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Human sensory neurons derived from pluripotent stem cells for disease modelling and personalized medicine

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ABSTRACT

In this concise Mini-Review we will summarize ongoing developments of new techniques to study physiology and pathophysiology of the peripheral sensory nervous system in human stem cell derived models. We will focus on recent developments of reprogramming somatic cells into induced pluripotent stem cells, neural differentiation towards neuronal progenitors and human sensory neurons.

We will sum up the high potential of this new technique for disease modelling of human neuropathies with a focus on genetic pain syndromes, such as gain- and loss-of-function mutations in voltage-gated sodium channels. The stem cell derived human sensory neurons are used for drug testing and we will summarize their usefulness for individualized treatment identification in patients with neuropathic pain. The review will give an outlook on potential application of this technique as companion diagnostics and for personalized medicine.

1. Sensory neuropathies – Hard to access, hard to translate

Patients suffering from human sensory neuropathies often present with impaired sense of temperature, disturbed somatosensation and most importantly, intense burning pain. This creates a high societal burden (about 7–8% of the general population are affected by neuropathic pain) (Bouhassira, 2019). Current pain therapy is often ineffective, the effect fades over time or is accompanied by severe side effects. It is common that patients need to sequentially test one treatment option after the other before finding an individual pharmacological or non-pharmacological intervention which alleviates the pain with bearable side effects or resign and bear the pain as is. Unfortunately, over the last decade virtually no new analgesics were released to the market, stressing the need for new approaches and better tools for more effective drug development.

The voltage-gated sodium channels Nav1.7 and Nav1.8 have been identified as two of the most promising targets for the development of new analgesics. Both channels are highly expressed in sensory neurons, including nociceptors and are necessary for the generation of action potentials. The importance of Nav1.7 in human pain was impressively demonstrated by patients, who completely lack this channel subtype and who are unable to feel pain (Cox et al., 2006). Their lack-of-pain-syndrome is called chronic insensitivity to pain (CIP). Gain-of-function mutations in Nav1.7, on the other hand, lead to chronic inherited pain syndromes (Lampert et al., 2014, Bennett et al., 2019). Research focuses on development of drugs specifically targeting Nav1.7 function. Although some drugs turned out to be effective in rodent models (Deuis et al., 2016) and there have been some encouraging phase II clinical trial results, other results have been negative (Zakrzewska et al., 2017, Kingwell, 2019) and definitive evidence of efficacy is still awaited.

One explanation for this lack of translation into clinical application may be that research is commonly performed using rodent models or heterologous expression systems, with the assumption that the findings can be easily translated from one species to the other. Unfortunately, as outlined below, this is questionable, especially in the pain field. Comparing the Na^+-channel subtype expression in rodent and

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postmortem human dorsal root ganglia neurons (DRGs) reveals specific differences (Rostock et al., 2018), and also, sensory fiber subtypes differ significantly between rodents and humans, which will be summarized below. Sodium channel subtypes may also have different roles in rodent and human action potential generation, leading to distinct pathophysiological and also pharmacological effects in rodents and humans. Hence, hurdles in translating data acquired in rodent models to humans are prominent.

Therefore, a human sensory neuron model is necessary for the development of new, more effective analgesics and to study human specific disease mechanisms on a cellular, molecular level. However, primary human sensory neurons are only rarely accessible, and *post mortem* tissue is often flawed. Induced pluripotent stem cells (iPSC) offer a highly promising way out of this dilemma. iPSCs can be reprogrammed from almost any somatic cell and have the potential to differentiate into virtually every cell type of the human body, including sensory neurons *in vitro*. Such cells harbor the complete genetic code of the patients/probands, including disease related mutations, but also SNPs and smaller variations which could affect their function by their interactions. Thus, this system allows for studies on the individual disease mechanisms and therapies, permitting preclinical screening of treatment options and thus offering the potential to identify personalized medicine for pain patients (Fig. 1).

2. Generating human sensory neurons

2.1. *Reprogramming towards pluripotency and direct lineage conversion into neurons*

Reprogramming of somatic cells to iPSCs requires the expression of specific reprogramming transcription factors, comprising OCT4, SOX2, KLF4 and c-MYC (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Once the pluripotent state is established, the ectopic expression of the reprogramming factors is no longer required since the endogenous

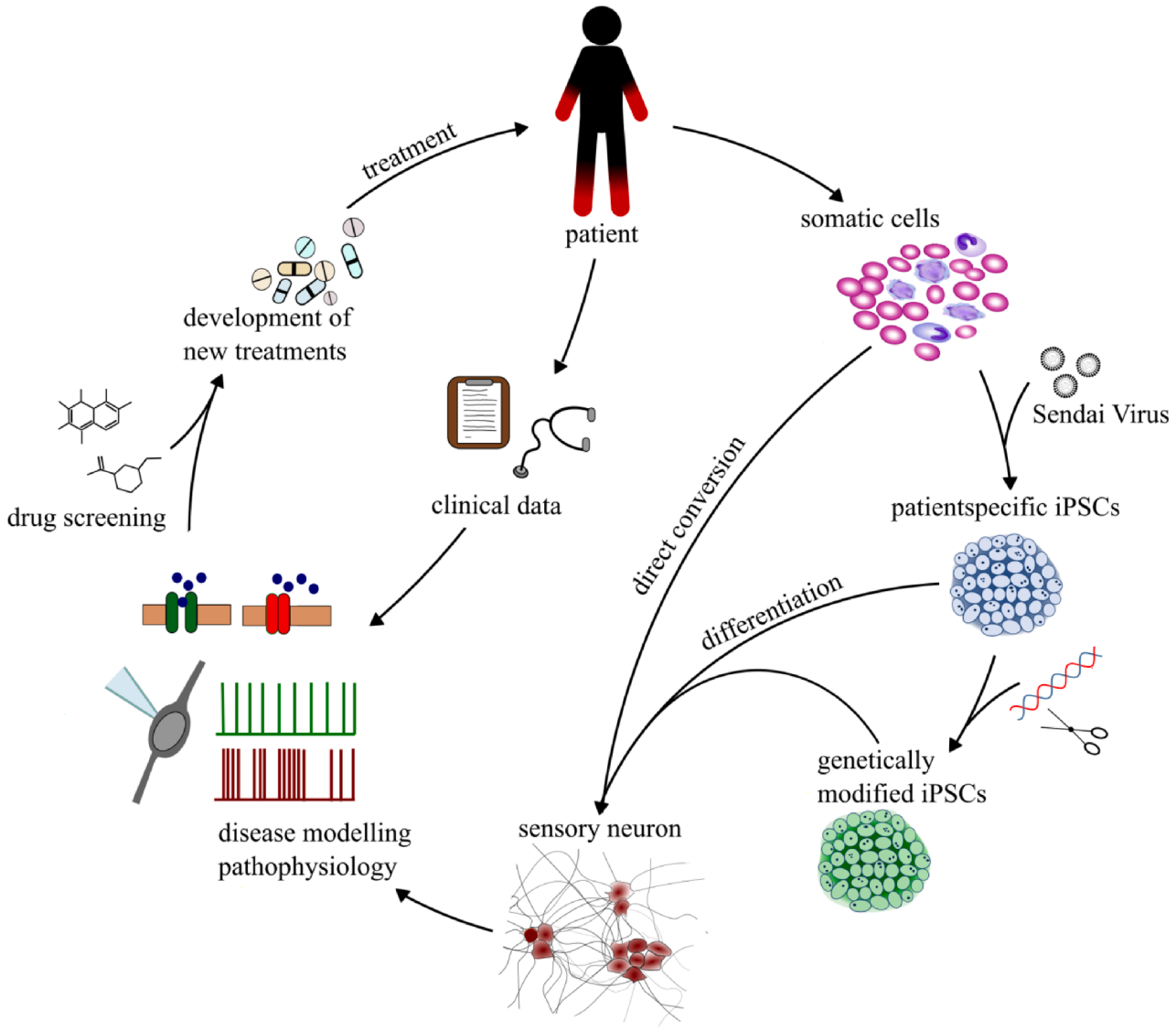

Fig. 1. Patient-specific iPSCs for disease modelling and personalized treatments of pain patients. Somatic cells of pain patients are reprogrammed to induced pluripotent stem cells (iPSCs) by e.g. sendai virus mediated ectopic expression of reprogramming factors and iPSCs are further differentiated to sensory neurons. iPSCs can be genetically modified to repair or introduce gene mutations followed by sensory neuron differentiation. Alternatively, direct conversion of somatic cells to sensory neurons was described. Sensory neurons serve for disease modelling in a dish. Clincal data of the specific patient are essential to pin-down potential *in vitro* pathophysiological phenotypes as being important for the patients' symptoms. Having identified pathophysiological alterations, *in vitro* drug screenings are performed to investigate potential treatments that ameliorate the observed phenotyp *in vitro*. Like that, patients might receive a personalized medication specific for their individual pain disease.

pluripotency gene network takes over and maintains the induced pluripotent state. Thus, transient expression of reprogramming transcription factors is sufficient for inducing a pluripotency state. Depending on the reprogramming method, the generated iPSCs are not genetically modified, but solely epigenetically reprogrammed. Further to this, iPSCs retain the patient-specific genetic background, including disease specific and/or associated mutations (Mall and Wernig, 2017; Meents et al., 2019). Additionally, iPSCs are readily subjected to genome engineering by CRISPR/Cas9 (as described below) to precisely introduce or repair mutations (Hotta and Yamanaka, 2015; Hockemeyer and Jaenisch, 2016; Sontag et al., 2017). This makes patient and disease specific iPSCs particularly appealing for disease modeling and drug discovery (Rowe and Daley, 2019).

Today, mostly plasmid or Sendai virus vectors are used for transient expression of reprogramming transcription factors (Okita et al., 2008; Fusaki et al., 2009; Ban et al., 2011). Sendai virus vectors are particularly attractive, since they (i) exhibit a high transduction efficiency and (ii) have a life cycle, which is completely confined to RNA, thereby precluding any genetic modification of target cells.

Fibroblasts are the most frequently employed somatic cells for reprogramming (Takahashi and Yamanaka, 2006; Takahashi et al., 2007, Mall and Wernig, 2017; Rowe and Daley, 2019). More recently peripheral blood mononuclear cells (PBMCs) have also been used, since obtaining PBMCs from patients is clinical routine and less invasive than a skin biopsy. Fibroblasts and PBMCs require the expression of all 4 reprogramming transcription factors (OCT4, SOX2, KLF4 and c-MYC). Often c-MYC is omitted due to its potential oncogenic activity or replaced by l-MYC, which has no oncogenic potential (Nakagawa et al., 2010). Target cells that endogenously express some of the reprogramming transcription factors, such as SOX2 in neural stem cells, require fewer transcription factors for reprogramming into iPSCs (Kim et al., 2008, 2009).

An important point to be considered when choosing patient cells for reprogramming is that iPSCs frequently contain somatic memory as remnants of the somatic cells used for reprogramming (Polo et al., 2010; Ohi et al., 2011). Somatic memory is epigenetically encoded and causes a differentiation bias towards lineages of the somatic cells used for reprogramming. However, with continuous passaging the somatic memory of iPSCs is erased (Kim et al., 2010; Nishino et al., 2011; Tesarova et al., 2016; Kim and Costello, 2017).

In the context of obtaining neurons, direct reprogramming of somatic cells into induced neurons (iNs, (Vierbuchen et al., 2010) or induced neural progenitors and induced neural stem cells (iNSC)) is particularly attractive (Han et al., 2012; Lujan et al., 2012; Thier et al., 2012). Obviously, iPSCs offer the advantage of obtaining the complete compendium of cells of our body, including neurons and auxiliary neural cells, such as Schwan cells. But reprogramming of somatic cells directly into iNs or iNSC might be the preferred way to go if only neuronal cells are to be worked with in follow-up studies.

2.2. *Differentiation into sensory neurons*

The starting point of mammalian nervous system development is the neural tube consisting of a single layer of epithelial cells enclosing a lumen that then expands to the ventricular zone (VZ) from which neurogenesis begins (Rakic, 1995). Modelling neurogenesis can be performed *in vitro* by starting from pluripotent stem cells. These can also organize as neural rosettes *in vitro*, which exhibit a strikingly similar architecture to that of the neural tube. Specifically, progenitor cells are located apicobasally and form a lumen-like structure (Zhang et al., 2001; Shi et al., 2012). These early methods reinforced the idea of "self-organization" in which pluripotent cells are capable of generating the above described complex structures without external cues, e.g. for cortical development (Gaspard et al., 2008). Major advancements came from adding specific factors (e.g. growth factors) to instruct the precursor structures to undergo a directed differentiation initiated via an "induction effect." These experiments also led to understanding and modeling mechanisms of neuronal diversification towards neurons of specific neurotransmitter phenotypes.

Compared to neuronal differentiation protocols for the central nervous system e.g. dopaminergic (Perrier et al., 2004) or cortical neurons (Muotri et al., 2005) it took comparatively long to develop effective strategies to obtain a high yield of neural crest cell (NCC) precursors as prerequisite to derive sensory neurons from hESCs or iPSCs. The early protocols relied on sorting-based methods for NCC-markers (Lee et al., 2010) to enrich NCC precursors spontaneously developing at the margins of neural rosettes (Lee et al., 2007; Zhou and Snead, 2008), in EBs (Jiang et al., 2009) or later in the population of neuralized cells generated by dual SMAD inhibition (Chambers et al., 2009; Lee et al., 2010). These multipotent NCC precursors give rise to multiple neural crest lineages including sensory neurons by adding neurotrophic factors.

The major and still most relevant method used for disease modeling of peripheral neuropathies was described by Chambers et al in 2012 (Chambers et al., 2012) (examples: (Cao et al., 2016)[IEM]; (Clark et al., 2017)[inflammatory demyelinating neuropathies]; (McDermott et al., 2019) [CIP]; (Mis et al., 2019) [IEM], (Meents et al., 2019) [IEM]; (Namer et al., 2019) [SFN]; (Zeltner et al., 2016) [FD]). The basic principle of this "induction effect" protocol is dual SMAD inhibition based neuralization of iPSCs using the BMP-Inhibitor LDN-193189 (LDN) and TGFβ-Inhibitor SB431542 (SB) later on paralleled by induction of a NCC fate by activation of WNT-signaling by GSK3-inhibition and inhibition of Notch and FGF-receptor signaling. Application of the three small-molecule inhibitors CHIR99021 (CHIR), SU5402 (SU) and DAPT leads to emergence of mostly NTRK1 (TRKA) positive nociceptive sensory like neurons in only ten days. The revolutionary discovery of these three inhibitors to promote NCC development not only accelerated differentiation of sensory neuron like cells but also replaced sorting-based methods. Of these three inhibitors CHIR seems to be the key factor to induce NCC identity and peripheral neuronal differentiation (Qi et al., 2017) whereas SU and DAPT further enhance the efficiency of sensory neuron differentiation to more than 75% and partially accelerate neuronal differentiation when combined with CHIR (Chambers et al., 2012). After transition through the NCC state, nociceptor-like cells are matured with the addition of neurotrophins (β-nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF] and glia cell-derived neurotrophic factor [GDNF]) to the media. Using this method, peptidergic and non-peptidergic subtypes of nociceptors can be grown, and ganglia-like clusters emerge after 30 days of differentiation. Importantly, the Studer group could confirm the identity of the nociceptor-like cells obtained by this protocol not only by expression of specific markers but also by electrophysiological and pharmacological characterization of mature and functional peripheral neurons including tetrodotoxin (TTX)-resistant sodium currents as a hallmark of nociceptive neurons and responses to ATP and Capsaicin (Blair and Bean, 2002; Chambers et al., 2012).

Up to now, there have been several minor modifications of this differentiation method by different groups. These changes address neuroectodermal induction by increasing the concentration or duration of application of SMAD inhibitors (Young et al., 2014; Zeltner et al., 2016) or the duration of CHIR application (Clark et al., 2017) that has been suggested to correlate with selectivity for the generation of nociceptors (Chambers et al., 2012) and/or timing in NCC induction (Maury et al., 2015). Also use of antimitotic agents (e.g. Mitomycin C or Cytosine β-D arabinofuranoside [ARAC]) has been reported to reduce the number of proliferating non-neuronal cells at the beginning of maturation (Young et al., 2014; Clark et al., 2017). Laminin or fibronectin seem to diminish disattachment of sensory-like neurons when included in the maturation media (Chambers et al., 2016; Zeltner et al., 2016; Clark et al., 2017; McDermott et al., 2019). However, the maturation period initially described may not be sufficient to yield highly mature sensory-neuron like cells (Eberhardt et al., 2015) and many groups changed to prolonged intervals of 8 weeks or more for functional analysis (Cao et al.,

2016; McDermott et al., 2019; Meents et al., 2019; Mis et al., 2019). Also including other factors like NT3 (Young et al., 2014; Schrenk-Siemens et al., 2015; Clark et al., 2017; McDermott et al., 2019), ascorbic acid (Young et al., 2014; Eberhardt et al., 2015) or retinoic acid (Schrenk-Siemens et al., 2015) might be beneficial but up to now the significance of individual changes for efficiency and subtype specificity in generation of sensory neuron like cells is incompletely understood. More recently, chemically defined approaches have been further developed to not only derive either a more heterogenous population of sensory neurons from hESCs (Alshawaf et al., 2018) but also to enrich specific subpopulations of sensory neurons e.g. proprioceptors derived from iPSCs (Dionisi et al., 2020).

Differences in the efficacy and reproducibility of differentiation also depend on genetic variations in iPSC lines (Popp et al., 2018), initial iPSC density (Chambers et al., 2009) and culture conditions (Schwartzentruber et al., 2018) at the start of the experiment and minute differences in small compounds, coating and other factors (e.g. experimenters and facility regulations) and might be a cause for adaptations of the protocol.

Besides this frequently used protocol, two other strategies have been followed to generate highly mature sensory neuron-like cells: (I) direct conversion of fibroblasts (Blanchard et al., 2015; Wainger et al., 2015) or of CD34+ mononuclear cells from peripheral blood (Lee et al., 2015; Vojnits et al., 2019) into sensory neurons and (II) generation of migrating NCCs that further differentiated to sensory neurons via neuroectodermal spheres, which attach spontaneously (Schrenk-Siemens et al., 2015).

Two direct conversion approaches (I) of murine and human fibroblasts were described using retroviral transformation either continuously expressing five transcription factors to obtain nociceptive sensory neuron-like cells (Wainger et al., 2015) or transiently expressing Brn3a in combination with either Neurogenin 1 or Neurogenin 2 to differentiate various subtypes of sensory neuron-like cells including pain- and itch-sensing neurons (Blanchard et al., 2015). One drawback however is the comparatively low efficiency in direct differentiation approaches from fibroblasts albeit the protocols seem to be robust enough to induce a comparatively mature and functional state of sensory neurons. To obtain sensory neurons from peripheral blood, CD34 expressing mononuclear cells are reprogrammed to an intermediate neuronal precursor state (iNPCs) using lentiviral delivery of Oct4 in combination with dual SMAD- and GSK3- inhibition. These iNPCs can further be differentiated into different subtypes of central nervous neurons, astrocytes and oligodendrocytes (Lee et al., 2015) as well as into sensory neurons. This strategy offers the advantage of comparatively uncomplicated precursor expansion and its sensory neurons have mainly been used in drug-screening formats investigating chemotherapy-induced neurite damage so far.

The second method (II) mimics embryonal development by taking advantage of the spatial separation of NCC-like populations that migrate out of human iPSC-derived neuroectodermal spheres. Maturation of these cells by neurotropic factors boosted by transient expression of Neurogenin 2 gives rise to mechanosensitive sensory neuron-like cells but could also be a promising strategy to adapt for other sensory neuron subtypes (Schrenk-Siemens et al., 2015).

Genetic variability of different individuals but also genetic variability of different iPSC lines (Popp et al., 2018) renders human cell-based experiments challenging. It is crucial to perform quality controls at the levels of pluripotency, including genetic stability and expression of pluripotency markers as well as testing the potential to differentiate into the desired lineage at comparable percentages between lines (Table 1). iPSC clones need to be tested and the most suitable clones should be selected. To reduce variabilities at different levels, inclusion of many patients if possible, e.g. when investigating common or sporadic diseases, or genome editing, e.g. generation of isogenic lines using CRISPR/Cas9 see 2.3, can help to solve these issues.

Taken together these differentiation strategies could not only be useful for disease modelling but also help our understanding of human subtypes of sensory neurons. However previous studies indicate that there seems to be an unmet need of functional evidence of maturity of sensory neuron-like cells (Eberhardt et al., 2015) and subtype confirmation/ characterization would be desirable for disease modeling (Table 1).

Table 1
Suggested quality control steps during differentiation of iPSCs into peripheral sensory neurons.

Cell type	Quality feature	Expected characteristics
iPSCs	Morphology	Round cells with prominent nucleoli and high ratio of nucleus to cytoplasm Densely packed colonies
	Pluripotency	Marker expression (e.g. TRA1-60) Differentiation potential (spontaneous or directed to the desired lineage or trilineage differentiation)
	Genetics	Karyotyping or Copy-Number-Variation-Analysis Sequencing of disease relevant genes Presence of desired gene variant/ mutation
Neural crest like cells	Marker Expression	SOX10, p75
Nociceptors	Marker Expression	*Sensory neurons*: Tuj1, Peripherin, ISL1, BRN3A *Nociceptor early genes*: NTR1, NGF, PRDM12 *Mature Nociceptors*: - surface expression of Nav1.7* and Nav1.8* - Substance P and/or cGRP - TRPV1* - TRKA
	Morphology	Neuron-type morphology with long processes, growth within ganglia
	Electrophysiology	Resting membrane potential <-40mV Mature action potentials with overshoot Robust sodium and potassium currents in voltage-clamp Tetrodotoxin (TTX)-resistant sodium currents in voltage-clamp with kinetics of Nav1.8 Responses to ATP and/or capsaicin

*indicates marker, that can be detected by immunostainings or, with higher sensitivity, by electrophysiology.

2.3. Genetic modification

Genome engineering offers researchers the ability to precisely delineate phenotype from genotype through the targeted manipulation of the genome. The technique significantly advances *in vitro* disease modelling by permitting the introduction and or/ correction of pathogenic mutations and thus holds promise for the study of rare hereditary pain disorders, where the patient population and patient-derived cellular resources are limited.

iPSCs are a challenging cell type to genetically engineer and initial approaches suffered from extremely low rates of efficiency which was compounded by difficulties associated with the clonal isolation of stem cells (Zou et al., 2009). The development of CRISPR-Cas technology has significantly increased the ease of iPSC-genome engineering (GE) and has revolutionized the GE field.

The endogenous CRISPR-Cas9 system serves as a bacterial and archaeal adaptive immune response intended to prevent the invasion of foreign DNA elements through the targeted nucleolytic destruction of invading DNA (Marraffini and Sontheimer, 2010). However, CRISPR-Cas9 has now been co-opted and repurposed for eukaryotic gene editing with great effect and has been widely adopted by the research community (Wang et al., 2013; Kleinstiver et al., 2015). Cellular expression of the Cas9 endonuclease protein along with a sequence

specific chimeric nucleotide single guide RNA (sgRNA) is sufficient to precisely target double stranded genomic DNA breaks. The simplicity of the system offers a number of advantages over early site specific nuclease (SSN) methods such as those that used transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs), both of which required laborious construction and validation of the bi-partite proteins for each genomic loci targeted (Durai et al., 2005; Sanjana et al., 2012). For CRISPR-Cas9, the researcher only needs to modify a short 20 nucleotide sequence appropriate to the target for efficient and precise cleavage, and site selection is constrained only by the requirement of a short, flanking 2–5 bp sequence known as the proto adjacent motif (PAM) (Mojica et al., 2009; Westra et al., 2013). The most commonly used Cas9 variant derived from *Streptococcus pyogenes* (spCas9) uses the PAM 'NGG', which is thought to occur on average every 8 bp in the human genome (Hsu et al., 2014).

A common concern of all gene editing tools is the potential for off-target activity. Although the targeting of Cas9 to DNA loci is thought to be tightly controlled by the specific sgRNA and the requirement for the PAM motif, a number of early papers demonstrated potential for off-target activity (Pattanayak et al., 2013; Lin et al., 2014). Subsequent work however has suggested that Cas9 DNA binding is more promiscuous than actual DNA cleavage (Wu et al., 2014) and several engineered Cas variants have now been developed with increased specificity and significantly decreased levels of off-target activity e.g. SpCas9-HF1 and HypaCas9 (Kleinstiver et al., 2016; Chen et al., 2017).

Once Cas9 has produced double stranded breaks (DSB) the repair can be manipulated through the provision of a DNA template containing user-defined nucleotide edits. The successful incorporation of this template into the genome via homology directed repair (HDR) means disease associated SNPs can be rapidly introduced into control cell populations. Equally, patient derived iPSC lines can be genetically 'corrected' and the mutations reverted to control sequences. In this manner, 'parental' and edited cells can be directly compared under the assumption that they differ only at the targeted locus, creating isogenic controls. This was demonstrated in SCN9A CIP iPSC patient lines in McDermott et al., 2019 (discussed further below). IPSC-derived nociceptors from patients with compound heterozygous loss of function mutations in SCN9A were found to have an increased rheobase (the current threshold required to elicit an action potential). Genome engineering of these patient lines was used to definitively ascribe this reduction in excitability to the SCN9A locus and indeed genetic correction of a c.2488c > t in exon 15 of SCN9A was sufficient to reverse the increased rheobase observed in CIP iPSC-derived nociceptors. This concept of isogenic comparison is particularly powerful for iPSC-based disease models where considerable clonal heterogeneity can significantly confound cellular phenotypes (Cahan and Daley, 2013), particularly those of neuronal excitability which can be relatively subtle *in vitro*.

The CRISPR-Cas toolbox has recently been further expanded through the development of a number of epigenetic and transcriptional modifier Cas variants, including catalytically dead dCas9 fused to a variety of repressor/activator domains e.g. VP64, P65, and KRAB (Thakore et al., 2015; Xu et al., 2019). The use of these variants allows for the fine dissection of protein dosage effects through targeted knockdown or gene activation. This approach may prove useful in establishing the expression levels required for phenotypic activity and thus may bear relevance for translational studies involving for instance ion channel blocker development.

Technical challenges remain in increasing rates of homology directed repair in iPSCs where the DNA repair pathway is strongly biased to non-homology directed end joining (NHEJ) - particularly a subtype known as microhomology mediated end joining (MMEJ) (van Overbeek et al., 2016; Taheri-Ghahfarokhi et al., 2018). The non-homology-independent repair pathway operates concurrently and competitively with HDR and leads to the formation of random insertion/deletion products. This phenomenon can be exploited experimentally for the rapid generation of gene knockouts through indel mediated frameshifts (McDermott et al., 2019). However, as the most frequent source of disease-causing variants are single point mutations, efforts to increase the efficiency of high-fidelity repair are important. This is also relevant to pain given that one of the most common cause of Mendelian pain disorders are single nucleotide changes in Nav1.7, resulting in gain of function and clinical phenotypes such as Erythromelalgia, Paroxysmal Extreme Pain Disorder and Small Fibre Neuropathy which are being modelled with iPSC-derived nociceptors, including the investigation (Meents et al., 2019; Mis et al., 2019) of genetic modifiers. Anzalone et al have recently described the development of a 'prime editor'. This system consists of a catalytically impaired Cas9 fused to a reverse transcriptase domain allowing for the simultaneous targeting and insertion of nucleotide edits (Anzalone et al., 2019). This approach significantly improves the efficiency and throughput of SNP editing, however this method has yet to be trialled in iPSCs.

Further developments aimed to increase the efficiency of larger edits will also be important to facilitate the study of sensory neuron proteins *in vitro*. Genetic knock-in of epitope and fluorescent tags can provide significant insights into intracellular protein trafficking and regulation (Fig. 2). Pursuing this strategy also circumvents problems with antibody specificity, which has afflicted the study of many neuronal proteins and in particular the voltage gated sodium channel family (owing to significant shared amino acid homology). Endogenous tagging is also preferable to the overexpression of proteins often seen in heterologous systems. Genetically tagged proteins remain under tight control of endogenous regulatory elements and promoters, better recapitulating *in vivo* patterns of expression.

3. Disease modelling

3.1. Gain of pain

Once iPSCs are generated and differentiated into peripheral sensory

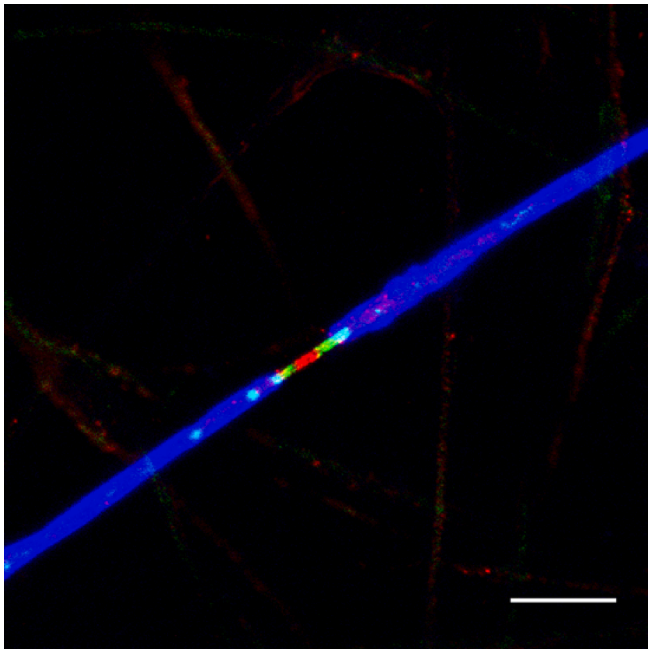

Fig. 2. Sensory neurite and Schwann cells form nodes of Ranvier. CRISPR/Cas9 was used to knock-in a HA tag to the c-terminus of $Na_v1.7$. Representative image showing Nav1.7-HA trafficked to the nodes of Ranvier in iPSC-derived nociceptors and rodent Schwann cell cocultures. Myelin Basic Protein (blue), CASPR (green), $Na_v1.7$ red. Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neurons they offer eclectic potential for studying disease mechanisms and to search for individualized or population therapies. iPSCs derived from patients suffering from genetic pain syndromes offer the unique possibility to study the effect of genetic variations directly on the affected cells, the nociceptors. Gain-of-function mutations in Nav1.7 are known to cause at least three separate pain syndromes: inherited erythromelalgia (IEM), paroxysmal extreme pain disorder (PEPD) or small fiber neuropathy (SFN) (Lampert et al., 2014; Bennett et al., 2019). Although clinically distinct, all three syndromes result in severe burning pain, which significantly impairs the patients' quality of life. Microneurography recordings of sensory nerve C-fibers of IEM or SFN patients revealed that their fibers are spontaneously active, and this activity correlates with the clinical symptoms (Kleggetveit et al., 2016). Thus, iPSCs derived sensory neurons of patients carrying mutations are likely to show a disease related phenotype.

Indeed, the first study on nociceptors derived from five IEM patients revealed increased spontaneous activity and a decreased rheobase for IEM nociceptors (Cao et al., 2016). Symptoms of IEM are typically enhanced by increased ambient temperature, and indeed application of heat significantly reduced the action potential threshold in IEM derived nociceptors. In a small clinical trial, the authors of this study tested a Nav1.7-specific blocker, which showed a positive effect on rheobase in the patient-derived nociceptors. Some of the five participating patients showed a mild pain reduction 4–5 h following treatment (Cao et al., 2016).

From experiments in HEK cells it was known that most IEM mutations lead to a significant alleviation of Nav1.7 channel opening, i.e. a smaller depolarizing stimulus is sufficient to open the channels (Lampert et al., 2014; Bennett et al., 2019). This is reflected by a leftward shift of the voltage-dependence of channel activation. As sodium channels are very fast gating, good voltage-clamp is necessary for reliable recordings. IPSC-derived nociceptors show long neurites which impair adequate recording conditions, and thus good measurements of voltage-dependence of activation is hard to achieve. These problems can be circumvented by using a pre-pulse protocol to inactivate sodium channels in the neurites (Milescu et al., 2010, Meents et al., 2019). With this technique it was shown that Nav1.7 indeed activates at more negative potentials when carrying an IEM mutation (Meents et al., 2019), thus, mechanistically explaining why patient derived neurons have a lower threshold for action potential firing.

To single out the currents contributed by Nav1.7, the selective blocker ProTx-II was applied to IEM derived nociceptors and indeed, the difference to WT nociceptors in sodium channel activation was abolished (Meents et al., 2019). The remaining TTX-sensitive sodium channels are activating at more negative potentials compared to WT Nav1.7, suggesting that Nav1.7 is not active during subthreshold depolarization, but sets the threshold for action potentials, thus regulating sensory neurons excitability. This finding is likely to have implications for future drug development.

There are about 28 known mutations in Nav1.7 linked to IEM, and all carriers show symptoms, albeit to a varying degree. In a study investigating iPSC- derived nociceptors of two carriers of the same mutation, these individual differences were also reflected in the excitability of the individuals' neurons (Mis et al., 2019). In addition, the authors identified a variant in Kv7.2 which seems to be protective, and thus may reduce the severity of the symptoms of one of the investigated patients. Thus, patient data and data generated with sensory neurons derived from iPSCs from a single patient can provide insights into the disease pathomechanisms of that individual patient with his/her specific genetic background.

3.2. Loss of pain

Congenital insensitivity to pain (CIP) is a striking clinical phenotype in which individuals are born unable to perceive pain in response to noxious mechanical, thermal and chemical stimuli applied anywhere to the body (Cox et al., 2006; Goldberg et al., 2007; Bennett and Woods, 2014). These patients also have absent itch in response to pruritic stimuli such as histamine and can have thermosensory deficits (McDermott et al., 2019). CIP patients accrue severe orthopedic and soft tissue injuries and have a higher rate of mortality compared to the general population, emphasizing the importance of acute pain as an adaptive phenomenon. The identification of the gene mutations responsible for CIP provides fundamental insight into the neurobiology of the nociceptive system and has also revealed novel analgesic drug targets. The genetic causes of CIP can be broadly divided into mutations in genes required for the early development and survival of nociceptors (NTR1, NGF and PRDM12) and genes that encode voltage gated ion channels regulating the functional properties (bi-allelic loss of function mutations in SCN9a encoding Nav1.7 and heterozygous gain of function mutations in SCN11a encoding Nav1.9) (Schon et al., 2018). Given the relatively selective expression of Nav1.7 in the peripheral nervous system and the complementary finding (described above) that gain of function mutations can cause inherited pain disorders, this channel has been a great focus for analgesic drug discovery. Using human iPSC-derived nociceptors provided the opportunity to directly assess the impact of Nav1.7 on the regulation of human nociceptor excitability. McDermott et al. generated iPSC lines from 2 CIP patients from independent pedigrees. Using CRISPR/Cas9 technology applied to a control line, they also generated a Nav1.7 knockout clone from a healthy control cell line. They showed that bi-allelic loss of function mutations in Nav1.7 resulted in reduced nociceptor excitability, both in the form of increased rheobase and also reduced action potential firing in response to suprathreshold current injection. Importantly, these effects could be largely reversed following genomic correction of one allele (as described earlier in the section on genome engineering). These findings *in vitro* were complemented by the demonstration that the same patients had an absence of functional nociceptors when using microneurography *in vivo*.

In mouse models it has been suggested that increased endogenous opioid expression in DRGs secondary to ablation of Nav1.7, contributes to pain insensitivity (Minett et al., 2015). Although this has not been replicated in a subsequent study using a rat Nav1.7 loss of function model (Chen et al., 2020). In the described *in vitro* system, reduction of excitability in human nociceptors as a consequence of reduced Nav1.7 currents was independent of endogenous opioid expression. Demonstrating the translational potential of the model, the authors also used iPSC-derived nociceptors lacking functional Nav1.7 to show that some small molecule compounds developed as Nav1.7-selective analgesics, are not, in fact, selective for this channel and show promiscuous off-target activity at clinically relevant concentrations. In sum, the advantages of using iPSCs in this context has been: (I) the confirmation that Nav1.7 function is a key regulator of excitability of human nociceptors; (II) that this effect can be achieved independently of endogenous opioids and finally, (III) this system can be used to screen target engagement of drugs acting on Nav1.7 with the reassurance of a negative control lacking this ion channel.

The use of human iPSCs to model CIP is not without its challenges. As in any *in vitro* cellular model, axonal length is much shorter than *in vivo*, the ability to test interaction with other cell types is limited (although some co-culture techniques are available (Clark et al., 2017)) and maturation time *in vitro* is also shorter. Whilst we found reduced epidermal innervation by nociceptors in skin biopsies of adults with Nav1.7 LOF CIP, we did not find a significant change in axon outgrowth of iPSC derived nociceptors *in vitro,* probably as a consequence of these differences. A further caveat is that the utility of the system depends on the expression of the gene of interest. Nav1.7 is highly expressed in the nociceptors generated by the "Chambers protocol" and generates sodium currents which are TTX sensitive in iPSC-derived nociceptors making this an excellent model system. Nav1.9 is a TTX-resistant voltage gated sodium channel and gain of function mutations in Nav1.9 can cause CIP through a proposed depolarizing block (Leipold et al., 2013; Huang et al., 2017). It would be interesting to test this hypothesis in a

human system, however it has been demonstrated that using the "Chambers protocol" with short differentiation times (as opposed to what would be expected in adult nociceptors), most of the TTX-R sodium current is derived from Nav1.5, reflecting the immaturity of the system (Eberhardt et al., 2015). This emphasizes the need to optimize differentiation protocols in terms of the heterogeneity of nociceptor sub-types and the generation of mature, adult nociceptors in order to fully realize the potential of this system in studying CIP.

4. Translational approaches

IPSC- derived sensory neurons offer a personalized treatment-approach which can be helpful to identify drugs specifically effective for an individual patient. Often neuropathic pain patients need to test several drugs over years, only to find that side effects are too severe, or efficacy is lacking. Subjects react individually and findings from population studies on drug efficacy may not apply to each specific patient. Thus, improvement in individual patient care is highly desirable. In order to build a platform for individual drug testing, it is required that the patient's clinical phenotype is recapitulated *in vitro*, and as outlined above we have good evidence that clinical symptoms of neuropathic pain are reflected faithfully in iPSC- derived sensory neurons of the patients investigated.

In a recent study on a patient suffering from SFN this asset was used and iPSC- derived sensory neurons were plated on multi-electrode arrays (Namer et al., 2019). In this setting increased spontaneous activity of iPSC- derived sensory neurons was recorded, which very nicely correlated with microneurography findings of spontaneously active C-fibers in this patient. The patient suffered from intense neuropathic pain over the past ten years (7,5 on a visual analog scale for pain (VAS scale) where 0 is no pain, and 10 the worst imaginable pain) and tested several drugs without satisfactory effect. The sodium channel modifier lacosamide was not part of the drugs prescribed to the patient. When tested on the iPSC- derived sensory neurons of the patient, lacosamide significantly reduced spontaneous activity *in vitro* (Namer et al., 2019). Following prescription of lacosamide to the patient, it dramatically reduced the pain levels to 1,5 on a VAS. Microneurography recordings of the patient under lacosamide revealed a significantly reduced number of spontaneously active C-fibers, which was now comparable to healthy age-matched controls. Therefore, iPSC- derived sensory neurons helped to identify an FDA-approved drug *in vitro* as an effective treatment option for that specific patient (Namer et al., 2019).

In order to be able to offer this approach to more pain patients, it is clear that the human model systems are needed at larger scales, and there are activities aiming at producing iPSCs from patients in a faster and less cost-intensive manner (e.g. stem cell factory, https://www.stemcellfactory3.de/en/). Differentiation into sensory neurons to date is laborious in that it takes a long time and is subject to high variability. Future attempts will need to simplify differentiation methods. Functional readout of cellular activity of iPSC- derived sensory neurons can comprise multi-electrode-arrays, high-content microscopy, and other higher throughput methods, such as fluorometry.

Availability of patient derived iPSCs will allow for individual drug screening, repurposing of already available drugs, but will also help the development of population-based medicine, can provide companion diagnostic tools, and help to stratify patient subgroups. This approach will likely help identify treatment not only for patients suffering from treatment resistant pain. To overcome inter-individual variability and find broader applicable treatment options, patient databases and corresponding iPSC-banks are necessary that allow the standardization and correlation of patient and *in vitro* data to identify more general disease mechanisms and predict the therapy outcome via iPSC-data combined with patient data in the future.

CRediT authorship contribution statement

Angelika Lampert: Conceptualization, Writing - original draft. **David L. Bennett:** Conceptualization, Writing - original draft. **Lucy A. McDermott:** Conceptualization, Writing - original draft. **Anika Neureiter:** Conceptualization, Writing - original draft. **Esther Eberhardt:** Conceptualization, Writing - original draft. **Beate Winner:** Conceptualization, Writing - original draft. **Martin Zenke:** Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper. David Bennett has acted as a consultant in the last 2 years for Amgen, Bristows, CODA therapeutic, LatigoBio, Lilly, Mundipharma, Orion, Regeneron and Theranexus on behalf of Oxford University Innovation. David Bennett has an Industrial Partnership grant with Astra Zeneca funded by the BBSRC. Angelika Lampert has an Industrial Partnership grant with Hofmann-La Roche and, together with Martin Zenke, with Grünenthal funded by the BMBF (Germany Ministry for Education and Research).

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