Comparison of three congruent patient-specific cell types for the modelling of a human genetic Schwann-cell disorder

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Patient-specific human-induced pluripotent stem cells (hiPSCs) hold great promise for the modelling of genetic disorders. However, these cells display wide intra- and interindividual variations in gene expression, which makes distinguishing true-positive and false-positive phenotypes challenging. Data from hiPSC phenotypes and human embryonic stem cells (hESCs) harbouring the same disease mutation are also lacking. Here, we report a comparison of the molecular, cellular and functional characteristics of three congruent patient-specific cell types—hiPSCs, hESCs and direct-lineage-converted cells—derived from currently available differentiation and direct-reprogramming technologies for use in the modelling of Charcot–Marie–Tooth 1A, a human genetic Schwann-cell disorder featuring a 1.4 Mbp chromosomal duplication. We find that the chemokines C–X–C motif ligand chemokine-1 (CXCL1) and macrophage chemoattractant protein-1 (MCP1) are commonly upregulated in all three congruent models and in clinical patient samples. The development of congruent models of a single genetic disease using somatic cells from a common patient will facilitate the search for convergent phenotypes.

Disease modelling that uses stem cell technologies, including human-induced pluripotent stem cells (hiPSCs), allows the precise analysis of human diseases that harbour inherited genetic mutations as a causative factor, particularly those in which animal models of molecular and cellular pathophysiology are not fully established1. Previous studies that used patient somatic-cell-derived hiPSCs recapitulated disease symptom-relevant cell types with individual genetic characteristics and identified new pathologic mechanisms at a cellular level in vitro2. Cells derived from hiPSCs are often considered to be a developmentally early stage, but most human diseases (even genetic ones) are progressive over years to decades. This property of hiPSC-derived cells often limits the modelling of neurological disorders even though it is a promising tool for modelling congenital abnormalities. Direct reprogramming has been suggested as an alternative to hiPSCs to better model mature cellular phenotypes with ageing signatures of converted cells by circumventing the pluripotent state3. It can therefore be considered a tool for the modelling of late-onset diseases as directly converted cells exhibit similar aged cellular features to hiPSCs. In spite of advanced cell-fate manipulation tools, it is still challenging to accurately model chronic diseases using a single methodology because each is limited by methodology-dependent stage-specific cellular characteristics.

Charcot–Marie–Tooth 1A (CMT1A) is caused by the duplication of a 1.4 Mbp region on chromosome 17p. It is a hereditary peripheral neuropathy in which Schwann-cell pathology results in dysmyelination and demyelination of the peripheral nerves4,5. The development of hiPSC models of CMT1A is critical as it is the most common hereditary peripheral neuropathy and currently has no targeted treatments. Furthermore, although rodent models recapitulate many clinical features of CMT1A, they have thus far been limited in their ability to predict the therapeutic efficacy of pharmacological treatments. Ascorbic acid proved robustly effective in treating CMT1A in rodent models6 but had much more muted effects in CMT1A clinical trials7–11.

Here we report the development of a protocol for the direct derivation and prospective isolation of Schwann cells from human pluripotent stem cells (hPSCs) and the use of CMT1A patient hiPSC-derived Schwann cells to model CMT1A pathogenesis, as well as the independent validation of those results with CMT1A Schwann cells from preimplantation genetic diagnosis—human embryonic stem cells (PGD–hESCs) and human-induced neural...
crest (hiNC) cells. We identified shared molecular and cellular features as a pathologic mechanism of CMT1A by comparing three different methodology-derived congruent Schwann-cell models, which suggested integrative analysis using various cell engineering tools as an advanced strategy in disease-modelling studies.

Results

Direct differentiation and prospective isolation of Schwann cells from hESCs.

We devised multiple reprogramming methods for the generation of Schwann-cell lineage populations and used them to model CMT1A. We initially created a defined protocol for the direct derivation and prospective isolation of putative Schwann-cell precursors (SCPs) from SOX10-reporting hPSCs (Fig. 1a,b). The protocol features a 21 day differentiation course and allows for the isolation of putative SCPs through α4-integrin CD49d expression (Fig. 1c,d). The purified population features differential global gene expression profiles for hESCs and hiNC cells, expresses peripheral glia-specific genes and exhibits gliogenesis-related gene ontology results (Fig. 1e,f and Table 1). After replating of isolated CD49d+ cells, our protocol permits in vitro culture for up to 80 d with abundant expression of Schwann-cell lineage proteins (Supplementary Fig. 1a). When the resulting SCPs are cultured beyond 35 d, there is segmental expression of myelin basic protein when co-cultured with neurons, and integrate in vivo when transplanted into the murine tibial nerve (Supplementary Fig. 1d,e).

Furthermore, in rat models of chronic peripheral nerve denervation, which causes a contractured hindpaw13,14, injecting hiPSC-derived TUJ1+ neurons, and integrate in vivo when transplanted into the murine tibial nerve (Supplementary Fig. 1d,e). Catwalk gait analysis reveals improved animal standing time, maximum paw contact area and paw print length and width, demonstrating that transplanting hiPSC-SCPs can also improve functional neuroregeneration in vivo (Supplementary Fig. 1f).
Modelling CMT1A with patient hiPSC-derived Schwann cells. We then created hiPSCs from the skin fibroblasts of four CMT1A patients (two parent-child pairs: 5146–5148 and 5165–5167) and healthy controls, and differentiated into SCPs (Fig. 2a and Supplementary Fig. 2a–c). No significant differences were found in the SCP yields (Fig. 2b) or the Schwann-cell marker gene enrichment on differentiation (Fig. 2c) and rTPCR for PMP22, the causal gene in CMT1A, revealed upregulation in the CMT1A hiPSC–SCPs (Fig. 2d). The observed in vitro variability in PMP22 gene expression is also in line with clinical observations57. Global differences in gene expression between CMT1A hiPSC–SCPs and controls were identified by submitting four independently differentiated samples from CMT1A hiPSCs (three samples from one clone from patient 5148 and one sample from one hiPSC clone from unrelated patient 5165) and controls for microarray analysis (the samples from the remaining CMT1A patients were used for the subsequent validation of microarray findings). There is a global pattern of upregulated gene expression in the CMT1A hiPSC–SCPs relative to the controls and, notably, PMP22 and HS3ST3B1 (both genes from the CMT1A duplicated region) are upregulated in the CMT1A hiPSC–SCPs (Fig. 2e). To better understand the broader patterns characterizing the differentially expressed genes, all genes that differentially expressed at least 3 s.d. from the mean were analysed with IPA (canonical pathways function) and DAVID (biological process, cellular components and Biocarta functions). Gene ontology analysis reveals that multiple genes in inflammatory signalling pathways (Supplementary Table 1), among unrelated pathways such as extracellular matrix production, were differentially expressed in CMT1A hiPSC–SCPs versus controls. Genes involved in inflammatory responses, the complement system, humoral immunity, granulocyte and agranulocyte adhesion and diapedesis all received statistically significant scores (Fig. 2f). To validate the microarray results, several genes were selected (Fig. 2e, marked genes) and evaluated with qRT–PCR using biologically independent samples from additional CMT1A hiPSC clones and genotypes (Fig. 2g). This raises the question of whether the upregulated inflammatory gene expression translates into increased inflammatory protein expression. Cytokine-array analysis of CMT1A and control hiPSC–SCP secretomes demonstrated that CMT1A hiPSC–SCPs secrete greater amounts of 14 of the 36 pro-inflammatory proteins profiled (Fig. 2h). Pro-inflammatory cytokines have multiple functions in different contexts, and a common function among those upregulated in the CMT1A hiPSC–SCPs is the ability to recruit monocytes/macrophages or neutrophils to areas of tissue damage63,37. The role of PMP22 gene duplication and increased PMP22 protein expression in CMT1A pathogenesis is a key question in CMT1A research. Intriguingly, we noticed that extended culture of CMT1A hiPSC–Schwann cells for 35 d further increased PMP22 protein expression (Supplementary Fig. 3a–c) and this correlated with increased inflammatory gene transcription, particularly of macrophage chemoattractant protein-1 (MCP1) and C-X-C motif ligand chemokine-1 (CXCL1). This raises the question of whether there may be a correlation between PMP22 expression, inflammatory cytokine upregulation and CMT1A pathogenesis, and we continue to explore these ideas using additional cellular models of CMT1A.

Establishment of congruent disease models with different methodology-derived CMT1A Schwann cells. To validate and build on the observations made in CMT1A hiPSC–SCPs, two complementary models of CMT1A Schwann cells were subsequently employed (Supplementary Table 2): CMT1A PGD–hESCs (hESCs isolated from two embryos with a preimplantation genetic diagnosis of CMT1A; Fig. 3a, Supplementary Fig. 4a–c) and CMT1A hiNC (CMT1A patient fibroblasts directly reprogrammed into Schwann-cell lineage via a neural crest intermediate; Fig. 3e). CMT1A PGD–hESCs and healthy controls were differentiated into CD49d+ SCPS using the same methods used for CMT1A hiPSCs (Fig. 3b–d). Next, we used a direct conversion technology with the same CMT1A and healthy control patient fibroblasts that were used for hiPSC generation and disease modelling. A single transcription factor, SOX10, was used in combination with WNT activation to convert human fibroblasts into induced neural crest and subsequently Schwann cells14 (hiNC–Schwann cells). As we observed inflammatory signatures in CMT1A hiPSC–SCPs, we confirmed that hiNC–Schwann cells are Schwann-cell lineage with glial marker expression that is distinguished from astrocytes, and are responsible for inflammation in central nervous system (Supplementary Fig. 5a,b). hiNC–Schwann cells were generated from fibroblasts of three CMT1A patients, two of which were included in our earlier hiPSC study. After the differentiation of CMT1A and control hiNCs towards the Schwann cells (Fig. 3e,f), CMT1A hiNC–Schwann cells demonstrated increased PMP22 gene expression (Fig. 3g) and the resulting cells from CMT1A and control hiNCs exhibit morphological characteristics stereotypical of Schwann cells and immunoreactivity for Schwann-cell lineage markers S100B and glial fibrillary acidic protein (Fig. 3h) with peripheral glia-related gene ontology results from transcriptome profiling (Supplementary Fig. 5c,d).

Next, we analysed the RNA sequencing of 21 samples across our three congruent CMT1A patients and control Schwann-cell models in search of disease-relevant phenotypes as well as global differences between hiPSC–, hESC- and hiNC-derived Schwann-cell lineage cells. Directly reprogrammed Schwann cells (hiNC–Schwann cell) show notable enrichment of ageing-related genes and features of DNA damage compared to differentiated Schwann cells (hiPSC–Schwann cell; Supplementary Fig. 6a,b), which suggests that hiNC–derived Schwann cells may represent a more mature or aged state than differentiated SCPS. We compared transcriptome profiles of hiPSC–Schwann cells and hiNC–Schwann cells from healthy control and CMT1A patients to validate the different features of each Schwann-cell population. Schwann-cell lines were clustered by methodology even when they are differentiated or converted from identical patient fibroblasts (Supplementary Fig. 7a). Furthermore, in conventional gene ontology analysis, each model exhibits distinct genetic signatures reflecting their disparate epigenetic origins. Genes involved in early development are upregulated in the hESC–Schwann-cell model, features of early development and glial/neuronal commitment are present in the hiPSC–Schwann-cell model and markers of ageing, cellular stress and inflammation are enhanced in the hiNC–Schwann-cell model (Supplementary Table 3). Gene ontology analysis for differentially expressed transcriptomes between hiPSC–Schwann cells and hiNC–Schwann cells indicates the enrichment of compact myelin and anatomic structures in directly converted Schwann cells (Supplementary Fig. 7b); compact myelin-related genes are upregulated in hiNC–Schwann cells whereas genes related to myelin sheath formation are upregulated in identical patient-derived hiPSC–Schwann cells (Supplementary Fig. 7c). Subsequent analysis with CMT1A

Table 1 | Gene ontology and pathway analysis

| GO terms (analysis) | P value |
|---------------------|---------|
| Erbb signalling (IPA) | 7.58578×10⁻⁶ |
| Neuregulin signalling (IPA) | 0.000436 |
| Gliogenesis (DAVID) | 0.000430 |
| PDGF signalling (IPA) | 0.0120 |
| CNTF signalling (IPA) | 0.0135 |
| Erbb signalling pathway (DAVID) | 0.0370 |

The upregulated gene list (CD49d+ cells over hESCs) was used. IPA, Ingenuity Pathway Analysis.
Fig. 2 | Modelling CMT1A with human Schwann cells from patient-derived hiPSCs. a. A schematic of the reprogramming methods used to generate CMT1A patient hiPSC-derived SCPs from CMT1A patient fibroblasts. b–d. Fluorescence-activated cell sorting (FACS) purification of LSB2i-treated CMT1A and control hiPSCs reveals similar yields of CD49d+ SCPs (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 36; CMT1A: n = 9; independent samples)). b. Post-FACS evaluation of Schwann-cell lineage gene enrichment in CD49d+ SCPs versus matched CD49d− samples from remaining CMT1A patients were used for subsequent validation of microarray findings non-SCPs, by qRT–PCR (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 11 for CDH19 and 15 for MPZ; CMT1A: n = 28 for CDH19 and 37 for MPZ; independent samples)). c. qRT–PCR for PMP22 in CMT1A and control CD49d+ hiPSC− SCPs (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 29; CMT1A: n = 76; independent samples); P values were calculated by unpaired t-test). d. Analysis includes five control hiPSC lines representing four genotypes and seven CMT1A hiPSC lines representing four genotypes. e. Volcano plot representation of microarray expression profiling of four biologically independent CMT1A SCP samples and four biologically independent control hPSC− SCP samples. Select genes of interest are highlighted and designated for validation with qRT–PCR. f. Results from DAVID gene ontology and IPA of differentially expressed genes. Data expressed as the P value of each pathway. g. qRT–PCR validation of the microarray results in biologically independent samples from five hiPSC clones representing three CMT1A patients. Data are expressed as the ratio of gene expression in CMT1A versus control hiPSC-derived SCPs. Data from independent clones are displayed as different colours; dots of the same colour are independent assays from the same clone. Data are expressed as the ratio of gene expression in CMT1A SCPs versus matched control (05148.1: n = 4; 05148.4: n = 4; 05165.7: n = 5; 05167.5: n = 2; 05167.8: n = 2; independent samples); P values were calculated by ratio paired t-test. h. Cytokine-array profiling of cell-conditioned medium from day 10 post-FACS CMT1A and control SCPs. Data are expressed as the ratio of protein expression in CMT1A SCPs versus a matched control (05148.1: n = 4; 05165.5: n = 2; independent samples) and P values were calculated by ratio paired t-test. Data from independent differentiations are displayed as different colours; dots of the same colour are from the same differentiation. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor.
Fig. 3 | Development of congruent CMT1A disease models by employing different cell-fate manipulating methods. **a.** A schematic of the method used to generate CMT1A PGD–hESC-derived SCPs from CMT1A blastocysts. **b–d.** FACS purification of LSB2i-treated CMT1A and control PGD–hESCs reveals similar yields of CD49d+ PGD–hESC–SCPs (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 27; CMT1A: n = 13; independent samples)) (**b**). Post-FACS evaluation of Schwann-cell lineage gene enrichment in CD49d+ SCPs versus matched CD49d− non-SCPs by rtPCR (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 9 for CDH19 and 9 for MPZ; CMT1A: n = 7 for CDH19 and 9 for MPZ; independent samples)) (**c**). rtPCR for PMP22 in CMT1A and control CD49d+ SCPs (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 32; CMT1A: n = 25; independent samples); P values were calculated by unpaired t-test) (**d**). Analysis includes two control hESC lines representing one genotype and two CMT1A PGD–hESC lines representing two genotypes. **e.** A schematic of the method used to directly convert CMT1A patient fibroblasts into CMT1A hiNC–Schwann cells. **f,g.** FACS purification of CMT1A and control hiNCs using CD34 antibody (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 12; CMT1A: n = 24; independent samples); P values were calculated by unpaired t-test) (**f**). rtPCR for PMP22 in CMT1A and control CD34+ Schwann cells (horizontal lines represent the mean, and error bars ±1 s.d. (control: n = 19; CMT1A: n = 22; independent samples); P values were calculated by unpaired t-test) (**g**). The analysis includes two control fibroblast lines that represent two control patients and three CMT1A fibroblast lines that represent three CMT1A patients. **h.** Representative immunofluorescence images for glial fibrillary acidic protein and S100b in hiNC–Schwann cells from two control fibroblasts and two CMT1A fibroblasts (scale bars, 50 μm). DAPI, 4,6-diamidino-2-phenylindole.

hiNC–Schwann cells and hiPSC–Schwann cells versus healthy controls revealed that six groups of ontologies (cell proliferation, multi-organismal process, behaviour, response to stimulus, metabolic process and presynaptic process involved in chemical synaptic transmission) were inversely expressed in CMT1A versus healthy samples. For example, all genes categorized under plasma lipoprotein particle clearance were oppositely expressed in healthy and CMT1A Schwann cells (Supplementary Fig. 7d,e), which is interesting given that a clinical manifestation of CMT1A is blebbing and rupture of the plasma membrane of Schwann cells and the formation of lipid droplets inside nerve tissues due to the metabolic dysfunction of Schwann cells.
Signs of DNA damage in directly reprogrammed cells were validated in Supplementary Fig. 6b, and even when the same starting fibroblast was used to make Schwann cells via hiPSC differentiation versus direct reprogramming, only the directly reprogrammed cells have increased γ-H2AX. γ-H2AX helps to remodel chromatin and repair double-stranded DNA breaks; it is phosphorylated by ATM/ ATR, which also activates cell cycle check point kinases CHK1/ CHK2 to initiate DNA repair. However, none of our cells—derived by either method—showed any ATM activity, despite directly reprogrammed cells having high levels of γ-H2AX (Supplementary Fig. 8a–c). Surprisingly, the phosphorylated forms of CHK1 and CHK2 were detected at high levels in healthy control lines but not CMT1A lines, and only when using the differentiation method rather than the reprogramming method (Supplementary Fig. 8c). It is unclear what mechanism is responsible for the DNA damage observed, but these observations represent a feature of direct-lineage-converted cells that may skew their phenotype, underscoring the importance of using multiple methodologies to minimize biased results from flaws inherent to each methodology. We used the recently developed POWER KIT19 to further analyse our data as our RNA sequencing dataset comprises multiple clones and individuals. Gene ontology analysis with this second approach showed distinct separation between models: themes of development/morphogenesis emerged in the hESC model, nervous system development in the hiPSC model, and cell cycle-related differentiation in the hiNC model (Supplementary Table 4). Interestingly, we observed WNT and Notch signalling-related pathway enrichment in the hiPSC model, whereas SMAD signalling-related pathways appeared in only the hiNC model, which is consistent with earlier observations that WNT and Notch are important signalling pathways for early gliogenesis and SMAD signalling functions in dedifferentiation, proliferation and death of matured Schwann cells, respectively20,21.

Immune cell recruitment as a shared pathologic phenotype of CMT1A in congruent models. We further analysed the transcriptional changes between the three CMT1A Schwann-cell models and controls using both DAVID and IPA (Supplementary Tables 5 and 6) to identify candidate pathologic pathways in CMT1A. Intriguingly, the differentiation-based hESC and hiPSC models showed pathology in the developmental signalling pathways and cell cycle-related signalling, whereas the direct-reprogramming-based hiNC model showed distinctive changes in endocytic trafficking that have been recently proposed in CMT1A research (Supplementary Fig. 8a and Supplementary Table 6). Despite these findings, we could not detect any differences in the efficiency of SCP generation between CMT1A and controls (Figs. 2b and 3b, f). Commonly misregulated pathways among all three models include NRF2 signalling, GnRH signalling, retinoic acid signalling and many others that play roles in inflammatory signalling. Schwann-cell function and neuronal function, at least in part via NF-kB signalling, a master regulator of both inflammation and myelination25–28 (Supplementary Fig. 8a and Supplementary Table 6).

To investigate these inflammatory transcriptional changes, we profiled the secreted proteins from independent genotypes of CMT1A and control hESC−Schwann-cell and hiNC−Schwann-cell models (Supplementary Fig. 9a–d), as done previously with hiPSCs (Fig. 2g, h), and we again found statistically significant upregulation of several pro-inflammatories proteins in CMT1A PGD−hESC−Schwann-cell models and CMT1A hiNC−Schwann-cell models (Supplementary Table 7). By comparing across the CMT1A and control hiPSCs, PGD−hESC and hiNC systems, two proteins, CXCL1 and MCP1, were found to be commonly upregulated among all three congruent models, resulting in a converged phenotype (Fig. 4a). CXCL1 is a cytokine involved in neutrophil recruitment and more recently monocyte migration in a concentration-dependent manner46. MCP1 expression has also been reported to be upregulated in a rodent model of CMT1A, lending support to our findings23,25. To determine whether this increased expression is sufficient to induce monocyte chemotraction, a transwell migration assay was used to evaluate THP1 human monocyte migration towards CMT1A and control SCP-conditioned medium (Fig. 4b). In all three congruent models, significantly higher numbers of THP1 monocytes migrated towards CMT1A SCP-conditioned medium than controls, and this recruitment is disrupted by inhibition of MCP1 signalling through a neutralizing antibody (Fig. 4c).

**Discussion**

The use of hPSCs and direct-lineage-conversion technology is a powerful approach for probing disease pathophysiology in a patient-specific manner. Our study describes an efficient strategy for modelling a human genetic disease by employing cell-fate plasticity. In agreement with data from model organisms2,23 and patient biopsies (Fig. 5i–k), we found that CXCL1 and MCP1 involved in monocyte/macrophage recruitment may be part of her primary pathology rather than a secondary reaction to abnormal myelin. Whether CXCL1 and MCP1 upregulation is directly responsible for the migration of monocytes/macrophages into the intercostal nerve of a third CMT1A patient requires further investigation. Similarly, further studies are needed to more precisely delineate the role of immunopathology in CMT1A pathogenesis.
proteins were upregulated with an associated infiltration of immune cells into nerve tissue in three congruent human models of CMT1A Schwann cells.

Our protocol for the directed differentiation and prospective isolation of SCPs from hPSCs (hiPSCs or hESCs) features a chemical pathways to enable the fate of Schwann cells to be determined in compound-based activation/inhibition of developmentally relevant pathways. In our protocol for the directed differentiation and prospective isolation of SCPs from hPSCs, we have found that Schwann cells robustly express GPR126, a gene critical for the initiation of myelination, further myelination studies should yield positive outcomes under in vitro and in vivo settings. The three healthy human Schwann-cell models profiled here still have distinct genetic signatures, reflecting their disparate epigenetic origins. This is evidenced by the results of the gene ontology analyses, through early developmental characteristics in the hESC-derived Schwann-cell model, features of glial and neuronal commitment in the hiPSC-derived Schwann-cell model and markers of ageing, such as substantial immune and inflammatory gene expression, in the hiNC-derived Schwann-cell model.

Intriguingly, most of the queried genes associated with ageing were upregulated in the hiNC-derived Schwann-cell model relative to other models, which is comparable to previous studies on improved cellular maturation in direct-converted models (Supplementary Fig. 6a) \textsuperscript{36}. Given that dysmyelination involves impaired myelination by developmentally immature Schwann cells, and this has been shown to play an important role in several CMT1A rodent models \textsuperscript{35}, our hPSC-derived SCPs may be more useful for studying the dysmyelination seen in CMT1A, as these cells are in early developmental stages. In contrast, our hiNC-derived Schwann cells are probably

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**Fig. 4 | Validation of the converged CMT1A phenotype from congruent Schwann-cell models.**

- **a** A Venn diagram of the upregulated secreted proteins in CMT1A hiPSC-, CMT1A PGD–hESC- and CMT1A hiNC-derived Schwann-cell models, with a converged phenotype of CXCL1 and MCP1 protein upregulation.
- **b** An illustration of how THP1 monocyte recruitment is assessed in a co-culture with Schwann cells.
- **c** THP1 human monocyte transwell counting assay results, using a conditioned media of SCPs derived from CMT1A and control hiPSC lines for each model. Horizontal lines represent the mean, and error bars ± s.d. of migrated THP1 cells (hiPSC–SCP control, n = 6 and CMT1A, n = 6, for each line; hESC–SCP control, n = 9 and CMT1A, n = 9, for each line; hiNC–Schwann cell control, n = 9 and CMT1A, n = 3, for each line; independent samples and independent assays); P values were calculated by one-way ANOVA.
**Fig. 5 | Association between PMP22 gene dosage and inflammatory signatures.**

**a.** The targeting sequences of TALEN-based exon editing for PMP22 (top). The sequencing result of TALEN-based second exon inactivation indicated that target sequences harbouring the start codon were eliminated (bottom). The efficiency of TALEN-based exon targeting with no off-target mutations in 16 predicted regions is 47% for clones with modifications and 12% for clones with two copies of wild-type and one copy of knockout alleles. **b.** Representative FACS plots from the purification of CD49d+ SCPs from CMT1A hiPSCs (CS67iCMT-n4) and their isogenic control hiPSCs (CS67iCMT-n4.ISOC4). **c.** The efficiency of differentiation from both lines. Horizontal lines represent the mean, and error bars ±1 s.d. (CMT1A: n = 5; isogenic control: n = 5; independent samples). **d.** The relative level of mRNA transcription of PMP22 in CMT1A and isogenic control SCPs. Horizontal lines represent the mean, and error bars ±1 s.d. (CMT1A: n = 3; isogenic control: n = 3; independent samples); P values were calculated using an unpaired t-test. **e, f.** The protein expression level of PMP22 in undifferentiated hiPSCs and hiPSC–Schwann cells from the CMT1A hiPSC line and its isogenic control (e and Supplementary Fig. 11) and the validation of protein band intensity (f). GAPDH was used as an internal control. Horizontal lines represent the mean, and error bars ±1 s.d. (CMT1A: n = 3; isogenic control: n = 3; independent samples); P values were calculated using an unpaired t-test. **g.** The protein expression level of MCP1 in undifferentiated CMT1A hiPSCs and their isogenic control. Horizontal lines represent the mean, and error bars ±1 s.d. (CMT1A: n = 6; isogenic control: n = 3; independent samples); P values were calculated using a paired t-test. **h.** A chemotaxis assay with THP1 monocytes was performed with a media control, CMT1A Schwann cells, CMT1A Schwann cells with antibody neutralization and isogenic control Schwann cells. Representative image of monocytes migrated into the Schwann-cell side of the chamber is available in Supplementary Fig. 10i. Horizontal lines represent the mean, and error bars ±1 s.d. (CMT1A: n = 6; isogenic control: n = 3; independent samples); P values were calculated by one-way ANOVA. **i.** The protein expression level of CXCL1 in CMT1A and control patient nerve biopsies. Horizontal lines represent the mean, and error bars ±1 s.d. of cytokine dot array intensity (n = 4 for each sample; independent biopsies); P values were calculated by one-way ANOVA. **j.** The protein expression level of MCP1 in CMT1A and control patient nerve biopsies. Horizontal lines represent the mean, and error bars ±1 s.d. of cytokine dot array intensity (n = 4 for each sample; independent biopsies); P values were calculated by one-way ANOVA. **k.** CD68 staining (a marker for monocytes/macrophages; immune-labelled cells appear as brown stained) in an intercostal nerve biopsy from a CMT1A patient (scale bar, 100 μm).
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a better model for studying impaired neuronal Schwann-cell signalling in CMT1A, as transcriptome analysis reveals enriched responsiveness to extracellular stimuli and robust expression of the cell death pathway, which may be related to inflammation and Schwann-cell death in clinical CMT1A patients. Although further studies will be needed to establish a robust and reproducible in vitro myelination by human Schwann cells, our CMT1A Schwann cells should provide a reliable model for investigating the various CMTA1 pathophysiological mechanisms in effect in both early and late developmental stages.

CMTA1 is not considered to be a clinically inflammatory disorder, although there is some laboratory evidence to support this idea. Our data might suggest that neutrophils or monocytes could be recruited to the nerves of CMTA1 patients, potentially via a mechanism mediated by CXCL1 and MCP1. This could be corroborated by rodent studies, which have found that CMTA1 mouse models overexpressing or mutating PMP22 have increased macrophage infiltration in the peripheral nerves and that this is largely mediated by MCP1. In this study, we exploited the plasticity of cellular identity through different reprogramming approaches—our results present an alternative method by which future hiPSC-based studies may also be validated through the use of congruent model systems.

Methods

Purification of OCT4+ embryonic stem cells and SOX10+ neural crest. hiPSCs were purified by FACs after 21–23 days of differentiation using a PE-conjugated antibody to CD49d (R&D Systems, FAB1354V). After FACs, SCPS were replated on tissue culture plates treated with fibronectin (R&D Systems, 1918-FN-02M) and laminin (Cultrex, R&D Systems, 3400–010–1)–coated plates. Plates were coated with 1 μg/ml–laminin and 2 μg/ml–fibronectin in PBS for 24 h. Cells were maintained in culture for up to 80 days using Neurobasal medium (Life Technologies, 21103–049) supplemented with 1-glutamine (Life Technologies, 25030–081), B-27 supplement (Life Technologies, 12587–070), N2 supplement (Life Technologies, cat. no. 17502–048) and 1% FBS (HyClone, SH30070.03).

Immunofluorescence. Cells were fixed in 4% paraformaldehyde for 20 min, then blocked and permeabilized in 0.5% BSA and 0.1% Triton X-100 in PBS for 30 min. Primary antibodies were diluted 1:100 to 1:500 in 0.5% BSA and 0.1% Triton X-100 in PBS and the samples were incubated overnight at 4°C. Secondary antibodies were diluted 1:1000 in 0.5% BSA and 0.1% Triton X-100 in PBS and incubated for 2 h at room temperature. Primary antibodies can be found in the Supplementary Table 8. Appropriate 488, 568- and 647-conjugated Alexa Fluor secondary antibodies were used.

RNA extraction and rTPCR. Cells were homogenized in Trizol reagent (Life Technologies, 15596–026) and the manufacturer’s protocols were followed to extract the total RNA. The total RNA (0.5 μg) was reverse transcribed into complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4387406). Real-time PCR was performed on an Eppendorf Mastercycler Ep gradient using the SYBR Green method. Cycling conditions were 95°C, 2 min, 40 cycles (95°C, 3 sec; 60°C, 30 sec). The primer probes can be found in the Supplementary Table 9. Data from the rTPCR for the microarray validation probes in hiPSCs, P D–hESCs and hNC–Schwann cells were displayed as fold change in CMT1A sample expression versus median gene expression in the controls, and analysed with the ratio paired t-test.

Microarray analysis. For the microarray analysis, the total RNA from Oct4+ embryonic stem cells (n = 3), SOX10+ neural crest (n = 3) and CD49d+ SCPS (n = 3) was collected from three independent differentiations, hybridized to the Affymetrix Primeview Human Gene Expression Array by the Johns Hopkins Deep Sequencing and Microarray Core and analysed in Partek. Transcripts that were upregulated or downregulated 2-fold or more with a P value greater than 0.05 were further analysed using DAVID and IPA.

Microarray analysis (CMT1A hiPSCs versus control hPSCs). Four independently differentiated samples from CMT1A hiPSCs (three samples from one clone from patient 5148, one sample from one hiPSC clone from unrelated patient 5165) and controls (two samples from one clone from control patient 2623, two samples from hESC line H9) were submitted for microarray analysis. Samples were collected immediately after FACS purification and were selected for a minimum of 1.5-fold PMP22 gene upregulation by qRT–PCR, to model the physiologically expected PMP22 upregulation in CMT1A. The total RNA was extracted and hybridized to the Affymetrix Primeview Human Gene Expression Array by the Johns Hopkins Deep Sequencing and Microarray Core and analysed in Partek. All genes differentially expressed at least 3 s.d. from the mean (corresponding to a fold change of 2.9–fold higher expression in CMT1A samples) were further analysed in IPA (canonical pathways function) and DAVID (biological process, cellular components and Bucafrunc functions).

RNA sequencing. The total RNA from 21 samples of hiPSC-derived SCPS (CMT1A: n = 4; control: n = 4), hESC-derived SCPS (CMT1A: n = 4; control: n = 2) and hNC-derived Schwann-cell lineage cells (CMT1A: n = 3; control: n = 4) were analysed by HiSeq Throughput Sequencing using a TruSeq library according to the Illumina Construction Kit (Illumina). The samples were performed to compare the gene transcripts across the three differentiation methodologies using control Schwann-cell models to search for general differences between the three models, first with conventional methods and then with POWER KIT. Conventional data analysis was performed by Macrogen (Cambridge) and catarized using Partek and DAVID for gene ontology analysis. For the analysis using POWER KIT, RNA-seq raw reads were mapped onto the NCBI GRCh38 release 89 transcriptome using TopHat2 and read counts for each transcript were measured using HTSeq-count. Two-step voom transformation was used to identify differentially expressed genes between samples (P < 0.05 and fold change <0.5 or >2.0); gene ontology enrichment of the differentially expressed genes was analysed with DAVID. Two subsequent analyses were performed to compare the CMT1A Schwann-cell transcripts with the control Schwann-cell transcripts in each differentiation model to find disease-specific transcriptional changes. Data analysis was performed by Macrogen (Cambridge) and catarized using Partek, DAVID and IPA for pathologic gene ontology and pathway analysis. RNA sequencing data has been uploaded to GEO under accession code GSE85599.

Acute nerve crush transection model and histology. GFP+ CD49d+ SCPS were isolated from B6, a hiPSC line constitutively expressing GFP (courtesy of L. Cheng, Johns Hopkins University). Nerve crush transection methodology was performed in accordance with and under protocols approved by the Johns Hopkins University IACUC. Four-month-old male NOD: CD17–PrKdcscid/I mice (The Jackson Laboratory, 001303) were anaesthetized under inhalation anaesthesia (1–3% isoflurane) using the VetEquip system. The left tibial nerve was exposed and a 5 mm segment just distal to the sciatic trifurcation underwent freezing/thawing twice with dry ice to kill all live cells (frozen with dry ice for 30 s and thawed for 2 min). The damaged area was marked with two sutures on the surface of the tibial nerve, flanking the distal and proximal ends of the damaged stump. The left tibial nerve was then transected 2 mm above the proximal end of the damaged stump and secured using United States Pharmacopeia 10/0.
sutures. Finally, 2μl of GFP+ CD49d+ cells (40,000 per μl) were transplanted to the freeze/thawed damaged stumps. Six weeks after GFP+ CD49d+ cell transplantation, the animals were anaesthetized and perfused with 4% paraformaldehyde. The previous surgical site was exposed; a portion of the tibial nerve (between the two suture sites) was harvested for immunohistochemistry and further fixed in 4% paraformaldehyde overnight. Paraformaldehyde-fixed nerves were cryoprotected by placing them in a solution of 15% sucrose for 24 h followed by a further 24 h in 30% sucrose. After embedding the tissue in OCT, serial 14-μm cross-sections were cut on a cryostat and stored at −20 °C for later use. Sections were blocked in 0.2% Triton X-100/PBS with 5% normal goat serum for 1 h. They were subsequently incubated with the TUJ1 (BioLegend, 801202; 1:1000) antibody overnight at 4 °C in 0.2% Triton X-100/PBS containing 5% normal goat serum. After the primary antibody incubation, the sections were washed thoroughly in 1× PBS and incubated with TETAX RED-conjugated secondary antibody (1:200, Vector Laboratories) in PBS with 5% normal goat serum for 45 min.

Chronic denervation transplantation model and catwalk gait analysis. All surgical procedures were performed on 6–8 week-old Lewis rats. The anastomosis of freshly anastomosed peroneal nerve to chronically denervated tibial nerve stump was performed as previously described.19,20 Comparison groups included control hiPSC–SCP injection during anastomosis (n = 4 animals) versus PBS sham treatment (n = 3 animals). The treatment group received 80,000 control hiPSC–SCPs (derived from patient 01582) in 6 μl injections in the distal nerve stump (2 μl per suture treatment) using a 33-gauge needle attached to a custom made 10 μl syringe. After injecting the cells, the needle was kept in place for 2 min and withdrawn slowly. To prevent acute rejection episodes of transplanted hiPSC–SCPs, 50 μg ml−1 of oral cyclosporine (Neoral, Novartis) was added to the drinking water from 3 d before transplantation until the end of the study. The CatWalk XT (Noldus Information Technology) assesses gait changes in rodents based on quantitative recordings of gait patterns as well as paw print characteristics. To evaluate the functional outcomes while walking, footprints were captured and recorded by a camera located beneath the glass plate. Testing occurred ten weeks after hiPSC–SCP transplantation. Each animal was recorded four times with the device. Recorded parameters include stand phase mean (average duration of the contact of paws with the glass plate), the width and length of the paw print on the glass plate, and the maximum contact area (paw surface area in contact with the glass at the moment of maximum contact). Results were analysed by examining the ratio of each parameter in the transplanted limb to the contralateral uninjured limb. All parameters had a pre-surgery ratio of one, signifying that both limbs were functioning equally before surgery.

CMT1A-induced pluripotent stem cell generation. Fibroblasts from four CMT1A patients were obtained from the Coriel Biorepository (GM05146, GM05148, GM05165 and GM05167). Fibroblasts were reprogrammed into induced pluripotent stem cells using previously reported methods1 and the Cytotune Sendai Reprogramming Kit (LifeTech A1378001). After two weeks of reprogramming, IPS cells were identified by their colony morphology and replated onto mouse embryonic fibroblast feeder layers. They were maintained on mouse embryonic fibroblast feeder layers through weekly passaging for up to 50 passages. To regularly assess to confirm they were free of mycoplasma contamination. The 18 hiPSC clones initially generated were screened for expression of the genes HHIL1 and LinCOR and microRNA 371 cluster expression. Yamanaka’s group showed that HHIL1 and LinCOR predict whether clones will be differentiation-effective versus differentiation-competent and microRNA 371 has been shown to predict differentiation towards neuronal lineages.21,22 Five of the 18 CMT1A hiPSC clones were chosen for further studies on the basis of the results of our screening (data not shown). Due to the variability in PMP22 expression levels, matched pairs of CMT1A hiPSC–SCPs and control hiPSC–SCPs with at least 1.5-fold upregulation of PMP22 were used for subsequent experiments to best model CMT1A physiology.

Generation and genome editing of CMT1A hiPSCs. Line C67/C67iCMT−n4 was generated at the Cedars-Sinai hiPSC core facility as previously described23, and is available at https://www.cedars-sinai.edu/Research/Research-Cores/Induced-Pluripotent-Stem-Cell-Center-Stem-Cell-Lines.aspx (IRB protocol no. Pro00031100). Confirmation of the PMP22 duplication was performed using multiplexed ligation-dependent probe amplification analysis of the parental line. To generate the isogenic control line, a pair of TALENs was designed targeting the start codon region of PMP22 (Celltech Bioresearch) and 2A-GFP and 2A-RFP were then cloned into the left and right TALENs, respectively. Using the NEON Transfection System (Thermo Fisher), TALENs were transfected into C67/C67iCMT−n4. After transfection (48h), GFP and RFP double-positive cells were sorted and cultured for 7–10 d. Healthy hiPSCs were dissociated into single cells using Accutase and plated at 10,000 cells per 10 cm dish. Rock inhibitor (Y-27632) was added at each passage. Following colony formation, individual clones were picked and plated in individual wells in a 96-well plate. After the colonies grew large enough to cover half the area of a well, colonies were split between two plates via manual dissection. One plate was used to confirm the genotype via Sanger sequencing and multiplexed ligation-dependent probe amplification. The other plate was either expanded or frozen until genome editing was confirmed. When the editing of one of the three copies of PMP22 was confirmed, the lines were expanded and characterized by karyotyping.

Secreted protein expression profiling. Pairs of CMT1A SCPs and matched controls with at least 1.5-fold PMP22 upregulation were identified. FACS-purified CD49d− hiPSC–SCPs and hiPS–hESC–SCPs were maintained in culture for 10 d post-FACS. For secreted protein profiling from hiNC–Schwann cells, the medium was changed to NB plus 1% FBS for the last 3 d of culture. The cell-conditioned medium was profiled with the Human Cytokine Array Kit (R&D Systems, ARY005).

Transwell migration assay. THP1 human monocyte chemotaxis assays were performed by using the HTS transwell plate system (5 μm pore size, Corning). Conditioned medium was collected from day 10 post-FACS for hiPSC–SCPs and hPGE–hESC–SCPs, and 2–5 weeks post-FACS for hiNC–Schwann cells. 100 μl of Schwann-cell-conditioned medium was placed in the bottom well with or without anti-MCP1 (R&D). THP1 monocytes were resuspended in Neurobasal medium (Gibco) plus 1% FBS to a concentration of 2.5 million cells ml−1 and 100 μl was placed in the upper wells. Migrated THP1 cells were counted after incubation for 3 h at 37°C with 5% CO2 n was defined by independent assays.

CMT1A PGD–hESC cell line generation. Two embryonic stem cell lines with a preimplantation genetic diagnosis of CMT1A were obtained from the NIH Human Embryonic Stem Cell Repository (HUES PGD11 and 12; NIH registration numbers 94 and 95; NIH approval numbers NIHhESC-11–0094 and NIHhESC-11–0095). Embryonic stem cell lines were generated from blastocysts that were determined to have the CMT1A duplication through preimplantation genetic diagnosis. The hESC line (H9 (WA09)) was used as a control. Lines were regularly assayed to confirm they were free of mycoplasma contamination. Matched pairs of CMT1A PGD–hESC–SCPs and H9-derived control SCPS with at least 1.5-fold upregulation of PMP22 were used for subsequent experiments.

CMT1A-induced neural crest-derived Schwann-cell generation. Three CMT1A patient fibroblasts (from patients S146, S148 and S1465) and two matching controls (from patients 0498 and 5756) were reprogrammed into induced neural crest and FACS-purified using published methods. hiNCs were maintained in Neurobasal medium containing 1 μM of doxycycline for 3 d, followed by 1 week of incubation with hiNC-derived Schwann cell-conditioned medium containing CNTF (10 ng ml−1) and NGF1 (20 ng ml−1), without doxycycline. Cells were maintained in culture for 2–5 weeks post-FACS.

CMT1A patient nerve biopsy procurement and analysis. To evaluate CXCL1 and MCP1 protein expression, de-identified sural nerve biopsies from two patients with genetically proven CMT1A (two women, aged 26 and 59) and two historically normal samples from non-CMT1A patients (two women, aged 55 and 79) were obtained from the Johns Hopkins Neuromuscular Histology Laboratory under appropriate Institutional Review Board supervision with informed consent. Lysates were obtained by sonicating nerve biopsies in RIPA buffer, and equal amounts of lysate were then used for secreted protein expression analysis (R&D Systems, ARY005).

To evaluate the presence of CD68+ monocytes/macrophages, a diagnostic biopsy of the intercostal nerve was performed in a 28-yr-old female patient with genetically confirmed CMT1A to evaluate any enlargement of her nerves and to rule out schwannomatosis (under the same IRB as above). She was diagnosed with CMT1A only 1 yr before biopsy when she presented with pain and enlarged nerve roots and lumbo-sacral and brachial plexus. The biopsy showed the classic features of an inherited demyelinating neuropathy with axonal loss and many mature onion bulbs. CD68 immunostaining was performed in paraffin-embedded sections with anti-CD68 antibody (DAKO, M071801–5) using standard immunohistochemical methods (Vectastain kit).

Constructs and electroporation. To validate the effect of PMP22 or HSST3B1, each gene was amplified from a CDNA library of H9 and inserted into the pcDNA3.1/V5-His plasmid using the TOPO TA system (Thermo Fisher). To induce overexpression of these genes in hiPSC-derived Schwann cells or peripheral neurons, each cell population was prepared in 24-well plates with serum-free medium including 10 μg of the plasmid; the plasmid was then transferred into the cells by using an adherent cell electrode and NEPA21 (Nepa Gene, Japan).

Statistical analyses. Data were analysed using Graphpad Prism 7. Means ± s.d. are plotted and P values were calculated using the unpaired t-test, ratio-paired t-test and one-way ANOVA, as noted. A normal distribution was assumed; n refers to cells from independent differentiation experiments, unless otherwise specified.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions
R.H.B., G.B., A.H., L.S. and G.L. conceived the study. B.M.-C., Y.I.K., K.E., R.H.B., G.B., A.H. and L.S. designed the study. B.M.-C., Y.I.K., R.M., B.K., I.Y.C., H.I., Y.O., B.L., K.J.K., S.B., J.H.K., W.I., O.H., Y.I.H.C. and G.L. performed experiments. B.M.-C., Y.I.K., R.M., B.K., I.Y.C., H.I., Y.O., B.L., K.J.K., S.B., J.H.K., W.I., O.H., Y.I.H.C., L.S. and G.L. analysed the data. B.M.-C., Y.I.K. and G.L. contributed to the data assembly. B.M.-C., Y.I.K. and G.L. interpreted the results. B.M.-C., Y.I.K. and G.L. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

| n/a | Confirmed |
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| ☐   | ☑         |
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The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

The statistical test(s) used AND whether they are one- or two-sided

A description of all covariates tested

A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection No software was used.

Data analysis Prism v7; commercial software for statistical analysis (Fig. 1d, Fig. 1f, Fig. 2b-d, Fig. 2g-h, Fig. 3b-d, Fig. 3f-g, Fig. 4c, Fig. 5c-d, Fig. 5f-j, Suppl.Fig. 1c, Suppl.Fig. 1f, Suppl.Fig. 3c, Suppl.Fig. 5a-b, Suppl.Fig. 6a, Suppl.Fig. 9a-d, and Suppl.Fig. 10a-f).

Partek Genomics Suite v7; commercial software for RNA-sequencing analysis (Fig. 1e, Suppl.Fig. 5c-d, Suppl.Fig. 7a-b, and Suppl.Fig. 7d-e)

DAVID; open-source software for gene-ontology analysis (Fig. 1g, Fig. 2f, Suppl.Table 1, Suppl.Table 3, Suppl.Table 4, and Suppl.Table 5).

Ingenuity; commercial for pathway analysis (Fig. 2f, Suppl.Fig. 8a, Suppl.Table 1, Suppl.Table 3, Suppl.Table 5, and Suppl.Table 6)

POWER KIT; open-source software for RNA-sequencing analysis (Suppl.Table 4).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that all data supporting the results in this study are available within the paper and its Supplementary Information. RNA sequencing data from CMT1A and control hESC-SCPs, hiPSC-SCPs, and hiNC-Schwann cells has been uploaded to GED under accession code GSE85598.
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
Although pre-study sample size calculations were not made, the majority of experiments were performed using multiple cell lines, multiple clones, multiple batches of differentiation, and multiple technical repeats, to ensure robustness. In addition, three independent human Schwann-cell models of CMT1A were used, generating further confidence in the findings.

**Data exclusions**
Pre-established exclusion criteria exclude samples with abnormal karyotypes and samples exhibiting poor Schwann-cell differentiation (measured by Cadherin19 and Myelin Protein Zero gene enrichment). Pre-established inclusion criteria include CMT1A and control Schwann-cell pairs with a PMP22 transcript expression difference of 1.5-fold.

**Replication**
The majority of experiments in this study were performed using multiple cell lines, multiple clones, multiple batches of differentiation, and multiple technical repeats, to ensure robustness.

**Randomization**
Whereas randomization was not feasible for this study, batch effects were minimized through simultaneous processing of cases and controls for all experiments.

**Blinding**
Blinding was not applicable to this study. Because primarily molecular techniques were used, investigator bias should play a minimal role in data acquisition for this particular study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

TUJ1; Covance; MRB-435P
CD49d; R&D; FAB1354P
MCP-1; R&D; MAB679
GFAP; DAKO; 20334
S100b; DAKO; 20311
GalC; Millipore; MBA342
PMP22; Assaybiotech; C0306
GAPDH; Cell Signaling; 2118
MBP;Abcam; ab7349
NANOG; Cell Signaling; 3580
TRA-1-60; Cell Signaling; 4746
TRA-1-81; Cell Signaling; 4745
SSEA3; DSHB; MC-631
SSEA4; DSHB; MC-813-70
V5; Serotech; MCA1360GA
CXCL1; Invitrogen; PAS-79101

**Validation**

All the antibodies in this study were commercial, and clones, targets and hosts were validated in previous studies and by the...
providers. Most of the manufacturers of the antibodies used in this study provide relevant information, which can be found from
the company name and catalogue number.

### Eukaryotic cell lines

**Policy information about [cell lines](#)**

| Cell line source(s) | GM05146 (Coriell), GM05148 (Coriell), GM05165 (Coriell), GM05167 (Coriell), GM01582 (Coriell), GM02036 (Coriell),
| | GM00498 (Coriell), GM05756 (Coriell), WA09 (WiCell), HUES PGD11 (NIH Human Embryonic Stem Cell Repository), HUES
| | PGD12 (NIH Human Embryonic Stem Cell Repository), CS67iCMT-n4 (Cedars-Sinai iPSC core facility) and CS67iCMT-n4.ISOC4
| | (Cedars-Sinai iPSC core facility) |

**Authentication**

Our primary skin fibroblasts (not cell lines) are purchased from Coriell, and human embryonic stem cell line was purchased
from WiCell. HUES PGD11 and HUES PGD12 were obtained from NIH Stem cell registry (NIH registration numbers 94 and 95;
NIH approval numbers NIHhESC-11-0094 and NIHhESC-11-0095). CS67iCMT-n4 and CS67iCMT-n4.ISOC4 were established at
Cedars-Sinai Medical Center (IRB protocol #Pro00031100).

**Mycoplasma contamination**

We have tested for mycoplasma contamination every other week, and have continuously maintained a mycoplasma-free
laboratory for over seven years.

**Commonly misidentified lines**

(See ICLAC register)

No commonly misidentified cell lines were used.

### Animals and other organisms

**Policy information about [studies involving animals](#) / ARRIVE guidelines recommended for reporting animal research**

| Laboratory animals | Four-months-old male NOD.CB17-Prkdcscid/J mice (001303) were purchased from the Jackson Laboratory. |
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight | All animal procedures were carried out in accordance with and under protocols approved by the Johns Hopkins University IACUC. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Flow Cytometry

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

| Sample preparation | Single cells were dissociated, filtered and labeled by using appropriate antibodies. |
| Instrument | FC500 (Beckman Culter), FACScalibur (BD), FACS ariall (BD), SH800 (Sony) |
| Software | FlowJo version 10 |
| Cell population abundance | Every sorting result was confirmed by re-test with cell post sorting for over 90% of positive population. |
| Gating strategy | All the FACS gating was made by comparing to population of iso-control every time. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.