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Modelling the dorsal root ganglia using human pluripotent stem cells: A platform to study peripheral neuropathies

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Modelling the Dorsal Root Ganglia using Human Pluripotent Stem Cells: A Platform to Study Peripheral Neuropathies

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Key words: Human pluripotent stem cells, dorsal root ganglia, sensory neurons, directed differentiation, direct reprogramming

Abstract

Sensory neurons of the dorsal root ganglia (DRG) are the primary responders to stimuli inducing feelings of touch, pain, temperature, vibration, pressure and muscle tension. They consist of multiple subpopulations based on their morphology, molecular and functional properties. Our understanding of DRG sensory neurons has been predominantly driven by rodent studies and using transformed cell lines, whereas less is known about human sensory DRG neurons simply because of limited availability of human tissue. Although these previous studies have been fundamental for our understanding of the sensory system, it is imperative to profile human DRG subpopulations as it is becoming evident that human sensory neurons do not share the identical molecular and functional properties found in other species. Furthermore, there are wide range of diseases and disorders that directly/indirectly cause sensory neuronal degeneration or dysfunctionality. Having an *in vitro* source of human DRG sensory neurons is paramount for studying their development, unique neuronal properties and for accelerating regenerative therapies to treat sensory neuropathies. Here we review the major studies describing generation of DRG sensory neurons from human pluripotent stem cells and fibroblasts and the gaps that need to be addressed for using *in vitro*-generated human DRG neurons to model human DRG tissue.

Introduction

The fundamental purpose of the sensory nervous system is to receive and transmit information from skin, muscle and sensory organs to the brain, which initiates how we interpret our external and internal world and consequently influences what response will be made. The sensory nervous system includes the visual, auditory and olfactory systems as well as the peripherally located cranial sensory and dorsal root ganglia (DRG). The DRG consists of a multitude of sensory neuronal subtypes that function to relay sensory stimuli, including temperature, pressure, pain and position to the central nervous system.

Not surprisingly, there are vast ranges of diseases and conditions (Melli and Höke, 2009), usually progressive, which can affect DRG sensory neurons. The underlying causes of DRG degeneration may be either directly intrinsic to DRG neurons or indirectly associated with other pathologies. Some inherited genetic diseases inducing DRG degeneration include Friedreich's Ataxia (Delatycki and Corben, 2012) and Charcot Marie Tooth Disease (d'Ydewalle et al., 2012). Other conditions that may affect DRG neuronal functionality are autoimmune disease (Sederholm, 2010), infections (such as AIDS) (Schütz and Robinson-Papp, 2013) or unknown idiopathic conditions (de Schryver et al., 2011). DRG degeneration is also observed in cases relating to drugs (Manji, 2011) and alcohol toxicity (Chopra and Tiwari, 2012). DRG neurons can also be severely damaged by physical trauma (Lim et al., 2015). People affected by DRG peripheral neuropathy report different symptoms, including mild to chronic pain, loss of feeling and intense itchiness. Such symptoms can be severely debilitating depending on the nature and cause. Often the degeneration is progressive and, for some conditions, treatments are geared to damping the symptoms and slowing progression of nerve damage. In many cases, there is no treatment available. Having an *in vitro* source of human sensory neurons would be invaluable for developing neuroregenerative therapies to treat specific neuropathies.

Classification of DRG sensory neurons

Somatosensory neurons of the DRG are traditionally classified into three sub-populations: nociceptors, mechanoreceptors and proprioceptors. This classification is based on characteristics relating to their function and morphological features such as cell body size, axon diameter, degree of myelination, types of afferent endings and the laminar targets of their efferent terminals in the spinal cord (Kandel et al., 2013). However, studies conducted on transgenic mice and more sophisticated molecular techniques have provided a breakthrough in this field, revealing a more complex and subtle diversity among these three subpopulations.

Emerging new knowledge enables the possibility to improve the traditional classification and adopt new criteria to classify the somatosensory neurons based on in-depth transcriptional profiling matched with functional analyses. A general overview of the three major classes of DRG sensory neurons are:

Nociceptive neurons respond to painful or pruritic (itch) stimuli and thermoception (Dubin and Patapoutian, 2010; Zhang and Bao, 2006). They innervate peripheral tissues such as cutaneous epithelial tissue, as well as muscle and other internal organs, where molecular receptors located on sensory terminals react to noxious stimuli (Proske and Gandevia, 2012). They are classified as small diameter (<30 μm) and can be distinguished several subtypes depending on the rate of myelination: unmyelinated (C) or lightly myelinated (Aδ) fibres. Unmyelinated C fibre neurons are characterized by slow conductivity (~2m/s) and are able of responding to different combination of stimuli, including temperatures, pruritogens, tissue damage, chemical irritants (Laing and Dhaka, 2016). Myelinated Aδ fibre neurons have faster conduction (up to 30m/s) and, as consequence of this property, are specialized for detecting localized fast pain, repeated stimulation as well as tissue injury (Laing and Dhaka, 2016). The neurotrophic receptor expressed by nociceptors is TrkA, that has high affinity for Nerve Growth Factor (NGF). There is a smaller population of nociceptive neurons that transiently express TrkA during development, and eventually express Ret (Luo et al., 2007). Nociceptors are predominantly excitatory neurons and release glutamate as their primary neurotransmitter. They can be further sub-classified into peptidergic and non-peptidergic neurons. Peptidergic nociceptive neurons release neuropeptides such as substance P or calcitonin gene related-peptide (CGRP), instead non-peptidergic neurons are identified by the expression of isolectin IB4 (a histological marker) (Mandge and Manchanda, 2015).

Proprioceptive neurons transmit information about conscious sensations, such as limb position, balance and movement. They consist of large (12-20μm) and medium (6-12μm) diameter myelinated A fibres (Aα, Aβ). Proprioceptors express TrkC neurotrophic receptors, activated by Neurotrophin 3 (NT-3) (Fariñas et al., 1998; Henion et al., 1995; Liebl et al., 1997; Tessarollo et al., 1997). Due to their degree of myelination and diameter size, they have a fast conduction velocity (72-120 m/s for large proprioceptors and 30-70m/s for medium ones) (Le Pichon and Chesler, 2014). Proprioceptors innervate specialised organs called Golgi tendon organs and muscle spindle. They function to sense muscle tension, contraction and limb position. Their efferent projections mostly end in laminae III-V of the spinal cord. However,

some also make connections in deeper laminae in order to communicate with motor neurons of reflex circuits (Le Pichon and Chesler, 2014).

Mechanoreceptors innervate skin tissue and detect cutaneous touch sensation and vibrational stimuli. Most mechanoreceptor fibres are large diameter (Aβ) (Abraira and Ginty, 2013; Le Pichon and Chesler, 2014) myelinated and usually distinguished by their expression of TrkB receptor which has high affinity for Brain-derived growth factor (BDNF) (Shimizu et al., 2007). Mechanoreceptors project to the dermal and epidermal regions of the skin and function to detect mechanical stimuli via specialised mechanosensory end organs such as Meissner's corpuscles, Pacinian corpuscles, Ruffini corpuscules and Merkel cell endings (Fleming and Luo, 2013; Gilman, 2002; Zimmerman et al., 2014).

These classification systems have served well but with the advent of better molecular techniques we know that they may not adequately cover the true diversity or the lineage origin. New emerging studies using single cell gene expression profiling of adult rodent DRGs have identified novel subpopulations, as well as re-defined gene expression profiles of more commonly known populations (Li et al., 2017, 2016; Usoskin et al., 2015). To date most of the molecular and functional profiling of sensory DRG subclasses has been driven by animal studies and less is known about human DRG neurons. The few studies describing human DRG sensory neurons show inconsistencies in molecular and functional properties compared to that described in the rodent. Some examples include: P2X3 receptors that are involved in mediating chronic pain show different potencies in their response to antagonists between rodent and primate DRG neurons (Serrano et al., 2012); pharmacological agents that effectively block $GABA_A$ receptor currents in rodent DRG neurons are not effective in human DRGs (Valeyev et al., 1996); the family of mas-related G-protein-coupled receptors (MRGPRS) associated with nociceptor neurons consist of 4 genes in the human genome but over 30 genes within the mouse genome (Zylka et al., 2003); there are differences in the proportion of DRG neurons that co-express TRPV1/TRKA and RET/TRKA between adult human and mouse DRG tissue (Rostock et al., 2017); NF200 expression is detected in most human DRG neurons but only in a subset of mouse DRGs (Rostock et al., 2017). These studies and others strongly highlight the need to work with human sensory neurons, particularly for screening candidate pharmaceutical compounds that modulate adverse sensations, such as chronic pain.

Human sources of dorsal root ganglia sensory neurons

There is a strong need to obtain an *in vitro* source of human sensory neurons to study their unique molecular and functional profiles, myelination properties, regenerative capacities and, in particular, for establishing high through-put drug screening platforms. Whilst there are reports describing human sensory neurons derived from human DRG tissue (Davidson et al., 2014; Rostock et al., 2017; Valtcheva et al., 2016; Zhang et al., 2017), this source is impractical for many laboratories. With the discovery of human pluripotent stem cells (hPSC), it is now feasible to derive human sensory neurons in abundance and minimize the need to access human DRG tissue for advancing therapies and knowledge about the human sensory system. The review below details some of the major studies describing generation of human DRG neurons from hPSC, outlining the approaches used, characterization of the neurons and their applications. An alternative approach, whereby sensory neurons are generated directly from human fibroblasts, is also described.

Human pluripotent stem cells

Pluripotent stem cells are stem cells that have the ability to differentiate to all cell types of body. There are two different avenue by which human pluripotent stem cells (hPSC) can be generated; (a) from embryos (human embryonic stem cells, hESC), and (b) via genetic reprogramming of somatic cells to induce pluripotent stem cells (iPSC) (Hirschi et al., 2014; Takahashi and Yamanaka, 2006). IPSC technologies have allowed the possibility of modifying patient-derived cells to regain their plasticity and pluripotency, which can then be used to generate the appropriate cell type for that disease. For this reason, in the last decade, medical research is focused on the possible application of iPSC in regenerative medicine, using iPSC disease models for elucidating disease pathologies and drug discovery research.

To date there have been two main approaches for deriving sensory neurons from hPSC (Figure 1). One approach is to recapitulate stages of DRG embryonic development in the culture dish, whereby hPSC are initially differentiated to neural crest progenitors followed by their subsequent differentiation to DRG neurons. The second approach is to direct hPSC differentiation to mature functional sensory neurons, bypassing the progenitor stage. Both systems have their pros and cons, which are outlined below.

HPSC differentiation to mixed populations of DRG sensory neurons

Developmentally, all DRG neurons arise from neural crest progenitor cells that migrate from the embryonic dorsal neural tube to the presumptive DRG regions and upregulate

expression of Islet1/2 and Brn3A (Dykes et al., 2011; Raible and Ungos, 2006). There are numerous publications describing hPSC differentiation to neural crest (Table 1) and include evidence of DRG sensory neuronal differentiation as demonstrated by co-expression of BRN3A and ISLET1 (Alshawaf et al., 2018; Brokhman et al., 2008; Deng et al., 2014; Denham et al., 2015; Dykes et al., 2011; Goldstein et al., 2010; Hoelting et al., 2016; Lee et al., 2012). This included studies from our laboratory describing efficient generation of neural crest from hPSC using small molecule inhibitors of activin/nodal and GSK3β signalling pathways, SB431542 and CHIR99021, respectively, followed by treatment with bone morphogenetic protein 2 (BMP2) (Denham et al., 2015). In this same study, we also reported DRG neuronal differentiation by culturing neural crest progenitors in media supplemented with BDNF, NT3 and NGF. Our initial study was not extensive in charactering DRG neuronal subtypes found in differentiated cultures but instead showed neurons expressing pan-sensory neuronal markers, BRN3A and ISLET1 as well as Peripherin (Denham et al., 2015). This was addressed in a more recent report whereby we examined expression of sensory neuronal subpopulation markers in the hESC-derived neural crest differentiated cultures (Alshawaf et al., 2018). We identified the sensory neuronal cultures consisted of heterogeneous neuronal subtypes, expressing TRKA (25%), TRKB (17%) and TRKC (24%) receptors, which are classic markers of nociceptors, mechanoreceptors and proprioceptors respectively. Using Usoskin et al.'s (Usoskin et al., 2015) study as reference, we also examined co-expression of markers to distinguish several subtypes of sensory neurons within the same subpopulation. By immunostaining, we observed neurons double positive for TRKB and NECAB2 as well as TRKC and FAM19A1, which correspond to different subtypes of mechanoreceptive neurons. In addition, we identified different subtypes of nociceptors, peptidergic and non-peptidergic neurons. This included neurons co-expressing TRKA and TRPV1 or PLEXIN C1 and somatostatin, which are found in distinct populations of non-peptidergic neurons. Peptidergic neurons expressing TRKA and FAM19A1 or TRKA and TAC1 were also detected. Characteristic markers of proprioceptive neurons, such as Osteopontin and Parvalbumin, were also found within the cultures. Hetereogeneity within the sensory neuronal cultures was further confirmed by functional analyses using multi-electrode arrays, whereby neurons responding to chemical, heat and osmotic pressure stimuli were demonstrated (Alshawaf et al., 2018).

This report was one of the first to show a multitude of sensory neuronal subclasses derived from hPSCs using the same differentiation protocol. Having a differentiation system that enables the generation of diverse DRG sensory neuronal populations may be resourceful

for modelling DRG tissue to study neuronal/neuronal and neuronal/glial interactions between different subtypes. It also provides a platform to reveal and characterize sensory neuronal populations that may exist within human DRG tissue. Further in-depth analyses, such as single cell RNA-sequencing, will provide further insight about the subclasses of sensory neurons found within the human DRG and be able to compare this to other species, particularly rodent DRGs that are extensively studied.

HPSC differentiation to nociceptors

Whilst having heterogeneous cultures of sensory neurons may be useful for modelling the DRG in its entirety, it is also advantageous to have cultures consisting of homogenous sensory neuronal subtypes for performing in-depth phenotypic and functional analyses to characterize a specific population.

The first report describing direct hPSC differentiation to a specific DRG lineage, nociceptors, was by Chambers et al. (Chambers et al., 2012). Using a combination of five different small molecules, LDN-193189, SB431542, SU5402, CHIR99021, and DAPT, at least 60% TRKA expressing nociceptors were generated from hPSC. RUNX1 expression supported the derivation of peptidergic neurons, which was confirmed by immunostainings for Substance P and CGRP. Interestingly, their results revealed a difference between what reported in literature about the expression of RUNX1 in mouse DRG sensory neurons and those derived by hPSCs (Chambers et al., 2012). RUNX1 expression was found to decrease at later stages of differentiation, whereas in mice, its expression persists in non-peptidergic neurons (Huang et al., 2015; Samad et al., 2010). Nociceptive identity in hPSC differentiated cultures was also supported with functional studies, demonstrating functional expression of SCN10A sodium channels and neuronal responsiveness to capsaicin and α,β Methylene-ATP, which mimic inflammatory pain. Interestingly, the majority of hPSC-derived nociceptors expressed P2RX3 rather than TRPV1.

Chamber's protocol for deriving nociceptors neurons was adapted by other groups to further explore the molecular and functional profiles of hPSC-derived nociceptors. Eberhardt et al. (Eberhardt et al., 2015) examined molecular and functional expression of tetradotoxin-sensitive (TTXs) and TTX-resistant (TTXr) voltage-gated sodium channels (NAVs) in hPSC-derived nociceptors. Their findings showed that hPSC-derived neurons expressed pan-neuronal NAVs, NAV1.1, NAV1.2, and NAV1.6, as well as NAVs specific to peripheral sensory neurons, NAV1.8, NAV1.9 and NAV1.5. The expression and electrophysiological properties

of NAV1.5 within hPSC-derived nociceptors was akin with embryonic rodent DRG neurons, indicating their developmental stage. Young et al. (Young et al., 2014) reported comprehensive transcriptome profiling of hPSC-sensory nociceptors at the single cell level and at different time points during the differentiation allowing them to better define their identity. Of significance, the data was compared to human sensory neurons obtained from three different adult donors. A significant difference in gene expression emerged between day 16 and 32 of hPSC sensory neuronal differentiation compared to human DRG tissue. Authors hypothesized that this difference is related to genes that are expressed in myelin-producing Schwann cells and infiltrating immune cells found in the human DRG. However, no relevant differences were observed at later time points of differentiation, suggesting maturation of hPSC-derived sensory neurons. Single cell qPCR analyses examined the degree of heterogeneity in hPSC-derived sensory neurons, with a particular focus on specific channels involved in pain sensation. The RNA based analyses were supported with matching functional data obtained by patch-clamp electrophysiological analyses. These results confirmed presence of functional $GABA_AR$ (subunits $\alpha2$, $\alpha5$, $\gamma2$), which correspond to those found in human DRG neurons. Isoforms of HCN (HCN1), KCNQ2/3 and ASIC (ASIC3) ion channels were also detected in hPSC-derived sensory neurons. HCN channels play an important role in regulating the action potential frequency in DRG neurons, KCNQ2/3 regulate neuronal resting membrane potential and ASIC channels are involved in pain sensation mediated by acidosis.

Overall, these findings demonstrate key comparisons between hPSC-derived sensory neurons and human DRGs. This knowledge helps set up the stage for using hPSC-derived sensory neurons to establish drug discovery platforms that target nociceptor populations.

HPSC differentiation to mechanoreceptors

Generation of mechanoreceptors from hPSC-derived neural crest was described by Schrenk-Siemens and colleagues (Schrenk-Siemens et al., 2015). Their report suggested that GDNF treatment, a RET receptor ligand, was sufficient for biasing neural crest differentiation to mechanoreceptors. The differentiated cultures consisted of ~2.8% of TRKB positive neurons, with detected expression of other specific mechanoreceptor markers including MAF, MAFA, and PIEZO2. Few TRKC positive neurons were observed and no expression of TRKA or other nociceptor markers were detected. The low percentage of TRKB positive neurons highlights the need to develop alternative methods to enrich for specific sensory neuronal populations. Relevant to this, the authors demonstrated that forced expression of

NEUROGENIN2 (NGN2) in hPSC-derived neural crest further increased expression of MAFA as well as pan-sensory neuronal markers, ISLET1 and NF200 (Schrenk-Siemens et al., 2015). Functional characterization of hPSC-derived mechanoreceptors was investigated by patch clamp recordings and calcium imaging. Firing properties of hPSC-derived mechanoreceptors were akin to rodent mechanoreceptors, showing functional evidence of Nav1.7 channels, while channels typically expressed in nociceptive neurons (such as NAV1.8 and NAV1.9) were absent. This study also used CRISP/CAS9 gene-editing to abolish expression of PIEZO2, a mechanically activated ion channel, in hPSC to determine its role in mechanoreceptors. The authors showed mechanotransduction currents where depleted in the absence PIEZO2 expression despite persistent expression of other mechanoreceptive markers. These findings are significant in showing a critical functional role of PIEZO2 in human mechanoreceptors. Such studies also reflect the value of deriving sensory neuronal subtypes from hPSC for interrogating ion function in human neurons.

Human sensory neurons derived directly from fibroblasts

An alternative to using hPSC as a source for generating human sensory neurons is to directly reprogram human fibroblasts to a neuronal fate (Figure 1). In 2015, Blanchard and colleagues reported direct conversion of human embryonic and mouse fibroblasts to DRG sensory neurons using ectopic expression of key transcription factors associated with DRG sensory neuron fate (Blanchard et al., 2015). The neurogenic basic helix-loop-helix protein NGN1 and NGN2, play an important role during the lateral migration of embryonic neural crest cells from the region between the epidermis and the neural tube to the future DRG regions (Blanchard et al., 2015; Hari et al., 2002; Ma et al., 1999) The subsequent expression of BRN3A in the neural crest progenitors differentiates the precursors to sensory neurons (Dykes et al., 2010, 2011; Eng et al., 2007; Zou et al., 2012). Using a doxycycline (dox)-inducible lentiviral vector system, fibroblasts were transduced to exogenously express NGN1/BRN3A or NGN2/BRN3A upon drug treatment. At least 90% of fibroblasts were reprogrammed to neurons, with either combination of NGN1/BRN3A and NGN2/BRN3A factors, as shown by expression of β tubulin III and MAP2ab. Expression of pre-synaptic markers, synaptobrevin and synapsin were also detected. Induced neurons were pseudounipolar, a characteristic morphological feature of sensory neurons, with a range of different cell body sizes, indicating different neuronal subtypes. Molecular markers of peripheral sensory neurons were also detected, including NF200, Peripherin, TRKA, TRKB and TRKC. Expression of TRKA,

TRKB, TRKC were observed in approximately 25-30% of the transduced cultures for each receptor. Calcium imaging and patch clamp analyses confirmed that the induced neurons were functional. Further functional characterization analyses of nociceptor neuronal subtypes were performed using agonists of TRP ion channels, such as capsaicin, menthol and mustard oil, as well as pruritogenic stimuli, histamine, chloroquine and SL1GRL.

Direct conversion of human and mouse fibroblast to nociceptor neurons was also described by Wainger and colleagues (Wainger et al., 2015). Assessing twelve candidate transcription factors, the authors identified five of them that were critical for direct reprogramming; ASCL1, MYT1L, KLF7, NGN1 and ISL2. The resultant reprogrammed cultures consisted of 14% neurons. Conversion to nociceptors was determined using a mouse embryonic fibroblast TRPV1 reporter cell line as well as expression of Peripherin, CGRP, TRPM8 and TRPA1. Interestingly, the proportion of neurons expressing specific ion channels was similar to that identified in primary adult DRG neurons. Functional evidence of ion channel expression was also demonstrated using calcium imaging and MEA analyses to measure neuronal responsiveness to noxious agonists, menthol, mustard oil and capsaicin. In this same study, the authors also generated induced nociceptive neurons from familial dysautonomia (FD) fibroblasts and showed reduced neurite outgrowth and branches compared to induced nociceptive neurons from healthy controls. This data is proof-of-concept in using direct reprogramming methodology to generate patient-derived sensory neurons for modelling peripheral sensory neuropathies.

One main advantage of reprogramming fibroblasts directly to neurons is that it is direct and therefore the protocol is potentially less likely to generate heterogeneous cell types, as observed with hPSC differentiation. The disadvantage, however, is that fibroblast cell lines may have a limited capacity to be maintained in culture. Nevertheless, studies involving direct reprogramming are highly informative for determining the combination of transcription factors needed to specify DRG sensory neuronal subtypes, which will help pave new approaches in generating human sensory neurons in a direct and consistent manner.

Advantages and limitations in using *in vitro* generated sensory neurons

One of the major advantages of generating human sensory neurons *in vitro*, particularly from hPSCs, is the capacity to generate high numbers of well-characterized human neuronal lineages in a consistent manner. This helps the development of high through-put screening assays and significantly reduces experimental animal usage. Another advantage is their use for *in vitro* disease modelling. Examples of these different applications are mentioned below.

However, there are still significant challenges that need to be addressed before *in vitro*-generated human sensory neurons can entirely model human DRG tissue. Such challenges include establishing co-culture systems whereby afferent and efferent processes of hPSC-derived sensory neurons form connections with appropriate target cells, as well as axonal myelination of larger sensory neuronal subtypes. In this respect, nociceptors are somewhat relatively easier to generate from hPSC since they do not require myelination and can be stimulated in the absence of innervating target tissue. Indeed, the literature to date reflects this as there are significantly more reports describing generation of nociceptors from hPSC than mechanoreceptor and proprioceptor subtypes.

Other challenges fall back on general issues relating to hPSC biology. It is well documented that hPSC show great variability between different cell lines as well as in the robustness of the protocol within the same line (Kim et al., 2007; Osafune et al., 2008; Pal et al., 2009). Generation of sensory neuronal reporter cell lines may partially address this issue whereby specific neuronal cell types can be identified amongst heterogeneous cultures and isolated for phenotypic and functional analyses. Saying this, given the complexity of phenotypic signature markers it may not be simple to identify a sensory neuronal lineage using 1-2 markers only. Nevertheless, using reporter cell lines is still a significant start for phenotyping major populations based on traditional markers, such as the TRK receptors. Another important issue to consider is relying on markers characterized in rodents or other species to identify human sensory populations. Ultimately, phenotypic and functional characterization of *in-vitro* generated sensory neurons needs to be compared with human fetal and adult DRG tissue as much as possible to determine consistencies.

Applications of hPSC-derived sensory neurons

Aside from current limitations associated with hPSC-derived sensory neurons, there is significant progress in recent years for their application in disease modelling, drug screening, toxicity screening and studying ion channels.

For high through-put screening of compounds, large numbers of homogeneous populations of sensory neurons are needed. The Chambers protocol for deriving nociceptors, albeit it is a mixed population of nociceptor subtypes, is appropriate given that it is direct and relatively efficient. Indeed, there have been at least a couple of publications that have adapted the Chambers method to establish high through-put screening systems of sensory neurons (Hoelting et al., 2016; Stacey et al., 2018). Hoelting and colleagues described a high through-put system for screening potential peripheral toxins (Hoelting et al., 2016). Interestingly, the

immature state of hPSC-derived sensory neurons was used as an advantage for the screening process based on the rationale that growing neurites are more susceptible to peripheral toxins than established neurites. Using fully automated live cell microscopy, large numbers of compounds were screened for effects on neurite outgrowth and cell viability. This assay, known as the 'PeriTox test', was trialled against known toxins and also compared with another toxicology screening assay. Consistent results were obtained showing proof-of-concept for using hPSC-derived sensory neurons for evaluating toxins. The other study, reported by Stacey and colleagues, described the application of hPSC-derived sensory neurons for screening candidate compounds to treat chronic pain (Stacey et al., 2018). A chemogenomics library consisting of 2700 small molecules were screened for their ability to inhibit veratridine-evoked excitability in hPSC-derived neurons using a 384-well calcium flux assay. Validation of the functional assay was also performed. Both studies demonstrate the capability of using hPSC-derived sensory neurons for large scale screens, although the differentiation protocols needed to be adapted to make it accessible and practical for multi-well systems. For example, both papers mentioned benefits of cryopreserving neurons for scaling up cell numbers prior to their use. Also, in addition to the differentiation protocol, the assay readout needs to be robust and reliable. Overall, these studies published to date are promising for using hPSC-derived sensory neurons to establish platforms with multi-purpose capabilities and large-scale screening capacities.

Complimentary to establishing high through-put screens, hPSC-derived sensory neurons are also purposeful for studying human ion channel function in both normal wild type and mutant states. Prior to hPSC technology, many studies used the approach of expressing human ion channels in oocytes or mammalian cell lines to characterize their pharmacological activities (Rosenbaum et al., 2002; Sangameswaran et al., 1996; Trezise et al., 1998). A significant step forward was the development of methods to transfect rodent primary neurons with human ion channels, thereby using a more appropriate cell type to study downstream pathways of ion channel activation/silencing (Dib-Hajj et al., 2009). Whilst these approaches have been successful in identifying unique functional properties and potential drug targets for mutant human ion channels, such as those described for mutations in sodium channel Nav1.7 (Dib-Hajj et al., 2005; Geha et al., 2016; Yang et al., 2016), generating sensory neurons from patient-derived iPSCs may yet be the closet model to the human *in vivo* scenario. Cao and colleagues (Cao et al., 2016) generated iPSC from a group of patients carrying different mutations in Nav1.7, which results in a condition called erythromelalgia whereby patients experience chronic pain particularly in response to heat stimuli. Sensory neurons derived from

different Nav1.7 mutant iPSC lines showed hyperexcitability and aberrant responses to heat, which is consistent with the clinical phenotypes. The Nav1.7 mutant sensory neurons were also used to assess efficacy of a candidate Nav1.7 channel blocker, PF-05089771, in reversing the neuronal hyperexcitability. Promising results were obtained, which led the authors to test the compound in erythromelalgia patients and similar positive findings were obtained. These studies are very encouraging for future developments in using hPSC-derived sensory neurons for disease modelling, particularly to investigate ion channel function, and assess candidate therapeutic compounds.

Conclusions

Progress in the areas of stem cell differentiation and reprogramming are rapidly advancing such that the gap between comparing hPSC-derived sensory neurons and human DRG tissue is closing. Clearly, there are still many challenges ahead before hPSC-derived sensory neurons can completely model the human DRG tissue. The immense value in having an ongoing *in vitro* source of human sensory neurons outweighs current challenges we face. Also, rapid advancements in stem cell and reprograming technologies will inevitably address these current challenges. The benefits, together with the potential clinical and pharmaceutical applications of *in vitro*-derived human sensory neurons, are all strong motivating factors for improving methodologies to generate human sensory neurons in the culture dish.

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Figure Legend

Figure 1. Different approaches for generating human sensory neurons *in vitro*. Differentiation of human ESC and iPSC either via neural crest progenitor (A) or directly (B) to sensory neuronal lineage. An alternative approach is direct reprogramming of fibroblasts to sensory neurons (C).

Table 1. Summary of main publications to date that describe generation of DRG sensory neurons from hPSC.

Paper	Differentiation factors	DRG subtypes	Characterization
Pomp et al., 2008 Brain Research	bFGF, NIM, NGF, BDNF, NT-3	Nociceptors	Immunostainings Q-PCR Electrophysiology (patch-clamp)
Chambers et al., 2012 Nature Biotechnology	SB431542, LDN-193189, SU5402, CHIR99021, DAPT, NGF, BDNF, GDNF	Nociceptors	Immunostainings Flow cytometry Microarray analysis Electrophysiology (Patch-clamp) Calcium Imaging
Lee et al., 2012 Plos One	SU5402, RO4929097, CHIR99021, A83-01, LDN-193189, NT-3, BDNF, NGF, GDNF, ascorbic acid, dibutyryl cAMP	Pan-sensory neuronal markers	Immunostainings Flow Cytometry Electrophysiology (patch-clamp)
Guo et al., 2013 Biomaterials	Noggin, SB431542, Wnt1, bFGF, hEGF, BDNF, NT-3, GDNF, NGF, cAMP, ascorbic acid	Pan-sensory neuronal markers and proprioceptors	Immunostainings Electrophysiology (patch-clamp)
Young et al., 2014 Mol. Ther.	SB431542, LDN-193189, SU5402, CHIR99021, DAPT, NGF, BDNF, GDNF, ascorbic acid	Nociceptors	Immunostainings Q-PCR Microarray Single-cell Q-PCR Electrophysiology (patch-clamp)

Blanchard et al., 2015 Nature Neuroscience	Inducible lentiviral: Brn3A/NGN1 (BN1) Brn3A/NGN2 (BN2)	Nociceptors Mechanoreceptors Proprioceptors	Immunostaining, Electrophysiology (Patch-clamp) Calcium Imaging
Boisvert et al., 2015 Scientific Report	NIM (neural induction media), bFGF, heparin, cAMP RA, BMP4 + Inducible lentivirus	Nociceptors	Immunostainings Q-PCR Calcium Imaging Electrophysiology (Patch-clamp)
Denham et al., 2015 Stem Cells	SB431542, CHIR999021, BMP2, FGF2, diff media	Pan-sensory neuronal markers	Immunostainings FACS Q-PCR
Eberhardt et al., 2015 Stem Cell Reports	LDN-193189, SB431542, CHIR99021, SU5402, DAPT, human-b-NGF, BDNF, GDNF, ascorbic acid	Nociceptors	Immunostainings Q-PCR Electrophysiology (patch-clamp)
Schrenk-Siemens et al., 2015 Nature Neuroscience	FGF, EGF, NGF, NT-3, BDNF, GDNF, RA + Inducible lentivirus: NGN2	Mechanoreceptors	Immunostainings In situ hybridization Q-PCR Deep-sequencing analysis Electrophysiology (patch-clamp) Calcium Imaging Generation of PIEZO2 Knockout hES cells

			Generation of hiPS cells
Wainger et al., 2015 Nature Neuroscience	Retroviruses for 5 transcription factors: Ascl1, Myt1l, Ngn1, Isl2, Klf7 + FGF, BDNF, CNTF, GDNF, NGF	Nociceptors	Immunostainings Electrophysiology (patch-clamp)
Cao et al., 2016 Sci. Transl. Med.	SB431642, LDN-193189, CHIR99021, SU5402, DAPT, NT-3, GDNF, BDNF, NGF, ascorbic acid	Nociceptors	Immunostainings Q-PCR Electrophysiology (patch-clamp)
Hoelting et al., 2016 Stem Cells Transl. Med.	Noggin, dorsomorphin, SB431642, CHIR99021, SU5402, DAPT, Y-27632, β-mercaptoethanol, FGF, BDNG, NGF, GDNF	Pan-sensory neuronal markers, Nociceptors	Immunostainings PeriTox test Q-PCR
Cai et al., 2017 Stem Cells Translational Medicine	LDN-193189, A83-01, CHIR99021, RO4929097, SU5402, RA, NT-3, NGF, BDNF, GDNF	Pan-sensory neuronal marker	Immunostainings Flow cytometry WB Q-PCR

Alshawaf et al., 2018, Scientific Reports	SB431542, CHIR99021, FGF2, BMP2, NGF, BDNF, NT3	Nociceptors Mechanoreceptors Proprioceptors	Immunostaining Electrophysiology (MEAs)
Guimaraes et al., 2018 BioRxiv	LDN-193189, SB431542, CHIR99021, FGF, EGF, BDNF, ascorbic acid, GDNF, NGF, NT-3, cAMP	Nociceptors	Immunostaining ELISA RNA-seq
Stacey et al., 2018 SLA Discov. Adv. Life. Sci	SB431542, LDN-193189, SU5402, CHIR99021, DAPT, NGF, BDNF, GDNF	Nociceptors	Immunostainings Chemogenomics Screening