PERSPECTIVE

Cellular reprogramming and inherited peripheral neuropathies: perspectives and challenges

Inherited peripheral neuropathies (or Charcot-Marie-Tooth disease, CMT) are a phenotypically and genetically heterogeneous group of disorders, which are currently untreatable. They are the most common inherited neuromuscular disorder, affecting around 1 in every 2,500 people (over 120,000 people in the US). Based on clinical neurophysiological and histopathological features, inherited neuropathies can be divided into two major forms: demyelinating (type 1) and axonal (type 2) CMT (Saporta, 2014). From a biological standpoint, these two major forms of CMT are associated with mutations in different sets of genes, affecting Schwann cell development and myelination (type 1) or peripheral axon physiology (type 2), although some overlap does exist (**Figure 1**). To date, over 70 genes have been associated with a CMT phenotype, making CMT an attractive natural model to study peripheral nervous system biology. Despite significant advances made in our knowledge of disease mechanisms in CMT, findings from animal models have so far translated poorly in clinical trials, underscoring the need for innovative methods to investigate the pathophysiology of these human disorders. Induced pluripotent stem cells (iPSCs) offer an unlimited source of patient specific, disease-relevant cell lines that can be used as a platform for identification of disease mechanisms, discovery of molecular targets and development of phenotypic screens for drug discovery (Saporta et al., 2011). iPSC-based models of neuromuscular disorders, including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA) and inherited peripheral neuropathies, have successfully reproduced pathophysiological findings from previous animal and cellular models and have also identified new disease mechanisms with potential therapeutical implications.

The first inherited neuropathy modeled by cellular reprogramming techniques was familial dysautonomia (or hereditary sensory autonomic neuropathy type III; OMIM: 223900), a severe autosomal recessive disorder with increased prevalence in the Eastern European Jewish population. Familial dysautonomia (FD) is characterized by the early onset of feeding difficulty, orthostatic hypotension and decreased pain and temperature perception, with only one-half of patients reaching adulthood. FD is caused by mutations in the *IKBKAP* gene that lead to reduced transcriptional elongation of several target genes, some of which are required for cell motility. Lee et al. (2009) generated iPSC lines from three patients with FD and demonstrated misregulated *IKBKAP* expression, defective neuronal differentiation and a decrease in neural crest precursor migration *in vitro*. Genes involved in peripheral neurogenesis and neuronal differentiation were found to be differentially expressed in FD cells, providing insight into the molecular mechanisms of the disease. Using iPSC-derived neural crest cells as a drug screening platform, a partial rescue of the disease phenotype was achieved after administration of kinetin, a plant hormone previously shown to reduce levels of the mutant IKBKAP splice form in FD-derived lymphoblast cell lines. Kinetin treatment of patient's cells significantly reduced the mutant IKBKAP splice form and increased the number of differentiating neurons; however, the level of increased IKBKAP did not lead to rescue of cell motility.

We have recently established an iPSC bank from 14 patients with multiple forms of inherited peripheral neuropathies as a human platform to study CMT. We have also derived motor neuron lines from two patients with early onset axonal forms of CMT associated with *MFN2* (CMT2A) and *NEFL* (CMT2E) point mutations and used them to study disease mechanisms in CMT2 (Saporta et al., 2015). iPSC-derived motor neurons exhibited gene and protein expression, ultrastructural and electrophysiological features of mature motor neurons (**Figure 2**). Immunostaining for intermediate filaments revealed an increased content of neurofilaments in the neuronal body of CMT2E spinal cord motor neurons. Similar inclusions were found in the spinal cord and brain of knockin mice expressing the same *NEFL* point mutation (N98S) as our patient-derived motor neurons (Adebola et al., 2015). Reduced mitochondrial mobility was found in axons from human CMT2E neurons, compared to age-matched controls, confirming previous data suggesting that mutant neurofilaments can interfere with mitochondrial trafficking along axons. The same phenomenon, albeit in a lesser degree, was observed in CMT2A motor neurons. CMT2A is caused by mutations in *MFN2*, a gene encoding an outer mitochondrial membrane protein involved in mitochondrial fusion and trafficking along axons. CMT2A and CMT2E iPS-derived motor neurons also generated action potentials at lower membrane potentials compared to control motor neurons, suggesting that these cells are hyperexcitable. Analysis of ion channel properties by voltage clamp demonstrated an increased density of sodium channels in CMT2A motor neurons, which also inactivated less efficiently, when compared to controls. Both CMT2A and CMT2E motor neurons also demonstrated an impaired inactivation of calcium channels. Taken together, this data suggest that ion channel dysfunction may play a role in the pathogenesis of axonal CMT, providing novel therapeutical targets for inherited neuropathies.

Demyelinating CMT (CMT1) is the most common type of inherited neuropathy in which a final molecular diagnosis is reached. Approximately 55% of patients with CMT and a positive genetic test will have CMT1A, caused by a duplication of a 1.4 Mb segment of chromosome 17, containing the myelin gene *PMP22*. The second most common form of CMT is caused by mutations in a gap junction protein, Connexin 32, expressed by Schwann cells in areas of non-compact myelin. Therefore, using cellular reprogramming to generate patient-derived Schwann cells will provide an important tool for the study of inherited neuropathies. Co-culture systems of patient-derived Schwann cells and peripheral neurons can be used to study *in vitro* myelination, Axon-Schwann cell interactions and Schwann cell intracellular events associated with the biology of inherited neuropathies. In this regard, Liu et al. demonstrated the feasibility of generating human embryonic stem cell (hESC)-derived neural crest and Schwann cells and created a co-culture system with rat dorsal root ganglia neurons to study *in vitro* myelination (Liu et al.,

2012; also revised by Ma et al., 2015). These findings are very promising, but it remains to be demonstrated whether *in vitro* myelination can be accomplished using both Schwann cells and neurons derived from patients' iPSC. This would be a required step in the process of generating a human *in vitro* platform to study demyelinating forms of CMT.

Another important structure of the peripheral nervous system implicated in the pathogenesis of inherited neuropathies is the neuromuscular junction (NMJ). This specialized cholinergic synapse between spinal cord motor neurons and their target skeletal muscle fibers is a complex and metabolic active region. Non-myelinating terminal Schwann cells and NMJ capping cells (kranocytes) also interact with the presynaptic motor nerve terminal and the skeletal muscle fiber, adding another layer of intricacy to this system. Neuromuscular junctions are of particular interest to the study of inherited neuropathies, as these conditions are usually length-dependent (*i.e.*, regions of the axon more distant to the neuronal cell body are affected before and more severely than the more proximal segments), and preferentially affect the distal regions of the peripheral nerves that help to form the NMJ. Therefore, studying how neuromuscular junctions are affected by the inherited neuropathies may provide important insights into early disease mechanisms and identify common therapeutical targets to different forms of CMT. hESC-derived motor neurons can form morphologically and functionally mature neuromuscular junctions in co-culture systems with muscle cells (Umbach et al., 2012). Similarly to co-culture systems to study myelination, it will be necessary to confirm that iPSC-derived motor neurons and muscle cells can also generate functional neuromuscular junctions *in vitro*, but the prospect of having such a powerful tool is encouraging.

There are still many challenges to overcome to establish cellular reprogramming as a consistent and useful platform for the study of human diseases. One of the main issues to be solved is the variability of results observed between research groups using cellular reprogramming to model neurodegenerative disorders. In this regard, recent studies investigating diseases mechanisms of a specific form of familial amyotrophic lateral sclerosis (ALS) associated with mutations in the *C9ORF72* gene, using patient-derived neuronal cell lines, found conflicting results concerning the presence of repeat-associated non-ATG-dependent (C9-RAN) protein products in these cells. One possible explanation for these contrasting results is related to differences in the differentiation protocols used to derive the neuronal lines studied. While one study using a motor neuron specific protocol (Sareen et al., 2013) did not find evidence of C9-RAN in their motor neurons, another study using a more "general" neuronal differentiation protocol (Donnelly et al., 2013) did find cytoplasmic poly-(Gly-Pro) RAN protein in *C9ORF72* ALS iPSC-derived neurons. These differences support the notion that, to correctly model a neurodegenerative disorder, one must take into consideration the specific subtype(s) of neuronal and non-neuronal cells affected by the condition in question. Therefore, it is of paramount importance that differentiation protocols are refined to the point where specific neuronal subtypes (both functionally and topographically) can be generated consistently and in enough numbers as to allow for their use in high content/high throughput platforms. Recent work by Maury et al. (2014) have demonstrated an elegant, high content-based approach to refining differentiation protocols. By screening various combinations of growth factors and small molecules at different concentrations using multiparametric, high content microscopy, the authors were able to optimize differentiation protocols for spinal and cranial motor neurons, spinal interneurons and sensory neurons. Investigators working in the development of other differentiation protocols should use similar strategies and the resulting protocols should be standardized and shared with the international community so that results from different groups can be more easily compared.

The ultimate goal of cellular reprogramming is the generation of patient-derived cells for use in cell replacement therapy. Recent advances in gene editing techniques, such as TALEN (transcription activator-like effector nuclease) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) allow for targeted correction of point mutations in patient-derived cells, which could be used to replace the original impaired cells. The use of gene edited, patient-derived Schwann cells could be an interesting strategy to treat demyelinating forms of CMT and may be beneficial for some axonal forms in which impaired axon-Schwann cell interactions could be optimized.

In summary, cellular reprogramming offers a new approach for the study of inherited peripheral neuropathies by enabling the study of human, patient-derived cell types directly related to their pathophysiology (neurons and glia) *in vitro*. Many challenges remain for investigators in the field, including developing subtype-specific differentiation protocols and co-culture systems to study myelination and neuromuscular junction pathology *in vitro*. Cell replacement therapy using patient-derived cells corrected by gene editing techniques may be a feasible treatment strategy especially for demyelinating forms of CMT.

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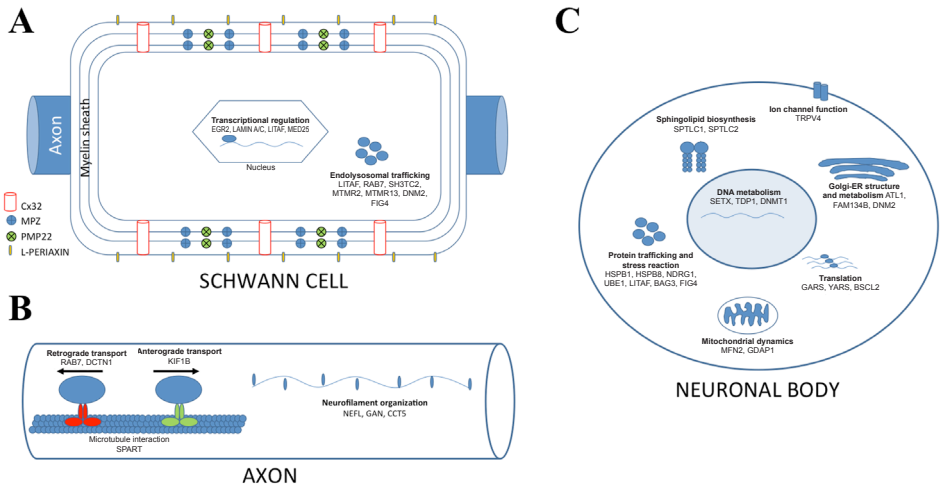

Figure 1 Main cellular compartments and functions affected by genes associated with Charcot-Marie-Tooth disease: (A) Schwann cell; (B) axon; (C) neuronal body.
ER: Endoplasmic reticulum. Modified from Saporta MA, Shy ME (2015) Peripheral Neuropathies. In: Neurobiology of Brain Disorders: Biological Basis of Neurological and Psychiatric Disorders (Zigmond MJ, Rowland LP, Coyle JT, eds), pp167-188. Academic Press. ISBN: 9780123982704.

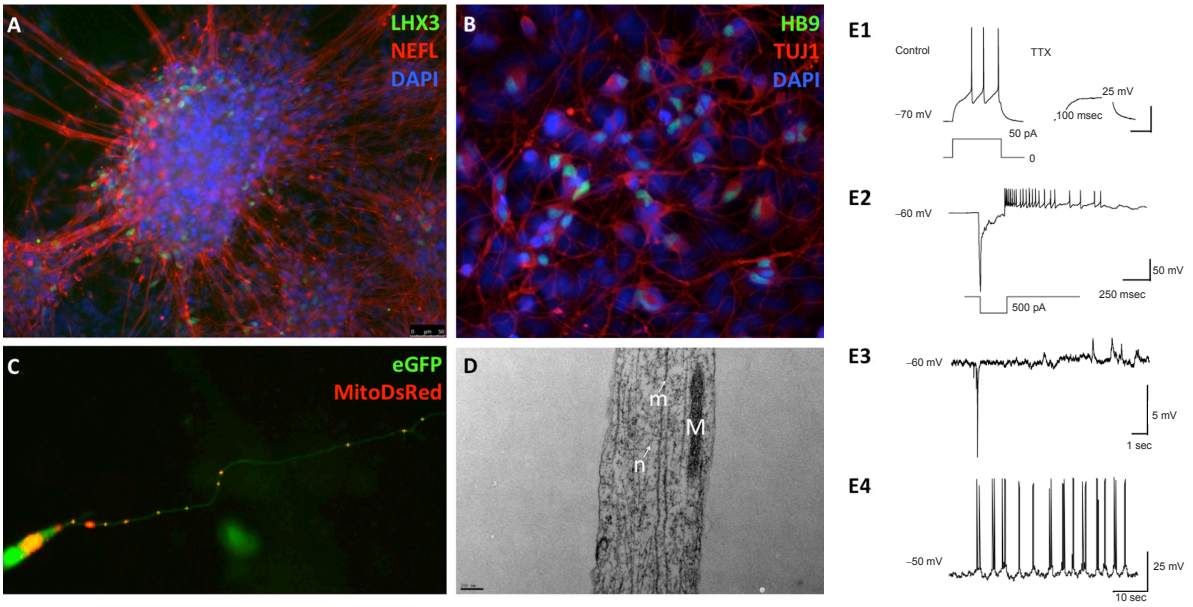

Figure 2 Induced pluripotent stem cells (iPSC)-derived motor neurons exhibited protein expression, ultrastructural and electrophysiological features of mature motor neurons.
After long-term culture (over 45 days), expression of the medial motor column marker LHX3 (A) and the somatic motor neuron transcription factor HB9 (B), as well as neuronal cytoskeletal proteins (NEFL and TUJ1), could be observed in most of the neurons. Note that, at this time point, spinal cord motor neurons tend to cluster (scale bar for A = 50 μm). (C) Motor neuron cultures can be transfected with enhanced green fluorescent protein (eGFP) and mitoDsRed plasmids to study axonal mitochondrial trafficking in axonal (type 2) inherited neuropathies. (D) Ultrastructural imaging of an axon revealed typical features including the presence of cytoskeletal proteins (*n* = neurofilament and m = microtubules) and mitochondria (M) (scale bar = 0.5 μm). Current clamp recordings of iPSC-derived motor neurons demonstrate tetrodotoxin (TTX) sensitive action potentials elicited by depolarizing current step (E1) and Ih current elicited by hyperpolarizing current step (E2). Spontaneous IPSPs and excitatory post-synaptic potentials (E3) and spontaneous bursts of action potentials (E4) could also be identified in these cultures. From Saporta et al. (2015).

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