Within the Ascending Aorta and the Brain.

NC cells should engraft in the appropriate locations for NC derivatives. In avian embryos, the fate of the neural crest is well established. The removal of the dorsal neural tube between the otic vesicle and

the third somite in chicken embryos results in a variety of defects of the derivatives of the arch arteries [74]. Chicken embryos have previously been used as a host for studying the differentiation potential of human stem cells [36,75,76]. To demonstrate that the NC cells have the potential to migrate and differentiate to their specific locations in vivo, and that freeze/thaw cycles do not affect the potential of the NC cells, we performed several experiments engrafting or injecting NC GFP+ cells into chicken developing embryos.

Fluorescent GFP+ HESC-derived NC P1 cells were thawed, harvested and passed to NC P2 cells. Those cells were split, counted and embedded into Matrigel for chicken embryo engraftment. Clumps of 50-100 cells were engrafted between the otic vesicle and the third somite, adjacent to the neural tube at Hamburger and Hamilton (HH) stage 9-10. Some embryos were harvested 20 and 42 hours post engraftment (HH16-17) to monitor the success of the engraftment. Observation under an inverted epifluorescent microscope showed they exhibited a lump of GFP+ cells in the region of the otic vesicle. Furthermore, migrating GFP+ cells were detected in all the embryos (Fig.4A and B). 4.5 days post engraftment (HH29), whole-mount confocal immunofluorescence imaging of the developing heart and ascending aorta was performed. A significant number of GFP+ cells were found in the ascending aorta expressing ACTA2 (Fig.4C). Furthermore, GFP+ cells were not found in the aorta following engraftment of GFP+ HESC-derived endoderm embedded into Matrigel. Additionally, we could not detect any NC-derived GFP+ cells in the epicardium or myocardium (Fig.4C). Taken together, this data demonstrates that the engrafted human NC cells can migrate into the ascending aorta and contribute to the SMC population within the chicken embryo.

We also utilized an alternative method that we have previously validated for using chicken embryos as a host to study the differentiation and integration of HESC-derived cells [36]. GFP+ NC cells were injected (500-1000 cells) into the extraembryonic circulation of chicken embryos at HH24. Embryos were harvested at HH34 and whole-mount confocal immunofluorescence was used for imaging of the brain, ascending aorta and heart. We detected 10-20 GFP+ NC cells per embryo, around the ascending aorta (Supplementary Fig.3A-F) and 40-50 GFP+ NC cells per embryo associated with the cerebral cortex vasculature (Supplementary Fig.3G-J). We could not detect any human NC cells in the subepicardium either the myocardium (Supplementary Fig.3K), areas we have previously seen the localization of HESC-derived epicardial cells [36]. HESC-derived NC cells could be clearly discriminated from the host cells by their cell size, high green fluorescence (Supplementary Fig.3A, D, G) and distinct nuclei (Supplementary Fig.3B, E, H). Some of the engrafted NC cells around the ascending aorta also expressed ACTA2, suggesting the onset of EMT and SMC differentiation in situ within the ascending aorta (Supplementary Fig.3C). Together, these results suggest that HESC-derived NC cells have access to ascending aorta in vivo and have the potential to contribute to tissue development in this location.

In Vitro Differentiation of NC to a Variety of Cell Types

NC cells can differentiate in vivo into a wide range of cell types such as neurons, melanocytes [77-79] mesenchymal stem cells (MSC) [80], adipocytes [81], chondrocytes, osteocytes [82,83] and vascular SMC [84]. We, therefore, followed a wide range of established differentiation protocols to confirm that our purified NC cells (NC P7 and further passages) were able to differentiate into their known derivatives.

To assess the capability of the NC to differentiate into neurons, we plated cells on polyornithine-laminin-coated culture dishes [21]. After ten days of differentiation, these cells spontaneously differentiated into beta III Tubulin^{+} neurons (Fig.5A). Neuronal morphology was confirmed by microscopy (Supplementary Fig.4B). Differentiation to SMC from NC was performed following our previous protocol [34]. SMC proteins such as ACTA2, SM22A, and CNN1 were detected by immunocytochemistry at twelve days of differentiation (Fig.5B). *ACTA2, TAGLN* and other SMC markers such as *MYH11, SMTN-B* and *MYOCD* were also upregulated in differentiated SMC as assessed by qRT-PCR (Supplementary Fig.4H).

Melanocyte differentiation was achieved following a previously reported protocol [38]. qRT-PCR assessed differentiation for melanocyte markers *KIT* and *MITF* [38] and NC markers such as *P75* and *FOXD3* (Fig.5C). NC-derived melanocytes showed statistically significant upregulation of *KIT* and *MITF* expression and a marked downregulation of *P75* and *FOXD3*. Typical melanocyte morphology was confirmed by microscopy (Supplementary Fig.4C). Mesenchymal stem cells (MSC) were derived from the NC using a previously reported protocol [21]. Differentiated MSC were positive for CD44 and negative for P75 as assessed by flow cytometry (Fig.5D), and expressed the MSC marker *CD105 (ENG)* by qRT-PCR (Supplementary Fig.4D) [85]. Chondrocytes, adipocytes, and osteocytes were successfully differentiated from NC using specific commercial differentiation media, following the manufacturer's instructions (ThermoFisher Scientific). Chondrocyte differentiation was demonstrated by Alcian Blue-positive staining of NC-derived chondrocytes (Fig.5E). *ACAN* expression [86] also confirmed chondrocyte lineage (Supplementary Fig.4E). Adipocyte differentiation was determined by positive oil red O staining (Fig.5F) and *PPARG* [87] expression (Supplementary Fig.4F). Osteocyte differentiation was shown by positive alizarin red staining (Fig.5G). COL1A1 [88] and Osteocalcin [89] were detected by immunocytochemistry in osteocytes derived from NC (Supplementary Fig.4G, top panel). Furthermore, the expression levels of the osteocyte genes sclerostin (*SOST*) and *COL1A1* were measured by qRT-PCR, revealing robust upregulation in NC-derived osteocytes (Supplementary Fig.4G, bottom panel).

Finally, we examined the ability of these cells to function as NC cells after freeze-thawing. NC cells were frozen at passage 7 then defrosted and cultured for 2 further passages and retained their NC marker expression comparable to passage 9 NC cells that had not undergone freeze-thawing (Supplementary Fig.4I, top panel). Similarly, freeze-thawing had no effect on the ability of the NC cells to generate derivatives such as smooth muscle cells (Supplementary Fig.4I, bottom panel). Furthermore, viability assays performed in thawed NC P2 and NC P7 cells [39] shown a recovery of 89±6.5% and 92.5± 6.25 % respectively from frozen vials (Supplementary Fig.4J). The ability to freeze and store these cells is important for the practical utilization of these cells, which can now be bulked up for storage and use as required, without losing NC properties.

Down-Regulation of Treacle Expression by siRNA impairs cell migration in NC and MSC.

Haploinsufficiency of TCOF1 in humans is associated with TCS; a condition characterized by craniofacial abnormalities thought to be due to impaired NC development [90]. To determine whether TCOF1 haploinsufficiency results in NC defects in humans, we initially investigated its role in HPSC-derived NC by siRNA-mediated knockdown. Transfection of TCOF1-targeted siRNA reduced mRNA expression in both NC and MSC as assessed by qRT-PCR (Supplementary Fig.5A), and at the protein level in the NC

by flow cytometry (Supplementary Fig.5B). To investigate whether TCOF1 has any role in the differentiation of NC to MSC, we differentiated NC P7 cells transfected with siRNA against TCOF1 or Scramble siRNA to a MSC fate as previously reported [21]. We observed no difference in the capacity for MSC differentiation from TCOF1 knockdown NC with upregulation of CD44 and downregulation of P75 observed in both knock-down and control conditions (Fig.6A).

In mice, Treacle plays a role in NC development and proliferation [32]. We assessed if siRNA-mediated knockdown of TCOF1 impaired proliferation of HPSC-derived NC by performing an MTT assay. As in the mouse, we observed decreased proliferation in siRNA-mediated TCOF1 knockdown cells when compared controls (Fig.6B). It has previously been suggested that migration is not impaired in Tcof1$^{+/-}$ mice [32]. To assess the role played by TCOF1 in human NC, we performed a wound healing scratch assay following siRNA-mediated knockdown of TCOF1. We observed impaired migration and scratch closure in TCOF1 knockdown NC when compared to controls (Fig.6C). Furthermore, whilst the migratory defect in this assay could be attributed to the documented impaired proliferation (Fig.6B), the migration phenotype was detected as early as 6 hours post-scratch, suggesting that the phenotype was indeed due to impaired migration. We performed further validation of this migratory defect using a Cytoselect™ transwell cell migration assay (Fig.6D). siRNA-mediated knockdown of TCOF1 produced a significant reduction in NC migration compared with controls (Fig.6D). This migration impairment was observed in FSB medium alone and observed in response to different NC chemo-attractors such as FGF8B [19] or 10% fetal bovine serum (Fig.6D). NC-derived MSC transfected with siRNA against TCOF1 also showed a similar migratory defect in a wound healing scratch assay (Fig.6E). Taken together, these findings demonstrate that TCOF1 may play a role in both the proliferation and migration of the human NC.

Modeling TCS in Vitro with TCOF1$^{+/-}$ HIPSC using CRISPR/CAS9 technology.

To further confirm the results observed using TCOF1 siRNA in our NC model and to try to model TCS in vitro, we created heterozygous TCOF1$^{+/-}$ KO HIPSC lines using CRISPR/Cas9 technology to accurately target Exon 1 of the TCOF1 gene in wild-type HIPSC (Fig.7A). Cas9-induced double-strand breaks in TCOF1 genomic DNA were repaired by the non-homologous end joining pathway leading to insertions or deletions (INDEL) in different HIPSC clones (Fig.7B). HIPSC Clones 12 and 24 (C12 and C24) had the same single nucleotide deletion in Exon 1 as observed by Sanger sequencing. This removal created a premature stop codon, resulting in a heterozygous KO for TCOF1 (Fig.7B). HIPSC Clone 8 (C8) was also transfected, but no INDELs were detected in the TCOF1 gene (data not shown). We, therefore, used C8 as an isogenic wild-type HIPSC line to compare with the TCOF1$^{+/-}$ clones (C12 and C24). NC was generated from all three clones, and NC P7 cells were used for subsequent studies. Immunoblotting for Treacle confirmed a significant reduction of protein levels in NC derived from TCOF1$^{+/-}$ HIPSC clones (C12 and C24) compared with isogenic wild-type clone 8 NC and wild-type HESC-derived NC (Fig.7C).

The downstream effect of defective ribosomal biosynthesis in mutant Tcof1$^{+/-}$ mice is a lower proliferative index than wild-type NC cells [32]. TCOF1$^{+/-}$ NC derived from clones C12 and C24 had significantly lower proliferation rate than similar wild-type NC cells derived from the C8 line and WT HESC, validating the findings observed in the Tcof1$^{+/-}$ mouse (Fig.7D).

The underlying cause of the reported craniofacial anomalies in the Tcof1 $^{+/-}$ mice is increased apoptosis in the neuroepithelium [32]. This developmental stage overlaps with the period of NC induction and migration from the neural plate border during embryogenesis [27]. To investigate whether our in vitro TCS model recapitulated this phenotype, we differentiated WT and TCOF1$^{+/-}$ HIPSC lines to neuroectoderm [34]. Flow cytometric analyses for Annexin V confirmed a marked induction of apoptosis in neuroectoderm cells derived from TCOF1$^{+/-}$ HIPSC (C12 and C24) compared with neuroectoderm derived from WT HIPSC Clone 8 or parental WT HESC (Fig.7E).

As we unveiled a previously unreported migratory defect in NC and NC-derived MSC deficient for TCOF1 by siRNA (Fig.6C and 6E), we performed the same analysis on the generated TCOF1$^{+/-}$ lines. A wound healing scratch assay demonstrated impaired migration in both NC (Supplementary Fig.6A), and MSC (Supplementary Fig.6B) derived from TCOF1$^{+/-}$ HIPSC C24. To further investigate this migratory defect uncovered by the wound healing scratch assay, we performed single cell analysis of cell migration and directionality of movement using real-time imaging of cells over a 12-hour time course. This analysis demonstrated that whilst WT HIPSC Clone 8 derived NC cells migrated freely in multiple directions over a 12-hour period, TCOF1$^{+/-}$ HIPSC derived NC cell show considerable impairment of migration at a single cell level (Fig.7F). These findings further validate the reduced migration phenotype of TCOF1-deficient NC cells in the human context.

DISCUSSION

This study first describes a simple and efficient method of generating NC cells from HPSC using chemically defined media. Importantly, this in vitro system has great potential for investigating the molecular and cellular defects in neurocristopathies such as TCS. We have established a model of Treacher Collins Syndrome using CRISPR/Cas9 gene editing and identified novel NC and MSC migration defects in our in vitro model.

Development of a Differentiation Protocol to Generate NC from HPSCs using a Chemically Defined Media

Several methods have been reported to produce NC from HPSCs, although further work is required to allow the production of cells in a fully chemically defined media and to reduce the complexity of the differentiation conditions. The limitations of the previously reported protocols include the use of biological substrates such as Geltrex™ [21] or Matrigel™ [23] that display original batch to batch variability. Other protocols used media enriched with complex Supplements such as N2™ and B27™ [23,43].

We have demonstrated that we can generate NC cells from HPSC using minimal cytokines when compared with previous protocols [18-21] or WNT pathway activation [23]. This reduction of cytokines makes our protocol simpler and more economically viable. Furthermore, we do not need to perform cell sorting to generate our populations, as we have observed that serial passaging effectively purifies the populations generated. We have also demonstrated that our cells can undergo freeze-thawing with up to 90% recovery rates and viability, permitting these cells to be used to generate stocks of NC cells at the same passages (Supplementary Fig.4J). We have used thawed NC cells for multiple

differentiations and have not seen any alterations in their maintenance or differentiation capacity following thawing. Therefore, our protocol permits the generation of a high number of NC cells which can be banked and used at later dates.

The differentiation of NC from HPSCs using this approach recapitulates, to some extent, the normal embryonic developmental stages. In our protocol, the passage of neuroepithelial neuroectoderm at low density in FSB appeared to select the NC population in vitro. The genome-wide mRNA expression studies suggested that earlier passages of NC populations were a mixed population of neural progenitor cells and premigratory NC cells. Premigratory NC genes such as *RHOB, CDH6, FOXD3* and neural progenitor genes such as *AXIN2* [55], *LHX2* [91], *DVL2* [92] and *OLIG2* [93], were upregulated in these populations (Fig.3B). It has been previously reported that the patterning of neural versus NC lineages is based on the plating density of early progenitors [18]. Serial passage of our cells at low density, selected against the neural population, with a significant downregulation of neural genes by later passages (P7). Meanwhile, the P7 cells expressed markers of migratory NC cells, including *SOX9, SOX10, P75,* and *TWIST,* and downregulated pre-migratory markers such as *WNT1, PAX3,* and *RHOB*. Further validation of in vitro selection upon serial passaging is provided by the pattern of expression of *SOX10* and *WNT1*. In the mouse, *Sox10* expression follows *Wnt1* expression and marks virtually all NC cells immediately after their delamination from the neural tube [94]. In our system, *WNT1* is lowly expressed in mixed NC population, while it increases alongside *SOX10* expression in further passages, confirming NC purification in vitro with passaging.

In contrast to other in vitro protocols [18,19,21,43] we did not need to modulate WNT or BMP signaling exogenously. We hypothesized that the endogenous activation of WNT and BMP signaling, as evidenced by our microarray analysis, was sufficient in early passages to induce NC differentiation. This endogenous signaling may reflect the cross-talk and local gradients between a mixed population of the neural plate and non-neural plate ectoderm. Upon further passage of the mixed NC P2 population, we observed downregulation of both BMP and WNT signaling cells in these populations, suggesting that the combination of endogenous BMP and WNT signaling at the early stages of the differentiation protocol was sufficient to specify NC differentiation. The protocol imitates, in part, the steps involved in the development of NC cells in the embryo [9].

The properties of NC cells generated with this protocol were broadly characterized both in vitro and in vivo. We demonstrated the expression of NC markers from passage 7 to beyond. NC cells could proliferate, self-renew and migrate to appropriate cues such as FGF8B has previously been reported [54]. Furthermore, we successfully demonstrated that these NC cells differentiate into their derivatives lineages including MSC, melanocytes, peripheral neurons, smooth muscle cells, adipocytes, chondrocytes, and osteocytes (Supplementary Fig.4) [21,23,95].

Importantly, in vivo characterization provides a critical test of a progenitor population's developmental capacity. To determine whether HPSC derived NC cells demonstrated functionality in vivo, we evaluated the integration and migration of these cells in the developing chick embryo. We found the engrafted NC cells localized in the wall of the ascending aorta (Fig.4), the aortic arch and the meningeal vessels of the brain (Supplementary Fig.3). The absence of NC cells in the epicardium and sub-epicardial regions of the heart confirmed the specificity of these cells in localizing correctly to NC in vivo developmental location. There could be several factors that mediate the migration and

localization of human NC cells in vivo including paracrine signals from chicken NC-derived tissues or cell-cell and cell-matrix interactions at particular NC-derived locations. Whilst we have demonstrated that our HIPSC-derived NC can contribute to the aortic SMC population in vivo, we have not explored the broad differentiation of these cells using this approach. The ability to transplant HIPSC-derived cells in ovo provides an opportunity to further our understanding of the developmental potential of human progenitor cells within an in vivo context. Analysis of NC, neuron/glial and pericyte marker expression in the transplanted cells may provide additional insight into the differentiation potential of these cells in vivo and their cell fate.

Modeling Treacher Collins Syndrome In Vitro

The ability to generate NC from patient-derived HIPSC lines provides a unique tool to study neurocristopathies in the affected cell lineage. A complementary approach to study specific diseases is the *de novo* creation of a particular mutation in wild-type lines of HPSC using CRISPR/Cas9 gene editing. This method permits the investigation of multiple diseases causing mutations, without the requirement of patient tissue. Moreover, this approach removes the influence played by various genetic backgrounds from different patient samples.

A vast number of NC-related diseases have been reported in the literature, including TCS, although studies using human models are lacking. Treacle protein depletion has been attempted previously using knock-in of a destabilization domain-tagging TCOF1 in HEK293 cells, resulting in impaired cell proliferation [87]. Furthermore, mice haploinsufficient for Treacle have been reported and demonstrate robust developmental NC phenotypes [32].

To finally confirm the quality and utility of the HPSC-derived NC using this protocol we attempted to model Treacher Collins Syndrome in vitro. We created TCOF1$^{+/-}$ HIPSC lines from wild-type cells using CRISPR/Cas9 technology. The differentiation of TCOF1$^{+/-}$ HIPSC clones with our protocol provided the opportunity to study the functionality of the affected NC cells. TCOF1$^{+/-}$ HIPSC derived NC cells displayed significant depletion of Treacle protein and their phenotype corresponded to the mutant phenotype found in the Tcof1 $^{+/-}$ mouse model [32].

Furthermore, neuroectoderm derived from TCOF1$^{+/-}$ HIPSC showed a dramatic increase in apoptosis, replicating the aberrant cell death in the neural plate in Tcof1 $^{+/-}$ mice [32]. The haploinsufficiency of Treacle also leads to reduced proliferation of NC cells in Tcof1 $^{+/-}$ mice [32]. NC derived from TCOF1$^{+/-}$ HIPSC also showed a significant reduction in proliferation compared with wild-type HIPSC-derived NC cells. Strikingly, we also demonstrated a previously unappreciated impairment in the migration of both TCOF1$^{+/-}$ NC and NC-derived MSC in our in vitro model. Models of Treacher Collins syndrome have been reported in both the zebrafish and the mouse [32,96]. Both systems demonstrate extensive craniofacial abnormalities, yet the developing NC populations are shown to migrate and populate the maxillary and frontonasal regions normally. It is of interest to note that whilst there is no reported difference in the migration behavior of the NC, there is an overall reduction in the number of migrating NC cells, and this has been demonstrated to be due to a proliferation and cell death defect at the early stages of NC delamination from the neuroepithelium. However, it is possible that the finding of fewer migrating NC cells in this model may also be due to a proportion of cells which fail to migrate, in addition to the clear cell survival defects reported [32,97]. Whilst we report a migration defect in our

TCOF1$^{+/-}$ HIPSC-based NC system, it is important to acknowledge that this is an in vitro model and so is limited by the many missing developmental cues and triggers which are present in vivo. Therefore, it is possible that the migratory phenotype we report here may not be recapitulated during human in vivo development. Further engraftment studies using TCOF1$^{+/-}$ HIPSC-derived NC within the cranial NC may provide novel insights into the mechanisms involved during the migration of the human NC and their contribution to cells of the facial prominences and subsequent craniofacial compartments. Nevertheless, our TCOF1$^{+/-}$ HIPSC-based NC model now adds to the limited information available regarding human NC development and its contribution to Treacher Collins syndrome.

CONCLUSION

We believe that this protocol will serve as a resource for researchers seeking to produce NC and NC-derived tissues and to model neurocristopathies in vitro. Furthermore, we have shown that NC cells derived from HPSCs can be differentiated into a wide variety of cell lineages which is of great interest to the field. The ability to study the mechanisms of NC biology and disease should also accelerate the development of innovative therapies to treat, or even prevent, NC disorders such as TCS.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Anna Osnato, Mr Muhammed Kaiser Abdul Karim, and Dr Mark Kotter for the provision of cDNA samples, and to Dr Alessandro Bertero for assistance in confocal imaging. This work was supported by the British Heart Foundation (FS/13/29/30024, FS/11/77/29327, RM/13/3/30159), the British Heart Foundation Cambridge Centre for Research Excellence and the Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute.

AUTHOR DISCLOSURE STATEMENT

The authors declare no competing financial interests.

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