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Getting in touch with your senses: Mechanisms specifying sensory interneurons in the dorsal spinal cord

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Abstract

The spinal cord is functionally and anatomically divided into ventrally derived motor circuits and dorsally derived somatosensory circuits. Sensory stimuli originating either at the periphery of the body, or internally, are relayed to the dorsal spinal cord where they are processed by distinct classes of sensory dorsal interneurons (dIs). dIs convey sensory information, such as pain, heat or itch, either to the brain, and/or to the motor circuits to initiate the appropriate response. They also regulate the intensity of sensory information and are the major target for the opioid analgesics. While the developmental mechanisms directing ventral and dorsal cell fates have been hypothesized to be similar, more recent research has suggested that dI fates are specified by novel mechanisms. In this review, we will discuss the molecular events that specify dorsal neuronal patterning in the spinal cord, thereby generating diverse dI identities. We will then discuss how this molecular understanding has led to the development of robust stem cell methods to derive multiple spinal cell types, including the dIs, and the implication of these studies for treating spinal cord injuries and neurodegenerative diseases.

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bone morphogenetic proteins, cell fate specification, dorsal sensory interneurons, regeneration, spinal cord

1 | INTRODUCTION

Somatosensation is essential for survival. It enables us to sense and respond to pain (nociception), heat (thermosensation), itch (pruriception), touch (mechanosensation/cutaneous) and the position of our body in space (proprioception). In this review, we will focus on the specification of the sensory interneurons in the dorsal spinal cord that process sensory information before it is relayed to the brain and/or motor neurons in the spinal cord (Osseward & Pfaff, 2019; West et al., 2015). There are six classes of dorsal interneurons, dI1–dI6 (Figure 1a,b), which relay or modulate distinct

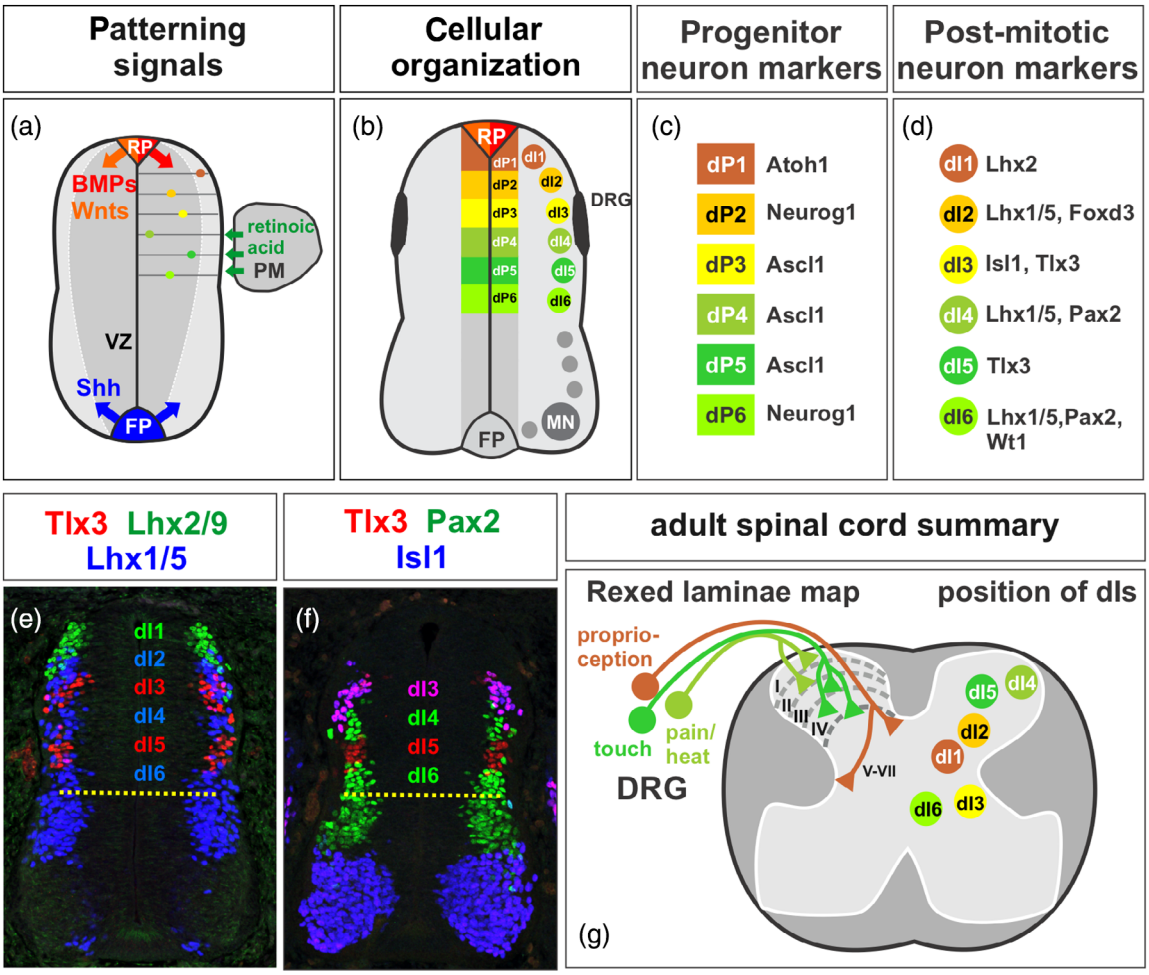

FIGURE 1 Patterning and functional organization of dorsal sensory interneurons (dIs). (a) The spinal cord is patterned along the dorsal–ventral axis using signals that include: the roof plate (RP)-derived Bmps and Wnts, presomitic mesoderm (PM)-derived RA and floor plate (FP)-derived Shh. (b) In the dorsal spinal cord, the combinatorial activities of RP- and PM-derived signals result in the specification of six progenitor domains (dP1–dP6) which differentiate to generate six classes of dI neurons (dI1–dI6s). (c,d) Both dorsal progenitors (dPs, c) and differentiated neurons (dIs, d) can be identified by their expression of specific transcription factors. (e,f) Hamilton Hamburger (HH) 24 chicken spinal cord section immunostained for the complements of transcription factors that permit the identification of six classes of dIs (dI1–dI6s). (g) Schematic depicting the organization and function of somatosensory circuits in the adult spinal cord. The dIs migrate from their embryonic positions to occupy different laminae in the adult dorsal horn (Rexed, 1954). Peripheral afferents carrying different sensory stimuli synapse onto specific layers, where the dorsal interneurons process this information before sending it to the brain or ventral spinal cord

somatosensory modalities via the release of excitatory or inhibitory neurotransmitters and various neuropeptides (Bourane et al., 2015; François et al., 2017; Prescott, 2015; Su et al., 2014). These dIs can be identified by the expression of specific transcription factors, and their position in the spinal cord (Figure 1c–f). Broadly speaking, pain, heat, and itch sensing dIs (dI4 and dI5) are located in the superficial layers of the dorsal horn while the mechanosensory and proprioceptive dIs (dI1–dI3, and dI6) are found ventrally in the dorsal horn (Lai et al., 2016; Figure 1g).

The diversity of dIs results from a tightly regulated series of lineage specification events that begins when the posterior neural plate acquires a spinal cord identity in the early embryo. Central to this process is the establishment of signaling centers which impart anterior–posterior and dorsal–ventral polarity to the developing spinal cord. Along the dorsal–ventral axis, secreted growth factors from key signaling centers, specifically the floor plate (FP), paraxial mesoderm and roof plate (RP) (Figure 1a), generate a striped pattern of progenitor domains that lays out the blueprint for the somatosensory circuitry in the dorsal spinal cord, and the motor circuitry in the ventral spinal cord. The mechanisms underlying fate specification in the ventral spinal cord has been extensively studied and reviewed (Briscoe & Novitch, 2008; Poh et al., 2002; Tanabe & Jessell, 1996; Ulloa & Briscoe, 2007). Here, we will focus on reviewing the mechanisms that specify dI identities, and how they differ from the ventral patterning process.

Understanding the mechanisms that direct dIs identity is critical for designing strategies to repair somatosensory circuitry using regenerative therapies. Damage to the spinal cord is common. Spinal cord injuries (SCI) are estimated to

affect ~1 million people in the United States alone (Armor et al., 2016; National Spinal Cord Injury Statistical Center, 2013). In addition, neurodegenerative conditions such as Charcot–Marie–Tooth disease (Koros et al., 2013) and diabetic neuropathies (Al-Nasser, 2012; Feldman et al., 2019) often lead to the loss of sensation due to disruption of sensory circuitry in the spinal cord. The dorsal horn of the spinal cord also contains inhibitory microcircuits that regulate the intensity of pain and itch sensation through gate control mechanisms (Melzack & Wall, 1965; Mendell, 2014). Disruption in these inhibitory circuits can lead to chronic neuropathic pain disorders such as hyperalgesia, where a patient develops heightened sensitivity to pain (Ravenscroft et al., 2000; Tsuda et al., 2017) or mechanical allodynia, where a light touch becomes painful (Lolignier et al., 2015; Szczot et al., 2018). Recent studies have co-opted the molecular details of spinal cord development to develop directed differentiation protocols to derive multiple neuron populations from stem cells (Adams et al., 2015; Amoroso et al., 2013; Andrews et al., 2017; Butts et al., 2017; Gouti et al., 2014; Gupta et al., 2018). These studies both provide critical neuronal classes for regenerative purposes and unprecedented resolution into transcriptomic events during spinal cord development. We will review the potential of these stem cell studies for understanding the molecular mechanisms of dI specification and their therapeutic use for neural repair.

2 | INDUCTION OF THE SPINAL CORD IN THE EARLY EMBRYO

The spinal cord emerges as an anatomically distinct structure when the neural ectoderm folds to form a neural tube. The anterior (rostral) region of the neural tube differentiates into brain structures, while the posterior (caudal) neural tube give rise to the spinal cord (Greene & Copp, 2009; Sadler, 2005). However, the segmental identities of the neural tube are specified at the molecular level before this morphological transformation. The central nervous system (CNS) is derived from a disc-shaped tissue known as the epiblast (Hemmati-Brivanlou & Melton, 1997). According to the Nieuwkoop activation-transformation model (Nieuwkoop, 1952, 1954), derived from studies in Xenopus embryos, the epiblast is first induced or "activated" to generate neural plate with an anterior forebrain identity through the inhibition of Bmp signaling during gastrulation (Piccolo et al., 1996; Zimmerman et al., 1996). Subsequently, a posterior signaling center called the node secretes additional signals, including wingless and integrase (Wnts) family, fibroblast growth factors (Fgf), and retinoic acid (RA). Together, these growth factors progressively transform the neural plate to generate more posterior compartments of the CNS, that is, the midbrain, hindbrain, and spinal cord (Blumberg et al., 1997; Niehrs, 2004; Stern et al., 2006).

The "activation-transformation" model posits that the spinal cord identity is molecularly fixed before neural tube closure and the specified compartments then expand to form the CNS. However, fate-mapping studies in chicken (Brown & Storey, 2000; Olivera-Martinez et al., 2012), mouse (Cambray & Wilson, 2007) and fish (Attardi et al., 2018) embryos have identified an additional mechanism. Signals from the node specify a key intermediate cell type fate, the neuromesodermal progenitors (NMPs), in the posterior neural plate (Henrique et al., 2015; Wilson et al., 2009). NMPs are bipotential, giving rise to either neural or mesodermal derivatives. They have the capacity to self-renew and thereby continuously contribute cells to the spinal cord and paraxial mesoderm during axis elongation while maintaining a pool of NMPs at the posterior end of the embryo (Attardi et al., 2018; Henrique et al., 2015). Wnts and Fgfs, secreted by the node, direct NMP induction and self-renewal (Takemoto et al., 2011), while somite-derived RA promotes NMPs to differentiate into spinal cord lineages (Cunningham et al., 2016). Once committed to the spinal cord lineage, the neural plate begins to express the Pax transcription factors, specifically Pax3, Pax6, and Pax7, and folds to form the neural tube. Pax proteins are initially present uniformly in the spinal cord neuroepithelium where they regulate the balance of proliferation versus differentiation (Blake & Ziman, 2014; Mansouri et al., 1996). However, they subsequently become restricted to dorsal progenitors (dP), as one of the earliest consequences of patterning along the dorsal–ventral axis.

Using this molecular understanding, NMP-like cells have been derived as an intermediate from both mouse (m) and human (h) embryonic stem cells (ESCs), leading to the development of efficient directed differentiation protocols to generate spinal cord cell types (Gouti et al., 2014; Turner et al., 2014). These in vitro derived NMP cells can be biased to generate either posterior spinal cord neurons or muscles in vitro, further confirming their dual potency (Gouti et al., 2014; Turner et al., 2014).

3 | DORSAL–VENTRAL PATTERNING OF THE SPINAL CORD

On neural tube closure, three major signaling centers emerge along the dorsal–ventral axis of the spinal cord which secrete distinct patterning signals (Figure 1a). The floor plate (FP) at the ventral midline secretes sonic hedgehog (Shh);

the RP at the dorsal midline produce multiple members of the bone morphogenetic protein (Bmp) and Wnt family, while somites adjacent to the intermediate neural tube secretes RA (Figure 1a). These inductive signals compete and collaborate to specify at least 11 progenitor (p) domains (dP1-dI6, p0-p3, pMN) along the dorsal–ventral axis of the neural tube. Progenitor domains become distinct from each other by the expression of specific basic helix loop helix (bHLH) (Figure 1b,c) and homeodomain (HD) transcription factors. These transcription factors then direct the progenitors to differentiate into a specific neural identity (Briscoe et al., 2000; Briscoe & Novitch, 2008; Jessell, 2000; Lai et al., 2016).

3.1 | The identity of the ventral spinal cord depends on Shh morphogen signaling

In the ventral spinal cord, FP-derived Shh functions as a morphogen (Figure 1a), that is, in a concentration dependent manner, to specify five progenitor (p) domains (p0-p3 and pMN) (Briscoe, 2009; Dessaud et al., 2008). The p3 and pMN fates are specified by high concentrations of Shh, while lower concentrations of Shh successively specify the intermediate ventral (v) interneuron identities (v3-v1) (Echelard et al., 1993; Ericson et al., 1997). Shh also functions as a temporal morphogen, where the fates of ventral neural progenitors depend on the duration of Shh signal (Dessaud et al., 2007; Kong et al., 2015). In this model, neural progenitor becomes progressively more ventralized, the longer it experiences Shh signaling. The ability of Shh to act as a temporal morphogen is mediated by Notch signaling, which stabilizes intracellular Shh signaling through cilia present on the luminal side of the neural progenitors (Kong et al., 2015).

3.2 | The identity of the dorsal spinal cord depends on multiple growth factors

In the dorsal spinal cord, the combinatorial activities of multiple growth factors, i.e. Bmps, Wnts and RA, specify six distinct dorsal progenitor (dP) domains—dP1–dP6—marked by stereotypical striped patterns of proneural bHLH gene expression (Figure 1b,f) (Helms & Johnson, 2003). Studies in both mouse and chicken embryos, have revealed the importance of the RP in patterning the most dorsal dPs (dP1–dP3) (Chizhikov & Millen, 2004; Lee et al., 2000; Liem et al., 1997). Signals emanating from RP, include the Bmps (Liem et al., 1995, 1997) and Wnts (Figure 1a) (Megason & McMahon, 2002; Muroyama et al., 2002), which regulate multiple processes ranging from fate specification, cell division, differentiation, and axon guidance (Andrews et al., 2019; Butler & Bronner, 2015; Comer et al., 2019). In contrast, the more intermediate dPs (dP4–dP6) are thought to be specified independently of the RP mediated signaling (Lee et al., 1998). In the following section, we will summarize the roles of the major signaling pathways that regulate dI specification.

3.2.1 | Role of the Bmp signaling pathway specifying the dorsal most interneurons (dI1–dI3s)

Bmps are secreted growth factors that belong to the transforming growth factor-β (TGFβ) superfamily. They signal through membrane-bound serine/threonine kinase receptors (Bmprs). The Bmp signal propagates inside the cell by phosphorylating Smad1/5/8, the receptor regulated (R) Smad secondary messengers. The R-Smads complex with the common (Co) Smad4, and then enter the nucleus to regulate gene transcription (Feng & Derynck, 2005). Bmp signaling has been shown to be reiteratively required for dI fate specification (Hazen et al., 2011, 2012; Le Dreau et al., 2012; Liem et al., 1995, 1997; Timmer et al., 2002; Wine-Lee et al., 2004), differentiation (Andrews et al., 2017) and axon guidance (Augsburger et al., 1999; Butler & Dodd, 2003; Yamauchi et al., 2008). Loss of function studies have demonstrated that both the RP (Lee et al., 2000) and Bmp signaling (Wine-Lee et al., 2004) are required for the specification of the dI1–dI3s. However, the mechanism by which Bmp signaling patterns the dorsal spinal cord had remained unresolved, until recently.

Multiple Bmp ligands are expressed at high levels by the RP (Andrews et al., 2017; Augsburger et al., 1999; Lee et al., 1998; Liem et al., 1995) in a species specific manner, and at lower levels in dPs (Andrews et al., 2017; Le Dreau et al., 2012). Thus, Bmp6, Bmp7 and growth/differentiation factor (Gdf) 7 (also known as Bmp12) are present in the rodent RP, while Bmp4, Bmp5 and Bmp7 are present in the chicken RP. By drawing parallels from ventral patterning,

Bmps were initially postulated to pattern the dI fates as a collective morphogen (Figure 2a,b) (Lee & Jessell, 1999). In this model, the RP derived Bmps would combinatorially generate a concentration gradient where high Bmp concentrations specify the RP and dorsal-most dI1s while lower Bmp concentrations successively specify the dI2 and dI3 fates (Figure 2a,b).

However, there has been no conclusive evidence for the morphogen model. Early studies observed that media conditioned by Bmp4- or Gdf7-expressing COS cells directed chicken neural plate explants towards a dI1 identity; however diluted Bmp4-conditioned medium failed to specify the dI3 fate, the predicted outcome for a canonical morphogen (Lee et al., 1998; Liem et al., 1997). Bmps have also been proposed to act as temporal morphogens, such that increasing the duration of Bmp signaling would progressively dorsalize dPs (Tozer et al., 2013). This model was also demonstrated using the chicken neural plate explant assay, where increasing the duration of Bmp4 exposure sequentially directed the dP2 identity, and then dP1 identity (Tozer et al., 2013). These studies suggest that spinal progenitors can register and respond to the duration of the Bmp signaling in the early neural tube. However, they do not address whether Bmps act as temporal morphogens during the relevant, later period of signaling when the Bmps are expressed in the RP and directing dI fate specification in vivo (Andrews et al., 2017). More recently, Bmp4 has been used to direct mESCs

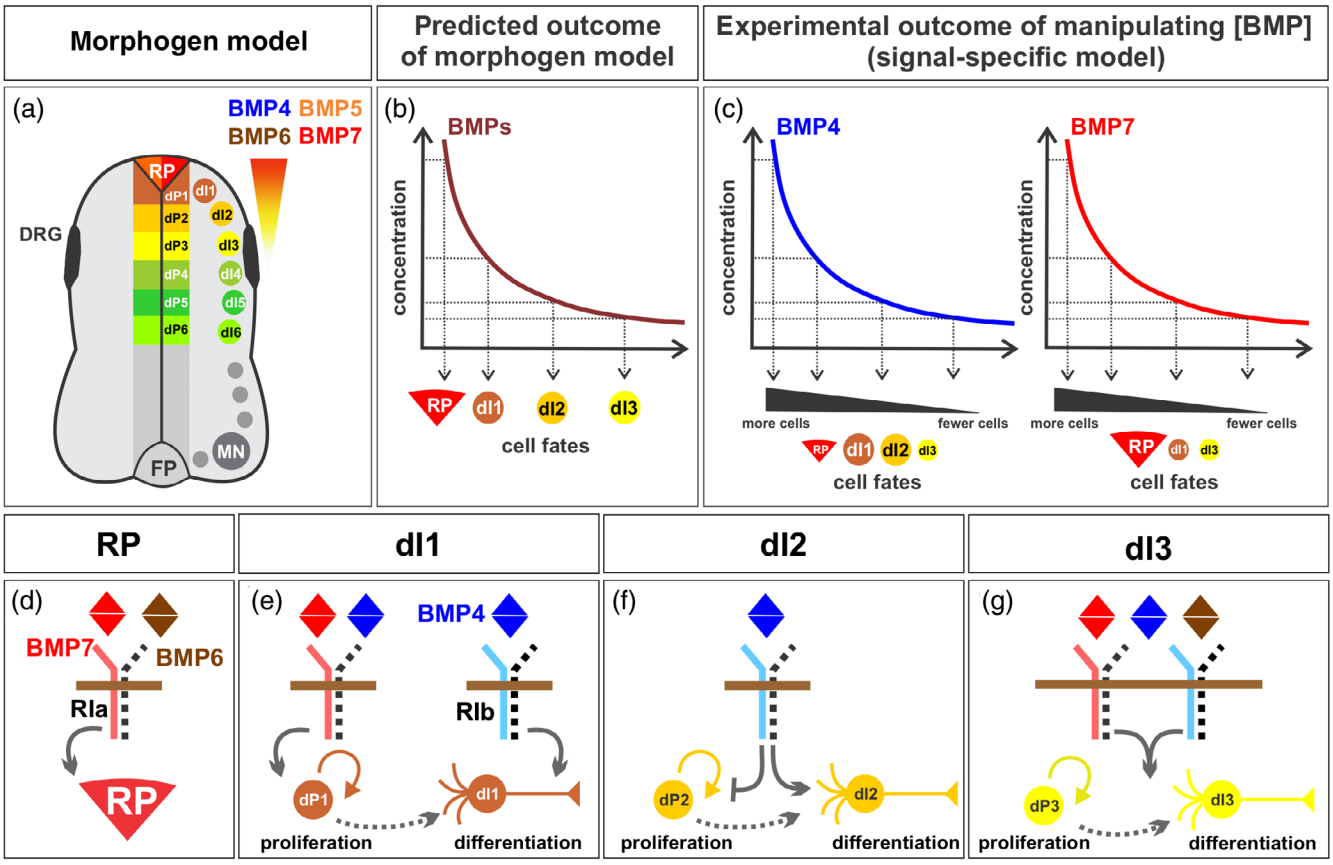

FIGURE 2 Models for Bmp-mediated patterning of the dorsal spinal cord. (a) In the morphogen model, RP-derived Bmps act in a concentration dependent manner to specify the RP itself, and the dI1, dI2 and dI3 populations of interneurons. (b) If Bmps function as a collective morphogen, then the highest concentration of Bmps would specify the RP and dI1 fates while successively lower concentrations of Bmps would progressively specify the dI2 and dI3 fates. (c) However, experimental observations using chicken embryos and mESCs as model systems, rather support an alternative "signal-specific model" of Bmp function. In this model, each Bmp specifies a range of cellular fates. For example, Bmp4 was shown to direct cells mostly towards the dI1 or dI2 fates, with some RP or dI3 fates, while Bmp7 directs cells towards the RP fate, with some dI1or dI3 fates. A high level of the Bmp is most effective at directing a specific range of cell types, whereas a lower level of the Bmp is less effective at directing the same range of cell types. (d–g) Schematics of a "mix and match" Bmp code that acts reiteratively to specify RP, dI1s, dI2s, and dI3s. In this model, Bmp7 (mouse) and Bmp7 (chicken) act though Bmpr1a to specify the RP identity (d). Both Bmp4 and Bmp7 act through Bmpr1a to direct dP1s to proliferate but only Bmp4 can promote dP1s to differentiate acting through Bmpr1b (e). However, only Bmp4 can block dP2 proliferation and promote them to differentiate into dI2s, through Bmpr1b (f). All tested Bmps can direct dP3s to proliferate and differentiate into dI3s (g)

towards dorsal spinal fates (Andrews et al., 2017; Duval et al., 2019). While Bmp4 can organize cells into a pattern similar to that observed in the dorsal spinal cord (Duval et al., 2019), changing the concentration and/or duration of Bmp4 treatment did not alter dI fate specification (Andrews et al., 2017), arguing against the spatial/temporal morphogen models. Finally, phosphorylated (p) (active) Smad1/5 staining in the dorsal spinal cord is observed in a dorsal high to ventral low gradient (Hazen et al., 2012), consistent with a model in which the Bmp morphogen gradient is interpreted by the quantitative activation of R-Smads. However *in ovo* electroporation studies, in the chicken spinal cord, have shown that the misexpression of either *Bmp4* or *Bmp7* can activate pSmad1/5 to the same level, yet these manipulations result in different cellular identities (Andrews et al., 2017). Thus, it seems unlikely that a gradient of R-Smad activation directs the specification of dI fates.

An alternative hypothesis is that RP-derived Bmps function in a signal-specific manner, such that each Bmp has a specific ability to direct a range of dorsal spinal fates (Figure 2c–g). This hypothesis was first suggested when mice mutant for Gdf7 gene were found to lack a specific subset of dIs (Lee et al., 1998). Further support came from studies in the chicken spinal cord, where knockdown of *Bmp4* or *Bmp7* expression led to different outcomes in dI fate specification (Le Dreau et al., 2012; le Dreau & Marti, 2013). Specifically, the loss of *Bmp4* reduces the number of dI1s, while loss of *Bmp7* reduces the number of dI1s, dI3s, and dI5s. Recent studies from our laboratory, using both chicken *in ovo* electroporation and mESC models, have unambiguously demonstrated that Bmps do not function as morphogens in the spinal cord, rather they have signal-specific activities (Andrews et al., 2017). We identified that Bmps differ in their ability to induce the RP, dI1, dI2 and dI3 fates, in a species-specific manner (Figure 2d–g). While Bmp6 (in mouse) and Bmp7 (chicken) can induce the RP most efficiently, Bmp4 sequentially promotes dP1 proliferation and dI1 and dI2 differentiation (Figure 2e,f). In contrast, Bmp7 can promote dP1 proliferation, but not dI differentiation (Figure 2e). All tested Bmps have a limited ability to promote dP3 proliferation and dI3 differentiation (Figure 2c,g). Bmps mediate their distinct activities by activating different type I Bmprs (Andrews et al., 2017; Panchision et al., 2001) (Figure 2d–g). Thus, Bmp patterning activities may arise from a combination of qualitative differences in the Bmp ligands, their specific domain of expression, and the activation of different type Bmprs. Together, this "mix-and-match" code may underpin the ability of Bmps to reiteratively direct different cell fate decisions.

Many questions still remain, including the mechanism by which individual Bmps activate specific Bmprs to specify different dI identities. Does each Bmp induce a distinct transcriptional response in dPs? Is the Bmp code conserved in the developing human spinal cord? These questions can be addressed using directed differentiation protocols to derive dIs from pluripotent stem cells (PSC). PSC models permit the quantities of growth factors to be precisely controlled, as well as generating a sufficiently large and synchronous population of cells to assess their transcriptional responses. Of the Bmps, Bmp4 has been repeatedly shown to be the most effective at inducing the dI1 and dI3 fates in human (Gupta et al., 2018) and mouse PSC models (Andrews et al., 2017; Duval et al., 2019). Thus, combining classical animal models with newly developed PSC-based approaches, will provide further mechanistic insights into how different Bmps reiteratively regulate dI fate specification in the dorsal spinal cord.

3.2.2 | Role of the Wnt signaling pathway specifying dorsal spinal identity

The Wnt signaling pathway is an evolutionarily conserved pathway that regulates numerous cellular processes in many organs during embryogenesis, including cellular proliferation and patterning (Clevers, 2006; Hikasa & Sokol, 2013; Logan & Nusse, 2004; Petersen & Reddien, 2009; Yang, 2012). Wnts signal through either the canonical β-catenin pathway, or the non-canonical planar cell polarity (Wnt/Pcp pathway) and Wnt/calcium pathways (Niehrs, 2012). Wnts regulate gene transcription mainly through the β-catenin pathway. Wnt binding to the frizzled receptor and Lrp5/6 co-receptors leads to the stabilization of β-catenin and its translocation into the nucleus where it acts as a transcription factor (Steinhart & Angers, 2018). In contrast, the noncanonical Wnt pathways regulate actin dynamics, either by activating Rho-associated protein and jun N-terminal kinases (Rock and Jnk) in the Wnt/Pcp pathway (Liu et al., 2014) or by regulating calcium release from the endoplasmic reticulum to activate calcium/calmodulin signaling in the Wnt/calcium pathways (De, 2011).

Like the Bmp family, multiple Wnt ligands, including Wnt1, Wnt3, Wnt3a, and Wnt4, are expressed by the RP (Figure 1a) and in dPs (Agalliu et al., 2009; Alvarez-Medina et al., 2008). Early studies postulated that Wnts function as mitogenic factors, controlling the size of the dorsal spinal cord, (Dickinson et al., 1994; Megason & Mcmahon, 2002). Overexpression of either Wnt1 or Wnt3a in the chicken spinal cord results in ∼2-fold increase in cells in the synthesis (S)-phase of the cell cycle. Wnts can control mitosis through the canonical β-catenin pathway, by inducing the

expression of cell cycle genes, such as cyclin D1 (Ille et al., 2007). Multiple lines of evidence have subsequently suggested that Wnts also regulate the process of dorsal patterning (Alvarez-Medina et al., 2008; Muroyama et al., 2002). Both dI1s and dI2s are lost in *Wnt1*;*Wnt3a* mutant mice, with a compensatory increase in the numbers of dI14-dI6s (Muroyama et al., 2002). Wnt signaling may regulate the dorsal–ventral boundary for neural progenitor identity. Mice lacking *β-catenin* show reduced expression of *Olig3*, which is normally present in dP1–dP3, while the ubiquitous expression of *β-catenin* leads to the expanded expression of *Olig3* (Zechner et al., 2007). Similarly, activating Wnt signaling by either overexpressing *Wnt1* or *Wnt3a*, or constitutively activating β-catenin, results in an expansion of dP identity, measured by *Pax6* and *Pax7* expression, at the expense of the ventral progenitor markers, that is, *Nkx6.1* and *Olig2* (Alvarez-Medina et al., 2008). Inhibition of Wnt signaling results in the converse phenotypes. Wnts may establish dP competence by inducing Gli3, a repressor of Shh, thereby preventing Shh signaling from expanding into the dorsal neural tube (Alvarez-Medina et al., 2008; Yu et al., 2008). Collectively, these studies suggest a critical role for Wnt signaling establishing a general dP identity.

The similarities in the expression patterns and the mode of signaling of the Wnts and Bmp families raises the possibility that these pathways interact in dorsal patterning. Studies in both chicken spinal cord and mESC assays, have shown that Bmp signaling induces Wnt1 expression (Chesnutt et al., 2004; Duval et al., 2019; Wine-Lee et al., 2004), suggesting that Wnt signaling is activated by the RP-derived Bmps. However, it remains unresolved whether Wnts support the proliferation of Bmp induced dPs (dP1–dP3s) or have a more direct role specifying dI1–dI3 identity. Moreover, further studies are required to assess the roles of different Wnt ligands in dorsal spinal cord development.

3.2.3 | The dI4–dI6 are specified independently of RP-derived signals during spinal cord development

The dI4–dI6s arise in the intermediate dorsal spinal cord. Their fates are thought to be specified independently from RP-derived signals, given that genetically ablating the RP does not affect the $Pax2^+$ dI4, dI6 populations (Lee et al., 2000). These dIs mediate the sensations of pain, itch, and heat (dI4/dI5s), and regulate gait (dI6s) (Andersson et al., 2012) through either inhibitory (dI4/dI6s), or excitatory (dI5) neurotransmission (Lai et al., 2016). These modalities are often lost in SCI patients, and the dI4–dI6 circuitry is also a target for the anti-nociceptive activities of the opioids (François et al., 2017; Wang et al., 2018), making it critical to understand their mode of action. According to gate control theory, the inhibitory neurons in the dorsal horn act as "gates" for incoming sensory information (pain, itch, and heat) to regulate the intensity by which it is relayed to the brain (Melzack & Wall, 1965; Mendell, 2014). Thus, a specific ratio of excitatory and inhibitory neurons is needed for normal somatosensation. An imbalance in this ratio can lead to chronic pain and itch disorders.

The dI4–dI6s are derived from Pax6/Pax7/$Pax3^+$ progenitors. In the early neural tube, the intermediate progenitors co-express *Pax6* and *Pax3*; they later segregate into nested expression domains of *Prdm* (PRDI-BF1 and RIZ homology domain containing) *13* and *Ascl1* and *Ptf1a*, two bHLH transcription factors. Together, Prdm13, Ascl1 (Mash1) and Ptf1a specify dP4-dP6 fates by combinatorial and cross-inhibitory activities (Borromeo et al., 2014; Chang et al., 2013; Mona et al., 2017), activating specific dI programs while repressing alternate dI fates. While it remains unresolved how the nested expression patterns of *Prdm13*, *Ascl1*, and *Ptf1a* are first established in the intermediate spinal cord, the mechanisms that then specify the inhibitory dI4s and excitatory dI5s have been more completely described (Helms et al., 2005; Mizuguchi et al. 2006; Mona et al., 2017; Nakada et al. 2004). Ptf1a directs the dP4 fate by inducing the expression of *Pax2*, *Lbx1* and *Lhx1/5*, while Prdm13 concomitantly represses *Ascl1* to inhibit the dI5 fate. In contrast, when Ptf1a is absent from dP5, Ascll induces the expression of *Tlx1* and *Tlx3,* which direct the dI5 fate while simultaneously suppressing the Lbx1-Pax2 dependent dI4 program (Chang et al., 2013). The cross inhibition of bHLH proteins thereby allows the correct ratio of inhibitory and excitatory neurons to be specified in the spinal cord.

Early studies in mouse and chicken systems have suggested that the specification of an intermediate dorsal identity also depends on RA, which is secreted from the adjacent paraxial mesoderm during spinal neurogenesis (Diez del Corral and Storey, Diez Del Corral & Storey, 2004). Reduced RA signaling, observed in vitamin A deficient quail embryos or *Raldh2* (RA synthesizing enzyme) loss of function mutant mice, leads to the reduced expression of *Pax3* and *Pax6* (Wilson et al., 2004). Mechanistically, RA functions by de-condensing chromatin around the *Pax* gene enhancers, since the chromatin remains condensed in *Raldh2* mutant embryos (Patel et al., 2013). Our studies, together with others, have shown that RA is sufficient to direct the dI4–dI6 fates in both mouse and human ESCs (Duval et al., 2019; Gupta et al., 2018; Ogura et al., 2018). Taken together, these data suggest that RA both establishes/maintains a broad dP

identity and initiates the dI4–dI6 fate specification program. However, it has been challenging to unravel the specific functions of RA, given its multiple roles during the spinal cord development (Lara-Ramírez et al., 2013). Newly developed stem cell models will be invaluable for dissecting the transcriptomic mechanisms by which RA regulates the dI4–dI6 identities in the intermediate spinal cord (Gupta et al., 2018).

In the adult spinal cord, the lineages of the dI4/dI5s diversify further into distinct microcircuits relaying different sensory submodalities. These submodalities depend on the expression of different neuropeptides and their receptors: for example, the neuropeptide Y $(NPY)^+$ dI4 subpopulation regulates mechanical itch (Bourane et al., 2015) while the dynorphin $(DYN)^+$ dI4 subpopulation (also called dIL_A) gates mechanical pain (Duan et al., 2014) and the sensation of chemical itch (Ross et al., 2010). The dI4/dI5 lineages also include locally acting interneurons (Todd, 2010; Todd & Sullivan, 1990), and the neurokinin1$^+$ projection neurons, which relay pain and heat to the brain through the anterolateral (AL) system (Almarestani et al., 2007; Todd, 2010; Todd et al., 2000). Further heterogeneity in the neural populations in the dorsal horn has been recently identified by coupling transcriptomic analyses with classic genetic manipulations in the mouse spinal cord (Häring et al., 2018; Sathyamurthy et al., 2018). For example, these studies revealed that there are six subpopulations of Vglut2$^+$ excitatory interneurons and five subpopulations of GABAergic inhibitory interneurons that selectively respond to heat stimuli (Häring et al., 2018). Additionally, many novel markers were identified for the AL tract projection neurons that selectively synapse onto the lateral parabrachial nucleus (LPb), a major pain processing center in the brainstem (Häring et al., 2018). These newly identified markers will permit neuronal populations encoding different sensory submodalities to be genetically targeted, as a means of understanding how they mediate diverse somatosensory signals. It will also be critical to refine the existing stem cell models (Andrews et al., 2017; Duval et al., 2019; Gupta et al., 2018) to derive the modality-specific dI4–dI6 subpopulations needed for therapeutic purposes. Understanding how the different dI4–dI6 populations are specified during development will facilitate therapies for SCI, chronic pain and itch disorders and as well as putatively mitigating against the devastating effects of the opioid addiction crisis (Singh et al., 2019).

3.3 | Differentiation of dorsal sensory interneurons

In the developing nervous system, neural progenitors both proliferate to sustain the progenitor pool and exit from the cell cycle to differentiate into neurons. The decision to self-renew versus differentiate is governed by the opposing actions of the notch signaling pathway and pro-neurogenic bHLH transcription factors, such as Ascl1 and Neurog1/2 (Kageyama & Ohtsuka, 1999; Ware et al., 2014). High notch activity is thought to maintain the proliferative state while high levels of bHLH proteins direct progenitors towards differentiation (Imayoshi et al., 2013; Ivanov, 2019). Both notch signaling components, such as Hes1/Hes5, and bHLH transcription factors are present in dPs (Baek et al., 2006; Helms et al., 2005; Sagner et al., 2018).

How does a neural progenitor choose when to exit the cell cycle to differentiate into a specific class of neuron? The oscillation model posits that the proliferative state correlates with the oscillatory expression of bHLH and Hes proteins, while the sustained expression of bHLH proteins drives progenitors to differentiate (Shimojo et al., 2008) (Figure 3a,b). The cyclical oscillation of Hes1/Hes5 and bHLH proteins arises as a result of cross-repression and feed-back loops. Elevated levels of Hes1/Hes5 repress bHLH gene expression and promote cell division (Barton & Fendrik, 2013; Kageyama et al., 2018). However, high levels of Hes factors also repress their own expression through a negative feedback loop, thereby allowing the levels of bHLH proteins to rise (Figure 3b,c). Elevated levels of bHLH proteins in turn activate notch signaling through a positive feedback loop, permitting the oscillations to continue (Figure 3c). Ultimately, stochastic imbalances arise in the patten of oscillations, such that pro-neurogenic bHLH proteins gradually accumulate in progenitors, which then exit the cell cycle and differentiate into neurons (Imayoshi et al., 2013) (Figure 3b). For example, the sustained expression of Ascl1 in ventral forebrain progenitors activates both cell-cycle regulators, such as Cdk1, Cdk2, and Ccd25b, and the factors regulating neurogenic program (Castro et al., 2011). The upregulation of distinct pro-neurogenic bHLH proteins in differentiating neural progenitors ensures neurons commit to a specific dI identity. Alternative fates are repressed, thereby preventing the specification of mixed neural identities, (Kutejova et al., 2016; Sagner & Briscoe, 2019). However, the elevated level of pro-neurogenic bHLH gene expression is transient (Figure 3b), quickly subsiding when progenitors transition to a mature neuronal identity, marked by the upregulation of the pan-neuronal marker tubulin-III (Tubb3) (Figure 3b) (Delile et al., 2019).

How is neurogenesis regulated in each dP to generate the number of dIs required to form somatosensory circuity? RP-derived signals, such as the Bmps, also regulate the rate at which dPs differentiate (Andrews et al., 2017; Ille

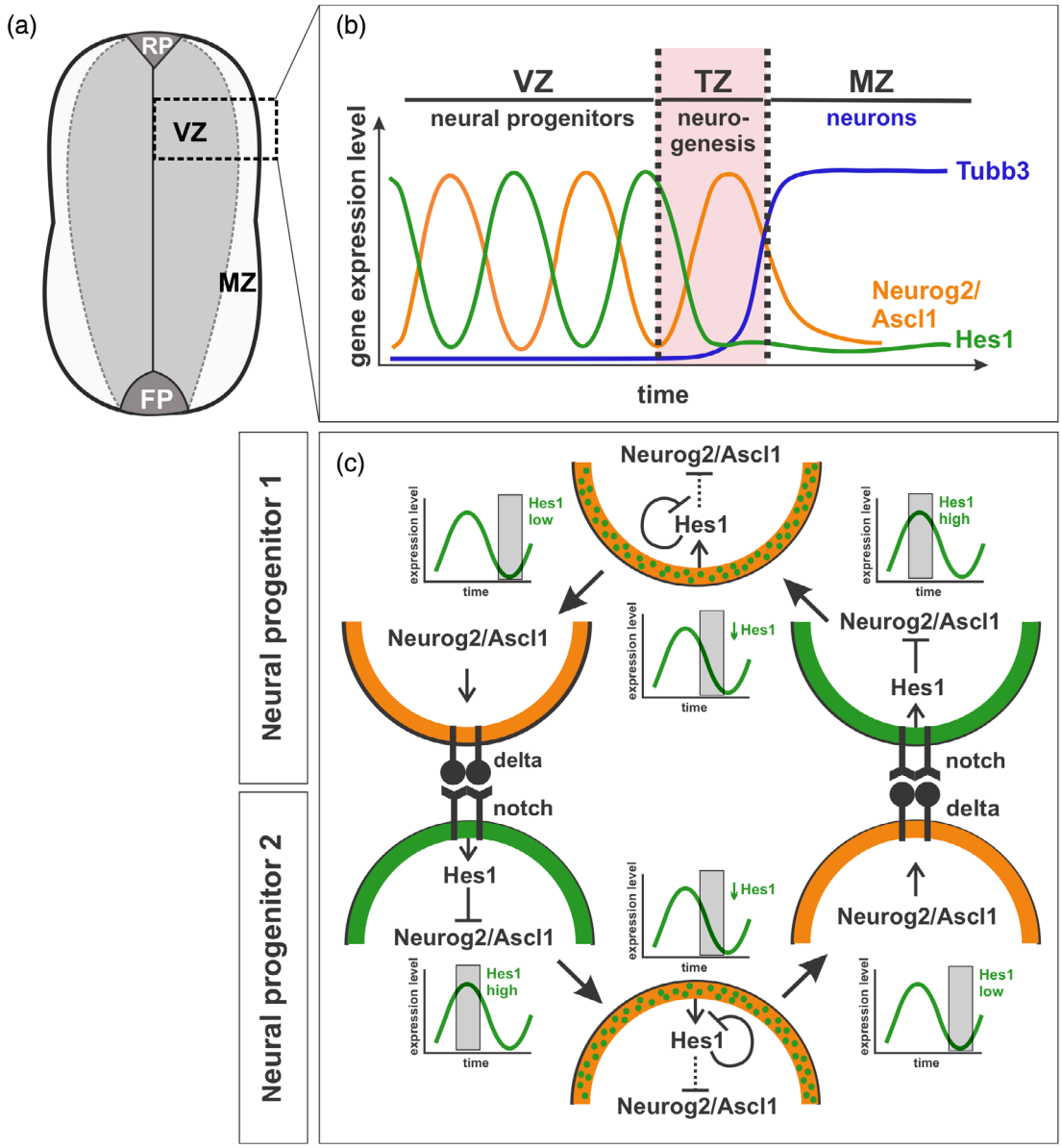

FIGURE 3 Oscillation model for dI differentiation. (a) Schematic of embryonic spinal cord depicting the ventricular zone (VZ) and marginal zone (MZ) where neural progenitors and mature neurons reside. (b) In the VZ, oscillatory expression of bHLH transcription factors such as *Atohl1*, *Ascl1*, and *Neurog1/2*, and *Hes1* maintains progenitors in a proliferative state. Sustained, elevated expression of the bHLH genes and decreased expression of Hes genes prompt progenitors to exit the cell cycle, migrate into the transition zone (TZ) and commit to neurogenesis. The levels of bHLH gene expression then rapidly diminishes as neurons finish their migration into the MZ. The transition of committed neural progenitors to mature neurons is marked by the upregulation of the pan-neuronal marker tubulin-III (Tubb3). (c) In dividing neural progenitors, the oscillatory expression of bHLH and Hes genes occurs by activating notch-delta signaling. High bHLH expression in progenitor 1 leads to delta accumulation on the cell surface which activates notch signaling in its neighbor, progenitor 2, and thereby elevates Hes1 expression. Through a negative feedback loop, high levels of Hes1 in progenitor 2 now suppresses Hes1, allowing bHLH gene expression to recommence, such that the cycle begins again. The periodic activities of notch-delta signaling, and the negative feedback loop that regulates Hes1, keeps the expression of Hes1 in oscillation with the bHLH genes and maintains the neural progenitors in a proliferative state

et al., 2007). Each Bmp has distinct effects on dP proliferation and/or neurogenesis by differentially regulating cell-cycle dynamics (Andrews et al., 2017; Panchision et al., 2001). Bmp4, Bmp7 (chicken) and Bmp6 (mice) can all drive dP1 proliferation by increasing the number of dP1 in S-phase of the cell cycle, but only Bmp4 can direct dP1s to exit the cell cycle. The Bmps also regulate neurogenesis by inducing different *inhibitor of differentiation* (Id) genes in specific dPs (Id1 in dP1, and Id2 in dP2-dP3) (Jen et al., 1997; Le Dréau et al., 2018; Wine-Lee et al., 2004). bHLH transcription

factors complex with E-proteins to direct DNA binding and transcription. In contrast, the Id proteins sequester E-proteins, thereby controlling the timing by which bHLH factors promote dI neurogenesis (Le Dréau et al., 2018). Thus, RP-derived Bmps employ multiple strategies to regulate that the dIs with the correct identity are generated in sufficient numbers to generate somatosensory circuitry.

4 | STEM CELLS: IMPLICATIONS IN UNDERSTANDING SPINAL CORD DEVELOPMENT AND TREATMENTS FOR SCI

Recent advancements in stem cell technologies have permitted multiple neural cell types to be derived from both mouse and human pluripotent stem cells (PSCs) (Asahina et al., 2006; Nizzardo et al., 2010; Rajala et al., 2011; Sneddon et al., 2018; Suzuki & Vanderhaeghen, 2015; Tsai et al., 2017). Genomic analyses suggest that stem cells can faithfully recapitulate in vivo developmental programs during differentiation (Delile et al., 2019; Gouti et al., 2017; Ostermann et al., 2019; Suzuki & Vanderhaeghen, 2015; Tsai et al., 2017). This finding provides an unprecedented opportunity to analyze the mechanisms of embryonic development at fine molecular resolution.

Stem cells also represent cellular therapeutics to treat debilitating SCI which currently have no cure. SCIs affects an estimated 1 million people in the United States alone (Armour et al., 2016) and result in long-term disability and increased mortality rates (Chamberlain et al., 2015; Ma et al., 2014). SCIs can affect both motor and sensory systems, leading to patients losing the ability to move in a coordinated way, and sense sensory warning signals, such as pain and heat. Additionally, patients often lose their sense of touch which can significantly reduce their quality of life. Current treatment options for SCI include neuromodulation and surgical decompression; however, these interventions have serious side effects and limited clinical efficiency (Cristante et al., 2012; Vismara et al., 2017). A more robust cure for SCI would require the regeneration of motor and sensory circuits through the regrowth of spinal tissue. This objective is most effectively achieved using PSC-derived spinal neurons to repopulate in the injured spinal cord.

A major milestone towards this goal was the establishment of directed differentiation protocols to derive motor neurons (MNs) from mouse (Gouti et al., 2014; Wichterle et al., 2002) and human PSCs (Karumbayaram et al., 2009). In the earliest methods, stem cells were first directed towards a posterior neural lineage by RA, and then ventralized by Shh agonists to generate MNs (Amoroso et al., 2013; Shimojo et al., 2015). Subsequent methods have derived motor neurons through the NMPs intermediate, which more accurately mimics the spinal cord development in vitro (Gouti et al., 2014; Turner et al., 2014), or enhanced the efficiency of MN derivation (Calder et al., 2015; Nizzardo et al., 2010; Soundararajan et al., 2007) and/or generate distinct MN types by overexpressing specific transcription factors (Adams et al., 2015; Lee et al., 2020; Soundararajan et al., 2006). In vitro-derived motor neurons are functional and can incorporate into the embryonic spinal cord after transplantation (Miles et al., 2004; Peljto et al., 2010; Umbach et al., 2012; Wichterle et al., 2002). These protocols provide a cellular platform to further understand the mechanistic basis of neurogenerative diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (An et al., 2019; Fuller et al., 2015; Naoki Suzuki et al., 2020; Ng et al., 2015). Additionally, they have also permitted a molecular exploration of the mechanisms by which transcription factors modulate chromatin dynamics and gene regulatory networks to direct motor neuron fates (Kutejova et al., 2016; Metzis et al., 2018). Spinal cord organoids and circuitoids have allowed further modeling of the motor circuits that mediate locomotion and neuromuscular junctions (Faustino Martins et al., 2020; Sternfeld et al., 2017).

While important advances have been made towards regenerating spinal motor function, the ability to restore somatosensation had lagged behind, since no methods were available to generate bona fide spinal sensory relay interneurons in vitro. Thus, towards this goal, we developed the first directed differentiation protocols to derive dIs from both mouse (Andrews et al., 2017) and human (Gupta et al., 2018) PSCs. In these protocols, the Shh agonist is replaced by Bmp4, along with RA, to dorsalize spinal neural progenitors. Central to success of this hPSC protocol was finding the correct time window in which human spinal progenitors are competent to respond to a dorsalising signal (Bmp4). Together, the RA and RA + Bmp4 protocols generate four key populations of dIs from both ESCs and induced PSC (iPSCs): dI1, dI2, dI3, and dI4–dI6 (Gupta et al., 2018).

Multiple challenges remain. Do in vitro derived dIs mimic their in vivo counterparts? Can they survive and assimilate into spinal circuits after transplantation? What is the optimal time window to transplant PSC-derived neurons into SCI patients, given the metabolic chaos and inflammation that occurs post-injury? (Giovanini et al., 1997; Li et al., 2016; Zholudeva & Lane, 2019). As a further caveat, the current protocols generate dIs as a mixed population with conversion efficiencies not yet high enough for clinical use. Additional mechanistic understanding of the process by

which dIs develop in vivo will guide the design of highly effective protocols. Single cell sequencing approaches will also identify novel molecular regulators of specific dI trajectories. These findings, in turn, will permit the development of more refined human stem cell-based protocols that generate pure populations of dIs. A critical objective is the generation of PSC-derived dI4/dI5s to further our understanding of pain disorders and develop the next generation of effective and non-additive analgesics. Understanding mechanisms that direct dI identity during embryonic development is crucial for developing both stem cell replacement therapies and drug discovery platforms.

5 | CONCLUSIONS

The diversity of sensory interneuron patterning is achieved through the reiterated actions of multiple growth factors during spinal cord development. These factors specify distinct interneuron identities by activating self-perpetuating gene regulatory networks to direct the terminal functionality of each neuronal class. Research over the last decade has identified that the Bmps and RA specify dI fates through mechanisms that are distinct from the Shh mediated ventral fate specification. These factors direct both dI patterning and differentiation to ensure that the dIs are generated in correct numbers to form functional somatosensory circuits. These molecular insights have permitted the development of novel stem cell methods to derive multiple classes of dIs in vitro. Stem cell derived dIs are the first step towards both replacing lost or damaged sensory spinal tissue and developing drug discovery platforms. For example, hPSC-derived dI4–dI6s, which mediate pain and itch can be used to identify non-opioid, non-addicting analgesics for the treatment of multiple chronic pain disorders, and thereby mitigate the devastating effects of opioid abuse. Future studies will determine the extent to which stem cell-derived dIs resemble their endogenous counterparts and the correct conditions for cellular replacement therapies, to permit patients to recover sensation.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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