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Generation of posterior motor neurons from human pluripotent stem cells.

By:

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Abstract

Motor neurons are essential for the propagation of signals which evoke muscle contractility and allow all bodily movements. An inability of motor neurons to function correctly, due to damage or degeneration, can result in multiple movement-based disorders. Generation of motor neurons from the *in vitro* differentiation of human pluripotent stem cells (hPSCs) has demonstrated a potential cell-replacement treatment for neurodegenerative diseases. However, existing *in vitro* hPSC differentiation protocols predominantly produce motor neurons of a hindbrain and cervical identity and fail to produce high yields of more posterior, thoracic and lumbar, identities.

Multiple studies have shown that thoracic and lumbar neural tissue have differing developmental origins to their anterior counterparts. During axis extension, a population of bipotential progenitors, termed Neuromesodermal Progenitors (NMPs), give rise to the neural and mesodermal lineages of the posterior axis. Therefore, we demonstrate that to efficiently generate a thoracic motor neuron identity, *in vitro*, there is an initial requirement to differentiate hPSCs via a NMP intermediate state. In doing so, we fully characterise the signals important for promoting both a neural and motor neuron identity downstream of NMPs. We show that WNT and FGF signals promote a dorsal neural state whilst the inhibition of TGFβ and BMP pathways enhance neuralisation. Further TGFβ and BMP pathways antagonism alongside the stimulation of Shh signalling are further implicated within dorso-ventral patterning to promote a ventral motor neuron identity. Whilst, continued FGF signals are required to promote a stable thoracic identity. To our knowledge, compared to existing protocols, our characterised protocol provides the highest yields of thoracic motor neurons and is reproducible within multiple hPSC cell lines. Furthermore, the hPSC-derived thoracic identity motor neurons are electrophysiologically functional and demonstrate the ability to integrate within the neural tube of chick embryos. Additionally, we have also compared the functionality of both hPSC-derived motor neurons of a hindbrain/cervical identity and thoracic identity. We find that both motor neuron subtypes display no electrophysiological differences. However, upon grafting into thoracic regions of chick embryo neural tubes it appears that hPSC-derived hindbrain/cervical motor neuron progenitors appear to display a greater degree of ectopic behaviours compared to their thoracic motor neuron progenitor counterparts. This suggests that acquisition of positional identity, prior to engraftment, can effect behaviour of transplanted cells *in vivo*.

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Chapter 1-Introduction

1.1- Formation of the spinal cord *in vivo*

The formation of the spinal cord has been deeply explored within existing literature. Early findings suggested that the formation of the neural tube began with the generation of the anterior neural plate, at the end of gastrulation, which was successively patterned by signals, such as Retinoic Acid, FGFs and Wnts, to generate more posterior neural character. This has been termed the activation-transformation model. However, more recently it has been suggested that there are two separate progenitor populations which give rise to the neural tissue of the entire body axis. It has been shown that during axis extension, a population of bipotential progenitors, termed Neuromesodermal Progenitors (NMPs), exist within the posterior regions of the extending embryo which give rise to the neural and mesodermal lineages of the posterior axis (Cambray and Wilson, 2002; Tzouanacou *et al.*, 2009a). Therefore, suggesting two divergent neural progenitor populations which give rise to anterior and posterior neural tissue.

Activation-Transformation Model: Anterior Neural Plate Formation

Early work suggested that neural tissue was induced and specified by the "activation-transformation" model (Nieuwkoop and Nigtevecht, 1954). Within this model, an early neural identity was induced (activation) which was then successively caudalised (or transformed), through exposure to signalling molecules, to pattern the neural tissue and generate posterior identities. Findings within amphibian embryos identified that the Organiser region was essential for the initial neural activation step during gastrulation as transplantation of donor Organiser regions into ectopic locations resulted in secondary neural axis formation (Spemann and Mangold, 2001). Homologous regions were identified to have similar neural inducing effects within different species, for example, the node within mice, Hensen's Node within chick and the shield region within zebrafish embryos (Beddington, 1994; Shih and Fraser, 1996; Boettger *et al.*, 2001).

After the identification of the Organiser/Node as the source of neural activation a lot of experiments aimed at elucidating the key signalling pathways which were essential for neural induction. The Organiser was shown to express and secrete high levels of the TGF-β

family antagonists; Noggin, Follistatin, Chordin and Cerberus (Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1996; Zimmerman, De Jesús-Escobar and Harland, 1996; Fainsod *et al.*, 1997). Emphasising the role of BMP inhibition in neural induction, it was shown that the expression of dominant-negative BMP4 receptors within Xenopus embryos resulted in ectopic neurectoderm formation (Hawley *et al.*, 1995). Conversely, the depleted function of chordin, noggin, and follistatin in Xenopus embryos resulted in an ablation of neural plate formation (Khokha *et al.*, 2005). Similar effects were observed in mice embryos lacking the BMP antagonists which resulted in the failure to allow correct neural induction and failure to form forebrain structures (McMahon *et al.*, 1998; Bachiller *et al.*, 2000).

Upon the activation of anterior neural identity, there are successive transformations of the anterior neural pate to generate more posterior neural identities. Retinoic Acid, FGF and WNT activities have all been suggested to have a key role within this patterning process. During neurulation, Retinoic Acid is synthesised within RALDH-2 expressing mesoderm which is located posteriorly to the anterior neural plate. Whilst the anterior neural plate expresses CYP26 which is an enzyme involved in Retinoic Acid degradation (Niederreither *et al.*, 1997; Swindell *et al.*, 1999). This results in high levels of Retinoic Acid posteriorly and low levels of Retinoic Acid within the anterior, allowing for a gradient of Retinoic Acid to act as a morphogen to pattern the anterior neural plate. The transforming ability of Retinoic Acid was initially shown when the addition of exogenous Retinoic Acid, within Xenopus and mice embryos, resulted in the transformation of anterior forebrain neural tissue to a posterior hindbrain identity (Durston *et al.*, 1989; Marshall *et al.*, 1992a). Equally, the antagonism of Retinoic Acid receptors, within chick embryos, resulted in the posterior expansion of anterior forebrain/midbrain neural identity and failure to form posterior hindbrain neural structures (Dupé and Lumsden, 2001). Furthermore, enhanced retinoid receptor activity was shown to reduce Otx2 expressing forebrain domains and cause anterior expansion of posterior hindbrain neural identity whilst the expression of dominant negative retinoid receptors was shown to enhance anterior forebrain neural identity (Blumberg et al., 1997). Manipulation of retinoid receptor activity appeared to have no effect on spinal cord Hoxb9 expression domains (Blumberg *et al.*, 1997). Similarly, treatment of chick caudal neural late explants with exogenous Retinoic Acid resulted in the formation of hindbrain identity and a failure to induce spinal cord identities (Nordström, Maier, Thomas M. Jessell, *et al.*, 2006). All these results favour Retinoic Acid activity as an inducer of posterior neural identity by

transforming anterior forebrain identity to gradually more posterior hindbrain identities. However, Retinoic Acid signals show little impact on inducing posterior spinal cord identity.

FGF and Wnt signals have both been shown to have a role in transforming the neural plate to a posterior spinal cord identity. FGFs have been shown to have a key role in the posterior patterning of the neurectoderm during neurulation. Treatment of prospective forebrain explants with bFGF caused the induction of hindbrain identity whilst prospective hindbrain explants gained spinal cord identity (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). Conversely, the forced expression of dominant negative FGF-receptors resulted in anterior gene expansion within posterior domains within Xenpous and Zebrafish embryos. (Koshida *et al.*, 1998; Kudoh, Wilson and Dawid, 2002). Further experiments identified that treatment of Xenopus ectodermal cells with low levels of bFGF resulted in generation of anterior neural identity whilst higher levels of bFGF resulted in progressively more posterior spinal cord neural identities (Kengaku and Okamoto, 1995). Supporting the role of FGF as a posterior morphogen in patterning the neurectoderm.

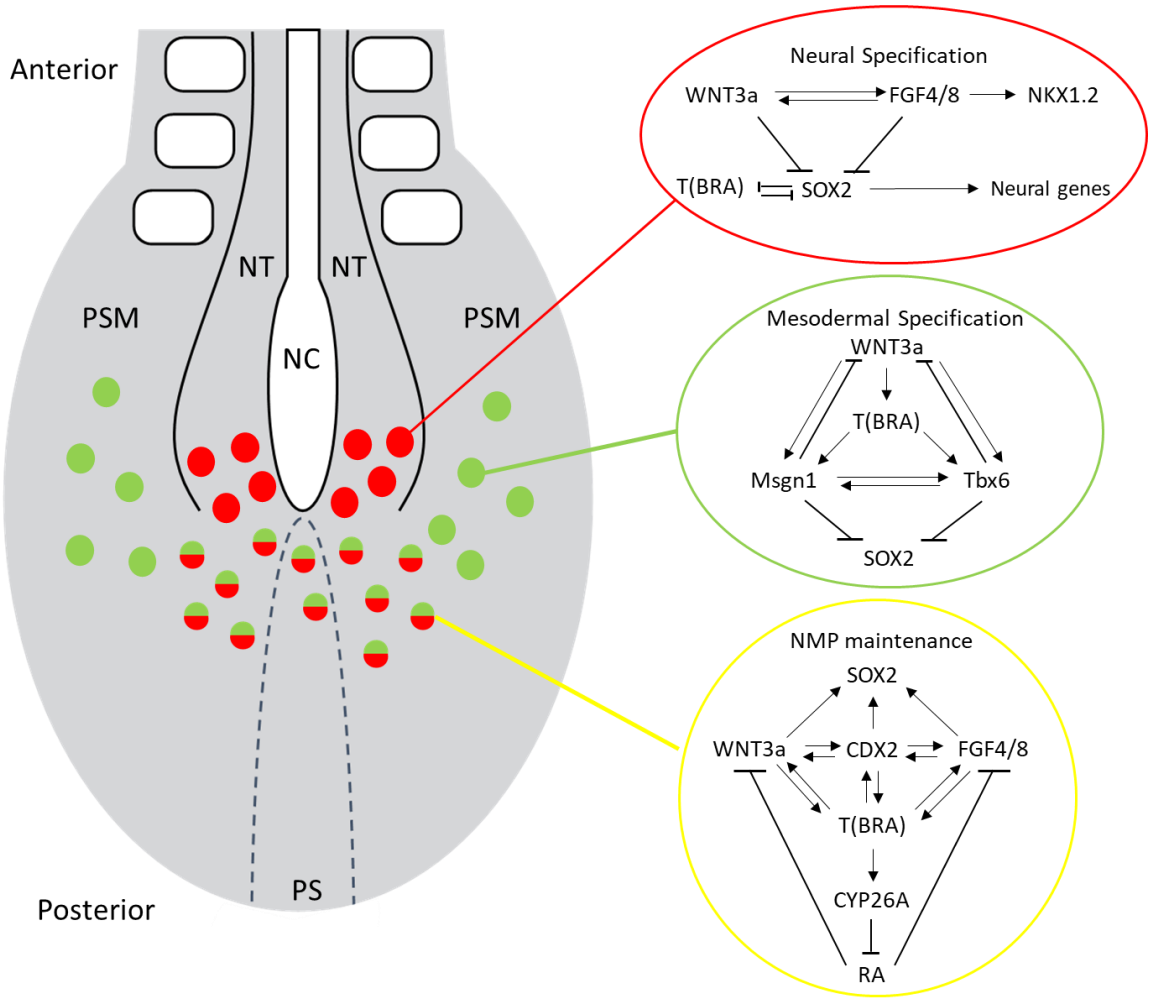
The role for Wnt in "transforming" the anterior neural plate to a posterior identity is also well documented. During axis formation the expression of Wnt antagonists within the Anterior visceral endoderm, which underlies the anterior neurectoderm, and expression of Wnt ligands within the posterior embryo and primitive streak results in an anterior-posterior morphogenetic gradient of high Wnt expression posteriorly to low expression anteriorly (Liu *et al.*, 1999). Indeed, the secretion of Wnt inhibitors from the anterior visceral endoderm have been shown to be essential to induce anterior forebrain identity (Thomas and Beddington, 1996). Treatment of Xenopus neural explants with Wnt3a resulted in the loss of forebrain identity and enhanced levels of spinal cord gene expression (McGrew, Lai and Moon, 1995). Within zebrafish embryos the application of endogenous Wnt8 resulted in anterior neural defects and expanded posterior neurectoderm identity (Kelly GM *et al.*, 1995). These findings also show the importance of Wnt signals in forming morphogenetic gradients and acting as a "posteriorising signal". Together, Wnt and FGF signals have been shown to be vital for the induction of posterior spinal cord identity. Chick caudal neural explants which were cultured in the presence of FGF and Wnt inhibitors fail to generate spinal cord and instead generate $OTX2^{+}$-forebrain character (Nordström, Maier, Thomas M. Jessell, *et al.*, 2006). However, culturing these same explants with FGF and Wnt resulted in

the formation of spinal cord character and a suppression of forebrain and hindbrain identity (Nordström, Maier, Thomas M. Jessell, *et al.*, 2006).

Neuromesodermal Progenitors: A source of posterior neural tissue

During the exploration of early neural induction multiple findings suggested that not all neural structures were derived from the anterior neural plate, elucidating a potential secondary source of neural tissue. For example, mouse mutants deficient in either Chordin, Noggin or Cerberus failed to induce anterior neurectoderm, failing to form anterior brain structures, and instead only express posterior neural genes (McMahon *et al.*, 1998; Bachiller *et al.*, 2000). Early clonal analysis and fate mapping studies suggested that there was an early separation between the progenitors which give rise to the anterior and posterior CNS (Brown and Storey, 2000; Mathis and Nicolas, 2000). Further clonal analysis studies identified that, at around E7.5 in mice embryos, cells located anteriorly to the node give rise to the anterior hindbrain regions whilst cells located closer and lateral to the node gave rise to posterior neural tissue and paraxial mesoderm (Forlani, 2003). This shows a divergence within the origins of anterior and neural tissue. This has been further enhanced through the discovery of a population of axial progenitors, termed Neuromesodermal Progenitors (NMPs), which are capable of generating mesoderm and neural tissue which populate the post-cranial axis (Tzouanacou *et al.*, 2009a). NMPs exist until later stages of embryonic development, long after the formation of the anterior neural plate. Lineage tracing studies, in a variety of species, have identified the Node Streak Border and Caudal Lateral Epiblast as the location of NMPs during pre-tailbud stages (Psychoyos and Stern, 1996; Wilson and Beddington, 1996; Brown and Storey, 2000; Noemi Cambray and Wilson, 2007; Filip J. Wymeersch *et al.*, 2016) (shown in **Schematic 1.1**) . Recent in vivo imaging, within chick embryos, has also shown the presence of dual-fated NMP progenitors located at the interface of mesodermal and neural progenitor zones (Wood *et al.*, 2019). At later time points, NMPs from the Node Streak Border have been shown to be retained within the Chordoneural Hinge (CNH) region of the tail bud until the end of somitogenesis (Cambray and Wilson, 2002; Tzouanacou *et al.*, 2009a). NMPs located in the CNH can be serially transplanted into the anterior streak and continuously show the ability to generate cells of the posterior axis, found within mesoderm, the neural tube and cells which repopulate the

CNH (Cambray and Wilson, 2002). Derivatives of NMPs have been shown to give rise to the posterior neural stem zone and differentiated neural plate (Delfino-Machín, J. S. Lunn, *et al.*, 2005a; Wood *et al.*, 2019). This shows the ability of NMPs to retain their identity as axial progenitors until later stages of development, providing a constant supply of posterior neural tissue which is divergent in origin to that of the anterior neural plate.



Schematic 1.1. A schematic showing the presence of Neuromesodermal progenitors (red/green circles) which reside in the anterior primitive node streak border region and caudal lateral epiblast and have the ability to generate mesodermal (green) and neural (red) cell lineages of the posterior axis. Abbreviations: NT Neural Tube, PSM Presomitic Mesoderm, NC Notochord and PS Primitive Streak.

Signals driving the formation of NMPs

Due to their location within the posterior embryo and their contribution to the posterior axis it has long been hypothesized that Wnt and Fgf signals are involved in the induction and maintenance of an NMP identity (N. Cambray and Wilson, 2007; Wymeersch *et al.*, 2019a). *In vivo*, Neuromesodermal Progenitor identity is marked by the co-expression of T (Brachyury), a mesodermal progenitor marker, and SOX2, a pluripotency/neural progenitor marker (Olivera-Martinez *et al.*, 2012; Tsakiridis *et al.*, 2014).

As T (Brachyury), an important marker of NMP identity and mesodermal specification, is a direct target of Wnt3a (Yamaguchi *et al.*, 1999), Wnt signals have been suggested to be vital for the induction of an NMP state (Robert J. Garriock *et al.*, 2015). Multiple studies have shown that removal of Wnt3a expression attenuates T (Brachyury) expression and results in the failure to generate paraxial mesoderm alongside premature cessation of axis elongation (Takada *et al.*, 1994; Yoshikawa *et al.*, 1997; Yamaguchi *et al.*, 1999; Martin and Kimelman, 2008; Savory *et al.*, 2009). The failure to correctly form the entire posterior axis within these mutants has been linked to a failure to maintain the NMP population. It has been hypothesized that Wnt signals are important for maintaining the NMP progenitor niche to retain an NMP population (R. J. Garriock *et al.*, 2015; Filip J. Wymeersch *et al.*, 2016). This hypothesis, as clearly demonstrated in mesodermal progenitors downstream of NMPs, is based on the idea that Wnt and T(Brachyury) are linked within an auto-regulatory loop; with Wnt ligands being targets of T(Brachyury) and T(Brachyury) being regulated by Wnt (Yamaguchi *et al.*, 1999; Martin and Kimelman, 2008). This auto-regulatory loop therefore allows for the continued expression of T(Brachyury) and progenitor identity.

The maintained expression of T(Brachyury), by the Wnt-T(Brachyury) auto-regulatory loop, is also thought to be important to prevent Retinoic Acid signals from expanding posteriorly. T(Brachyury) has been shown to activate CYP26a expression which is an enzyme which degrades Retinoic Acid (Iulianella *et al.*, 1999). Initially, the presence of Retinoic Acid has been implicated in the early induction of SOX2 to allow specification of an early NMP identity (Cunningham, Kumar, *et al.*, 2015; Cunningham, Colas and Duester, 2016; Gouti *et al.*, 2017). However, Retinoic Acid Response Elements, with repressive activity, have been identified upstream of Wnt8a and Fgf8 which under high levels of Retinoic Acid signals can result in the loss of NMP identity and premature axial cessation (Sakai *et al.*, 2001; Kumar

and Duester, 2014; Cunningham, Kumar, *et al.*, 2015; Gouti *et al.*, 2017). Therefore, the presence of CYP26a expression within NMPs allows for the degradation of Retinoic Acid, to maintain low levels of Retinoic Acid and a maintenance of the NMP population to prevent premature axis cessation (Iulianella et al., 1999; Sakai et al., 2001; Martin and Kimelman, 2010).

CDX2, a known marker of the NMPs and the elongating trunk, has also been shown to have a vital role in inducing and maintaining an NMP identity. Cdx2 enhancer binding domains have been identified upstream of Fgf8 and Wnt5a which promote their caudal expressions (Amin *et al.*, 2016a). Cdx2 mutant mice have also been identified to have a reduction in Fgf and Wnt transcripts within the trunk regions and a resultant truncation within the posterior axis (Amin *et al.*, 2016a). CDX2 has also been shown to synergistically bind with T-Brachyury to activate NMP-associated gene regulatory networks which are reflective of the severe trunk mutations observed in Cdx2 and T-Brachyury null mutants (Yoshikawa *et al.*, 1997; Yamaguchi *et al.*, 1999; van de Ven *et al.*, 2011; Amin *et al.*, 2016a). This shows the importance in CDX2 for maintaining the NMP population and allowing axis extension.

FGFs have also been proposed to have a similar role in inducing and maintaining an NMP identity. Early expression analysis within chick embryos, at HH Stages 4-6 prior to neurulation, identified that multiple FGFs (FGF3, FGF4, FGF8, FGF13, FGF18) are expressed within the NMP region and surrounding tissues (Karabagli *et al.*, 2002; Delfino-Machín, J Simon Lunn, *et al.*, 2005). Whilst expression of FGF4 and FGF8 are retained within the NMP population up until much later tail-bud stages (Cunningham, Zhao and Duester, 2011; Olivera-Martinez *et al.*, 2012). Similar expression patterns have also been shown in both mice and zebrafish embryos (Gofflot, Hall and Morriss-Kay, 1997; Reifers *et al.*, 2000). FGF8 signaling plays an important role in activating known genes of an NMP identity. For example, FGF8 is known to activate the expression of the NMP marker *NKX1.2* (Bertrand, Médevielle and Pituello, 2000; Delfino-Machín, J. S. Lunn, *et al.*, 2005a). Conversely, the conditional knock out of Fgf4 and Fgf8 within mice embryos resulted in reduced expression of Wnt3a, Cyp26a1 and T(Brachyury) within the NMP region (Naiche, Holder and Lewandoski, 2011; Boulet and Capecchi, 2012). Similarly, dominant negative FGF-receptor1 expression resulted in a similar reduction in NMP gene expression (Wahl *et al.*, 2007). These results support the idea that FGF signals play an important role in NMP induction and maintenance.

Multiple studies have shown an essential role for Wnt and FGF signals working synergistically to maintain the NMP population. For example, within NMPs, SOX2 expression is driven under the control of the N-1 enhancer which is divergent from the anterior neural plate where it is under the control of the N-2 enhancer (Uchikawa *et al.*, 2003; Takemoto *et al.*, 2006). Mutational analysis identified that activation of the N-1 enhancer is dependent on both Fgf8 and Wnt8 signals (Takemoto *et al.*, 2006). Furthermore, FGF pathway and Wnt pathway components have been shown to be key targets of T(Brachyury) and CDX2 within NMPs, showing that maintaining these signals, via inter-regulatory pathways, is vital for maintaining an NMP population (Amin *et al.*, 2016b). Similarly, exposure of mouse embryo explants to small molecule inhibitors of Myc activity were shows to cause a downregulation of Wnt, Fgf and Sox2 transcripts (Mastromina *et al.*, 2018). Furthermore, activation of the Wnt, Fgf and Notch pathways were shown to enhance cMyc expression whilst the presence of Retinoic Acid resulted in the ablation of cMyc expression (Palomero *et al.*, 2006; Mastromina *et al.*, 2018). This suggests that a feedback loop between Fgf, Wnt and Myc activity plays an essential role within the maintenance of NMP identity.

Together, these findings suggest that there are a large number of interconnected pathways which play an essential role in inducing and maintaining the NMP population within the posterior of the embryo.

Signals driving the downstream differentiation of NMPs

As NMPs are bipotent it is important to understand the signalling mechanisms which are involved to allow correct fate restrictions to either a mesodermal or neural identity. Acquisition of a mesodermal fate is largely linked to Wnt signals. As previously discussed, Wnt signals are required to induce and maintain an NMP state so it is hypothesised that exposure to higher concentrations of Wnt signals is the requirement for mesoderm induction. *In vivo*, downstream of NMPs, high concentrations of Wnt have been shown to induce the expression of mesodermal specification genes such as Tbx6 and Msgn1 (Mesogenin1) (Wittler *et al.*, 2007; Bouldin *et al.*, 2015). Both Mesogenin1 and Tbx6 work within an auto-regulatory loop to maintain the expression of each other and to promote mesodermal identity and have hence been denoted as master regulators of mesodermal fate

(Nowotschin *et al.*, 2012; Chalamalasetty *et al.*, 2014; Koch *et al.*, 2017). Similarly, both Mesogenin1 and Tbx6 have been shown to inhibit Wnt3a gene expression (Takemoto *et al.*, 2011a; Nowotschin *et al.*, 2012; Bouldin *et al.*, 2015) which in turn results in a inhibition of T(Brachyury) expression (Chapman and Papaioannou, 1998), favouring a robust mesoderm identity and exit from a progenitor identity.

TBX6 is also believed to play an important role in preventing neural identity downstream of NMPs. Inhibition of Sox2, the neural progenitor marker, mediated by TBX6 has been demonstrated in both zebrafish and mice (Takemoto *et al.*, 2006, 2011b; Bouldin *et al.*, 2015). Tbx6 (-/-) mice and zebrafish embryos fail to form mesoderm and upregulate Sox2 (Kimmel *et al.*, 1989; Chapman and Papaioannou, 1998; Griffin *et al.*, 1998) with mouse mutants even forming ectopic neural tubes (Chapman and Papaioannou, 1998). This demonstrates that not only is TBX6 important for mesoderm specification, downstream of NMPs, but in mice the absence of Tbx6 expression may be sufficient to allow neural induction. FGF activity has also been shown to be important for mesodermal specification from NMPs as it has a role in regulating the expression of Msgn1 and Tbx6, which as discussed previously are the master regulators of mesodermal identity (Goto et al., 2017). Inhibition of FGF activity has also been shown to prevent Wnt activation and Notch activity which results in the loss of mesoderm segmentation and specification of paraxial mesoderm (Wahl *et al.*, 2007; Boulet and Capecchi, 2012). Importantly, FGF4 has been suggested to be essential for controlling the oscillations of the Notch pathway which determine mesodermal segmentation of the presomitic mesoderm (Boulet and Capecchi, 2012; Anderson *et al.*, 2020).

Neural induction from an NMP state is a 2-step dependent process; with the initial FGF-dependent induction of a pre-neural progenitor identity followed by induction of a definitive neural progenitor state (Del Corral *et al.*, 2003). FGF signals are vital for inducing the initial pre-neural progenitor identity. Activation of Sox2, via the N-1 enhancer, and Sox3 neural genes is dependent on FGF signals (Takemoto *et al.*, 2006; Nishimura *et al.*, 2012). Similarly, the expression of NKX1.2 within the NMP state and maintained expression within pre-neural progenitors, which are NMP-derived progenitors fated to become posterior spinal cord, is dependent on activation by FGF (Bertrand, Médevielle and Pituello, 2000; Delfino-Machín, J. S. Lunn, *et al.*, 2005a). FGF signals have also been shown to enhance *HDAC1* transcription

within pre-neural progenitors which has been suggested to alter chromatin organisation and allow the expression of neural-specific genes (Olivera-Martinez *et al.*, 2014).

The transition from a pre-neural progenitor to a neural progenitor of the spinal cord is largely regulated by high levels of Retinoic Acid signaling from the somites (Rossant *et al.*, 1991). Retinoic Acid has been shown to inhibit Wnt and Fgf signals (Shum *et al.*, 1999; Del Corral *et al.*, 2003) and downregulate the expression of the pre-neural marker NKX1.2 (Del Corral *et al.*, 2003; Olivera-Martinez *et al.*, 2014; Sasai, Kutejova and Briscoe, 2014). Upregulation of the neural progenitor markers *PAX6* and *IRX3* are activated by Retinoic Acid (Del Corral *et al.*, 2003) but also appear to be dependent on the loss of FGF signals to promote correct chromatin organisation (Olivera-Martinez *et al.*, 2014). This fits with the finding that the downregulation of FGF signals is essential for the progressive differentiation to both a mesodermal and neural identity downstream of NMPs whilst maintained FGF signals promote a progenitor identity (Dubrulle, McGrew and Pourquié, 2001; Del Corral *et al.*, 2003). Taken together, these results would suggest that neural identity, downstream of NMPs, requires low Wnt signals to prevent a mesodermal induction. FGF signals appear to promote a pre-neural identity and may have a role in allowing proliferation of these pre-neural progenitors. However, Retinoic Acid signals appear to be the vital signal to promote neuronal progenitor profile induction.

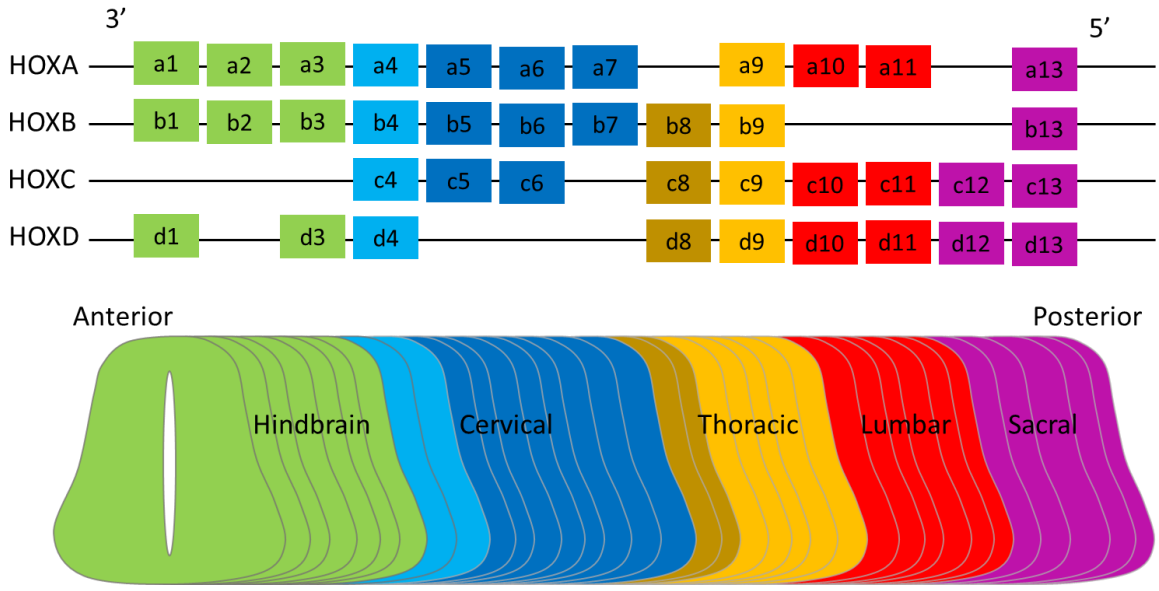
1.2- Exploring positional identity

Anterior-posterior patterning of the neural axis

During neural development it is essential that correct anterior-posterior patterning of positional identity is achieved so that correct bodily orientation is generated within an adult. Patterning of the anterior-posterior neural axis is regulated by a set of homeotic genes known as the HOX genes. In most vertebrates there are 39 HOX genes which are located in 4 separate gene clusters (HOXA, HOXB, HOXC and HOXD), with each cluster containing a set of genes numerically ordered HOX1-13 (as shown in **Schematic 1.2**). These highly conserved segmental homeobox genes are expressed in collinear order (Duboule and Dollé, 1989; Gaunt, Krumlauf and Duboule, 1989; McGinnis and Krumlauf, 1992). 3' HOX genes are initially expressed in the posterior primitive streak regions with successive activation of more 5' HOX genes within this region at later time points as the axis extends (reviewed by Deschamps *et al.*, 1999). This region of initial HOX expression contains NMPs which allows for the axial patterning of their descendants (Wymeersch *et al.*, 2019b). HOX genes have sharp boundaries of expression which results in unique HOX expression domains across the anteroposterior axis of the neural tube. Notably, the forebrain region is Hox-negative and during early development is marked by the expression of markers such as Otx2 (Simeone *et al.*, 1993). Following this the remainder of the anterior-posterior axis is marked by unique HOX expression domains: HOX1-3 paralogs correlates with a hindbrain identity, HOX3-5 reflect a cervical identity, the HOX6-7 paralogs are reflective of a brachial identity, thoracic locations are marked by HOX8-9 expression whilst the expression of Hox10-13 confer a lumbosacral identity (Duboule and Dollé, 1989; Gaunt, Krumlauf and Duboule, 1989; McGinnis and Krumlauf, 1992) (as shown in **Schematic 1.2**).

The initial activation and lineage-specific maintenance of robust HOX expression domains are regulated by morphogenetic gradients (FGF, Wnt and RA) but also through autoregulation. Many studies have shown that anterior HOX genes are able to activate more posterior 5' located HOX genes (Faiella *et al.*, 1994; Hooiveld *et al.*, 1999) whilst posterior HOX genes can conversely inhibit their more anterior located counterparts (Struhl and White, 1985; Miller *et al.*, 2001; Plaza *et al.*, 2008). Once the anteroposterior induction of

HOX expression has been induced each specific HOX domain activates a unique set of transcription factors which confers a specific positional and morphological identity (Dasen, Liu and Jessell, 2003a). Aberrations in the normal HOX transcriptional activation or patterning result in axial homeotic transformations (Rijli *et al.*, 1993; Favier, 1997). Thus, Hox genes are able to determine the patterning, identity and function of specific segments across the AP axis. Due to their conserved expression patterns and ability to reproducibly activate specific regional identify the Hox genes have become a notable marker to distinguish positional identity.



Schematic 1.2. A schematic showing the chromosomal HOX orientations and their reflective anteroposterior expression patterns which define specific axial neural identities.

Retinoic Acid is responsible for patterning hindbrain/cervical neural identity

Retinoic Acid has long been implicated as a candidate for the activation and patterning of anterior neural identity. Retinoic Acid, which is a metabolite of Vitamin A, has been shown to display sharp boundaries of synthesis within the mouse embryo; anteriorly at the level of the first somite and posteriorly at the level of the last forming somite (Rossant *et al.*, 1991; Colbert, Linney and LaMantia, 1993). Zebrafish and chick embryos also display the same sharp boundaries of Retinoic Acid synthesis within similar regions (Marsh-Armstrong *et al.*, 1995; Maden *et al.*, 1998). These sharp boundaries of expression are maintained due to the location-specific expression of retinoic acid synthesis and degradation enzymes. Retinaldehyde dehydrogenase 2 (RALDH2), an enzyme essential for the synthesis of retinoic acid, has been shown to be expressed by trunk regions and is notably missing from the hindbrain and tailbud (Niederreither *et al.*, 1997). Conversely $P450_{RA}$, enzymes which are capable of metabolically inactivating Retinoic Acid, are expressed within cranial and tailbud regions (Fujii *et al.*, 1997; Iulianella *et al.*, 1999). Specifically cytochrome P450 26 (CYP26), a member of the cytochrome $P450_{RA}$ family, has high levels of expression within the anterior head regions and the tailbud (Sakai *et al.*, 2001).

Disruption to normal levels of Retinoic Acid activity has been shown to have severe effects on HOX-positional patterning within the developing neural tube. Expansion of Retinoic Acid activity into hindbrain regions, by abolishing CYP26 degradative activity in *Cyp26* (-/-) mice, resulted in the ectopic anterior expansion of *Hoxb1* expression (Sakai *et al.*, 2001). These mutant mice also display anterior expansion of cervical *Hoxb4* expression (Sakai *et al.*, 2001). Similarly, treatment of anterior hindbrain regions with exogenous Retinoic Acid has also been shown to cause the anterior expansion of posterior HOX genes and the loss of Krox20 anterior rhombomere expression (Conlon and Rossant, 1992; Marshall *et al.*, 1992b). Reflectively, *Raldh2* (-/-) mice, which are incapable of synthesising Retinoic Acid, were shown to have abnormal expressions of KROX20, HOXB1 and HOXB2 and a complete loss of HOX3/4 expression. Morphologically these mice had an expansion of anterior neural identity with the presence of rhombomeres r3-r5 but a loss of r5-r8 (Niederreither *et al.*, 2000). Together, these findings show the importance of Retinoic Acid in activating the HOX1-4 paralogs and inducing hindbrain/cervical neural identity.

Many of the more anterior expressed HOX genes have been identified to be directly regulated by Retinoic Acid signals. Initial work identified the presence of a Retinoic Acid Response Element (RARE) within the mouse enhancer of *Hoxb1* which appeared to be evolutionary conserved to an enhancer found in the chicken and pufferfish (Marshall *et al.*, 1994). Point mutations within this Retinoic Acid Response Element results in the abolishment of *Hoxa1* expression caudal to rhombomere 4. Many other studies have since found Retinoic Acid Response Elements within other HOX gene enhancers (Frasch, Chen and Lufkin, 1995; Langston, Thompson and Gudas, 1997). Using a Cre-LOXP system for the conditional knock-out of *Hoxa1* Retinoic Acid Response Element enhancer resulted in cranial nerve abnormalities in mice (Dupé *et al.*, 1997). Whilst the Retinoic Acid Response element in the human *HOXD4* enhancer was found to be required for gene expression in response to Retinoic Acid in both human carcinoma cell lines and transgenic mice (Morrison *et al.*, 1996). Taken together the existing literature shows that Retinoic Acid works as a morphogen in the patterning and induction of anterior (hindbrain/cervical) neural identity via it's regulation of the HOX1-5 paralogs which contain Retinoic Acid Response Elements. Retinoic Acid diffuses from the hindbrain/spinal cord boundary, where it is synthesised and highly expressed (Marsh-Armstrong *et al.*, 1995; Maden *et al.*, 1998), to form a gradient across the hindbrain where it is antagonised/degraded by CYP26 (Niederreither *et al.*, 2000; Sakai *et al.*, 2001). In this manner higher concentrations of Retinoic Acid activate cervical HOX genes and hence cervical neural identity, whilst lower levels of activity activate hindbrain identity by the activation of 3' HOX genes (Conlon and Rossant, 1992; Marshall *et al.*, 1992b; Niederreither *et al.*, 2000; Sakai *et al.*, 2001).

WNT & FGF signals pattern posterior neural identity via CDX-HOX activation

Cdx genes are important for the regulation of trunk HOX genes (HOX6-10 paralogs) and are therefore required for the activation of thoracic neural identity. The Cdx homeobox genes are homologs of the Drosophila CAUDAL gene which is also expressed, during development, in a gradient emanating from the posterior (Hoey *et al.*, 1986). Within mice there are 3 CAUDAL homologs; *Cdx1* (Duprey *et al.*, 1988), *Cdx2* (Beck *et al.*, 1995) and *Cdx4* (Gamer and Wright, 1993) which are respectively termed xcad2, xcad1 and xcad3 in the Xenopus (Pillemer *et al.*, 1998). During gastrulation, *Cdx1* is initially expressed throughout the primitive streak and in later stages within the posterior neural tube, somites, mesoderm and limb buds (Meyer and Gruss, 1993). *Cdx2* is expressed in the caudal neural tube, neuromesodermal progenitor population and notochord and within the tailbud ectoderm, mesoderm and endoderm. *Cdx4* is similarly expressed within posterior structures during axial elongation (Gamer and Wright, 1993).

Many studies have shown the importance of the Cdx genes in regulating trunk spinal cord HOX expressions (HOX6-10 paralogs). Within the Xenopus it has been shown that the ectopic injection of xcad3 mRNA, the Cdx homolog, results in the activation of hoxc6, hoxc7, hoxb9 and hoxa9 whilst presence of a repressor of xcad3 results in the transcriptional repression of these HOX genes (Isaacs, Pownall and Slack, 1998). Xenopus embryos treated with the repressor of xcad3 also have severe posterior axial defects (Isaacs, Pownall and Slack, 1998). *Cdx1* (-/-) mutant mice were also observed to have a posterior shift in HOX expression domains and anterior axial transformations across their axis similar to those observed in Hoxa5 and Hoxc8 mutants (Subramanian, Meyer and Gruss, 1995). Conversely, the gain of function of thoracic Hox genes within Cdx mutants was able to rescue posterior axial defects (Young *et al.*, 2009). Cdx1(-/-)Cdx2(+/-) double mutant mice have enhanced axial defects and increased posterior shifts in HOX gene expression (van den Akker *et al.*, 2002). This suggests that not only are the Cdx genes important for early trunk-identity HOX patterning but there is potential functional redundancy between the Cdx genes. Strong evidence for the regulation of posterior Hox genes by CDX is also demonstrated through the presence of CDX-specific binding sites within the enhancers of 5' HOX genes (Taylor *et al.*, 1997; Charité *et al.*, 1998; Isaacs, Pownall and Slack, 1998; Gaunt, 2018). Mutations within these binding motifs have been shown to inhibit Hox gene activation and result in axial defects (Charité *et al.*,

1998; Isaacs, Pownall and Slack, 1998; Gaunt, 2018). These findings demonstrate the importance of CDX genes in the activation of trunk Hox expressions to allow correct patterning of trunk identity within the neural tube.

As CDX has been shown to directly bind and regulate Hox expression many studies have looked into the signals which are responsible for the activation and regulation of the Cdx genes. There is some evidence to suggest that Retinoic Acid could be responsible for the regulation of Cdx. Treatment of chick and mice embryos with Retinoic Acid has been shown to cause an increase in Cdx1 expression (Houle *et al.*, 2000; Prinos *et al.*, 2001). Production of mutant embryos with functionally inactive Retinoic Acid Response Elements have also been shown to cause reduced levels of CDX1 expression with some similar phenotypes to Cdx1 (-/-) mutants (Houle, Sylvestre and Lohnes, 2003). However, RARγ(-/-) mutants were shown to have no reduction in CDX1 expression and the phenotypes observed appeared to be restricted to the cervical region (Allan *et al.*, 2001). Similarly, in ovo electroporation of dominant negative RARα1 resulted in the loss of HOXB4 expression but had no effect on HOXB9 or posterior HOX (Bel-Vialar, Itasaki and Krumlauf, 2002). This data would suggest that Retinoic Acid can potentially regulate the early expression of Cdx but is more important for the direct regulation of anterior 3' HOX genes. Suggesting that other signals are involved in the regulation of posterior Cdx and Hox expressions.

One such candidate, for the regulation of Cdx, is WNT. During axial extension WNT is expressed within the posterior embryo (Yamaguchi, 2008; Zhao and Duester, 2009; Cunningham, Kumar, *et al.*, 2015). Defects within WNT signalling cause reduced expression of CDX1 in the primitive streak and tailbud and importantly mutants display homeotic transformations across the entire anteroposterior axis similar to those in Cdx1 (-/-) mutants (Ikeya and Takada, 2001; Prinos *et al.*, 2001). Exposure of both Embryonic Stem Cells (ESCs) to WNT and Xenopus animal cap explants to WNT8 is sufficient to cause the induction of Cdx genes (Lickert *et al.*, 2000; Keenan, Sharrard and Isaacs, 2006). Furthermore, there is evidence for the presence of TCF and b-catenin binding sites within the enhancer regions of Cdx1 and Cdx2 (Lickert *et al.*, 2000; Amin *et al.*, 2016a). Within the trunk-residing NMP population, activation of Cdx2 by Wnt signals have been shown to be important to maintain the population of posterior axial progenitors due to the CDX2-T(Brachyury)-WNT

autoregulatory network (Young *et al.*, 2009; Amin *et al.*, 2016a). Whilst the activation of Cdx2, by Wnt signals, has been shown to be important to allow unique chromatin remodelling to permit trunk neural induction (Nordström, Maier, Thomas M. Jessell, *et al.*, 2006; Mazzoni *et al.*, 2013a; Metzis *et al.*, 2018). Together, these findings demonstrate the necessity for WNT signals in the induction and maintenance of Cdx expressions to induce trunk Hox expressions and the induction of posterior neural identity.

FGF is another posterior deriving signal which has been implicated in the regulation of Cdx and hence posterior Hox genes. Initial work in the Xenopus showed that overexpression of eFGF and bFGF resulted in the upregulation and anterior expansion of xcad3, hoxc6, hoxa7 and hoxb9 expression. Expression of a dominant negative FGF-receptor resulted in the reduced expression of xcad3 and hoxa7 (Lamb and Harland, 1995; Pownall *et al.*, 1996). Treatment of animal cap explants with eFGF resulted in the rapid induction of xcad3, hoxa7 and hoxb9. This upregulation of hoxa7 and hoxb9 was lost by the presence of a repressor of xcad3 (Isaacs, Pownall and Slack, 1998). This shows that in the Xenopus FGF induces posterior Hox in the presence of Cdx homologs.
Similar findings within the chick embryo identified that treatment with FGF2/4 caused a rapid anterior expansion of *CDX* genes which was followed by an anterior expansion of HOXB9 within the neural tube (Bel-Vialar, Itasaki and Krumlauf, 2002). *HOXB6*, *HOXB7* and *HOXB8* were also shown to be sensitive to FGF treatment whilst *HOXB1*, *HOXB3*, *HOXB4* and *HOXB5* were not (Bel-Vialar, Itasaki and Krumlauf, 2002). Interestingly HOXB6-9 expanded no further anteriorly than the hindbrain. Similar to the role of WNT signals, FGF signals have also been shown to induce Cdx2 expression which promotes chromatin remodelling to promote the induction of thoracic Hox expression and thoracic spinal cord identity (Mazzoni *et al.*, 2013). These findings also demonstrate that FGF signals are required for the activation of CDX and thoracic HOX expressions to promote a trunk neural identity.

The strongest evidence presented would suggest that WNT and FGF signals, from the posterior of the embryo, work synergistically to initiate and regulate trunk Cdx expression (Keenan, Sharrard and Isaacs, 2006; Young *et al.*, 2009; Peljto *et al.*, 2010a). CDX genes are then responsible for the activation of thoracic HOX genes to promote a thoracic neural identity.

Regulation of lumbosacral identity

Lumbosacral neural identity is largely regulated by the expression of the Hox10-13 paralogs. During development, the formation of lumbosacral-identity neural tissue is derived from a population of Neuromesodermal progenitors which reside in the tail bud (Cambray and Wilson, 2002). Maintenance of this NMP population is dependent upon the presence of Lin28 as forced expression of Lin28 resulted in increased proliferation of tail-bud NMPs and caudal neural expansions (Aires *et al.*, 2019). Termination of axial extension and the conclusion of neural tube formation has been largely associated with the expression of the Hox13 paralogs (Young *et al.*, 2009; Aires *et al.*, 2019). Indeed, premature expression of the Hox13 paralogs within mice embryos resulted in the early termination of axial cessation (Young *et al.*, 2009). Whilst, Hoxb13(-/-) mice mutants were identified to have severely extended neural tubes and spinal structures (Economides, Zeltser and Capecchi, 2003). Recent findings have also shown that Gdf11 (-/-) mice embryos have enhanced Lin28 activity and extended caudal structures similar to Hox13 (-/-) embryos (Aires *et al.*, 2019). Therefore, suggesting that GDF11 signalling is required for the induction of the Hox13 paralogs to repress Lin28 expression and promote the termination of axis extension (Aires *et al.*, 2019).

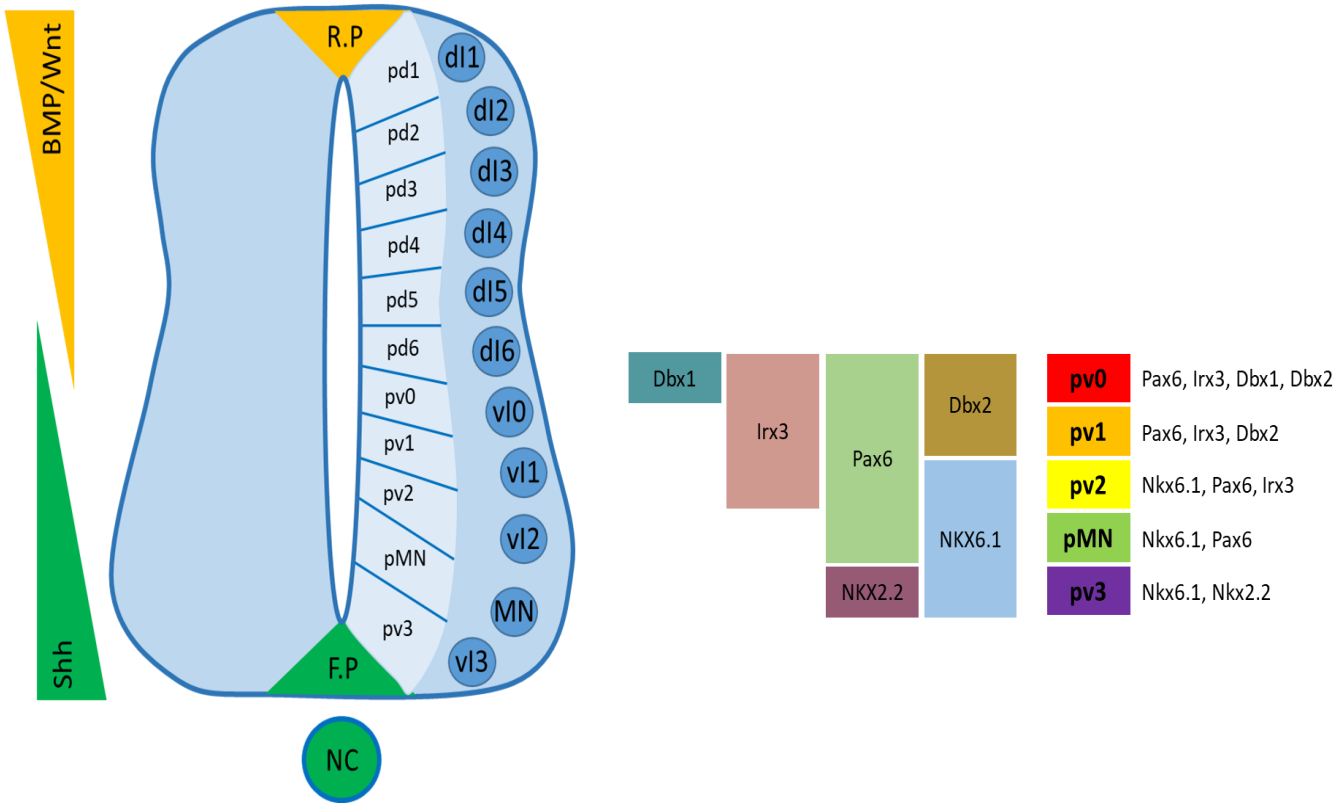
1.3- Patterning of the neural Tube

Dorsoventral patterning of the developing spinal cord

During gastrulation the induction of the anterior neural plate is regulated by a series of TGFβ and Wnt inhibitors, as described in **Section 1**. During neurulation this anterior neuroepithelium undergoes a series of morphological changes to generate the curvature of the neural groove and eventually a fully closed neural tube (reviewed in Nikolopoulou *et al.*, 2017). As the axis extends posteriorly Neuromesodermal Progenitors generate pre-neural progenitors, as described in **Section 1.1**, which form the posterior neuroepithelium at the caudal end of the developing neural tube. After exposure to Retinoic Acid these (NKX1.2+, SOX2+) pre-neural progenitors will become (PAX6+, SOX1+) neural progenitors and contribute to the posterior end of the neural tube (Diez del Corral, Breitkreuz and Storey, 2002; Delfino-Machín, J Simon Lunn, *et al.*, 2005). This process allows a continual source of neural tissue to extend the neural tube posteriorly across the entire anteroposterior (AP) axis. Across the anteroposterior axis, the patterning of the neural tube shows a highly conserved process which is dependent upon two distinct signalling centres. Dorsal emanating BMP/ TGFβ and Wnt signals which pattern the dorsal neural tube (Lee, Dietrich and Jessell, 2000; Millonig, Millen and Hatten, 2000) and Shh from the notochord and floor plate (Placzek *et al.*, 1991; Ericson *et al.*, 1996) which pattern the ventral neural tube are important for the induction and specification of unique neuronal subtypes located across the dorsoventral axis of the neural tube.

Multiple evidence has shown that BMP, Wnt and Shh signals activate specific expression patterns of homeodomain- specific transcription factors across the dorsoventral axis (Ericson, Briscoe, *et al.*, 1997; Timmer, Wang and Niswander, 2002). Each homeodomain transcription factor shows a specific nested domain of expression (Erskine, Patel and Clarke, 1998) (as shown in **Schematic 1.3**). Upon activation, these transcription factors are responsible for activating their own transcriptional network which leads to the formation of distinct neuronal subtypes across the DV axis (Briscoe *et al.*, 1999). The uniform homeodomain expressions result in the induction, from dorsal to ventral (as shown in **Schematic 1.3**), of six dorsal interneuron domain subtypes (dI1, dI2, dI3, dI4, dI5 and dI6), three ventral interneuron subtypes (V0, V1, V2), a motor neuron domain (MN) and a final

fourth ventral interneuron subtype (V3). To maintain their sharp boundaries of expression these homeodomain proteins have been shown to have cross-repressive action upon each other (Briscoe *et al.*, 2000). This allows for the inhibition of ectopic expansion of homeodomain expressions into ectopic adjacent homeodomains and allows for the correct specification of neuronal subtypes across the dorsoventral axis within the correct locations. This is clearly demonstrated during the restriction of the motor neuron progenitor domain in the ventral, pMN, domain of the neural tube. Within the pMN domain, NKX6.1 has been shown to have cross-repressive actions on IRX3 and NKX2.2 which are homeodomain proteins expressed in domains either side of the NKX6.1 expression domain (Briscoe *et al.*, 2000) .



Schematic 1.3. A schematic showing the robust progenitor domains located across the dorsoventral axis of the neural tube (on the left). Of note, motor neurons arise from the pMN domain within the ventral aspect. On the right the schematic shows the unique expression patters of the homeodomain transcription factors, within the ventral progenitor domains of the neural tube, which result in specific patterning of the unique progenitor subtypes.

Patterning of the dorsal neural tube

Patterning of the dorsal neural tube is largely due to Wnt and TGFβ/ BMP signals secreted from the dorsal Roof Plate and the surrounding tissues. The importance of the Roof Plate emanating signals was demonstrated in initial experiments where the ablation of the Roof Plate resulted in a failure to induce dorsal interneuron subtypes (Lee, Dietrich and Jessell, 2000; Millonig, Millen and Hatten, 2000).

Wnts, predominantly Wnt1, Wnt3a and Wnt4, are expressed in overlapping domains within the dorsal midline (or Roof Plate) of the neural tube (Parr *et al.*, 1993; Hollyday, McMahon and McMahon, 1995; Cauthen *et al.*, 2001). Many studies have looked at the importance of Wnt signals in patterning the dorsal neural tube and inducing dorsal phenotypes. Wnt1(-/-);Wnt3a(-/-) mutant mice embryos were shown to have a loss of the dI1 and dI2 dorsal interneuron subtypes (Muroyama *et al.*, 2002). Similarly, the loss of Wnt signals in mice embryos, by a β-catenin loss of function mutation, resulted in the loss of expression of the dorsal neural tube markers Olig3 and Ngn2 (Zechner *et al.*, 2007). Conversely, increased levels of Wnt signals in the mouse embryonic neural tube, by a β-catenin gain of function mutation, resulted in expanded expression of the dorsal neural tube markers Olig3, Ngn2 and Math1 (Zechner *et al.*, 2007). These results display the role of Wnt signalling in patterning the dorsal neural tube.

However, other studies have identified that Wnt signals had no effect upon cell identity patterning within the dorsal neural tube. Chick neural tubes which were electroporated with WNT3 and WNT3a or transfected with dominant active β-catetin displayed no alterations in their ability to obtain dorsal expression domains (Megason and McMahon, 2002; Chesnutt *et al.*, 2004). Instead, it was hypothesised that Wnt may be acting as a mitogen to promote proliferation within the dorsal neural tube. Electroporation of chick neural tubes with WNT1 and WNT3a results in the enhanced levels of BrdU labelled neuronal progenitors and a reduction in the number of mature neurons within the dorsal and ventral neural tube domains (Megason and McMahon, 2002; Chesnutt *et al.*, 2004). Transfection of dominant active β-catenin also resulted in enhanced levels of neural progenitors within chick neural tubes (Megason and McMahon, 2002). Conversely, the transfection of chick neural tubes with the Frizzled8 extracellular domain, to inhibit Wnt activity, resulted in the reduction of BrdU labelled progenitors (Chesnutt *et al.*, 2004). Enhanced Wnt signals were also shown to

enhance the levels of cyclin D1 within the neural tube (Megason and McMahon, 2002). Cyclin D1 is essential for the G1 transition. These results suggest that Wnt signals are likely involved in promoting neural progenitor proliferation and a different signal is required for the patterning of the dorsal neural tube.

The dorsal Roof Plate has been shown to be a rich source of BMP/TGFβ signalling molecules during development. Expression of *BMP4*, *BMP5*, *BMP7* and Activin have all been observed within the Roof Plate (Liem *et al.*, 1995; Liem, Tremml and Jessell, 1997; Lee, Mendelsohn and Jessell, 1998). Fittingly, these TGFβ signals have been shown to have an important role in patterning the dorsal neural tube to cause induction of the dorsal neuronal phenotypes. Treatment of chick neural tube explants with BMP4, BMP7 and Activin also resulted in the induced expression of dorsal neural tube markers such as *PAX3* and *MSX1* and also resulted in the generation of dI1 and dI2 dorsal interneurons (Liem *et al.*, 1995; Liem, Tremml and Jessell, 1997). Using transfection or electroporation to cause the endogenous expression of an active from of the BMP receptor resulted in the expanded expressions of the dorsal genes *PAX7*, *MSX1*, *MSX2* and *DBX2* (Timmer, Wang and Niswander, 2002). Conversely, the conditional knock-out of the Bmpr1a and Bmpr1b receptors in mice neural tubes results in the loss of Math1 expressing dI1 progenitors and a failure to form dI1 interneurons. There is also a failure to generate dI2 interneurons within these mutants (Wine-Lee *et al.*, 2004). These results show the importance in the BMP/TGFβ signals for the patterning of the dorsal neural tube and inducing dorsal interneuron phenotypes.

Taken together the prevailing model for dorsal neural tube patterning and specification is that Wnt signals are required to promote proliferation and expansion of neuronal progenitor populations whilst BMP/TGFβ signals pattern the dorsal neural tube and promote neuronal maturation (Megason and McMahon, 2002; Ille *et al.*, 2007). The strongest evidence to support this was shown as exposure of rat spinal cord-derived neurospheres to Wnt1 promoted proliferation with high levels of Cyclin D1 activation. However, when Wnt1 and BMP2 were exposed to the neurospheres there was a marked reduction in Cyclin D1 levels and increased generation of maturating neurons (Ille *et al.*, 2007).

Shh patterns the ventral neural tube

Initial experiments demonstrated the importance of the notochord and Floor Plate as signalling centres for setting up ventral neural tube identity. Grafting of notochord or Floor Plate to ectopic regions adjacent to the neural tube resulted in ectopic ventralisation within the neighbouring neural tube (van Straaten *et al.*, 1988; Placzek *et al.*, 1991; Pera and Kessel, 1997). *Ex vivo* culturing of neural tube explants alongside Floor Plate and notochord without contact resulted in the induction of ventral neural subtypes (Hynes *et al.*, 1995), suggesting that contact-independent induction of ventral identity is regulated by the secretion of a morphogenetic molecule. Shh is initially expressed within the axial mesoderm, or notochord, which underlies the neural tube ventral midline, or Floor Plate, in which Shh expression is secondly activated (Martí *et al.*, 1995; Roelink *et al.*, 1995). Due to the high levels of expression of SHH within these two structures it was hypothesised that SHH was the signalling factor important for ventralisation.

Multiple studies have shown the importance of SHH signals in inducing ventral identity. Exposure of frog embryos to exogenous Shh resulted in ectopic induction of ventral identity within neural tubes (Roelink *et al.*, 1994). Similarly, ectopic induction of Shh expression within mice dorsal neural tubes resulted in the ectopic induction of ventral identity genes, Nkx2.2 and Nkx6.1, within the dorsal neural tube (Rowitch *et al.*, 1999). Conversely, monoclonal antibodies specific for Shh were able to block the induction of ventral identity within neural explants by the notochord and exogenous Shh (Marti *et al.*, 1995; Ericson *et al.*, 1996). Whilst mutant mice deficient in Shh were unable to form ventral neural tube subtypes (Chiang *et al.*, 1996). Together, these results show the importance of SHH signals in setting up ventral neural tube identity. SHH is believed to exert its patterning effects in a concentration dependent manner with the highest SHH concentrations inducing the most ventral identities and lower concentrations responsible for gradually more dorsal identities. This was neatly displayed within neural tube explants where generation of ventral neuronal subtypes depended on graded SHH concentrations (Ericson, Briscoe, *et al.*, 1997).

SHH is believed to pattern the ventral neural tube by a two-step process, an initial step involves the inhibition of BMP-induced homeodomain (Class I) proteins and a second step involving the activation of Shh-responsive (Class II) proteins (Liem *et al.*, 1995; Ericson *et al.*, 1996; Pierani *et al.*, 1999). Various studies have displayed the ability of Shh in inhibiting the

expression of Class I proteins. *In vivo*, the expression of Msx1 and Pax3 have been shown to initially expand ventrally within the neural tube and then become restricted to their final dorsal expression domains (Liem *et al.*, 1995). Furthermore, dorsal neural tube explants were shown to downregulate and lose expression of Msx1, Pax3, Pax7 and other Class I proteins in response to culturing with notochord and Shh signals (Liem *et al.*, 1995; Ericson *et al.*, 1996; Pierani *et al.*, 1999). Similarly, ectopic grafting of notochord in dorsal regions resulted in the reduction of dorsal gene expression and upregulation of ventral gene expression (Fedtsova and Turner, 1997). The repression of Class I proteins has been shown to allow the activation and expansion of Class II protein expression domains (Price *et al.*, 1992; Ericson, Rashbass, *et al.*, 1997; Qiu *et al.*, 1998). This suggests that the repression of Class I proteins by SHH is sufficient to allow the de-repression of Class II proteins. Class II proteins include NKX2.2, NKX6.1 and OLIG2. As discussed previously, the domain-specific expression of homeodomain proteins (Class I and Class II), maintained by their cross-repressive activity to maintain sharp boundaries of expression, results in transcriptional upregulation of specific genes leading to the formation of distinct ventral neuronal subtypes (Briscoe *et al.*, 1999).

Recently, Notch signals have also been implicated within a role in the dorso-ventral patterning of the neural tube. Inhibition of Notch signals within chick neural explants prevented the induction of floor plate identity (Kong *et al.*, 2015; Stasiulewicz *et al.*, 2015). Whilst, the electroporation of the Notch intracellular domain, the Notch signalling transcriptional activation element, resulted in the ectopic ventralisation of more dorsal neural progenitors with no alteration in Shh expression levels (Stasiulewicz *et al.*, 2015). Importantly, it has been shown that treatment of cells with Notch intracellular domain or *cHairy2*, a Notch target, resulted in the increased levels of Smoothened within primary cilia. Alternatively, treatment with Notch inhibitors resulted in the reduced clearance of Ptch1 from the primary cilia (Kong *et al.*, 2015; Stasiulewicz *et al.*, 2015). These findings suggest an early role for Notch signalling in modulating the responsiveness of neural progenitors to Shh signals to allow induction of ventral identity.

1.4- Motor Neuron Specification

Transcriptional Specification of Motor Neurons

Motor neurons arise from a progenitor domain, the pMN domain (as shown in **Schematic 1.3**), located within the ventral neural tube. The early motor neuron progenitor domain is marked by the expression of the homeodomain proteins; PAX6, NKX6.1 and OLIG2 (Tanabe, William and Jessell, 1998; Novitch, Chen and Jessell, 2001; Vallstedt *et al.*, 2001). Ventrally they are bordered by the V3 domain, which is marked by the expression of Nkx2.2 and Nkx6.1, and dorsally by the V2 domain, in which progenitors express Pax6, Nkx6.1 and Irx3 (Ericson, Rashbass, *et al.*, 1997). Cross-repressive actions between PAX6 and NKX2.2, at the ventral boundary, and OLIG2 and IRX3, at the dorsal boundary, maintain the sharp boundaries of homeodomain protein expression (Ericson, Rashbass, *et al.*, 1997; Briscoe *et al.*, 2000; Chen *et al.*, 2011). These cross-repressive activities are essential as the loss of Olig2 expression has been shown to allow ventrally expanded Irx3 expression and the generation of V2 interneurons at the expense of motor neurons (Zhou and Anderson, 2002).

The MN progenitor domain is the location of a common progenitor which is responsible for the generation of both motor neurons and oligodendrocytes. The expression of Pax6, Nkx6.1 and Olig2 have been shown to be responsible for upregulating specific transcriptional networks which promote the specification to either motor neuron or oligodendrocyte identity (Sugimori *et al.*, 2007). PAX6 and OLIG2 have been shown to be responsible for the upregulation of Neurogenin2 (Ngn2) to promote motor neuron identity (Novitch, Chen and Jessell, 2001; Sugimori *et al.*, 2007). This was demonstrated as the ectopic expression of Olig2 resulted in the ectopic upregulation of Ngn2 within dorsal neural tube locations (Novitch, Chen and Jessell, 2001). Similarly, Pax6 mutant mice showed a severe downregulation of both Olig2 and Ngn2 and a failure to form motor neurons (Mizuguchi *et al.*, 2001). These findings suggested that co-expression of Olig2 and Ngn2 is important for the promotion of motor neuron identity over oligodendrocytes. This is heavily supported as Ngn2 has been shown to be upstream of multiple motor neuron maturity genes including Hb9/Mnx1, Islet1 and Lhx3 (Novitch, Chen and Jessell, 2001). Further supporting evidence showed that *in vivo* Olig2 and Ngn2 expressing progenitors give rise to mature motor neurons. However, progenitors which express Olig2 alone, in the absence of Ngn2, showed

upregulation of Mash1 and were specified to an oligodendrocyte identity (Mizuguchi *et al.*, 2001; Novitch, Chen and Jessell, 2001; Scardigli *et al.*, 2001; Lu *et al.*, 2002).

As motor neurons become more mature they upregulate genes such as Islet1 and Hb9/Mnx1 which are markers of a post-mitotic identity. Hb9/Mnx1 has been shown to be a downstream target of Olig2 and Ngn2 (Tanabe, William and Jessell, 1998; Lee *et al.*, 2009). However, it has also been shown to induced through Shh and Retinoic Acid signals (Tanabe, William and Jessell, 1998). Similarly, the LIM homeodomain transcription factor, Islet1, has also been shown to be SHH responsive (Thor *et al.*, 1991; Yamada *et al.*, 1991). Upon activation, ISL1 promotes motor neuron identity by forming a complex with LHX3 (Thaler *et al.*, 2002; Song *et al.*, 2009). ChiP-seq has shown that the ISL1-LHX3 complex is essential for the activation of genes responsible for the cholinergic neurotransmission ability of motor neurons (Cho *et al.*, 2014). Reflective of its role in motor neuron induction the loss of Islet1 expression within mice mutants resulted in the failure to generate motor neurons (Song *et al.*, 2009).

Columnar identities of Motor Neurons

Across the anteroposterior axis of the spinal cord motor neurons are arranged within orderly columnar locations, as summarised in **Table 1.1**. The specific columns display unique expression profiles whilst the neurons within these columns share a common function and project axons within a similar manner. At all axial levels there is presence of the Median Motor Column (MMC). MMC motor neurons are located within the most medial and ventral domains of the spinal cord and project axons for the innervation of the axial muscles (Gutman, Ajmera and Hollyday, 1993; Tsuchida *et al.*, 1994). MMC motor neurons are marked by the co-expression of Hb9/Mnx1, Islet1/2 and the continued post-mitotic expression of Lhx3 (Tsuchida *et al.*, 1994; Sharma *et al.*, 1998). Fittingly, the forced expression of Lhx3 within other columnar identity motor neurons is efficient to allow the conversion to a MMC identity (Sharma *et al.*, 1998). The persistent expression of Lhx3 is believed to make MMC motor neurons unresponsive to Hox patterning which is reflective of their uniform presence across all axial levels (Dasen *et al.*, 2005a, 2008; Jung *et al.*, 2010).

The motor neurons of the Lateral Motor Column (LMC) are important for the innervation of the muscles of the limbs and are therefore only present at brachial levels, for the innervation of the forelimbs, and lumbosacral levels, for innervation of the hindlimbs (Hollyday, Hamburger and Farris, 1977; Hollyday and Jacobson, 1990). LMC motor neurons arise from the most lateral regions within the ventral spinal cord (Bueker, 1944) and extend axons into the limbs (Landmesser, 1978; Tosney, Hotary and Lance-Jones, 1995; Matise and Lance Jones, 1996). Retinoic Acid signals from the paraxial mesoderm have been shown to be essential for the induction of LMC motor neurons within the ventral spinal cord. Upon exposure to Retinoic Acid early Islet1/2 expressing LMC motor neurons upregulate the expression of Raldh2 (Ensini *et al.*, 1998; Sockanathan and Jessell, 1998). For these reasons LMC motor neurons can be marked by the co-expression of Islet1, Raldh2 and Foxp1.

Within the thoracic regions of the spinal cord motor neurons have been shown to contribute to two thoracic-specific motor neuron columns. These are the Preganglionic Motor Column (PGC) and the Hypaxial Motor Column (HMC). Both of these motor neuron columnar subtypes arise from the same Islet1/2 expressing motor neuron precursors. However, the continued expression of Islet2 in mature motor neurons results in the generation of HMC motor neurons whilst the continuation of Islet1 expression results in PGC motor neuron formation (Thaler *et al.*, 2004). Motor neurons of the Preganglionic Motor Column innervate the sympathetic ganglia to allow innervation of the autonomic nervous system. Molecularly they are marked by the expression of Islet1, SMAD1, nNOS and low levels of Foxp1 (Dasen, Liu and Jessell, 2003a; Dasen *et al.*, 2008; Rousso *et al.*, 2008). Hypaxial Motor Column motor neurons arise from a more lateral location within the ventral spinal cord compared to the MMC and PGC motor neurons found at the same thoracic levels. HMC motor neurons are important for the innervation of the ventral axial musculature such as the abdominal and intercostal muscles (Prasad and Hollyday, 1991; Tsuchida *et al.*, 1994). The expression of Hb9/Mnx1, Islet1/2 and ETV1 are molecular markers of a HMC motor neuron identity.

Motor Column	Axial Location	Innervation	Unique Markers
Medial Motor Column (MMC)	All axial levels	Axial Muscles	HB9/MNX1, ISL1, ISL2, LHX3, HOX negative
Spinal Accessory Column (SAC)	Hindbrain-Cervical	Muscles of the neck and mastoid muscles	ISL1, RUNX1, PHOX2B
Phrenic Motor Column (PMC)	Cervical	Diaphragm	HB9/MNX1, ISL1, ISL2, HOX5, POU3F1, SCIP
Lateral Motor Column (LMC)	Cervical/Brachial (forelimb) Lumbar (Hindlimb)	Muscles of the limbs	ISL2, FOXP1, RALDH2
Preganglionic Motor Column (PGC)	Thoracic-Lumbar	Sympathetic Ganglia	ISL1, SMAD1, nNOS, SIP1, $FOXP1^{low}$
Hypaxial Motor Column (HMC)	Thoracic	Ventral Intercostal and Abdominal Muscles	HB9/MNX1, ISL1, ISL2, ETV1

Table 1.1. A table summarising the different motor neuron columnar identities found across the anteroposterior axis and the unique expression patterns which they express.

Positional Specification of Motor Neurons

Multiple studies have shown that reversal of spinal cord orientations or transplantation of spinal cord regions to ectopic locations at early stages of development can still result in motor neurons innervating the correct target muscles (Lance-Jones and Landmesser, 1980; Lance-Jones and Landmesser, 1981). Whilst this has been used as an argument for diffusible chemoattractant secretion by neuronal targets it can also be seen as the earliest evidence to suggest that motor neurons are patterned early to acquire a specific positional identity. This acquisition of a unique positional identity is important to determine many characteristics including columnar identity. Assignment of motor neurons within distinct columnar organisation during development allows for uniform and correct innervation and functionality of the diverse targets dispersed across the AP axis. Patterning of these differing neuronal subtypes, to generate correct columnar organisation, is therefore a vital process within motor neuron specification. As described previously, in **Section 1.2**, Hox genes play a vital role in patterning the AP axis and determining positional specific identity. Multiple studies have suggested that Hox genes act in a similar manner to confer positional identity and functionality in motor neurons. Axial level-dependent Hox expression can be observed within motor neurons with more posteriorly located motor neurons expressing successively more 5' located Hox genes. At brachial levels motor neurons express Hoxc6, at thoracic levels Hoxc9 expression is observed whilst in lumbosacral motor neurons there is expression of Hoxd10 (Carpenter EM *et al.*, 1997; Dasen, Liu and Jessell, 2003a; Jung *et al.*, 2014). Hox expression patterns clearly delineate motor neurons into separate AP domains. Furthermore, these Hox expressions were also shown to be specific to the different motor columns which originate at the different axial levels. Hoxc6 was shown to clearly coincide with Raldh2 expressing motor neurons of the brachial division of the Lateral motor column whilst Hoxc9 expression overlapped with motor neurons of the pre-ganglionic motor column and Hoxd10 was co-expressed with Raldh2 in lumbosacral identity Lateral motor column neurons (Dasen, Liu and Jessell, 2003a; Shah, Drill and Lance-Jones, 2004). This shows that segmental Hox expressions form part of the identity of differing axial level motor columns.

Further experiments then wished to explore if axial-level Hox expression was required and essential for determining the motor neuron columnar identity. Supporting of the instructive role of Hox genes, the forced expression of Hoxc9 within brachial motor neurons resulted in the downregulation of Hoxc6 and a switch to thoracic pre-ganglionic motor column identity (Dasen, Liu and Jessell, 2003a; Jung *et al.*, 2010). Reciprocal forced expression of Hoxc6 within thoracic motor neurons resulted in the loss of Hoxc9 expression and a conversion to a brachial HOXC6+ RALDH2+ lateral motor column identity (Dasen, Liu and Jessell, 2003a). This finding was similar to that of Hoxc9 mutant mice, which displayed a loss of thoracic motor neurons and an expansion in HOXC6+ RALDH2+ lateral motor column brachial identity motor neurons (Jung *et al.*, 2014). Supporting the role of Hoxc6 and Hoxc9 in specifying brachial and thoracic motor neurons, the knock-out of HoxC cluster genes resulted in the failure to form brachial and thoracic motor neurons whilst lumbosacral lateral motor column neurons, which are regulated largely by Hoxd10, are still formed (Jung *et al.*, 2014). *In ovo* electroporation of chick thoracic HOXC8+ spinal cords with Hoxd10 resulted in the ectopic transformation of thoracic motor neurons to a lumbosacral lateral motor column RALDH2+HOXD10+ identity (Shah, Drill and Lance-Jones, 2004). This demonstrates the dependency of lumbosacral motor neurons of the lateral motor column upon Hoxd10 expression. Interestingly, in snakes the absence of limbs is reflected by an absence of Hoxc6 expressing motor neurons and a vast amount of Hoxc9 expressing motor neurons (Jung *et al.*, 2014). Together these findings demonstrate the importance of axial level dependent Hox gene expression interacting with the motor neuron differentiation profile to confer a specific positional and functional identity during development.

Multiple studies have aimed to elucidate the divergent molecular profiles which the different Hox genes activate to dictate the specific motor neuron identities. For example, in the Lateral Motor Column (LMC) early extrinsic FGF8 signals act to pattern Hox gene expression (Dasen *et al.*, 2005a). LMC neurons located most rostrally acquire a Hoxc5 expression whilst those neurons located most caudally have an induced expression of Hoxc8 (Dasen *et al.*, 2005a). These Hox genes have been shown to then activate a specific transcriptome. Hoxc5 activates the Runx transcription factors whilst Hoxc8 activates Pea3. Similarly, it was shown that the expression of Hoxc6 is essential for the activation of Foxp1 to allow brachial LMC motor neuron specification. Electroporation of the Engrailed-

repressor of Hoxc6 (which inhibits Hoxc6 expression) resulted in a loss of Foxp1 and Raldh2 expression and a failure to form LMC brachial motor neurons (Dasen *et al.*, 2008). The findings demonstrate the requirement of Hox genes to allow intrinsic transcriptomic specification of the unique motor pools and branches of the LMC neurons to determine muscle innervation and maturation (Dasen *et al.*, 2005a, 2008).

1.5- *In Vitro* Generation of Motor Neurons

Existing protocols for the in vitro generation of anterior Motor Neurons

The generation of Pluripotent Stem Cell (PSCs) lines has allowed multiple developments within the field of regenerative and developmental biology. PSCs is a term used to encompass both Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSC). ESCs are derived by explanting the inner cell mass of pre-implantation blastocysts (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998; Reubinoff *et al.*, 2000)(Evans and Kaufman, 1981; Thomson *et al.*, 1998) whilst iPSCs are derived through the transfection of terminally differentiated cells with a set of transcription factors to induce a state of pluripotency (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). In theory PSCs have the ability to continuously self-renew and be differentiated in culture to generate any cell type of the body. These properties have resulted in multiple studies aiming to devise protocols for the differentiation of PSCs to generate motor neurons *in vitro*, as summarised in **Figure 1.1**.

Early protocols for the *in vitro* generation of motor neurons from PSCs relied on an initial TGFβ inhibition step to promote a rapid induction of an anterior neural state. In doing so, exposure to TGFβ inhibitors was shown to induce a very high yield, >80% of cells in culture, of PAX6+, SOX1+ neural progenitors (Smith *et al.*, 2008; Chambers *et al.*, 2009a; Zhou *et al.*, 2010). Upon generation of a stable neural identity the next steps within many existing protocols involves a period of exposure to Retinoic Acid and Sonic Hedgehog (Shh) signals (H Wichterle *et al.*, 2002; Hu and Zhang, 2009; M. W. Amoroso *et al.*, 2013). Presence of Retinoic Acid is essential to promote neural/spinal cord identity (Tonge and Andrews, 2010) whilst the presence of Shh signals are important for promoting a ventral spinal cord identity (H Wichterle *et al.*, 2002; Hu and Zhang, 2009; M. W. Amoroso *et al.*, 2013). Successive downstream differentiation of a motor neuron progenitor identity resulted in the generation of Islet1+, Hb9/Mnx1+ motor neurons (H Wichterle *et al.*, 2002; Lee *et al.*, 2007; Li *et al.*, 2008; Hu and Zhang, 2009; M. W. Amoroso *et al.*, 2013; Du *et al.*, 2015). The presence of neurotrophins within later stages of the differentiation protocol have been shown to be important to allow motor neuron maturation (M. W. Amoroso *et al.*, 2013).

Whilst the presence of NOTCH inhibition during motor neuron maturation was also identified to be important to enhance levels of post-mitotic motor neurons (Du *et al.*, 2015; Maury *et al.*, 2015).

Interestingly, Notch inhibition has also been implicated within

As discussed previously, in **Section 1.1**, the activation-transformation model of neural induction is dependent on an initial induction of an anterior neuroepithelium which is transformed to more posterior identities by the exposure to a caudalising signal; Retinoic Acid. However, under this model there is a failure to induce neural identities which lie posterior to cervical domains which is likely reflective of their different developmental origins *in vivo* (Tzouanacou *et al.*, 2009a). Many of the existing *in vitro* protocols for PSC-derived motor neuron induction reflect the activation-transformation model. An initial neural induction by TGFβ inhibitors generates an Otx2+ anterior neural state with the exposure to Retinoic Acid in order to promote more posterior identities (Lee *et al.*, 2007; Li *et al.*, 2008). The exposure to Retinoic Acid within these protocols is efficient to generate more posterior motor neuron identities, marked by the expression of cervical/brachial Hox expression (HOX1-5 paralogs) (H Wichterle *et al.*, 2002; Lee *et al.*, 2007; Li *et al.*, 2008; M. W. Amoroso *et al.*, 2013). However, there is a general failure to generate motor neurons which express thoracic or lumbosacral Hox (HOX6-13 paralogs).

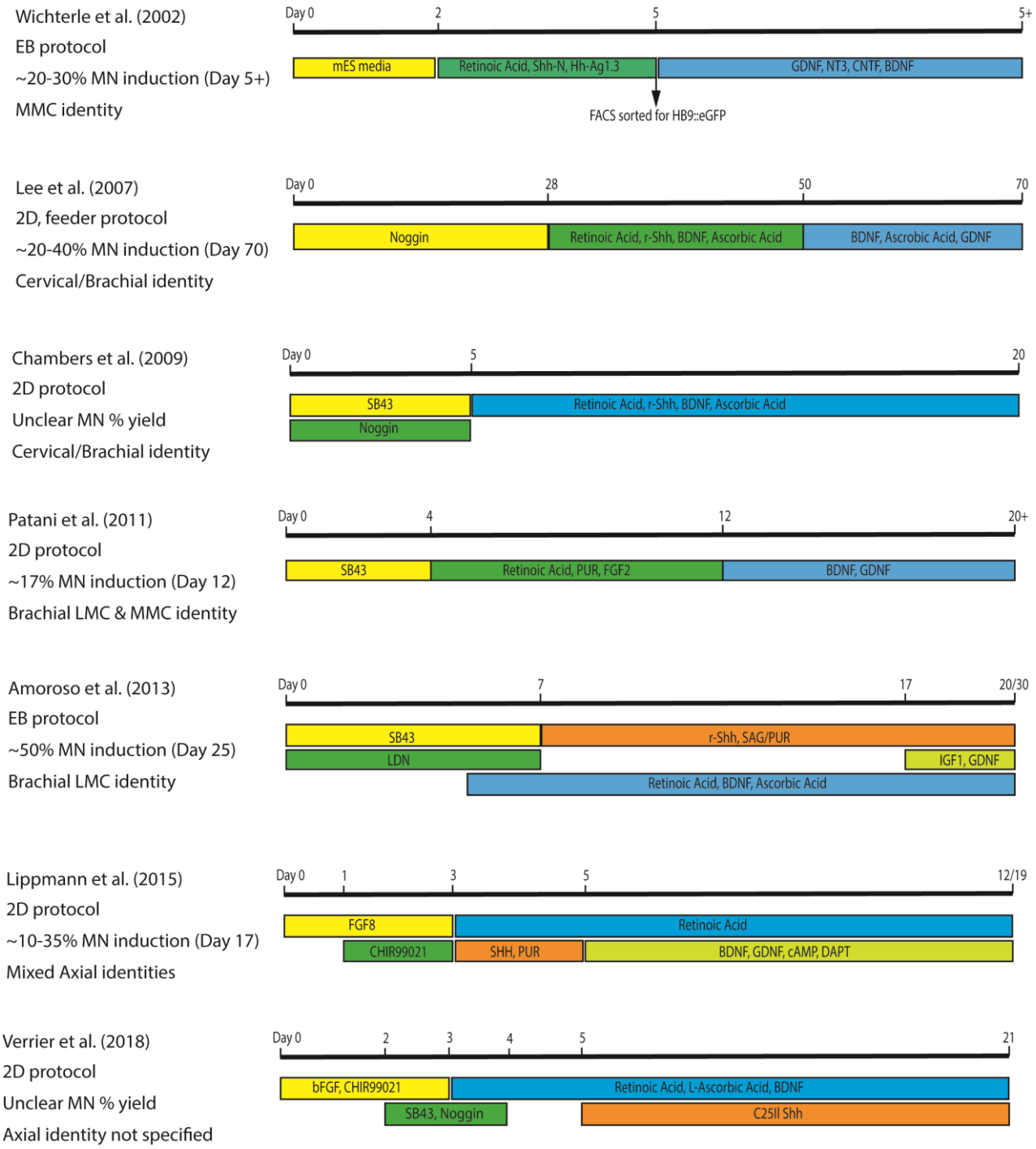


Figure 1.1. An overview of current existing differentiation protocols for the PSC-generation of motor neurons.

In vitro generation of posterior identity motor neurons

It is now understood that, *in vivo,* posterior neural tissue is derived from a divergent source, via Neuromesodermal Progenitors (NMPs), from that of anterior neural tissue (Tzouanacou *et al.*, 2009a). Therefore, multiple studies have focused on optimising a protocol for the generation of PSC-derived NMPs *in vitro* which could be further differentiated to generate posterior identity motor neurons.

Recapitulating the known signals which are required to induce NMPs *in vivo,* as detailed in **Section 1.1**, many groups have optimised the conditions for the differentiation of NMP-like axial progenitors from mouse and human PSCs (D. A. Turner *et al.*, 2014; Gouti *et al.*, 2014a; Tsakiridis *et al.*, 2014; Lippmann *et al.*, 2015a). Culturing mouse Epiblast stem cells (EpisCs) and mouse ESCs in the presence of FGF2 and CHIRON99021 (a Wnt signalling agonist) resulted in the generation of NMP-like identity at day 3 (David A Turner *et al.*, 2014; Gouti *et al.*, 2014; Tsakiridis *et al.*, 2014). Similarly, exposing human ESCs to a short period of Wnt and Fgf signals resulted in high yields of SOX2+/T(Brachyury)+ cell populations after 72 hours of culturing (Gouti *et al.*, 2014; Lippmann *et al.*, 2015a).

NMPs are a posterior axial progenitor with the ability to generate both neural, neural crest and mesodermal structures (Tzouanacou *et al.*, 2009a; Frith *et al.*, 2018a). Therefore, multiple studies have analysed the culturing conditions which best promote specific fate choices downstream of the PSC-derived Neuromesodermal progenitor state. Reflecting the findings from *in vivo* studies, culturing of PSC-derived (SOX2+/T(Brachyury)+) NMPs under the presence of Wnt agonists results in the generation of mesoderm which is marked by the mesodermal markers T(Brachyury), Tbx6 and Mesogenin1 (D. A. Turner *et al.*, 2014; Gouti *et al.*, 2014a; Tsakiridis and Wilson, 2015; Edri *et al.*, 2019; Diaz-Cuadros *et al.*, 2020). However, within the current literature there is a large disparity between the culturing conditions used to promote a neural identity downstream of PSC-derived Neuromesodermal progenitors. It has been suggested that continued culturing of PSC-derived NMPs with Fgf and Wnt signals results in the generation of a SOX2+/T(Brachyury)- neural biased population (Tsakiridis and Wilson, 2015). However, others have noted that continued culturing of PSC-derived NMPs within Fgf and Wnt signals resulted in a maintenance of an NMP identity (D. A. Turner *et al.*, 2014). A commonly agreed upon finding is that culturing PSC-derived NMPs

in Fgf signals in the absence of Wnt results in enhanced neural induction (D. A. Turner *et al.*, 2014; Tsakiridis and Wilson, 2015). The presence of Retinoic Acid within culturing conditions was also shown to be required to allow efficient neural induction from a PSC-derived NMP state (Gouti *et al.*, 2014a; Lippmann *et al.*, 2015b; Edri *et al.*, 2019; Rayon *et al.*, 2019; Diaz-Cuadros *et al.*, 2020). Whilst, in some cases the presence of TGFβ family antagonists were also reported to enhance neuronal induction (D. A. Turner *et al.*, 2014; Kumamaru *et al.*, 2018; Verrier, Davidson, Gierli, *et al.*, 2018). Together, these findings have suggested potential signalling molecules required to promote a neural identity downstream of a PSC-derived Neuromesodermal progenitors. However, there is still a requirement to fully explore the contributions of each signalling pathway and determine the optimum conditions required to promote neural conversion.

Multiple studies have attempted to further differentiate neural derived progenitors, obtained from a PSC-derived Neuromesodermal progenitor intermediate state, into motor neurons. Initial experiments showed that continued differentiation of mouse PSC derived NMPs, after Day 3 of differentiation, in the presence of Retinoic Acid, to promote neuralisation, and a Shh agonist (SAG), to promote ventralisation, resulted in the generation of an OLIG2 expressing motor neuron progenitor population which maintained HOXC9 thoracic identity (Gouti *et al.*, 2014c). More recently, work in human ESCs have suggested a role for the presence of TGF-β and BMP inhibition, alongside Retinoic Acid, to promote neural identity downstream of a NMP state which can be further differentiated to generate *in vitro*-derived posterior motor neurons (Kumamaru *et al.*, 2018; Verrier, Davidson, Gierlinśki, *et al.*, 2018). Downstream differentiation of a hESC-derived NMP state in the presence of TGF- β and BMP inhibition were shown to inhibit mesodermal and endodermal induction whilst FGF and Wnt signals are able to promote thoracic SOX2+ neural progenitor identity by day 10 of differentiation (Kumamaru *et al.*, 2018). Continued differentiation of these progenitors to day 20 of differentiation, in the presence of Wnt, TGF-b inhibition and Shh agonists, resulted in OLIG2 and Nkx6.1 induction within these cultures, albeit at low levels of expression (less than 25% of cells express Nkx6.1) (Kumamaru *et al.*, 2018). Furthermore, a separate group has shown that presence of dual SMAD inhibition alone, by SB43 and Noggin, had no impact on neural induction however dual SMAD inhibition alongside Retinoic Acid enhanced neural induction (Verrier, Davidson, Gierlin, *et al.*, 2018).

Extended differentiation, in the presence of Shh agonists and neurotrophins resulted in the generation of ISLET1/HB9 motor neurons at Day 21 (Verrier, Davidson, Gierlin, *et al.*, 2018). Whilst these protocols have clearly demonstrated that optimum *in vitro*-neural induction is best achieved in the presence of Retinoic Acid and dual SMAD inhibition the downstream yields of posterior motor neurons are still low. Therefore, there is a requirement to optimise these conditions for the generation of thoracic identity motor neurons downstream of an NMP intermediate state. Furthermore, there is a requirement to fully characterise the motor neurons derived from these protocols to confirm character and positional identity.

Existing protocols, for the generation of posterior motor neurons, often include the removal of both FGF and Wnt signals upon the generation of an NMP identity at day 3 of differentiation (Lippmann *et al.*, 2015a; Verrier, Davidson, Gierlinśki, *et al.*, 2018). It has even been argued that the application of Retinoic Acid is able to fix axial HOX identity and is efficient to generate thoracic motor neurons downstream of a thoracic neurectoderm (Lippmann *et al.*, 2015a). However, within their protocols Lippmann et al (2015) predominantly produced HOXC6+/HOXC9- motor neurons and fail to generate high levels of HOXC9+ motor neurons (Lippmann *et al.*, 2015a; Estevez-silva *et al.*, 2018a) whilst Verrier et al (2018) do not characterise the axial identity of their derived motor neurons. These protocols have been dependent upon 2D cultures however existing protocols have also aimed to generate thoracic identity motor neurons through 3D approaches (Faustino Martins *et al.*, 2020; Mouilleau *et al.*, 2020). hESCs cultured as Embryoid Bodies (EBs) can generate NMP-like populations and further downstream exposure to Retinoic Acid and Sonic Hedgehog agonism results in the generation of motor neurons (Faustino Martins *et al.*, 2020; Mouilleau *et al.*, 2020). Interestingly, the presence of FGF inhibitors after an NMP intermediate state prevented the formation of thoracic motor neuron identities and promotion of brachial identity (Mouilleau *et al.*, 2020). Conversely, the supplementation of FGF resulted in the promotion of thoracic motor neuron identity (Mouilleau *et al.*, 2020). Taken together, these findings from both 2D and 3D culturing systems would suggest that the presence of FGF signals after an NMP state are required to promote posterior motor neuron populations. However, this needs further exploration in 2D differentiation protocols where cells can be exposed to consistent levels of FGF signals.

1.5- Plasticity of positional identity

Many studies have utilised transplantation studies as a means to observe the behaviours of *in vitro* derived cell types. Engrafting of PSC-derived motor neurons into a host spinal cord allows observations as to whether the *in vitro* motor neurons are able to respond to environmental cues and function characteristically. Multiple studies have utilised the chick embryo spinal cord within transplantation studies (H Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006a; Peljto *et al.*, 2010b; Son *et al.*, 2011). Grafting pure populations of mouse PSC-derived Hb9+/Mnx1+ postmitotic motor neurons into the chick developing spinal cord resulted in some contribution to the ventral spinal cord with stereotypical ventral root axonal projections (Son *et al.*, 2011). Other studies have grafted mixed populations of mice PSC-derived motor neuron progenitors and postmitotic motorneurons into HH15-17 stage chick embryos. These studies resulted in large contributions of grafted cells to the ventral regions of the chick spinal cord after culturing to later stages (H Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006a; Peljto *et al.*, 2010b). Engrafted motor neuron progenitors were also shown to mature and differentiate in vivo and resulted in a large number of Hb9/Mnx1 expressing motor neurons which projected axons in a similar manner to the endogenous chick motor neurons (H Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006a). More recent experiments have shown that human PSC-derived motor neurons, with a Foxp1+ LMC identity, have the ability to integrate within the ventral neural tube and project axons when grafted into HH Stage 15-18 chick embryos (M. W. Amoroso *et al.*, 2013). Interestingly, when the grafted mice PSC-derived cells integrated incorrectly into the dorsal neural tube it would appear that axons were still capable of responding to chemoattractive signals to allow synapse formation with the correct targets (Soundararajan *et al.*, 2006a). Alternatively to the grafting of PSC-derived motor neurons into developing embryos, it was shown that the engraftment of mouse PSC-derived motor neurons into adult mice forelimbs resulted in axonal projections and muscle innervation (Yohn *et al.*, 2008).

For cell-replacement purposes, it is important to validate if positional identity obtained by PSC-derived motor neurons *in vitro* remains fixed upon grafting and determines behaviour upon grafting or if environmental cues can alter positional identity and functionality. Most of the current transplantation studies have involved placing PSC-derived motor neurons within a positional matched spinal cord location. The early chick grafting experiments done

by Wichterle *et al.*, (2002) involved the grafting of mouse PSC-derived MMC identity motor neurons marked by the expression of Hb9/Mnx1, Islet1 and Lhx3. MMC motor neurons are present at all axial levels which is reflective of their ability to integrate within brachial, thoracic and lumbosacral chick regions upon transplantation (H Wichterle *et al.*, 2002). This was further supported by experiments conducted by Soundararajan *et al.*, (2006) and Son *et al.*, (2011) who showed that grafting of mouse PSC-derived MMC identity motor neurons integrated within the chick lumbosacral spinal cord. Whilst grafting of brachial identity LMC motor neurons derived from hESCs at the level of the chick forelimb, a region defined as brachial identity, (somites 15/20) resulted in ventral integration and stereotypical axonal projections towards limb targets (M. W. Amoroso *et al.*, 2013). Taken together, these results show that PSC-derived motor neurons integrate well within a host if they are transplanted into a region which has a matched positional identity.

Very few studies have examined the effects of grafting PSC-derived motor neurons into opposing positional identity regions of the spinal cord. Peljto *et al.*, (2010) demonstrated that brachial identity Foxp1+ mouse PSC-derived motor neurons could integrate into ectopic thoracic regions of HH15-17 chick spinal cords. However, these neurons integrated within a lateral domain of the ventral spinal cord, characteristic of their LMC identity, and projected axons towards the limb (Peljto *et al.*, 2010b). Thoracic level motor neurons do not normally contribute to limb innervation which suggests that the Foxp1+ LMC motor neurons retained their identity upon grafting and did not obtain a thoracic identity. Within these studies the motor neurons are grafted at a postmitotic state which is likely less amenable to alterations in positional identity upon grafting. Therefore, future experiments should look at grafting earlier progenitor stages to observe the effects of environmental cues on positional identity.

The plasticity of positional identity has been largely explored for other cell lineages and through manipulations of *in vivo* tissue. For example, early work with neural crest identified that transplantation of chick neural crest from the level of the adrenomedullar into the quail at the level of the endogenous vagal neural crest (or vice versa) resulted in the cells altering their behaviours and generating neural crest derivatives reflective of their position of implantation (Le Douarin and Teillet, 1974; Le Douarin *et al.*, 1975). This suggests that within

the neural crest there is a degree of plasticity when it comes to positional identity. However, this could also be due to the neural crest being of an early developmental stage upon grafting as similar grafting with later stage neural crest derivatives resulted in lower levels of plasticity (Le Douarin and Dupin, 2018). Therefore, suggesting that at later developmental time-points positional identity becomes fixed. Reflective of this, reversing the location of the lumbosacral region of the neural tube at HH stage 15 resulted in a matched reversal of motor neuron projections to the muscles of the limb (Lance-Jones and Landmesser, 1980). However, reversal of the same lumbosacral region prior to HH Stage 15 results in no differences in motor neuron projections (Matise and Lance Jones, 1996). This suggests that at later developmental time points positional identity becomes fixed. Interestingly, the implantation of hPSC-derived neural progenitors of a forebrain identity were shown to be incapable of causing spinal cord regeneration when implanted into a cervical lesion site within rat spinal cords. Conversely, the transplantation of cervical hPSC-derived neural progenitors into the same location resulted in regeneration across the lesion site (Kumamaru *et al.*, 2018). These results would suggest that, after a certain point in development, to allow correct integration and functionality there is a requirement for transplanted tissue and the site of engraftment to have the same positional identity. Furthermore, it also suggests that at later developmental stages there is a loss of developmental and patterning cues which prevent the ability of the environment to "reprogram" positional identity of the engrafted cells which will result in ectopic behaviours or poor integration.

1.6- Summary and Global Experimental Aims

The elucidation of *in vitro* hPSC protocols for the generation of motor neurons is of great potential for regenerative medicine and disease-modelling. Current protocols predominantly generate motor neurons of a hindbrain/cervical and brachial identity but fail to generate high yields of clearly-defined thoracic identity motor neurons. Therefore, within my experiments I aimed to:

1) Define the optimal signal combination promoting the specification of posterior ventral neurectoderm and motor neurons from hPSC-derived NMPs
2) Compare the *in vivo* behaviours and positional plasticity of anterior and posterior identity hPSC-derived motor neuron progenitors following chick embryo grafting.

Chapter 2 Materials and Methods

2.1 hPSC Cell Culture

hPSCs were cultured on Laminin-521 (Biolamina #LN521, Lot:80119) coated 12.5cm^2 tissue culture flasks (UltraCruz #sc-200261). Cells were grown in Essential8 medium (made in house; see **Table 2.1**) at 37°C with humidified 5% CO_2 until they reached 70-80% confluency.

For passaging, the media was aspirated and the cells were quickly washed with PBS. 2ml of EDTA (Invitrogen #15575-038) was applied for 5 minutes at 37°C. EDTA was then aspirated and 1ml of Essential8 media was applied to lift the cells into small clumps. Cells were then seeded at densities from 1:3 to 1:8.

Essential8 Culture Media

Essential E8 media was made in house by generating a 50x concentrated solution which was frozen in 10ml aliquots at -20°C. For use in tissue culture the aliquots were thawed and diluted 1:100 with DMEM F-12 (Sigma #D6421) resulting in the following concentrations below;

Component	Final Concentration/ Volume per 1litre	Supplier
DMEM/F12	344ml	Sigma #D6421
L-ascorbic acid	64mg/l	Sigma #A8960
Sodium selenium	14µg/l	Sigma #S5261
Insulin	19.4mg/l	Thermo Fisher #A11382II
NAHCO3	543mg/l	Sigma #S5761
holo-transferrin	10.7mg/l	Sigma #T0665
Glutamax	5ml	Thermo Fisher #35050038
FGF2	100µg/l	Peprotech #100-18B
TGFB1	2µg/l	Peprotech #100-21

Table 2.1. A summary of the components used to make up 1 litre of Essential 8 medium used for hPSC tissue culture.

Cell Thawing

hPSCs were removed from liquid nitrogen or -80°C and quickly thawed by re-suspending in Essential8 medium. Centrifugation at 1300rpm for 3 minutes resulted in a pellet which was re-suspended in 2ml of Essential8 medium supplemented with 10µM Y-27632 2HCl (Adooq Biosciences #A11001). This suspension was placed into previously prepared Laminin-521 coated 12.5cm^2 tissue culture flasks at 37°C.

Cell Freezing

hPSCs with a confluency of 60-70% had media removed and were quickly washed with PBS. 2ml of EDTA (Invitrogen #15575-038) was applied for 5 minutes at 37°C. Essential8 medium was then used to dissociate the cells into small clumps which were then centrifuged at 1300rpm for 3 minutes. The resultant cell pellet was re-suspended in 1ml of Stem- cell banker (Zenoaq #11890) and placed in a cryovial. Cryovials were placed in a Mr Frosty at -80°C overnight before being transferred to liquid nitrogen for longer term storage.

2.2 Plate Preparation

Laminin

Laminin-521 (Biolamina #LN521, Lot:80119) with a stock concentration of 100 µg/ml was diluted with 1x DPBS (Thermo Fisher #14040-091) resulting in a concentration of 5 µg/ml. Tissue culture flasks and plates were set for 2 hours at 37°C prior to use.

Vitronectin

Vitronectin (Thermo Fisher #A31804) with a stock concentration of 0.6mg/ml was kept at -80°C in 200µl aliquots. Vitronectin was diluted to a concentration of 6 µl/ml with PBS and applied to plates for 1 hour at 37°C prior to use.

Geltrex

Geltrex (Thermo Fisher #A14132-02) was initially diluted 1:10 with cold DMEM F-12 (Sigma #D6421) to generate 1ml aliquots which were kept at -20°C. For use, a 1ml aliquot was further diluted 1:10 with cold DMEM F-12 (Sigma #D6421) and applied to plates for 1 hour at 37°C prior to use.

2.3 Neuromesodermal Progenitor Differentiation

hPSCs with a confluency of 60-80% had media removed and EDTA (Invitrogen #15575-038) was applied for 5 minutes at 37°C. EDTA was removed and 1ml of Essential8 media used to lift cells in small clumps. Cells were then centrifuged at 1400rpm for 3 minutes and re-suspended in 1ml of N2B27 (see **Table 2.3**) supplemented with NMP differentiation reagents (see **Table 2.2**) and 10µM Y-27632 2HCl (Adooq Biosciences #A11001).
Cells were counted and seeded at a density of 63,000 cells/cm^2, on Vitronectin or Laminin521 coated plates, in N2B27 supplemented with NMP differentiation reagents and 10µM Y-27632 2HCl on previously coated plates.
After 24 hours the media was aspirated and replaced with NMP differentiation medium.
After 72 hours T+/Sox2+ NMPs are obtained.

Component	Concentration	Supplier
FGF2	20ng/ml	Peprotech #100-18B
CHIR 99021	3µM	Tocris #4423
LDN-193189	100nM	Tocris #6053

Table 2.2. A summary of the reagents, at specific concentrations, supplemented into N2B27 basal media to promote 3 day NMP differentiation from hPSCs

Component	Final Volume/ Concentration for 200ml	Supplier
DMEM F-12	100ml	Sigma #D6421
Neurobasal Medium (1x)	100ml	Thermo Fisher #21103-049
N-2 Supplement (100x)	2ml	Thermo Fisher #17502-001
B-27 Serum Free Supplemet (50x)	4ml	Thermo Fisher #17504-001
MEM Non Essential Amino Acid	2ml	Thermo Fisher #11140-035
Glutamax (100x)	2ml	Thermo Fisher #35050-038
2-Mercaptoethanol	200µl	Thermo Fisher #31350-010
Penstrep (100x)	4µl	

Table 2.3. A summary of the components and dilutions used to make 210ml of N2B27 basal media.

2.4 Thoracic Motor Neuron Differentiation Protocols

Day 3 differentiated hPSC-derived neuromesodermal progenitors had differentiation media removed and Accutase (Sigma #A6964) applied for 5 minutes at 37°C. DMEM F-12 (Sigma #D6421) was applied to stop the Accutase reaction and lift as single cells. Cells were centrifuged at 1400rpm for 3 minutes and the resultant cell pellet was re-suspended in 1ml of N2B27 (see **Table 2.3**) supplemented with 10µM Y-27632 2HCl (Adooq Biosciences #A11001).

Cells were seeded at a density of 63,000 cells/cm^2, on Geltrex coated plates, in N2B27 supplemented with 10µM Y-27632 2HCl and unique combinations of reagents dependent upon the differentiation protocol followed, as cited in the results section (see **Table 2.4** for full reagent combinations).

24 hours later (day 4) the media was aspirated and replaced. Media replaced every 2 days. Cells were re-plated on day 7 differentiation using Accutase to lift and dissociate. Cells were seeded at 86,000 cells/cm^2, on Geltrex coated plates, in N2B27 supplemented with 10 µM Y-27632 2HCl and the unique reagent combinations listed in Table 5.4. Media replaced on day 8.

Protocol	Component	Concentration	Supplier
Prolonged FGF, WNT & Enhanced SMADi	FGF2	100ng/ml	Peprotech #100-18B
	CHIR 99021	3µM	Tocris #4423
	LDN-193189	100nM	Tocris #6053
	SB431542	10µM	Tocris #1614
	DMH1	1µM	Tocris #4126
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	
Dual SMADi	LDN-193189	100nM	Tocris #6053
	SB431542	10µM	Tocris #1614
	DMH1	1µM	Tocris #4126
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	
FGF, WNT, RA	FGF2	100ng/ml	Peprotech #100-18B
	CHIR 99021	3µM	Tocris #4423
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	
BMP inhibition	FGF2	100ng/ml	Peprotech #100-18B
	CHIR 99021	3µM	Tocris #4423
	LDN-193189	100nM	Tocris #6053
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	

Table 2.4. A summary of the components, and their concentrations, added to the N2B27 basal media from Day 3 to Day 10 of differentiation under the different thoracic motor neuron differentiation conditions.

On day 10 differentiation the media was aspirated and changed to N2B27 supplemented with posterior ventralisation reagents (detailed in **Table 2.5** for each protocol). Media was again replaced on day 12.

Protocol	Component	Concentration	Supplier
Prolonged FGF, WNT & Enhanced SMADi	LDN-193189	100nM	Tocris #6053
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	
Dual SMADi	LDN-193189	100nM	Tocris #6053
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	
FGF, WNT & RA	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	
BMP inhibition	LDN-193189	100nM	Tocris #6053
	SAG	0.5µM	Tocris #4366
	Purmorphamine	1µM	Sigma #SML0868
	All trans Retinoic Acid	0.1µM	

Table 2.5. A summary of the components, and their concentrations, added to the N2B27 basal media from Day 10 to Day 14 of differentiation under the different thoracic motor neuron differentiation conditions.

On day 14 differentiation cells were re-plated, using Accutase, and seeded on Geltrex coated plates at 100,000 cells/cm^2 in motor neuron maturation media (see **Table 2.6**) supplemented with 10µM Y-27632 2HCl. Media was replaced on day 15 differentiation and continually replaced every 2 days.

Component	Concentration	Supplier
BDNF	20ng/ml	Peprotech #450-02
GDNF	20ng/ml	Peprotech #450-10
L-Ascorbic Acid	200µM	Sigma #A8960
DAPT	10µM	Sigma #D5942

Table 2.6. A summary of the components, and their concentrations, added to the N2B27 basal media from Day 14 of differentiation onwards to promote neuronal maturation.

2.5 Anterior Motor Neuron Differentiation

hPSCs with a confluency of 60-80% had media aspirated and EDTA (Invitrogen #15575-038) was applied for 5 minutes at 37°C. EDTA was aspirated and 1ml of DMEM F-12 (Sigma #D6421) was used to lift and dissociate the cells as small clumps. Cells were centrifuged at 1400rpm for 3 minutes to create a pellet. The cell pellet was re-suspended in 1ml of N2B27 supplemented with anterior neuralisation reagents (detailed in Table **2.7**) and 10µM Y-27632 2HCl.

Cells were seeded at 63,000 cells/cm^2, on Geltrex coated plates, in N2B27 supplemented with anterior neuralisation reagents and 10µM Y-27632 2HCl. Media was replaced 24 hours later (day 1).

Component	Concentration	Supplier
LDN-193189	100nM	Tocris #6053
SB431542	10µM	Tocris #1614

Table 2.7. A summary of the components, and their concentrations, added to the N2B27 basal media from Day 0 to Day 3 to promote anterior neural induction

On day 3 differentiation the cells media was aspirated and Accutase (Sigma #A6964) applied for 5 minutes at 37°C. DMEM F-12 was used neutralise the accutase reaction and lift the cells as single cells which were then centrifuged at 1400rpm for 3 minutes. The resultant pellet was re-suspended in 1ml of N2B27 supplemented with anterior ventralisation reagents (see **Table 2.8**) and 10µM Y-27632 2HCl.

Cells were counted and seeded at 63,000 cells/cm^2, on Geltrex coated plates, in N2B27 supplemented with anterior ventralisation reagents and 10µM Y-27632 2HCl. Media was replaced on day 4 and day 6 of differentiation.

On day 7, the cells were lifted, using Accutase. They were then re-plated at a density of 86,000 cells/cm^2 on Geltrex coated plates in N2B27 supplemented with anterior ventralisation reagents and 10µM Y-27632 2HCl.

Component	Final Volume/ Concentration for 200ml	Supplier
LDN-193189	100nM	Tocris #6053
SB431542	10µM	Tocris #1614
DMH1	1µM	Tocris #4126
SAG	0.5µM	Tocris #4366
Purmorphamine	1µM	Sigma #SML0868
All trans Retinoic Acid	0.1µM	

Table 2.8. A summary of the components, and their concentrations, added to the N2B27 basal media from Day 3 to Day 10 to promote ventralisation.

On day 10 differentiation the media was changed to N2B27 supplemented with ventralisation reagents (detailed in **Table 2.8**). Media was again replaced on day12.

Component	Final Volume/ Concentration for 200ml	Supplier
SAG	0.5µM	Tocris #4366
Purmorphamine	1µM	Sigma #SML0868
All trans Retinoic Acid	0.1µM	
L-Ascorbic Acid	200µM	Sigma #A8960

Table 2.9. A summary of the components, and their concentrations, added to the N2B27 basal media from Day 10 to Day 14 of differentiation under the anterior differentiation conditions.

On day 14 differentiation, cells were re-plated using Accutase. Cells were seeded at a density of 100,000 cells/cm^2 onto Geltrex coated plates in N2B27 medium supplemented with motor neuron maturation reagents (see **Table 2.6**) and 10µM Y-27632 2HCl. On day 15 the media was replaced and continually replaced every 2 days.

2.6 Neural Conversion of NMPs Experiments

Day 3 hPSC-derived NMPs, generated as in **Section 2.3**, had media removed and Accutase applied for 5 minutes at 37°C. DMEM F-12 was used to stop the accutase reaction and lift the cells as single cells which were centrifuged at 1300rpm for 3 minutes. Cells were re-suspended in 1ml of N2B27 supplemented with 10 µM Y-27632 2HCl. Cells were counted and seeded at 86,000 cells/cm^2 on Geltrex coated plates under the different media conditions shown in Table 5.10.

On day 4 and of differentiation the respective medium were replaced.

On day 7 of differentiation cells were pelleted for RNA extraction and fixed for immunofluorescence.

Condition 1	3uM CHIR 99021 (Tocris #4423), 100ng/ml FGF2 (Peprotech #100-18B), 0.1µM RA
Condition 2	3uM CHIR 99021 (Tocris #4423), 100ng/ml FGF2 (Peprotech #100-18B)
Condition 3	3uM CHIR 99021 (Tocris #4423), 1µM PD03
Condition 4	1µM XAV, 100ng/ml FGF2 (Peprotech #100-18B)
Condition 5	1µM XAV, 1µM PD03, 0.1µM RA
Condition 6	3uM CHIR 99021 (Tocris #4423),1µM PD03, 0.1µM RA
Condition 7	1µM XAV, 100ng/ml FGF2 (Peprotech #100-18B), 0.1µM RA

Table 2.10. A summary of the components, and their concentrations, added to the N2B27 basal media, under each of the tested conditions, from Day 3 to Day 7 of differentiations to explore the effects of specific signalling pathways on neural induction and maintenance of thoracic HOX expressions.

5.7 Chick Embryo Grafting

All cells used for chick embryo grafting were GFP-positive to allow the distinction between host chick cells and hPSCs. On day 8 of trunk neural crest differentiation or day 13 of posterior motor neuron differentiation, cells were plated as hanging drops at concentrations of 1000–5000 cells/cm^2 on the under-side of a petri-dish lid. Cells were in 20µl drops in DMEM F12 media (Sigma) supplemented with N2 supplement (Thermo Fisher), MEM Non Essential Amino Acids (Thermo Fisher), Glutamax (Thermo Fisher). After 24 hours of incubation at 37°C the cells generated clumps with a diameter of ~50–100 mm.

Fertilised Bovan brown chicken eggs (Henry Stewart and Co., Norfolk, UK) were staged according to Hamburger and Hamilton (1951). Eggs were incubated for 36-48 hours prior to transplantation resulting in chick embryos staged at around HH Stage 10-13. Eggs were rotated 180° and shaken to orientate the embryo prior to placing upon an egg holder. A small window was generated directly above the embryo using a sharp blade. Roughly 1ml of albumin was removed from a small hole created in the lower area of the egg using a 19-gauge syringe. Blue food colouring was injected underneath the embryo using a 30-gauge syringe to allow a clearer observation of the embryo. A few drops of L-15 medium (Thermo Fisher #11415-049) was then applied to the chorion to allow easier removal of the membrane. Removal of the membrane was best achieved by piercing the membrane with a sharp blade and then sharply peeling away from the embryo using a tungsten needle.

Lesions were generated within the roof plate of the neural tube of HH stage 10-13 chick embryos at the level of the first 2-3 somites (anterior graft) or at the level of the newly developing somite's (posterior graft). Cell clumps were transferred directly ontop of the embryo using a P20 pipette. A tungsten needle of extremely sharp blade was then used to guide the clumps into the lesioned area. Parafilm was then used to seal the window and the embryos were placed back in the incubator at 37°C.

2-3 days' post-transplantation (HH stage 20-24) the embryos were harvested. Only those embryos which had survived (observed to have a heartbeat) or had just died (with clear

vasculature and staging) were harvested. To harvest the embryos were dissected out of the egg using forceps and dissection scissors and placed in a petri-dish containing L-15 medium. Harvested embryos were screened for presence of GFP-positive cells; those containing GFP were imaged whole mount. Embryos were then fixed in 4% PFA for 2/3hours at 4°C. Embryos were then transferred to 30% sucrose solution for 2-3 days at 4°C. The part of the embryos containing the GFP-positive cells were then embedded in OCT (VWR #361603E) prior to sectioning.

Sectioning was conducted using a (Leica CM1950). Sections of 15-20µm width were collected on Superfrost Plus microscope slides (Thermo Fisher #J1800AMNZ) in adjacent sections. The sections were left at room temperature for 2-4 hours to allow for a sufficient drying period.

Prior to staining the sections were washed 3 times using PBS to remove the OCT. Blocking buffer, PBS with 1% BSA (Millipore #821001), 0.3% Triton-X (Acros Organics #9002-91-1) and 3% Donkey Serum (Sigma #D9663), was then applied for 2-3 hours at room temperature to block and permeabilise the sections. Primary antibodies, diluted in blocking buffer, were applied to the sections overnight at 4°C. After removal of the primary antibody the sections were washed 3 times with PBS. Secondary antibodies were then applied for 2-3 hours at room temperature at a 1:1000 dilution in blocking buffer. Following the removal of the secondary antibodies the sections were again washed 3 times with PBS.

Excess PBS and fluid was removed from the microscope slides containing the sections. 4 small drops of Fluoroshield with DAPI (Sigma #F6057) was then applied to each microscope slide. A 22x64 mm cover glass (VWR #631-0880) was slowly lowered over the sections using forceps taking care to avoid the presence of any bubbles. These microscope slides were then left to dry in the dark before being placed at 4°C for long term storage until they could be imaged using the InCell 2200 (GE Healthcare).

2.8 Immunostaining

Cells for immunostaining were taken at specific time points for each of the differentiations. Cells had media aspirated and were quickly washed with 1xPBS. 4% PFA was then applied for 5 minutes at room temperature to allow fixation. Permeabilisation of the cells was achieved by incubating the cells for 10 minutes at room temperature with PBST, PBS with 0.3% Triton-X (Acros Organics #9002-91-1). After this the cells were incubated with blocking buffer, 1xPBS with 1% BSA (Millipore #821001), 0.3% Triton-X (Acros Organics #9002-91-1) and 3% Donkey Serum (Sigma #D9663), for 1-2 hours at room temperature. Primary antibodies, diluted to their respective dilutions in blocking buffer (stated in **Table 2.11**), were applied to the cells overnight at 4°C. After removal of the primary antibodies the cells were washed 3 times with 1xPBS. Secondary antibodies (stated in **Table 2.12**) were then applied, at a dilution of 1:1000 in blocking buffer, for 2-3 hours at room temperature. Upon removal of the secondary antibodies the cells were again washed 3 times with 1xPBS. Hoescht33342 (Invitrogen #H3770) in a dilution of 1:1000 in PBS was applied to the cells for 5 minutes. After removal of the Hoescht33342, a large amount of 1xPBS was applied to each of the wells containing cells to prevent the cells from drying out during long-term storage at 4°C. Stained plates were imaged as soon as possible using the InCell 2200 (GE Healthcare).

Processing of the images was conducted using CellProfiler. For each biological repeat 15-20 fields were imaged per condition with approximately 1,000-5,000 cells in each field. Secondary only antibody staining was used as a negative control to set the brightness values for all fields and to identify the percentage of expressing cells.

Antibodies	Details	Format	Dilution
Choline Acetyltransferase	Abcam #ab144P, Polyclonal Goat IgG	Purified Antibody	1:100
Hoxc9	Abcam #ab50839, Monoclonal Mouse IgG	Purified Antibody	1:50
Islet 1/2	DSHB #39.4D5, Monoclonal Mouse IgG2b	Hybridoma Concentrate	1:200
MNR2/HB9/Mnx1	DSHB #81.5C10, Monoclonal Mouse IgG	Hybridoma Concentrate	1:200
Neurofilament	Abcam #ab8135, Polyclonal Rabbit IgG	Purified Antibody	1:1000
Sox10	CSF #D5V9L, Monoclonal Rabbit IgG	Purified Antibody	1:500
Sox2	Abcam #ab92494, Monoclonal Rabbit IgG	Purified Antibody	1:400
TUJ1	Abcam #ab78078, Monoclonal Mouse IgG	Purified Antibody	1:1000
Pax6	Biolegend #901302, Polyclonal Rabbit IgG	Purified Antibody	1:200
Olig2	R&D #AF2418, Polyclonal Goat IgG	Purified Antibody	1:200

Table 2.11. A summary of the primary antibodies, and their dilutions, used during immunostaining.

Antibodies	Details	Dilution
Donkey anti Mouse IgG (H+L) AF 647	Invitrogen #A-31571	1:1000
Donkey anti Mouse IgG (H+L) AF 594	Invitrogen #A-21203	1:1000
Donkey anti Goat IgG (H+L) AF 594	Invitrogen #A-11058	1:1000
Donkey anti Rabbit IgG (H+L) AF 647	Invitrogen #A-31573	1:1000
Donkey anti Rabbit IgG (H+L) AF 555	Invitrogen A-32794	1:1000

Table 2.12. A summary of the secondary antibodies, and their dilutions, used during immunostaining.

2.9 Gene Expression Analysis

RNA extraction

Cells were lifted using Accutase. Media was removed from cells and accutase applied for 5 minutes at 37°C. DMEM F-12 was added to neutralise the enzyme reaction. The cells were then centrifuged at 1300rpm for 3 minutes to generate a cell pellet. These cell pellets were either stored at -80°C for later use or used immediately. To extract RNA a Total RNA purification kit (Norgen Biotek #37500) was used. Manufacturers protocol was followed. RNA was eluted in ddH_20. The concentration and 260/280 value of RNA which was eluted was measured using the Nanodrop Lite Spectrophotometer (Thermo Fisher). RNA is considered pure and can be used as for cDNA synthesis if the 260/280 ratio is between 1.8 and 2.0. RNA is stored at -80°C until use in cDNA synthesis.

cDNA Synthesis

A high-capacity cDNA reverse transcription kit (Invitrogen #4368814) was used to convert 1µg of RNA into 1µg of cDNA. Manufacturers protocol was followed. A final reaction volume of 20µl was placed in each PCR tube (see **Table 2.13** for components). This 20µl contained 5.8µl of reagents from the high-capacity cDNA reverse transcription kit (Invitrogen #4368814), a calculated volume of RNA for 1µg and a calculated volume of ddH_20 to make the total volume up to 20µl. Reverse transcription was conducted, as by the manufacturers protocol, within a Thermocycler (Applied Biosystems 2720). The cycle conditions used were 25°C- 10 mins, 37°C- 120 mins, 85°C- 5mins. Until use the cDNA was then stored at -20°C.

Component	Volume for 1 reaction (µl)
Reverse Transcriptase	1
Random Primers	2
10x RT Buffers	2
dNTPS (100nM)	0.8
RNA	Required for 1µg
ddH_20	14.2 – Vol. of RNA

Table 2.13. A table summarising the cDNA reaction components

qPCR

Quantitative Real Time PCR was conducted using the QuantStudio 12K Flex Real-Time PCR System (Life Technologies 4471087). Reactions were set up in 384 well plates with each well containing one reaction per gene of interest (see **Table 2.14** for reaction components). Amplicon amplification was measured using the Roche Universal Probe library system. Cycling conditions used are as follows: 95°C- 10 minutes for initial denaturing followed by 45 cycles of 95°C- 10 seconds and 60°C for 1 minute. All primers used were designed according to the Roche UPL primer design assign and are listed in **Table 2.15**. GAPDH was used as a housekeeping gene for normalisation of gene expression during obtaining 1/Delta CT values.

Component	**Volume for 1 reaction (µl)**
Taqman Master mix (Thermo Fisher 4352042)	5
10µM Forward/Reverse **Primers**	0.2
ddH_20	2.7
Roche Probe	0.1
5ng/ml cDNA	2

Table 2.14. A table summarising the qPCR reaction components

Gene	Forward	Reverse	Roche Probe
GAPDH	gcccaatacgaccaaatcc	agccacatcgctcagacac	60
Sox2	ttgctgcctctttaagactagga	taagcctggggctcaaact	35
T(Bra)	aggtacccaaccctgagga	gcaggtgagttgtcagaataggt	23
Cdx2	atcaccatccggaggaaag	tgcggttctgaaaccagatt	34
Nkx1-2	gtcgaagcggggaaagat	gatcctccgcatcctcct	78
Msgn1	agctcaggatgaggaccttg	ctggcctctctggctgtaga	87
Foxa2	cgccctactcgtacatctcg	agcgtcagcatcttgttgg	9
Sox1	gaagcccagatggaaatacg	ggacaaggaagggtgttgag	66
Foxg1	atgatccccaagtcctcgtt	gtggtggttgtcgttctgg	64
Otx2	ccatctccccactgtcagat	ggtcatgggataggacctctg	4
Olig2	agctcctcaaatcgcatcc	atagtcgtcgcagctttcg	12
HB9 (Mnx1)	ttacctgacttatgaaacttgaaacc	cccagagacgtaagcataaacc	50
Isl1	aaacaggagctccagcaaaa	aaaggactctttcagccaagg	4

HoxA1	gacgaccgcttcctagtgg	tcccggaagtctggtaggta	9
HOXB1	ccagctaggggcttgtc	atgctgcggaggatatgg	39
HOXA2	caagaaaaccgcacttctgc	tgtgttggtgtaagcagttctca	5
HOXB2	aaatcgctccattacataaatcg	aattcatggctttcaatggtg	3
HOXA3	gacagctcatgaaacggtctg	ggtttgacacccgtgagg	70
HOXB3	agctgctgaactgtccgttt	ccaggtccacgatgattttt	3
HOXD3	tcaagaaaacacacacatacataattg	tgctgaatcctgagagagctg	1
HoxA4	gttgccacccaagagagaac	ccaagtagtccttctcaggtatcc	20
hoxb4	aaaccaggccccttcctac	gcacacagatattcacacatacga	45
hoxd4	gggcgggattctctctctaa	cataagtttaatgactcgccaagat	51
Hoxc4	agccaattctcatccttctcc	caatgcaaaaggcctaagga	12
HoxA5	gcgcaagctgcacataag	cggttgaagtggaactcctt	1
Hoxb5	aagcttcacatcagccatga	cggttgaagtggaactccttt	1
HOXC5	cccgggatgtacagtcagaa	gcctgctcctctttgatctc	25
HoxA6	ggaaaacaagctcatcaattcc	cctgcccaggcatctactc	26
HoxB6	tggaagctgaagaagaaactgaa	gccgggtttatgatttgttg	12
HOXC6	tgaattcctacttcactaacccttc	atcataggcggtggaattga	87
HOXA7	cagtgacctcgccaaagg	caggggtagatgcggaaat	30
HOXB7	ctacccctggatgcgaag	caggtagcgattgtagtgaaattct	1
HOXB8	agctcttcccctggatgc	atagggattaaataggaactccttctc	1
HoxC8	tcccagcctcatgtttcc	tgataccggctgtaagtttgc	86
HOXD8	cccttgtaatcgcctgaaat	ctactgaaaataacggaacacagc	83
HOXA9	ccccatcgatcccaataa	caccgctttttccgagtg	66
HoxC9	tcctagcgtccaggtttcc	gctacagtccggcaccaa	70
HoxA10	gttttgcacaagaaatgtcagc	gacattgttgtgggataatttgg	32
HoxC10	aggagagggccaaagctg	agccaatttcctgtggtgtt	19
HoxA11	ttggaaagagttagggaaatgc	ggctttcccagatgagatcc	34
HoxC11	gagaggggcgcagatttc	actgggcagatagaggttgg	24
HoxD11	tgaacgactttgacgagtgc	gacggggccacatagtagg	71
HOXA13	cctctggaagtccactctgc	gcttctttctccccctcct	17
HOXB13	acgtgtctgtggtgcagact	gcaacagggagtcatgtcg	80
HOXD13	ggaacagccaggtgtactgc	cggctgatttagagccaca	5
IRX3	aaaagttactcaagacagctttcca	ggatgaggagagagccgata	57
NGN1	gcggatgtctcttggtctg	aaaaggaaaggccgtctagg	26
NGN2	aacgctgaggcacagttagag	ccgagcagcactaacacg	69
NKX6.1	gagatgaagaccccgctgta	gacgacgacgaggacgag	27
NKX6.2	agcacaaaccctcgaacttg	cccggattctgcaaaaatag	70
NEFL	atgaatgaagcgctggagaa	tctaatttgttgatcgtgtcctg	17
NEFH	ccgacattgcctcctacc	ggccatctcccacttggt	73

Table 2.15. A table summarising the primers used during qPCR analysis of the secondary antibodies, and their dilutions, used during immunostaining.

2.10 Electrophysiology

In all cases the hPSC-derived motor neurons used within the electrophysiology experiments were differentiated as stated in **Section 2.4**, for the thoracic motor neuron differentiation, or **Section 2.5**, for the anterior motor neuron differentiation. Extended differentiation of the neurons, from Day 24, to Day 34 of differentiation was conducted in the presence of N2B27 basal medium **(**see **Table 2.3)** supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 200µM L-Ascorbic Acid and 10µM DAPT to promote mature motor neuron identity. Media was changed every 48 hours.

On Day 34 of differentiation, Accutase (Sigma #A6964) was applied for 5 minutes at 37°C. DMEM F-12 (Sigma #D6421) was applied to stop the Accutase reaction and lift the neurons as single cells. Cells were centrifuged at 1400rpm for 3 minutes and the resultant cell pellet was re-suspended in 1ml of N2B27 (see **Table 2.3**) supplemented with 10µM Y-27632 2HCl (Adooq Biosciences #A11001). hPSC-derived neurons were plated onto 13mm coverslips at a density of 50,000 cells per coverslip within 12 well plates in N2B27 basal medium **(**see **Table 2.3)** supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 200µM L-Ascorbic Acid and 10µM DAPT. Neurons were maintained at 37°C with humidified 5% CO_2 until Day 36 where they were used within Patch Clamp.

Patch Clamp recordings were conducted by Dr Ke Ning (Senior lecturer, Medical School, University of Sheffield) and these methods are supplied by Dr Ke Ning. All recordings were performed at room temperature and all reagents for solutions were purchased from Sigma. Electrodes for patch clamping were pulled on a Sutter P-97 horizontal puller (Sutter Instrument Company) from borosilicate glass capillaries (World Precision Instruments). Coverslips were placed into a bath on an upright microscope (Olympus) containing the extracellular solution at pH7.4 composing of 150 mM NaCl, 5.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM Glucose osmolarity,~305 mOsm/Kg. Whole-cell current clamp recordings were performed using an Axon Multi-Clamp 700B amplifier (Axon Instruments, Sunnyvale, CA, USA) using unpolished borosilicate pipettes placed at the cell soma. Pipettes had a resistance of 4-6MΩ when filled with intracellular solution of 140mM K^+-gluconate, 10 mM KCl, 1 mM $MgCl_2$, 0.2mM EGTA, 9 mM NaCl, 10 mM HEPES, 0.3 mM Na^+-GTP, and 3 mM Na^+-ATP adjusted to 298 mOsm/Kg at pH7.4. For both solutions glucose, EGTA, Na^+-

GTP, and Na^+-ATP were added fresh on each day of the experiment. To identify neurons present, cells were visualised using the microscope x40 objective, and those with a triangular cell body and processes to indicate neuronal morphology were selected. To measure depolarized evoked action potential firing in the cells using a 15-step protocol for a duration of 500 miliseconds, injecting current from -80pA, every 10pA. Recordings were acquired at ≥10 kHz using a Digidata 1440A analogue-to-digital board and pClamp10 software (Axon Instruments). Electrophysiological data were analysed using Clampfit10 software (Axon Instruments). Firing magnitude 20mV and higher were included for analysis.

2.11 Solutions

4% PFA

Paraformaldehyde powder (Sigma 1582127) was dissolved within 1xPBS to prepare a 4x working solution (for 500ml of PFA: 20g of Paraformaldehyde powder was mixed with 500ml of 1xPBS). Upon mixing, the solution was heated until the powder was dissolved into solution. 4% PFA was then stores at -20°C until use.

1x PBS

PBS used was Dulbeccos's Phosphate Buffered Saline without magnesium chloride and calcium chloride (Sigma D1408). This 10x solution was diluted 1:10 with ddH_20 and sterilised by autoclaving.

Chapter 3- Optimising the in vitro generation of motor neurons utilising an NMP intermediary state

3.1 Introduction

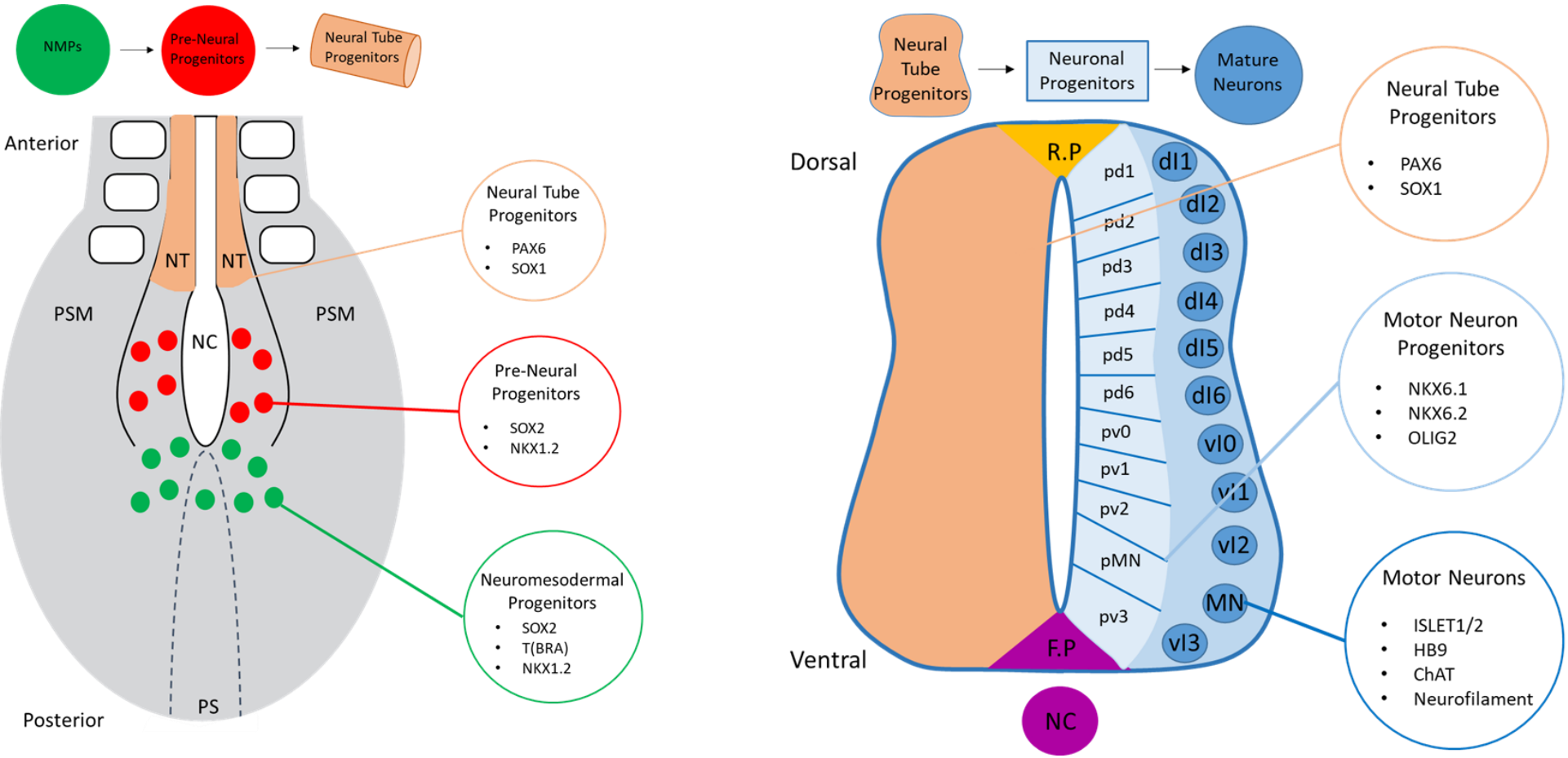
Bipotential Neuromesodermal Progenitors (NMPs) have been shown to exist during amniote axial extension and act as a source for neural and mesodermal derivatives of the posterior axis (Tzouanacou *et al.*, 2009b). *In vitro*, the exposure of differentiating PSCs to Wnt and FGF signals was sufficient to allow the generation of NMP-like cells which expressed the similar genes to their *in vivo* counterparts (David A. Turner *et al.*, 2014a; Gouti *et al.*, 2014a; Lippmann *et al.*, 2015b). However, there still remains a need to fully clarify the signalling pathways required to promote mesodermal and neural identity downstream of NMPs. It is commonly agreed that exposure of PSC-derived NMPs to Wnt agonists alone results in the specification to a mesoderm identity (D. A. Turner *et al.*, 2014; Gouti *et al.*, 2014a; Tsakiridis and Wilson, 2015; Edri *et al.*, 2019; Diaz-Cuadros *et al.*, 2020). However, the differentiation to a neural identity has been demonstrated through exposure to FGF and Wnt signals (Tsakiridis and Wilson, 2015; Edri *et al.*, 2019), FGF signals alone (David A Turner *et al.*, 2014) whilst others have reported a need for exposure to Retinoic Acid and TGFβ-family inhibitors (D. A. Turner *et al.*, 2014; Gouti *et al.*, 2014a; Lippmann *et al.*, 2015b; Kumamaru *et al.*, 2018; Verrier, Davidson, Gierli, *et al.*, 2018; Edri *et al.*, 2019; Rayon *et al.*, 2019; Diaz-Cuadros *et al.*, 2020). Therefore, there is a need to clearly define the signalling pathways which are required to promote a neuronal identity downstream of NMPs which will be important for further downstream differentiation to a motor neuron fate.

As NMPs are the known source of posterior identity motor neurons in vivo, multiple studies have tried to generate PSC-derived thoracic motor neurons by differentiating through an initial FGF and Wnt evoked NMP-like state (Gouti *et al.*, 2014a; Kumamaru *et al.*, 2018; Verrier, Davidson, Gierli, *et al.*, 2018; Rayon *et al.*, 2019; Faustino Martins *et al.*, 2020). Upon generation of an NMP-like state these protocols utilise Retinoic Acid and Shh signals to promote a ventral neural tube identity. Some of these protocols have also shown a requirement for TGFβ-family inhibitors to promote both neuralisation and ventral identity (Gouti *et al.*, 2014a; Lippmann *et al.*, 2015a; Kumamaru *et al.*, 2018; Verrier, Davidson,

Gierli, *et al.*, 2018). Due to the conflicting different small molecules and proteins used within these protocols there is a need to define the optimal culture conditions which allow for the generation of thoracic identity motor neurons. There is also a need to molecularly characterise the cells generated throughout the differentiation to confirm both axial and dorsoventral identity. In order to allow for the correct confirmation of cell identity within the differentiation a set of well-defined markers will be screened which are known to mark a specific identity (as clearly shown in **Schematic 3.1**). Due to the unclear roles of specific signalling pathways in directing thoracic motor neuron identity from NMPs, current protocols also yield low numbers of thoracic motor neurons (Lippmann *et al.*, 2015; Verrier, Davidson, Gierlinśki, *et al.*, 2018). Therefore, there is a requirement to fully characterise the downstream differentiation of NMPs to ensure the highest yields of thoracic motor neurons.

3.2 Aims

1. Defining the signals mediating the transition from NMPs to posterior ventral neurectoderm.
2. Optimise the culture conditions for the generation of posterior identity motor neurons.



Schematic 3.1. A schematic showing the different molecular markers which have been shown to be expressed within the different cell types *in vitro*. Within my experiments these markers will be screened for to characterise the identity of the cells obtained through the differentiation protocols. Abbreviations; NMPs-Neuromesodermal Progenitors, NT-Neural Tube, NC-Notochord, PS-Primitive Streak, PSM-Presomitic Mesoderm, RP-Roof Plate, FP-Floor Plate.

3.3 Results

3.3.1 Optimising a protocol for the *in vitro* development of hPSC-derived thoracic identity motor neurons

3.3.1.1 BMP inhibition promotes a neural bias in hPSC derived NMPs

In order to generate an efficient protocol for the generation of hPSC-derived thoracic motor neurons, which use NMPs as an intermediate stage, I was required to first demonstrate that an NMP identity could be achieved. Multiple existing protocols within the literature have clearly demonstrated that PSCs can be differentiated, over a period of 3 days, under the exposure to Wnt and FGF signals to robustly reproduce a SOX2+/T(BRA)+ NMP state (David A. Turner *et al.*, 2014b; Gouti *et al.*, 2014b; Lippmann *et al.*, 2015b). (Large amounts of the following data are taken from my undergraduate wet laboratory dissertation project and is therefore joint work conducted by myself, Dr Thomas Frith and Dr Anestis Tsakiridis). Reflective of published findings, when we differentiated hPSCs under the exposure to 20ng/ml FGF2 and 3µM CHIRON, a Wnt agonist, on Day 3 there are high yields of SOX2+/T(BRA)+ coexpressing cells. Differentiation of hPSCs on either Vitronectin or Laminin521 coated surfaces resulted in similar high yield induction of NMPs by day 3 (on average 90% induction and 86% induction respectively) (**Figure 3.1B &C**).

Multiple studies have suggested that the regulation of BMP signalling is important to allow for the correct downstream differentiation of NMPs. Largely, BMP signals have been implicated in the induction of mesodermal identity (Chambers *et al.*, 2009a; Neely *et al.*, 2012; Filip J Wymeersch *et al.*, 2016) and the inhibition of BMP signals are required to promote enhanced neuralisation of NMPs and prevent mesodermal specification (Chambers *et al.*, 2009a; Verrier, Davidson, Gierli, *et al.*, 2018). BMP signals are also vitally important for governing the specification of neural crest within the neural plate border region and dorsal neural tube (Stuhlmiller and García-Castro, 2012). For these reasons, we wished to investigate if endogenous BMP signals within culture, during the 3 day NMP differentiation, could be promoting a mesodermal, neural crest or neural bias within our obtained NMP cultures. Initially, we differentiated hPSCs in 20ng/ml FGF2 and 3µM CHIRON with the presence or absence of LDN, a BMP inhibitor. Immunofluorescence was used to observe the

yield of NMP induction. The presence of BMP inhibition resulted in a higher yield of SOX2+/T(BRA)+ NMPs by day 3 when culturing hPSCs on both Vitronectin and Laminin coated surfaces (**Figure 3.1B**).

Next, we wished to observe if the presence of endogenous BMP signals were having an effect on the potential bias towards a neural crest or mesodermal lineage within the NMP population. To do this, we observed the expression of SNAI2/SLUG within NMPs at Day 3 of differentiation. SNAI2 is upregulated within cells associated with an epithelial to mesenchymal transition and as such is associated with both mesodermal and neural crest identity (Sefton, Sanchez and Nieto, 1998; Nieto, 2002). When hPSC were cultured in FGF2 and WNT signals alone there is observed expression of SNAI2. However, when the BMP inhibitor DMH1 was included, alongside FGF2 and WNT, there is a significant reduction in the levels of SNAI2 expression at Day 3 (**Figure 3.1C**). These findings suggest that the presence of endogenous BMP signals in culture promote a bias towards mesodermal or neural crest lineages within the day 3 NMP population. This has been further expanded and is reflective of further findings demonstrated by our group (Frith *et al.*, 2018a). In parallel other groups have also demonstrated the requirement for BMP inhibition in the downstream differentiation of NMPs to a neural identity (Rosenzweig *et al.*, 2018a; Verrier, Davidson, Gierli, *et al.*, 2018). Taken together, this suggests that in future NMP differentiations the presence of BMP inhibition for Day 0 to Day 3 should be included as standard to overcome endogenous BMP signals and promote a neural bias.

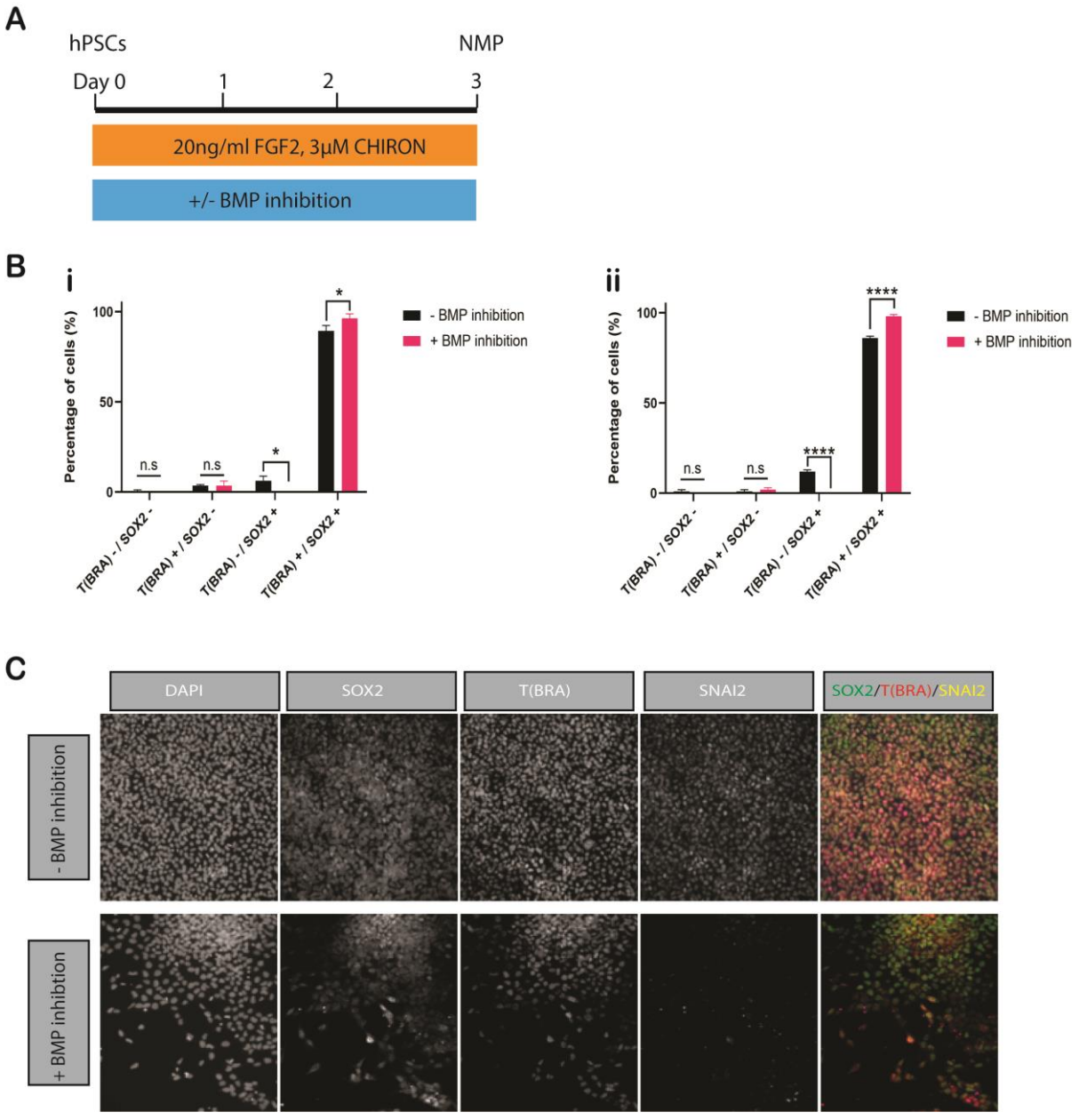


Figure 3.1 BMP inhibition promotes a neural bias in hPSC derived NMPs. (A) A Schematic showing the differentiation protocols followed. **(B)** Quantification of the immunofluorescence showing the expression of data of T(BRA) and SOX2 on Day 3 of differentiation in the presence or absence of BMP inhibition when cultured on **(i)** Vitronectin and **(ii)** Laminin521 coated surfaces. **(C)** Immunofluorescence showing the coexpression of SOX2, T(BRA) and SNAI2 in day 3 NMPs.

3.3.1.2 TGF-β and BMP antagonism enhances neural conversion of NMPs

Existing protocols for the *in vitro* generation of motor neurons from hPSCs predominantly produce motor neurons of a brachial and cervical identity and fail to produce HOXC9^{+} thoracic identity motor neurons (H Wichterle *et al.*, 2002; Lee *et al.*, 2007; Li *et al.*, 2008; M. W. Amoroso *et al.*, 2013). As Neuromesodermal progenitors have been shown to be the source of thoracic neural structures multiple studies have tried to generate PSC-derived thoracic motor neurons by differentiating through an initial FGF and Wnt evoked NMP-like state (Gouti *et al.*, 2014a; Kumamaru *et al.*, 2018; Verrier, Davidson, Gierli, *et al.*, 2018; Rayon *et al.*, 2019; Faustino Martins *et al.*, 2020). However, these protocols often result in low yields of motor neurons and often use conflicting combinations of proteins and small molecules. Therefore, I aimed to identify a robust protocol for the high-yield generation of PSC-derived thoracic motor neurons. As discussed previously BMP and TGFβ antagonism are essential for the induction of anterior neurectoderm (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou, Kelly and Melton, 1994; Sasai *et al.*, 1994; Zimmerman, De Jesús-Escobar and Harland, 1996; Fainsod *et al.*, 1997; Perea-Gomez *et al.*, 2002; Yamamoto *et al.*, 2004) and more recently BMP and TGFβ antagonism have been utilised within *in vitro* neural differentiations of NMPs (Kumamaru *et al.*, 2018; Verrier, Davidson, Gierlin, *et al.*, 2018). Within these in vitro-differentiation protocols there is often low yields of neural precursors and neural conversion can take a long period of time. Therefore, I aimed to characterise if the presence of BMP and TGFβ antagonism, within neural differentiation downstream of NMPs, and successive motor neuron induction, resulted in the generation of HOXC9+ thoracic motor neurons.

To address this, I differentiated both the hESC cell line; H9 and the hiPSC cell line; SFCi55-SsGr under standard NMP differentiation conditions to generate a stable NMP identity by day 3. NMP differentiation is conducted under the presence of BMP inhibition as this has been shown to promote neural bias (Frith *et al.*, 2018a). To identify the role of BMP and TGFβ antagonism within neural induction, the *in vitro*-derived NMPs were cultured for a further 5 days under the presence of BMP inhibitors (LDN193189 and DHH1) and a TGFβ inhibitor (SB43) (as shown in **Figure 3.2**). Retinoic Acid was also supplemented within the media as it has been shown to be essential for neuralisation (Del Corral *et al.*, 2003; Olivera-Martinez *et al.*, 2014). Addition of Sonic Hedgehog agonists (SAG, Smoothened Agonist, and

Purmorphamine) allowed for the promotion of ventral identity (Ericson, Briscoe, *et al.*, 1997).

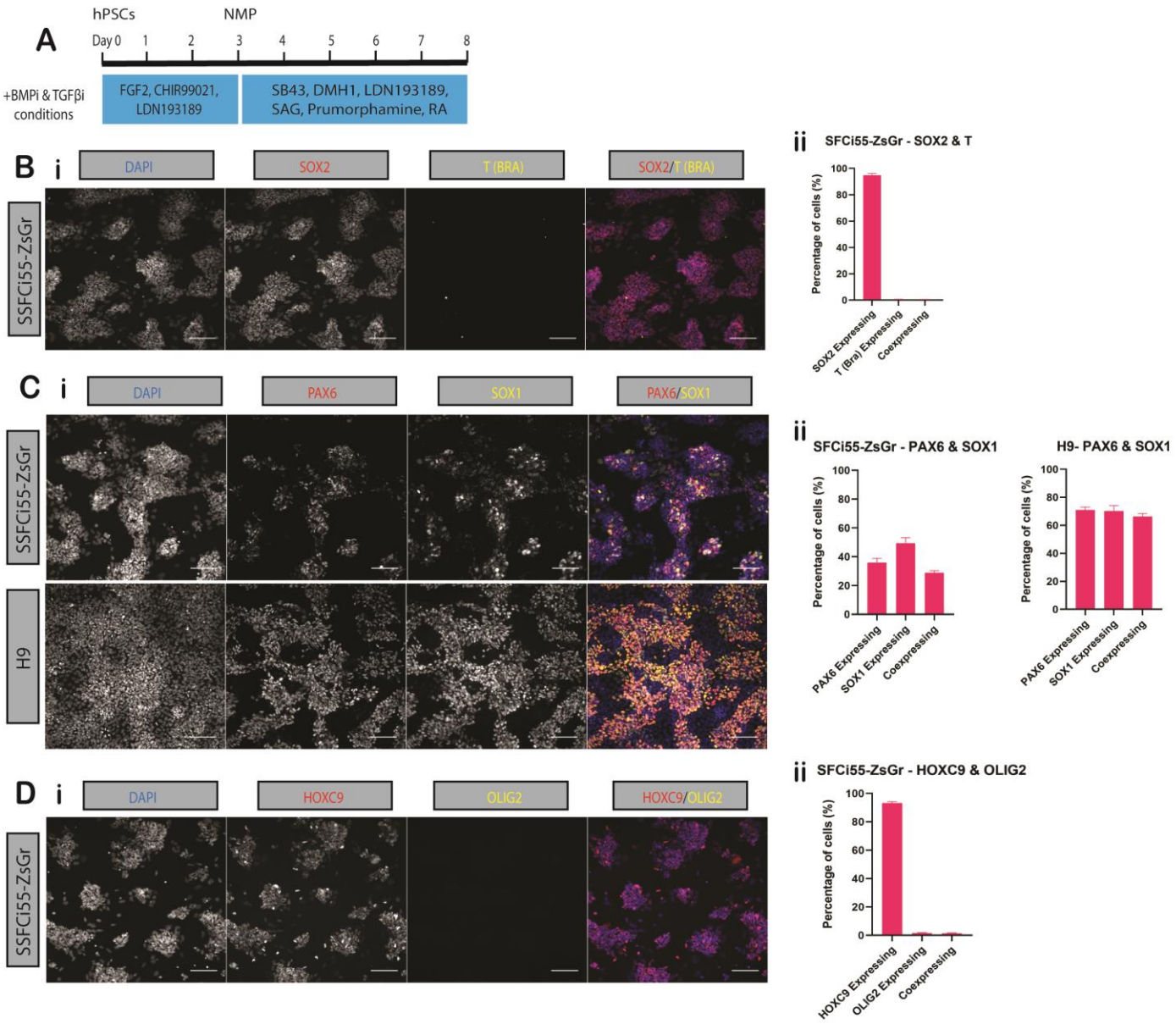


Figure 3.2 BMP and TGFβ inhibition promote neural identity downstream of NMPs. (A) A Schematic showing the differentiation protocol followed. Immunofluorescence showing the expression of **(B)** SOX2 and T(Brachyury) and **(C)** PAX6 & SOX1 and **(D)** HOXC9 and OLIG2 at Day 8 of differentiation. Scale bar, 100µm. **(ii)** Immunofluorescence quantification of the DAY 14 expressions (n=3).

In order to assess the effects of BMP and TGF-β antagonism on neural differentiation immunofluorescence was used to identify the efficiency of the expression of specific proteins. At Day 8 of differentiation, upon the exposure to BMP and TGFβ inhibition, the expression of T(Brachyury) was not maintained (**Figure 3.2B**). However, expression of SOX2, a neural progenitor marker, was observed in an average of 94% of cells in SFCi55-ZsGr-derived cultures (**Figure 3.2B**). In H9- derived cultures SOX2 was expressed in an average of 96% of cells (data not shown). This shows that the presence of BMP and TGFβ antagonism alongside Retinoic Acid signals is favourable to the promotion of a neural progenitor identity and is not compatible with a conversion to a mesodermal progenitor identity or maintenance of an NMP state.

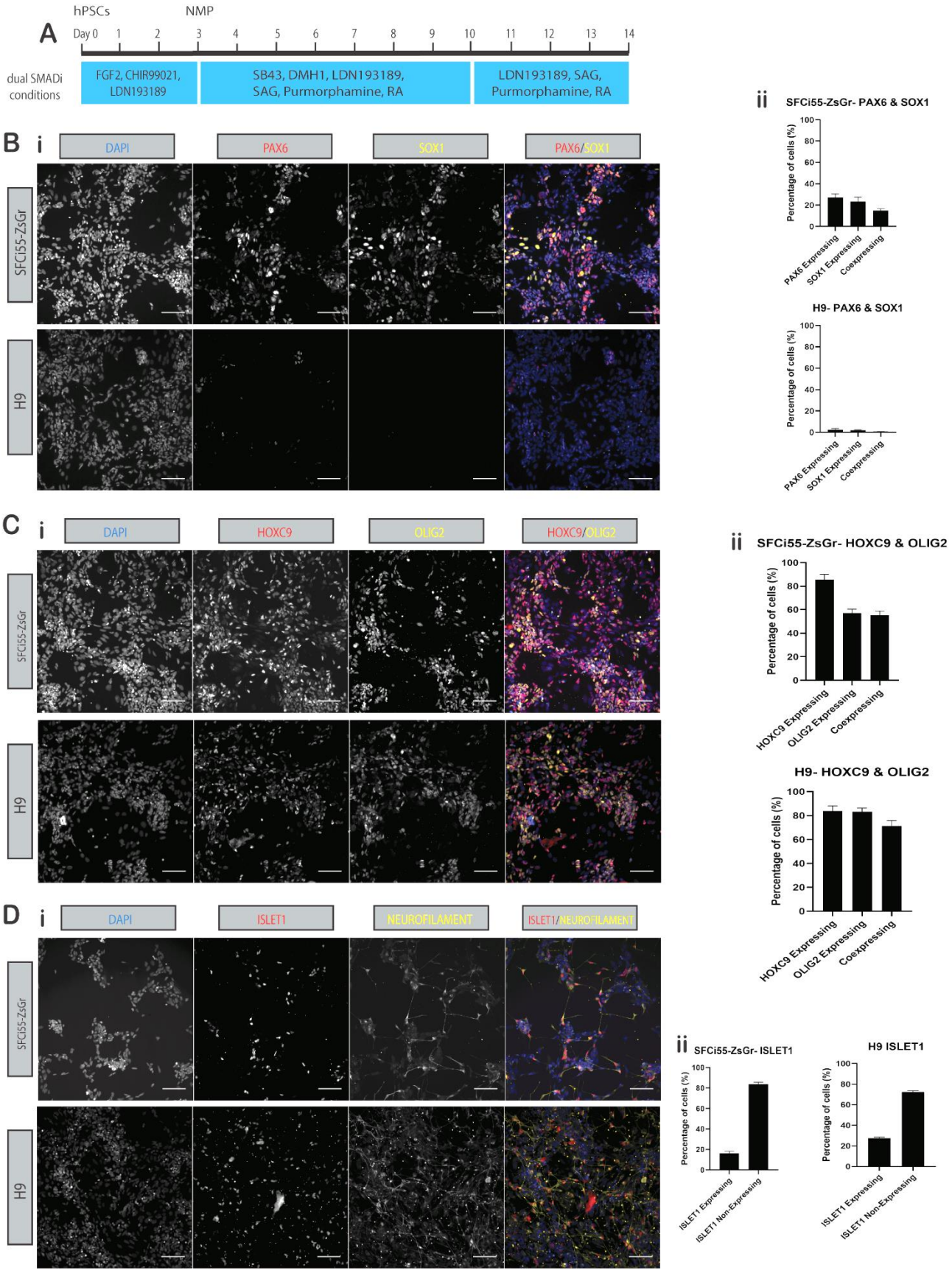
To further asses the formation of a, later stage, neural tube progenitor identity I examined the expression of the early neural determinant and pan-neural tube marker proteins; SOX1 and PAX6 (Walther and Gruss, 1991; Pevny *et al.*, 1998) by immunofluorescence. Upon exposure of NMPs to BMP and TGFβ antagonism in SFCi55-ZsGr -derived cultures, an average of 50% of cells express SOX1, 36% express PAX6 and 29% co-express PAX6 and SOX1 together (**Figure 3.2C**). Whilst in H9-derived cultures SOX1 is expressed in an average of 73% of cells, PAX6 is expressed in 71% and 66% of cells co-expressed SOX1 and PAX6 (**Figure 3.2C**). Interestingly, by Day 8 of differentiation there was no observed expression of the ventral motor neuron progenitor marker OLIG2 (**Figure 3.2D**). However, expression of the thoracic identity marker HOXC9 is expressed in an average of 92% of cells within SFCi55-ZsGr-derived cultures (**Figure 3.2D**) and an average of 90% of cells within H9 derived cultures (data not shown). This data shows that by Day 8 of differentiation there is a high yield generation of $PAX6^{+}/SOX1^{+}/HOXC9^{+}$ thoracic neural tube progenitors. This suggests that both BMP and TGFβ antagonism alongside Retinoic Acid play an important role within the promotion of a neural tube progenitor identity downstream of NMPs.

Recent protocols have shown that hPSC derived-posterior HOXC9+ neurectoderm, generated through similar exposure to BMP and TGF-β inhibition, can be further differentiated to generate motor neurons (Kumamaru *et al.*, 2018; Verrier, Davidson, Gierli, *et al.*, 2018). However, these protocols do not characterise the positional identity of their derived motor neurons. Therefore, I extended the differentiation of the $PAX6^{+}/SOX1^{+}/HOXC9^{+}$ neural tube progenitors (shown in **Figure 3.2**) with the aim of generating a population of posterior motor neurons. This protocol is denoted as the dual SMAD inhibition (dual SMADi) protocol (**Figure 3.3**). Within this protocol an initial NMP state was achieved by Day 3 of differentiation. As explained previously, from Day 3 to Day 10 of differentiation BMP and TGFβ signalling inhibitors, were supplemented alongside Retinoic Acid within the media to promote a thoracic neural tube progenitor identity. As the iPSC line appears to demonstrate a delayed differentiation compared to that in the hESC cell line the differentiation conditions were extended from Day 8 to Day 10 (shown in **Figure 3.3A**); with the intention that the iPSC line would then achieve similar levels of neural induction. Continued presence of a BMP pathway inhibitor (LDN193189) from Day 10 to Day 14 of differentiation was used to suppress the emergence of BMP-dependent dorsal neural tube lineages (Timmer, Wang and Niswander, 2002) whilst SB43 and DMH1 were removed at these time point as their role in inducing neural identity has been robustly achieved (as shown in **Figure 3.2**). Sonic-hedgehog agonists, Smoothened Agonist (SAG) and Purmorphamine, were also supplemented within the media from Day 3 to Day 14 as they have been shown to promote a ventral neural identity (Mackenzie W. Amoroso *et al.*, 2013).

At Day 14 of differentiation expression of PAX6 and SOX1 were detected by immunofluorescence. In SFCi55-ZsGr-derived cultures an average of 23% of cells expressed SOX1, 27% expressed PAX6 and 15% co-expressed both SOX1 and PAX6 (**Figure 3.3B**). This shows that at Day 14 of differentiation there is the retention of a population of neural tube progenitors. I next looked to identify the efficiency of the dual SMADi protocol at inducing a population of motor neuron progenitors by Day 14. To do this I observed the protein expression of the motor neuron progenitor marker; OLIG2 (Mizuguchi *et al.*, 2001; Novitch, Chen and Jessell, 2001; Lu *et al.*, 2002; Zhou and Anderson, 2002). In SFCi55-ZsGr-derived cell cultures an average of 57% of cells expressed OLIG2 (**Figure 3.3C**) whilst in the H9-derived cultures an average of 83% of cells expressed OLIG2. Importantly, these cell cultures

also appeared to retain a thoracic positional identity as on average 85% of cells in the SFCi55-ZsGr-derived cultures (**Figure 3.3C**) and 84% of cells in the H9-derived cultures expressed HOXC9. Due to the high percentages of cells expressing the motor neuron progenitor marker, OLIG2, I next wished to observe if the dual SMADi protocol could also generate a population of post-mitotic motor neurons by Day 14. An average of 16% of cells in the SFCi55-ZsGr derived cultures (**Figure 3.3D**) and 27% of cells in the H9-derived cultures expressed the post-mitotic marker ISLET1. Neuronal projections could also be observed in these cultures which were marked by Neurofilament staining during immunofluorescence (**Figure 3.3D**). These data show that by Day 14 in the dual SMADi protocol a high yield (an average >80% of cells) of motor neuron progenitors which express OLIG2 are generated. Within the cultures there is also the presence of some PAX6 and SOX1 expressing neural tube progenitors and a population of post-mitotic maturating neurons.

Figure 3.3 Characterising Day 14 of the dual SMADi protocol. (A) A Schematic showing the differentiation protocol followed. **(i)** Immunofluorescence showing the coexpression of **(B)** PAX6 and SOX1 and **(C)** HOXC9 and OLIG2 and **(D)** Neurofilament and ISL1 at Day 14 of differentiation. Scale bar, 100µm. **(ii)** Immunofluorescence quantification of the DAY 14 expressions (n=3).


A
hPSCs
NMP
Day 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
dual SMADi conditions
FGF2, CHIR99021, LDN193189
SB43, DMH1, LDN193189, SAG, Purmorphamine, RA
LDN193189, SAG, Purmorphamine, RA
B i
DAPI
PAX6
SOX1
PAX6/SOX1
SFCi55-ZsGr
H9
ii
SFCi55-ZsGr- PAX6 & SOX1
Percentage of cells (%)
PAX6 Expressing
SOX1 Expressing
Coexpressing
H9- PAX6 & SOX1
C i
DAPI
HOXC9
OLIG2
HOXC9/OLIG2
ii
SFCi55-ZsGr- HOXC9 & OLIG2
HOXC9 Expressing
OLIG2 Expressing
Coexpressing
H9- HOXC9 & OLIG2
D i
DAPI
ISLET1
NEUROFILAMENT
ISLET1/NEUROFILAMENT
ii
SFCi55-ZsGr- ISLET1
ISLET1 Expressing
ISLET1 Non-Expressing
H9 ISLET1

With a high yield of motor neuron progenitors obtained at Day 14 of differentiation the next step of the dual SMADi Protocol was to allow for motor neuron maturation. *In vivo* the role of Notch inhibition in promoting motor neuron maturation has been well documented (Marklund *et al.*, 2010; Rabadán *et al.*, 2012). The presence of neurotrophins has also been shown to be vitally important to promote survival, enhance proliferation and promote axonal outgrowth of motor neurons (Henderson *et al.*, 1994; Jungbluth, Koentges and Lumsden, 1997; Faravelli *et al.*, 2014; Cortés *et al.*, 2017). For these reasons, on Day 14 of differentiation, in the dual SMADi protocol, the cells were transferred to a neurotrophic media supplemented with Brain-Derived Neurotrophic Factor (BDNF), Glial cell line-Derived Neurotrophic Factor (GDNF), L-Ascorbic Acid and a Notch/ γ-secretase inhibitor, DAPT (**Figure 3.4A**).

To identify the efficiency of these conditions at inducing a mature motor neuron identity, I next observed the protein expressions within the cell cultures at Day 24 of differentiation. By immunofluorescence, in the SFCi55-ZsGr- derived cultures it was observed than an average of 64% of cells maintained the expression of OLIG2 (**Figure 3.4B, 3.4Fi**). However, there was a large increase in the expression of the post-mitotic motor neuron markers ISLET1 and HB9. ISLET1 was expressed by an average of 42% of cells in culture (**Figure 3.4C, 3.4Fii**) which is an increase of 26% of cells from the expression levels observed at Day 14. HB9, which had not been detected at Day 14 of differentiation, was expressed by an average of 14% of cells in culture at Day 24 (**Figure 3.4D, 3.4Fiii**). Cholinergic Acetyl Transferase was also detected by immunofluorescence (**Figure 3.4D**) with high levels of expression within the neuronal projections which were also marked by expression of Neurofilament (**Figure 3.4C**). This shows that the dual SMADi protocol conditions, from Day 14 to Day 24, were capable of promoting motor neuron maturation. However, at Day 24 of differentiation expression of HOXC9 was expressed at extremely low levels within the cell cultures (**Figure 3.4B**) suggesting that there is a loss in thoracic identity.

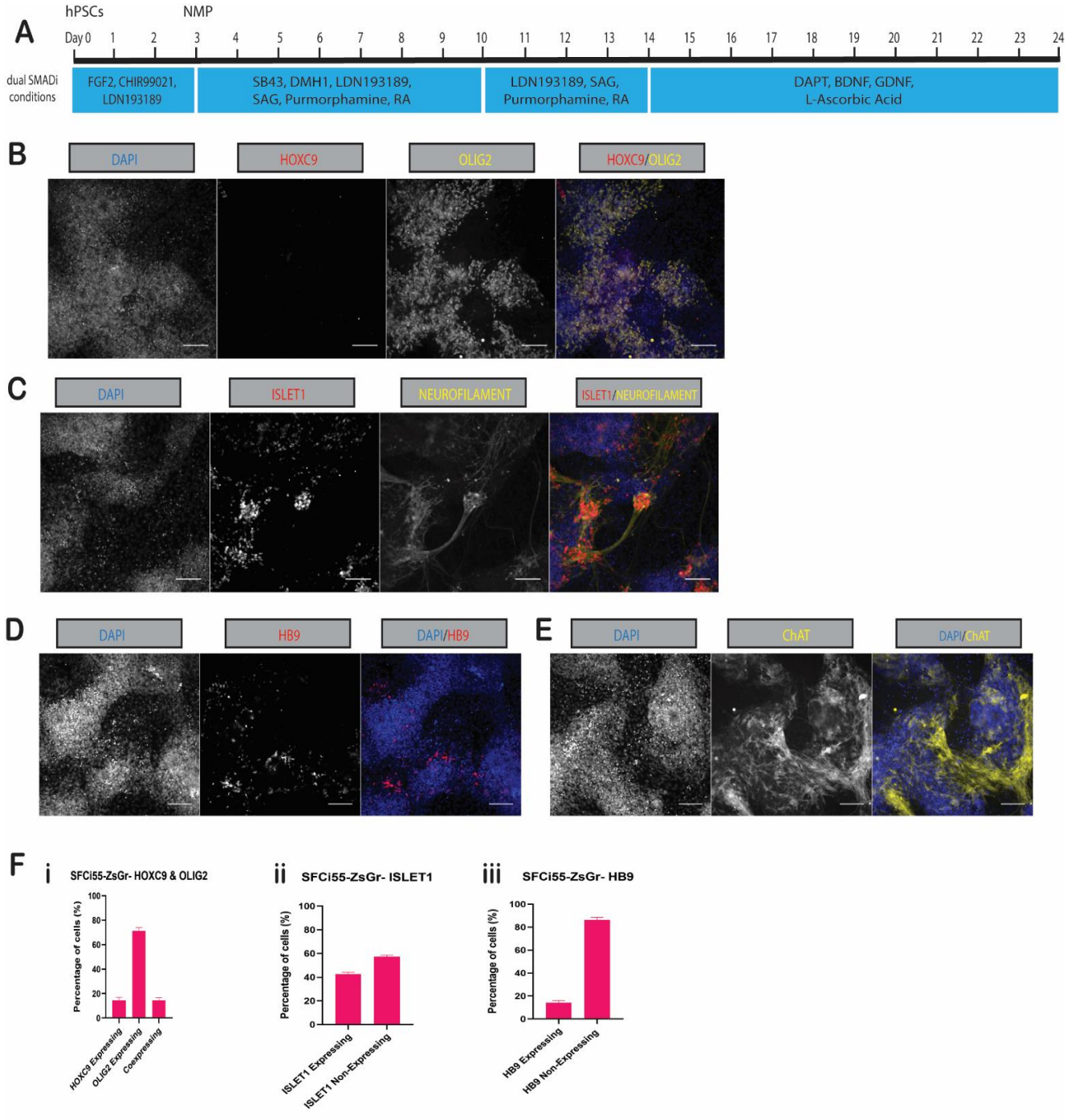


Figure 3.4. Characterising Day 24 of the dual SMADi protocol. (A) A Schematic showing the differentiation protocols followed. Immunofluorescence showing the expression of **(B)** HOXC9 and OLIG2 and **(C)** ISLET1 and Neurofilament and **(D)** HB9 and **(E)** ChAT at Day 14 of differentiation. Scale bar, 100µm. **(ii)** Immunofluorescence quantification of the DAY 14 expressions (n=3).

3.3.1.3 Discussion

The roles of BMP and TGF-β inhibition have been clearly defined within the generation of anterior neural identity (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou, Kelly and Melton, 1994; Sasai *et al.*, 1994; Zimmerman, De Jesús-Escobar and Harland, 1996; Fainsod *et al.*, 1997; Perea-Gomez *et al.*, 2002; Yamamoto *et al.*, 2004) whilst the addition of small molecule inhibitors of BMP and TGF-β signalling pathways have been widely used for anterior neurectoderm induction from hPSCs (Lee *et al.*, 2007; Li *et al.*, 2008; Neely *et al.*, 2012). Similarly, the inhibition of BMP signals within chick embryos has been shown to result in the posterior expansion of, the neural progenitor marker, SOX2 (Takemoto *et al.*, 2006). Here, I have shown that the presence of BMP and TGF-β antagonism also plays an important role within the neuralisation of *in vitro*-derived NMPs reflective of other recent *in vitro* differentiation findings (Verrier, Davidson, Gierli, *et al.*, 2018). Furthermore, I have clearly demonstrated that it is possible to generate motor neurons *in vitro* from hPSC-derived NMPs. The dual SMADi protocol utilises BMP and TGF-β signalling antagonism to promote an initial neural tube progenitor identity. Further exposure to Shh signals and BMP inhibition, allows for correct Dorsal/ventral patterning, to promote motor neuron identity. The ability of the dual SMADi protocol to generate mature motor neurons further shows the requirement for BMP and TGF-β inhibition for the neuralisation of NMPs (Kumamaru *et al.*, 2018; Verrier, Davidson, Gierli, *et al.*, 2018). However, further experiments which directly compare the presence or absence of BMP and TGF-β inhibitors after an NMP state would be required to definitively state that the inhibition of these signalling pathways promotes neural lineages over mesoderm and neural crest.

During the dual SMADi differentiation protocol there are clear cell line-specific differences between the SFCi55-ZsGr hiPSC line and the H9 hESC line. At Day 8 of differentiation the H9 cell line yields, on average, 26% more PAX6+/SOX1+ neural tube progenitors than in SFCi55-ZsGr. Similarly, at Day 14 the H9 cell line achieved higher yields of OLIG2+ motor neuron progenitors (on average 26% more) and ISLET1+ motor neurons (on average 11% more). The expression of HOXC9 are similar between the cell lines. These results could suggest a potential temporal difference in differential capabilities with the H9 hESC cell line differentiating at a faster rate. In order to observe this the differentiation of the SFCi55-ZsGr hiPSC line should be extended for longer time periods, under the same conditions, to

observe if it demonstrates the ability to achieve similar induction levels as the H9 line. Alternatively, multiple studies have demonstrated that iPSC cell lines can retain epigenetic modifications which hinder differential capabilities (Hu *et al.*, 2010; Kim *et al.*, 2010, 2011; Polo *et al.*, 2010; Bar-Nur *et al.*, 2011; Kajiwara *et al.*, 2012). Whilst other studies have discussed the variability within the endogenous BMP signalling levels between PSCs which are known to impact neuronal differentiation (Kattman *et al.*, 2011; Nostro *et al.*, 2011). Therefore, further manipulation of specific signalling pathways, such as BMP signalling, may be required to allow for enhanced neuralisation and dorsal/ventral patterning within the SFCi55-ZsGr hiPSC line.

Further manipulation of the dual SMADi protocol may also be required to enhance the yields of motor neurons. For example, at Day 14 of differentiation there is a yield of 57% OLIG2+ motor neuron progenitors within the SFCi55-ZsGr hiPSC line and 83% within the H9 cell line. Whilst these yields are higher than most existing protocols (Hynek Wichterle, Lieberam, *et al.*, 2002; Lippmann *et al.*, 2015b; Verrier, *et al.*, 2018) it suggests that there is generation of other cell types. Due to the presence of Shh agonists and BMP inhibition it is likely that the contaminating cell types are other ventral neuronal progenitor subtypes. This suggests that there is still room for optimisation within the protocol. Within the dual SMADi protocol NOTCH inhibitor is not added until Day 14 of differentiation to promote the maturation of motor neuron progenitors to mature motor neurons. However, NOTCH signalling has also been shown to be important in enhancing Shh signals within the ventral neural tube to enhance dorso-ventral patterning (Kong *et al.*, 2015; Stasiulewicz *et al.*, 2015). Therefore, suggesting that earlier manipulation of the NOTCH pathway could enhance the yield of motor neuron progenitors and eliminate contaminant phenotypes.

HOX expression patterns are essential for determining motor neuron positional identity and to determine functionality (Carpenter EM *et al.*, 1997; Dasen, Liu and Jessell, 2003a; Jung *et al.*, 2014). HOXC9 is classed as the master-regulator for thoracic motor neuron identity (Dasen, Liu and Jessell, 2003; Dasen *et al.*, 2008). Forced expression of HOXC9 can result in the ectopic transformation to a thoracic motor neuron identity (Dasen, Liu and Jessell, 2003; Jung *et al.*, 2010) whilst the downregulation of HOXC9 expression and expression of HOXC6 or HOXD10 can result in the transformation to a brachial or lumbosacral motor neuron

identity respectively (Jung *et al.*, 2014). Therefore, the lack of expression of HOXC9 within mature motor neurons obtained in the dual SMADi protocol, at Day 24, could suggest that they are not of a thoracic identity. As NMPs give rise to neural lineages of the entire post-cranial axis (Noemi Cambray and Wilson, 2007; Tzouanacou *et al.*, 2009b) it is possible that the motor neurons obtained represent either a brachial or lumbosacral identity. Notably, signals known to promote thoracic HOX gene activation (FGF and WNT) (Ikeya and Takada, 2001; Prinos *et al.*, 2001; Bel-Vialar, Itasaki and Krumlauf, 2002) and lumbosacral gene activation (GDF11) (Gaunt, George and Paul, 2013; Matsubara *et al.*, 2017; Aires *et al.*, 2019; Suh *et al.*, 2019) are not present for extended periods within the differentiation protocol suggesting that the motor neurons obtained likely represent a brachial identity. Furthermore, NMP-like cells have been shown to have the capability to derive $HOXC6^{+}/HOXC9^{-}$ brachial motor neurons upon downstream differentiation (Lippmann *et al.*, 2015b; Estevez-silva *et al.*, 2018b). This would suggest that whilst thoracic HOX expressions are induced by day 14 of differentiation, this thoracic HOX expression is not stable and results in an inability successfully derive thoracic HOXC9+ motor neurons. Chromatin remodelling and epigenetic changes exerted by CDX2 have already been shown to be important to allow stable thoracic HOX expressions (Mazzoni *et al.*, 2013a). FGF and WNT signals are required for the activation of CDX2. Therefore, suggesting that the production of thoracic identity motor neurons will depend upon longer lengths of FGF and WNT signals to promote CDX2-mediated epigenetic changes which will promote stable thoracic HOX expression.

Alternatively, it is important to note that, at thoracic levels, Medial Motor Column (MMC) motor neurons exist which are known to be HOX negative (Dasen *et al.*, 2005b, 2008). Therefore, suggesting that this protocol potentially generates high yields of thoracic MMC HOX^{-} motor neurons. Expression of ISLET1 and HB9 are highly detected within the derived motor neurons which are markers of MMC motor neurons. In future experiments, it will be interesting to use single cell sequencing to confirm both the axial and columnar identity of the derived motor neurons within this protocol. Examining for the expression of ISELT2 and LHX3 should also be used to clearly distinguish a MMC subtype from the other thoracic motor neuron columnar subtypes (Tsuchida *et al.*, 1994; Dasen *et al.*, 2005b). Although MMC motor neurons arise from thoracic regions they are not specific to thoracic domains as

they are present at all axial levels (Tsuchida *et al.*, 1994). Generation of MMC motor neurons which integrate within chick spinal cords has already been demonstrated (H Wichterle *et al.*, 2002; Peljto *et al.*, 2010b). However, there is still a requirement to generate HOXC9+ motor neurons which belong to the thoracic-specific Hypaxial motor column and Preganglionic motor columns.

3.3.2 Maintained FGF and WNT signals promote thoracic neural identity downstream of NMPs

3.3.2.1 Overview

In vitro- derived NMPs have the ability to be differentiated into $PAX6^{+}/SOX1^{+}/HOXC9^{+}$ thoracic neural tube progenitors, as I have shown in **Section 3.3.1**. However, downstream differentiation of these thoracic neural tube progenitors resulted in the generation of $HOXC9^{-}$ motor neurons by Day 24 of the dual SMADi protocol. Within the existing literature it has been clearly demonstrated that WNT and FGF signals are responsible for the induction of CDX2 and, in turn, thoracic HOX expression (Keenan, Sharrard and Isaacs, 2006; Nordström, Maier, Thomas M Jessell, *et al.*, 2006; Mazzoni *et al.*, 2013b; Mouilleau *et al.*, 2020). Interestingly, FGF and WNT signals have also been implicated within an early role for the restriction of NMPs to a neural identity (Bertrand, Médevielle and Pituello, 2000; Delfino-Machín, J. S. Lunn, *et al.*, 2005a; Takemoto *et al.*, 2006; Nishimura *et al.*, 2012). Therefore, I aimed to examine if the manipulation of FGF and WNT signals from Day 3 to Day 7 of differentiation, after an NMP intermediate state, resulted in;

1. a restriction to neural identity downstream of NMPs.
2. changes to the HOX expression and axial identity of the generated neural progenitors.

3.3.2.2 Interactions between FGF, WNT and RA signalling specify early neurogenesis

In order to correctly derive thoracic motor neurons, *in vitro*, there is a need to fully characterise the signalling pathways which are required to promote a neural restriction, resulting in the formation of a thoracic identity neurectoderm, after an NMP state. During axis extension, neuromesodermal progenitors give rise to an intermediary population of pre-neural progenitors which go on to from the posterior nervous system (Delfino-Machín, J Simon Lunn, *et al.*, 2005; Sasai, Kutejova and Briscoe, 2014). Multiple signalling pathways have been shown to be implicated within this specification process. The initial conversion of NMPs to pre-neural progenitors has been shown to be dependent on FGF signals (Bertrand, Médevielle and Pituello, 2000; Delfino-Machín, J. S. Lunn, *et al.*, 2005a). FGF and Wnt signals have also been shown to be important for the activation of the N1 enhancer for Sox2 (Takemoto *et al.*, 2006; Nishimura *et al.*, 2012). Determination of a neural tube progenitor from a pre-neural progenitor has then been associated with the actions of Retinoic Acid (Del Corral *et al.*, 2003; Sasai, Kutejova and Briscoe, 2014). BMP and TGF-β inhibition are also believed to be important in the conversion to a neural tube progenitor identity by promoting neuralisation and restricting mesodermal bias (Chambers *et al.*, 2009b; Verrier, Davidson, Gierli, *et al.*, 2018). Fittingly, I have demonstrated, in **Section 3.3.1**, that BMP and TGF-β alongside Retinoic Acid signals are important for the neural restriction of NMPs to a neural tube progenitor identity.

Despite current findings there is still a need to understand the effects of exposing *in vitro*-derived NMPs to different signals (FGF, Wnt, RA) in order to examine their effects on the formation of a robust neural identity downstream of NMPs. An increased understanding of the impact of these signals can then be used to allow more efficient generation of thoracic identity motor neurons.

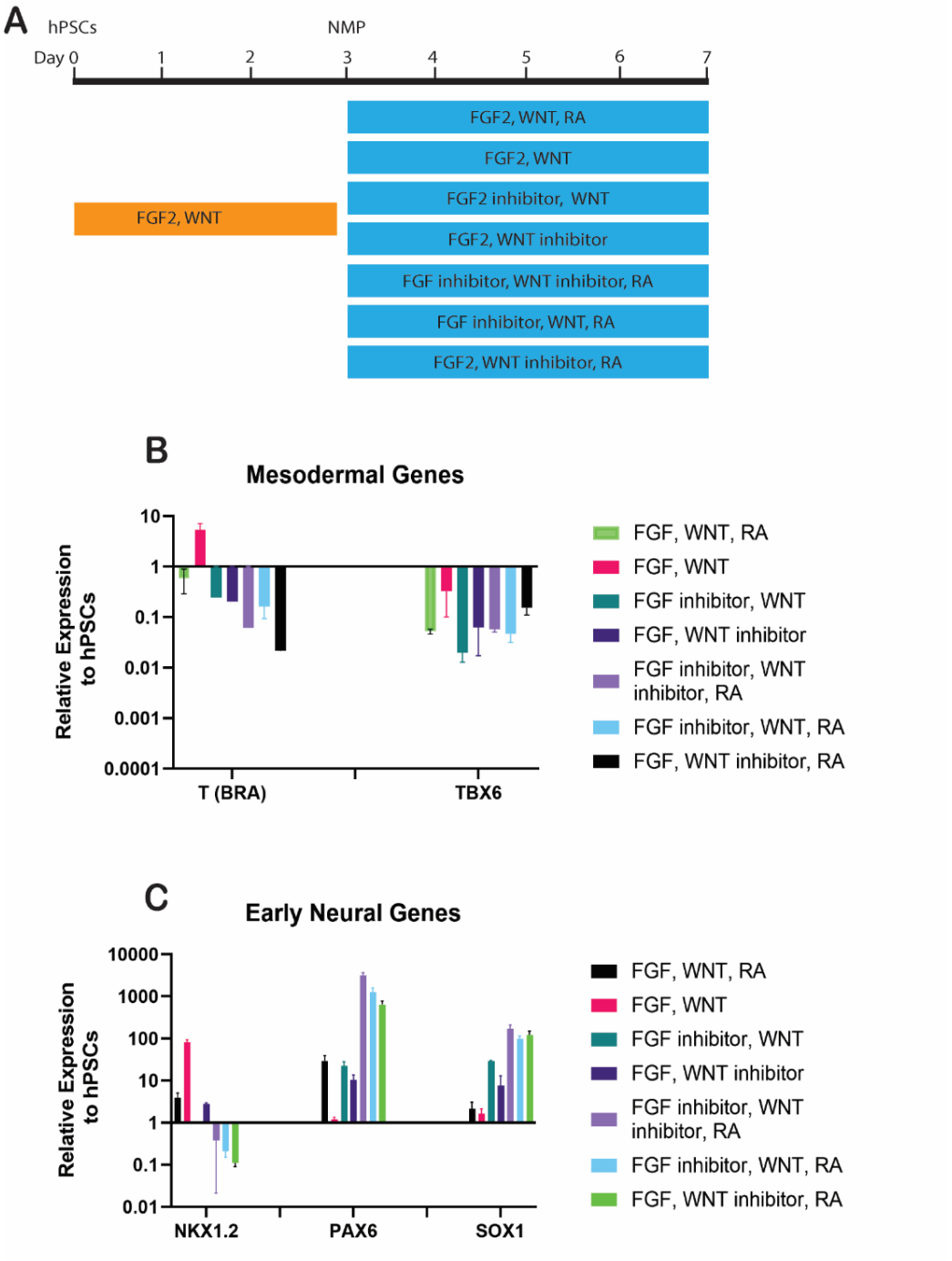


Figure 3.5 Impact of signalling pathways on fate decisions downstream of NMPs. A. A Schematic showing the different culture conditions which the *in vitro* derived NMPs were exposed to upon replating on Day 3 of differentiation. quantitative PCR data for the transcriptional expression of **B.** mesodermal markers and **C.** early neural markers within hPSC-derived NMPs exposed to the various culture conditions. Total RNA was extracted from the cells at Day 7 of differentiation. In all cases the qPCR data is shown as 2-DDCT values which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3).

Firstly, I aimed to examine the effects of culturing *in vitro*-derived NMPs with differing combinations of FGF, WNT and Retinoic Acid signals on cell fate decisions downstream of NMPs. To do this I exposed *in vitro*-derived NMPs, derived from a hiPSC cell line (SFCi55-ZsGr), to a range of culturing conditions. FGF and Wnt signals were manipulated by either; activation through the exposure to FGF2 and CHIR99021, a WNT pathway activator, or inhibition of endogenous signals by exposure to PD03, an FGF signalling inhibitor, and XAV939, a WNT signalling inhibitor. Application of Retinoic Acid under specific conditions was also used to mimic the *in vivo*-mesodermal derived signals (Niederreither *et al.*, 1997). On day 7 of differentiation, 4 days after a robust NMP state is formed, the expression of specific genes were examined which are markers of either a neural or mesodermal identity (**Figure 3.5**). As neuromesodermal progenitors are known to have the capacity to generate mesoderm, I screened for the expression of the early mesodermal determination genes *T(Brachyury)* and *TBX6* (Wilkinson, Bhatt and Herrmann, 1990; Chapman *et al.*, 1996; Chapman and Papaioannou, 1998; Uchiyama *et al.*, 2001). In all conditions observed, apart from one, there was no transcriptional expression of *T(Brachyury)* (**Figure 3.5B**) at Day 7. The only condition found to maintain minimal expression of *T(Brachyury)* was under the exposure to 100ng/ml FGF2 and WNT agonist, 3µM CHIR99021. However, the expression levels of *T(Brachyury)* under this condition are very low and extremely downregulated compared to the expression levels found in Day 3 NMPs. This condition, like all of the other conditions tested, does not result in the expression of *TBX6* (**Figure 3.5B**). As none of these conditions resulted in the expression of *TBX6*, by day 7, and there was a drastic decrease in the expression levels of *T(Brachyury)* it would suggest that these conditions are not compatible with the progression to a mesodermal state.

Next, I observed neural gene activation after the exposure of *in vitro*-derived NMPs to FGF, WNT and Retinoic Acid signals. *NKX1.2* is known to be expressed highly within NMPs and maintains a high expression within these trunk region-specific progenitors during axis elongation (Albors, Halley and Storey, 2018). Importantly, *NKX1.2* is also a marker of a pre-neural progenitor identity but is downregulated upon activation of a later-stage neural tube progenitor identity (Delfino-Machín, J. S. Lunn, *et al.*, 2005a; Sasai, Kutejova and Briscoe, 2014). On day 7, the expression of *NKX1.2* remains the most highly expressed under exposure to 100ng/ml of FGF and WNT activation (**Figure 3.5C**, red bar). The expression of

NKX1.2 under this condition is nearly 100-fold greater than under any of the other conditions tested (**Figure 3.5C**). Interestingly, of the other conditions tested there is not a high level of upregulation or downregulation in *NKX1.2* expressions relative to that in hPSCs. However, within these conditions there was enhanced upregulation of both *PAX6* and *SOX1* (**Figure 3.5C**). *PAX6* and *SOX1* are both expressed in early neurectoderm and act as determinants of neural fate (Walther and Gruss, 1991; Pevny *et al.*, 1998; Bylund *et al.*, 2003; Zhang *et al.*, 2010). Importantly, activation of *PAX6* and *SOX1* have previously been associated with a downregulation of *NKX1.2* as cells transition from a pre-neural progenitor identity to a neural tube progenitor identity (Sasai, Kutejova and Briscoe, 2014). This is reflected within my results as under exposure to 100ng/ml of FGF and WNT activation (**Figure 3.5C**, red bars) there is maintained *NKX1.2* expression and no upregulation of *PAX6* and *SOX1*; suggesting that these cells retain a pre-neural progenitor identity. However, under the other culturing conditions the NMPs appear to have transitioned through the pre-neural progenitor state and become neural tube progenitor fated, by downregulating *NKX1.2* expression and upregulating both *PAX6* and *SOX1*.

On day 7 of differentiation the conditions which resulted in the largest transcriptional upregulation of *PAX6* and *SOX1* where those conditions which included the addition of Retinoic Acid (**Figure 3.5C**, lilac, light blue and light green bars). Again, this is reflective of the understanding that Retinoic Acid is required to promote the upregulation of neural determinant genes (Del Corral *et al.*, 2003). The only condition containing Retinoic Acid which did not evoke as great an induction in *PAX6* and *SOX1* transcription was when Retinoic Acid was added alongside exogenous 100ng/ml FGF2 and WNT agonist; 3µM CHIR99021 (**Figure 3.5C**, black bar). This is likely due to the fact that Retinoic Acid is believed to activate the transition from pre-neural progenitor to a posterior neural tube progenitor by inhibiting Wnt and FGF signals and resulting in the downregulation of *NKX1.2* (Shum *et al.*, 1999; Del Corral *et al.*, 2003). Applying both exogenous FGF and WNT signals allows for the ability to counteract the inhibitory effects of Retinoic Acid (**Figure 3.5C**, black bar). Therefore, under the conditions where Retinoic Acid is supplemented alongside combinations of exogenous FGF or WNT inhibitors (**Figure 3.5C**, lilac, light blue and light green bars) there is less counteraction to the inhibitory effects of Retinoic Acid which results in a more rapid conversion to a *NKX2.1*^{low}/*PAX6*^{high}/*SOX1*^{high} neural tube progenitor identity.

Thus explaining why under the absence of Retinoic Acid but application of exogenous FGF and WNT signals (**Figure 3.5C**, red bar) a *NKX2.1*^{high}/*PAX6*^{low}/*SOX1*^{low} pre-neural progenitor identity was maintained.

Taken together, this novel *in vitro* differentiation data adds weight to existing arguments that activation of a *NKX1.2*^{high} pre-neural progenitor identity, downstream of NMPs, is dependent upon the presence of FGF and WNT signals. I also show that presence of WNT signals are compatible with the transition to a neural fate downstream of NMPs. Furthermore, I have demonstrated that the transition from a pre-neural progenitor identity is then dependent upon the presence of Retinoic Acid signals which can inhibit FGF and Wnt signals and promote a *NKX2.1*^{low}/*PAX6*^{high}/*SOX1*^{high} neural tube progenitor identity.

3.3.2.3 Discussion

Overall these findings have provided an *in vitro*-based insight on the signalling pathways which are required for neural induction downstream of NMPs. These data support existing findings, showing that a NKX1.2^{+} pre-neural progenitor identity is a necessary intermediate state in the eventual formation of a thoracic neurectoderm (Del Corral *et al.*, 2003; Delfino-Machín, J. S. Lunn, *et al.*, 2005b; Sasai, Kutejova and Briscoe, 2014). Similarly, I have shown, that alike *in vivo*, FGF signals are required during *in vitro* differentiation of NMPs to promote the formation of a NKX1.2^{+} pre-neural progenitor identity (Delfino-Machín, J. S. Lunn, *et al.*, 2005b; Sasai, Kutejova and Briscoe, 2014). Interestingly, Wnt signalling has long been implicated for its importance in mesodermal specification downstream of NMPs (Wittler *et al.*, 2007; D. A. Turner *et al.*, 2014; Gouti *et al.*, 2014a; Bouldin *et al.*, 2015; Tsakiridis and Wilson, 2015; Edri *et al.*, 2019; Diaz-Cuadros *et al.*, 2020). However, my results would suggest that Wnt signals are also permissive to the promotion of a neural identity. This is supported by the findings that the N1-enahncer of SOX2 is Wnt-responsive and that increased levels of Wnt signalling were not shown to be detrimental to neural development (Takemoto *et al.*, 2006; R. J. Garriock *et al.*, 2015; Edri *et al.*, 2019).

Transition from a pre-neural progenitor identity to a neural progenitor identity is dependent upon the downregulation of FGF signals and the loss of *NKX1.2* expressions (Sasai, Kutejova and Briscoe, 2014). Retinoic Acid has been shown to be important for the activation of *PAX6* and *SOX1* expression within neural tube progenitors (Del Corral *et al.*, 2003; Sasai, Kutejova and Briscoe, 2014). Retinoic Acid signals have also been shown to inhibit both FGF and Wnt activity (Sakai *et al.*, 2001; Cunningham, Brade, *et al.*, 2015). Here, I have shown that the presence of Retinoic Acid resulted in higher yields of *NKX2.1*^{low}/*PAX6*^{high}/*SOX1*^{high} neural tube progenitors in the presence of exogenous FGF and WNT inhibition (**Figure 3.5C**). Suggesting that *in vitro*, similar to *in vivo* findings, inhibition of FGF and WNT signals by Retinoic Acid is a requirement for the promotion of a *NKX2.1*^{low}/*PAX6*^{high}/*SOX1*^{high} neural tube progenitor identity. Importantly, the presence of Wnt signalling was able to counteract the inhibitory effects of Retinoic Acid and prevent the conversion to a neural tube progenitor identity suggesting that Wnt signals, alongside the already documented FGF signals, have an important role in maintaining a pre-neural progenitor identity.

3.3.2.4 FGF and WNT signals are essential for the maintenance of thoracic HOX during in vitro neural conversion

In **Section 3.3.1 and Section 3.3.2**, I have clearly demonstrated that *in vitro* hPSC-derived NMPs can be further differentiated to generate neural tube progenitors and mature motor neurons. However, the mature motor neurons obtained at Day 24 of differentiation within the dual SMADi protocol do not display a thoracic HOXC9^{+} identity (**Section 3.3.1**). CDX genes play an essential role in the regulation of thoracic HOX expression with multiple thoracic HOX genes containing CDX-specific binding sites within their enhancers (Taylor *et al.*, 1997; Charité *et al.*, 1998; Isaacs, Pownall and Slack, 1998; Gaunt, 2018). Importantly, CDX2 is highly expressed within NMPs and has been shown to be vitally important for the establishment of their posterior progenitor identity (van den Akker *et al.*, 2002; Amin *et al.*, 2016a). As CDX has been shown to directly bind and regulate thoracic HOX expressions it is important to understand which signals are responsible for the maintenance of CDX/thoracic HOX expression downstream of NMPs, as cells become neural-fated, in order to produce a protocol for the optimum generation of thoracic identity HOXC9+ motor neurons. In order to assess this Day 3 hPSC-derived NMPs were exposed to a range of differing culture conditions (**Figure 3.6A**) which in **Section 3.3.2.3** have been shown to promote both a pre-neural progenitor and a neural progenitor identity.

Within the existing literature is has been clearly demonstrated that WNT and FGF signals are responsible for the induction of *CDX2* and, in turn, thoracic HOX expression *in vivo* (Keenan, Sharrard and Isaacs, 2006; Nordström, Maier, Thomas M Jessell, *et al.*, 2006; Mazzoni *et al.*, 2013b). Initially, I examined the effects of FGF signals on the expression of CDX genes and thoracic HOX genes during neural conversion of NMPs after 7 days of differentiation. Both *CDX1* and *CDX2* showed the largest transcriptional upregulation under conditions with the presence of 100ng/ml FGF2 (**Figure 3.6B** black, red, purple and light green bars). When comparing the effects of FGF activation and FGF inhibition, provided by PD03 (a MEK/ERK inhibitor of the FGF signalling pathway), there was a clear >1000-fold increase in *CDX1* and *CDX2* expression in the presence of FGF2 (**Figure 3.6B** comparing black/ light blue bars and red/green bars). Similarly, when I examined the expression of the HOX6-10 paralogs at day 7 of culturing the highest transcriptional upregulation within the HOX6-10 paralogs was observed under conditions which included 100ng/ml FGF2 (**Figure 3.6C**, black, red, purple

and light green bars). Importantly, the expression of *HOXC9*, a key marker of thoracic neuronal identity, had a >1000-fold increased induction in the presence of FGF2 compared to in FGF inhibition (**Figure 3.6C** comparing black/ light blue bars and red/green bars). To further investigate the effects of FGF signalling during the neural conversion of NMPs I examined the day 7 protein expression of HOXC9 and CDX2 by immunofluorescence (**Figure 3.7**). Expression of HOXC9 and CDX2 were observed to be the highest in the conditions which contained 100ng/ml FGF2 and extremely low levels of expression were observed under the exposure to FGF inhibition (**Figure 3.7**). For example, when cultured alongside WNT signals the presence of FGF inhibition resulted in an average 31% reduction of CDX2, 37% reduction of HOXC9 and 38% reduction in CDX2 and HOXC9 co-expression (**Figure 3.7** comparing panel 1/ panel 6). These data clearly demonstrate that FGF signals are vital to maintain both CDX and thoracic HOX expression downstream of NMPs during *in vitro* neural differentiation. In the absence of FGF signals there is a resultant downregulation of CDX and thoracic HOX expressions. Importantly, FGF signals also display a similar effect on the transcriptional expression of other 5' located HOX genes.

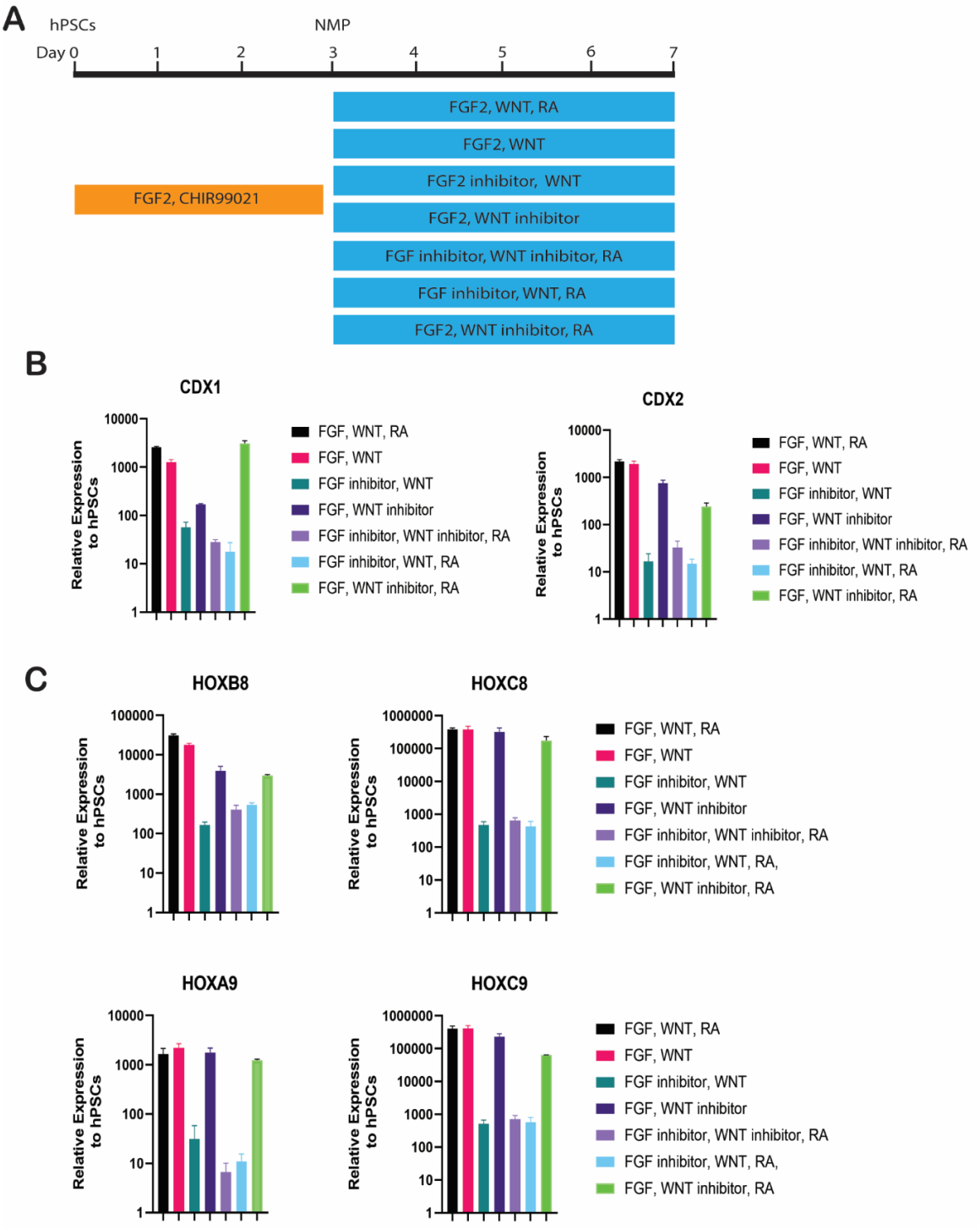


Figure 3.6 Impact of signalling pathways on HOX expression downstream of NMPs. A. A Schematic showing the different culture conditions which the in vitro derived NMPs were exposed to upon replating on Day 3 of differentiation. Quantitative PCR data showing the relative gene expression for **B.** CDX1 and CDX2 and **C.** HOX6-10 paralogous groups. Upon exposure to the different culture conditions total RNA was extracted from the cells at Day 7 of differentiation. In all cases the qPCR data is shown as log expression change calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3)

Next, I examined the CDX and thoracic HOX expressions upon the manipulation of WNT signals during the neural conversion of NMPs. Transcription of *CDX2* is significantly increased in the presence of WNT activation, by CHIR99021 a GSK3β pathway inhibitor which activates the WNT pathway, compared to when WNT signals are inhibited, by XAV939 (**Figure 3.6B** comparing red/purple bars and black/light green bars). WNT signals have a similar effect on *CDX1* transcription when NMPs are cultured with 100ng/ml FGF and a WNT agonist compared to 100ng/ml FGF2 and a WNT inhibitor (**Figure 3.6B** comparing red/purple bars). However, when NMPs are cultured with 100ng/ml FGF, 3μM CHIR99021 and RA compared to 100ng/ml FGF, 1μM XAV939 and RA there is no significant difference in *CDX1* expression (**Figure 3.6B** comparing black/light green bars). This suggests a potential role for Retinoic Acid in activating *CDX1* expression and compensating for the loss of WNT. WNT signals were also shown to have a role in the expression of the HOX6-10 paralogs (**Figure 3.6C**). However, the effect exerted on the transcription of the HOX6-10 paralogs by WNT is minimal compared to that of FGF. Whilst WNT activation significantly increased *HOXB8*, *HOXC8*, *HOXA9* and *HOXC9* expression, compared to when WNT signals were inhibited, it did so by less than a 10-fold expression change (**Figure 3.6C** comparing red/purple bars and black/light green bars). When initially observing the effects of WNT signals by immunofluorescence I found that the presence of WNT pathway activation or inhibition alongside FGF signals resulted in no significant difference in the expression of HOXC9 and CDX2 (**Figure 3.7** comparing panel 2 and panel 4). However, when NMPs were cultured in the presence of WNT activation in conjunction with 100ng/ml FGF2 and Retinoic Acid the levels of CDX2 expressing cells remained the same but there was an increase in the number of HOXC9 expressing cells compared to culturing in WNT inhibition, 100ng/ml FGF2 and Retinoic Acid (**Figure 3.7** comparing panel 1 and 7). Taken together these findings suggest that WNT signalling plays a role in maintaining the expression of CDX and thoracic identity HOX as NMPs undergo neural conversion. However, the action of WNT signals exert a minimal effect on the maintenance of CDX and thoracic HOX genes compared to that exerted by FGF signals.

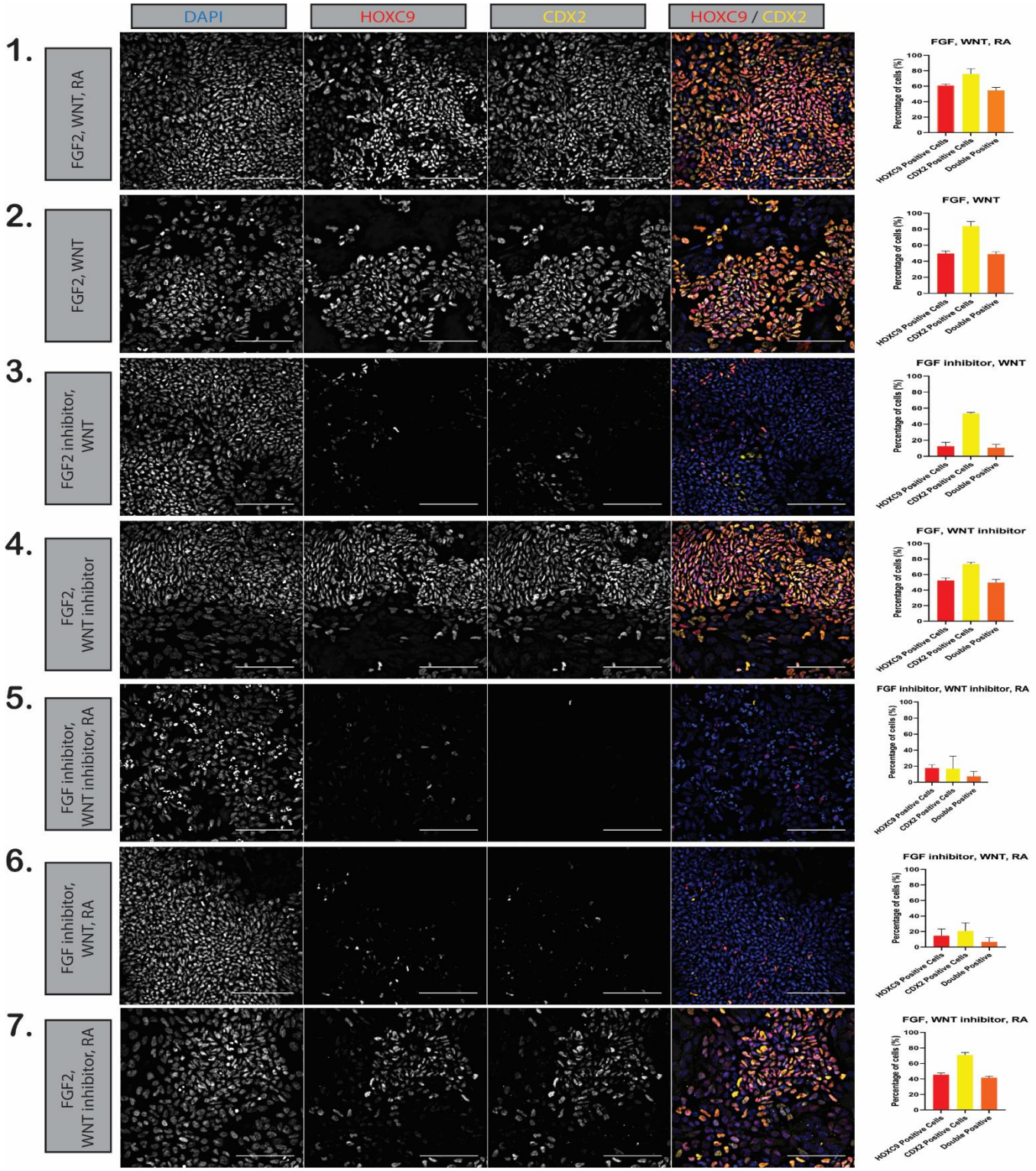


Figure 3.7 Impact of signalling pathways on HOXC9 & CDX2 expression downstream of NMPs. Immunofluorescence showing the expression of HOXC9 and CDX2 at Day 7 of differentiation, within the SFCi55-ZsGr cell line after exposure to different culture conditions. Scale bar, 100µm. Immunofluorescence quantification of the DAY 7 expression of HOXC9 and CDX2 are shown adjacent their respective images. (Error bars indicate SEM around the mean. n=3).

Lastly, I tested the effects of Retinoic Acid on CDX and thoracic HOX expressions during the *in vitro* neural conversion of NMPs. At Day 7 of differentiation, *CDX1* is significantly upregulated in the presence of Retinoic Acid compared to in its absence (**Figure 3.6B** comparing purple/green bars and red/black bars). However, expression of *CDX2* shows no significant difference in transcription in the presence or absence of Retinoic Acid (**Figure 3.6B**). Similarly, the presence or absence of Retinoic Acid resulted in no significant change in the expression of the *HOX6-10* paralogs (**Figure 3.6C**). Furthermore, when observing *HOXC9* and *CDX2* expression by immunofluorescence, at day 7, there was no significant difference in the levels of HOXC9 and CDX2 expressing cells in the absence or addition of Retinoic Acid (**Figure 3.7** comparing panels 4/7 and panels 2/1). These data suggest that Retinoic Acid has a role within the activation and maintenance of CDX1 expression during the neural conversion of NMPs. However, the presence of Retinoic Acid has no effect upon the activation and maintenance of CDX2 or thoracic HOX expressions.

The HOX1-5 paralogs are more commonly associated with hindbrain and cervical identity (Conlon and Rossant, 1992; Marshall *et al.*, 1992b; Niederreither *et al.*, 2000; Sakai *et al.*, 2001). However, I decided to examine the expression of the HOX1-5 paralogs to observe if a cervical/brachial identity was promoted under any of the NMP neural conversion conditions. Interestingly, the highest transcriptional upregulation of the *HOX1-5* paralogs was shown when NMPs were differentiated in the presence of Retinoic Acid (**Figure 3.8**, black, lilac, light blue and light green bars). In all conditions tested the presence of Retinoic Acid, compared to its absence, resulted in a >10-fold increase in expression of *HOXA1*, *HOXA2*, *HOXA4*, *HOXA5* and *HOXB5* (**Figure 3.8** comparing red/black bars and dark green/light blue bars and purple/light green bars). The presence of FGF signals or presence of FGF inhibition resulted in no statistical difference in the expression of the *HOX1-5* paralogs (**Figure 3.8** comparing red/dark green bars and black/light blue bars). This was similarly reflected in the presence or inhibition of WNT signals (**Figure 3.8** comparing red/purple bars and dark black/light green bars). These findings suggest that during neural conversion of NMPs, the activation of *HOX1-5* paralogs are primarily responsive to Retinoic Acid signals whilst FGF and WNT signals exert minimal effects on *HOX1-5* gene expression. Thus, suggesting that the *HOX1-5* paralog expressions observed in FGF and WNT conditions are due to endogenous Retinoic Acid signals.

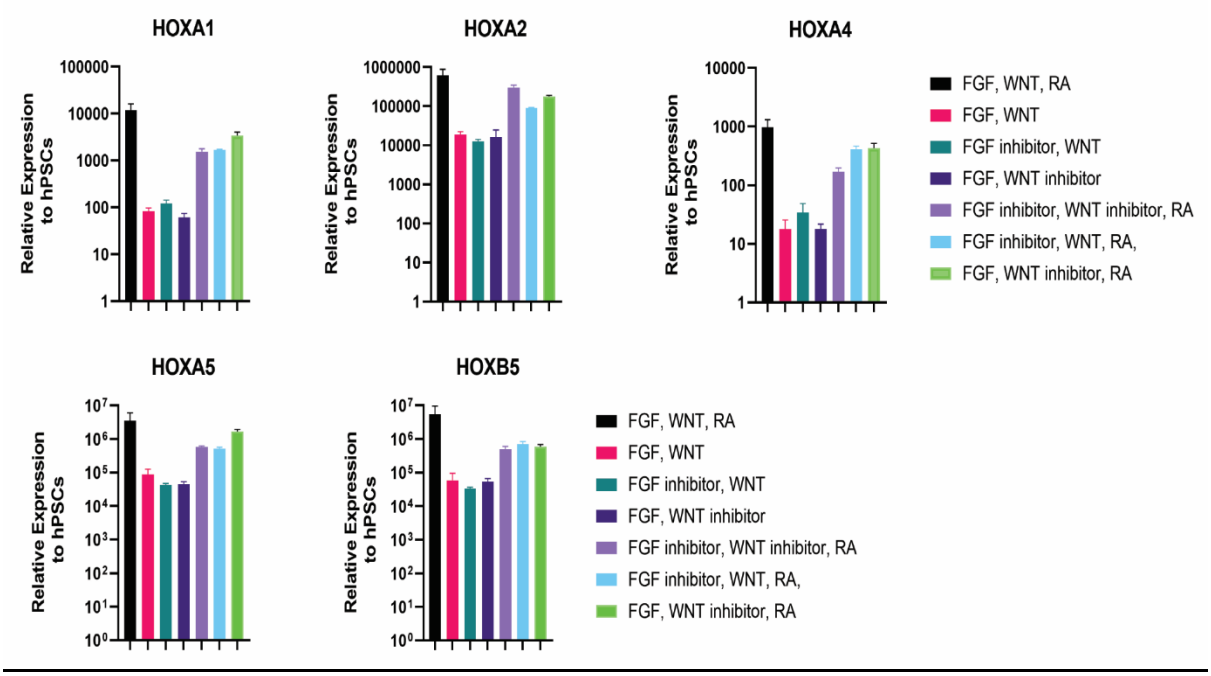


Figure 3.8 Impact of signalling pathways on HOX expression downstream of NMPs. Quantitative PCR data showing the relative gene expression for HOX1-5 paralogous groups. Upon exposure to the different culture conditions total RNA was extracted from the cells at Day 7 of differentiation. In all cases the qPCR data is shown as log expression change calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3)

3.3.2.5 Discussion

These data clearly demonstrate that FGF and WNT signals are essential for the maintenance of thoracic identity during the *in vitro* neural differentiation of NMPs. Supplementation of culturing conditions with exogenous FGF resulted in greater expression levels of CDX2 and reflectively these were the conditions which resulted in higher levels of HOXC9 expression. Reflective of the expanded CDX and HOX6-9 expressions upon treatment of chick embryos with FGF2/4 (Bel-Vialar, Itasaki and Krumlauf, 2002). Presence of WNT pathway activation also resulted in higher expression levels of CDX2 and HOXC9, albeit to a lower effect than that of FGF signals. These findings further support existing literature which have shown that CDX2-dependent activation of thoracic HOX is dependent upon synergistic FGF and WNT activation of CDX2 (Keenan, Sharrard and Isaacs, 2006; Nordström *et al.*, 2006; Mazzoni *et*

al., 2013; Amin *et al.*, 2016). Maintained activation of CDX2, by FGF and WNT signals, during the neural conversion of NMPs allows for the activation of thoracic HOX genes which have been shown to contain CDX-specific binding sites within their enhancers (Taylor *et al.*, 1997; Charité *et al.*, 1998; Isaacs, Pownall and Slack, 1998; Gaunt, 2018). This suggests that in future motor neuron differentiation protocols an extended period of FGF and WNT signals should be maintained to promote the maintenance of CDX and thoracic HOX expression which was not observed within the dual SMADi protocol.

Within my findings I have shown that Retinoic Acid signals had no impact on thoracic HOX expressions, during the neural conversion of NMPs, but did result in the enhanced expression of the 3' HOX1-5 paralogs. Similarly, multiple other studies have shown that HOX1-5 paralogs are Retinoic Acid-responsive whilst HOX5-9 paralogs are not (Bel-Vialar, Itasaki and Krumlauf, 2002; Houle, Sylvestre and Lohnes, 2003; Mazzoni *et al.*, 2013). HOX1-5 paralogs are dependent upon Retinoic Acid Response Element activity to allow chromatin re-modelling and gene activation (Allan *et al.*, 2001; Mazzoni *et al.*, 2013). Retinoic Acid has also been implicated within the early activation of CDX genes. Indeed, I identified that during the neural conversion of NMPs CDX1 expression was enhanced upon the exposure to Retinoic Acid. Furthermore, CDX1 has been shown to be a direct target of Retinoic Acid (Houle *et al.*, 2000; Bel-Vialar, Itasaki and Krumlauf, 2002), even being suggested to play a role in the Retinoic Acid-dependent activation of HOX1-5 paralogs (Houle *et al.*, 2000). However, I have also shown that activation of CDX2 is not enhanced upon exposure to Retinoic Acid signals. Instead, CDX2 activation is shown to be dependent upon WNT and FGF signals. Furthermore, the expression of CDX2 has a role in the repression of Retinoic Acid-dependent hindbrain and cervical identity genes (Metzis *et al.*, 2018). Suggesting that Retinoic Acid signals and CDX2 act antagonistically to pattern the neural axis.

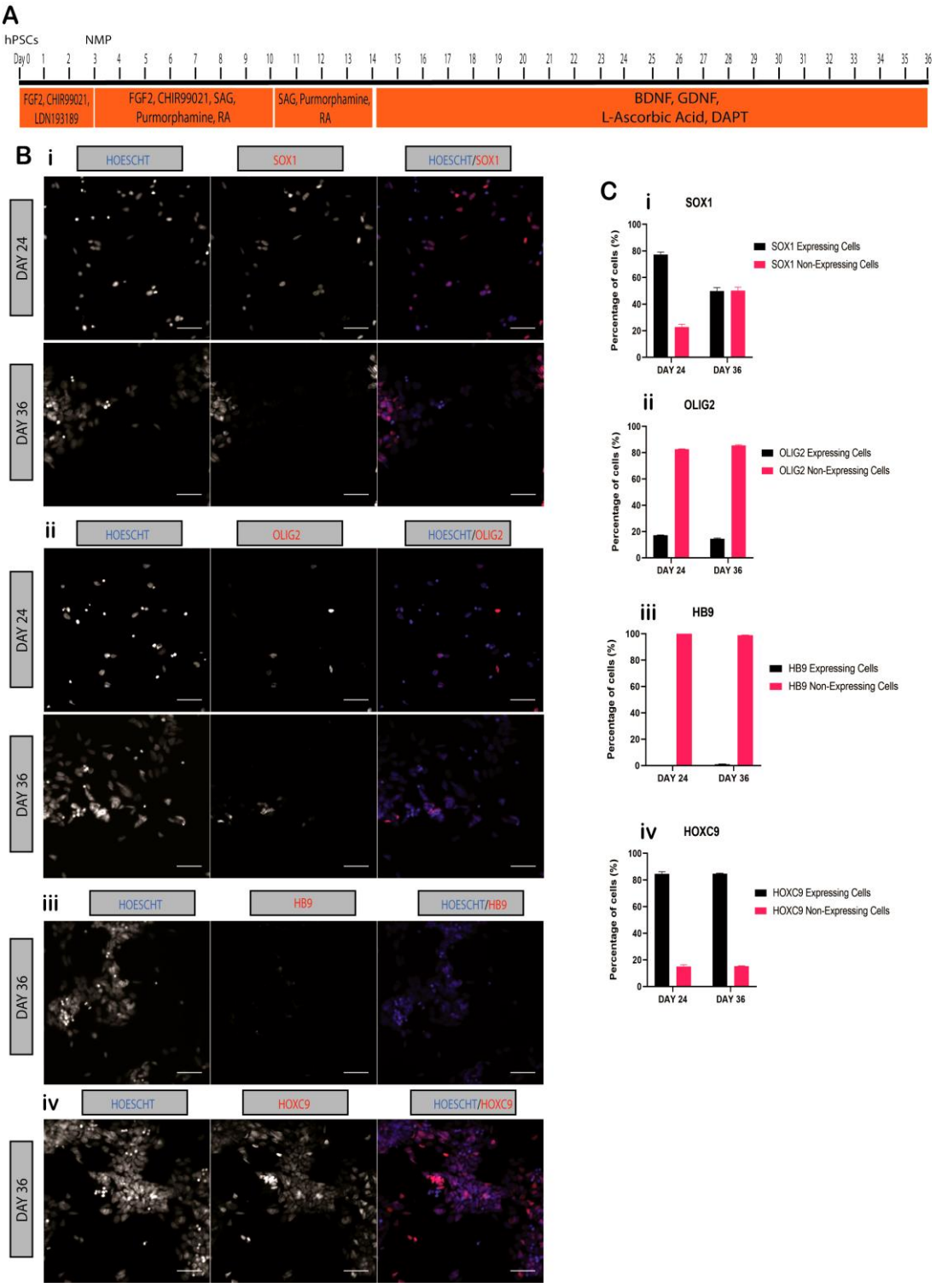
3.3.3 Exploring the effects of FGF-WNT and RA signals on posterior motor neuron generation

3.3.3.1 FGF, WNT & RA protocol induces neural tube progenitors of a dorsal character

In **Section 3.3.2**, I showed that FGF, WNT and Retinoic Acid signals are capable of promoting a population of *NKX1.2*$^{-}$/*SOX1*$^{+}$/*PAX6*$^{+}$ neural tube progenitors downstream of NMPs. Furthermore, I have demonstrated that the maintenance of a thoracic HOXC9+ identity during neural differentiation is dependent on continued presence of FGF and WNT signals. Therefore, taking these findings into account, I devised a hPSC differentiation protocol for the generation of thoracic identity motor neurons termed the FGF, WNT & RA protocol (**Figure 3.9A**). After an initial standard 3 day NMP differentiation, NMPs were exposed, for a further 7 days, to FGF, WNT and Retinoic Acid signals to promote a neural tube progenitor identity whilst also maintaining thoracic HOX expressions. Shh agonists, SAG and Purmorphamine, were also supplemented in the media between Day 3 and Day 14 to promote a ventral neural identity (Mackenzie W. Amoroso *et al.*, 2013). After Day 14 of differentiation the Shh agonists were retained in the media to continually promote ventral identity and motor neuron specification whilst the presence of BDNF and L-Ascorbic Acid were used to promote neuronal maturation (Henderson *et al.*, 1994; Jungbluth, Koentges and Lumsden, 1997; Faravelli *et al.*, 2014; Cortés *et al.*, 2017).

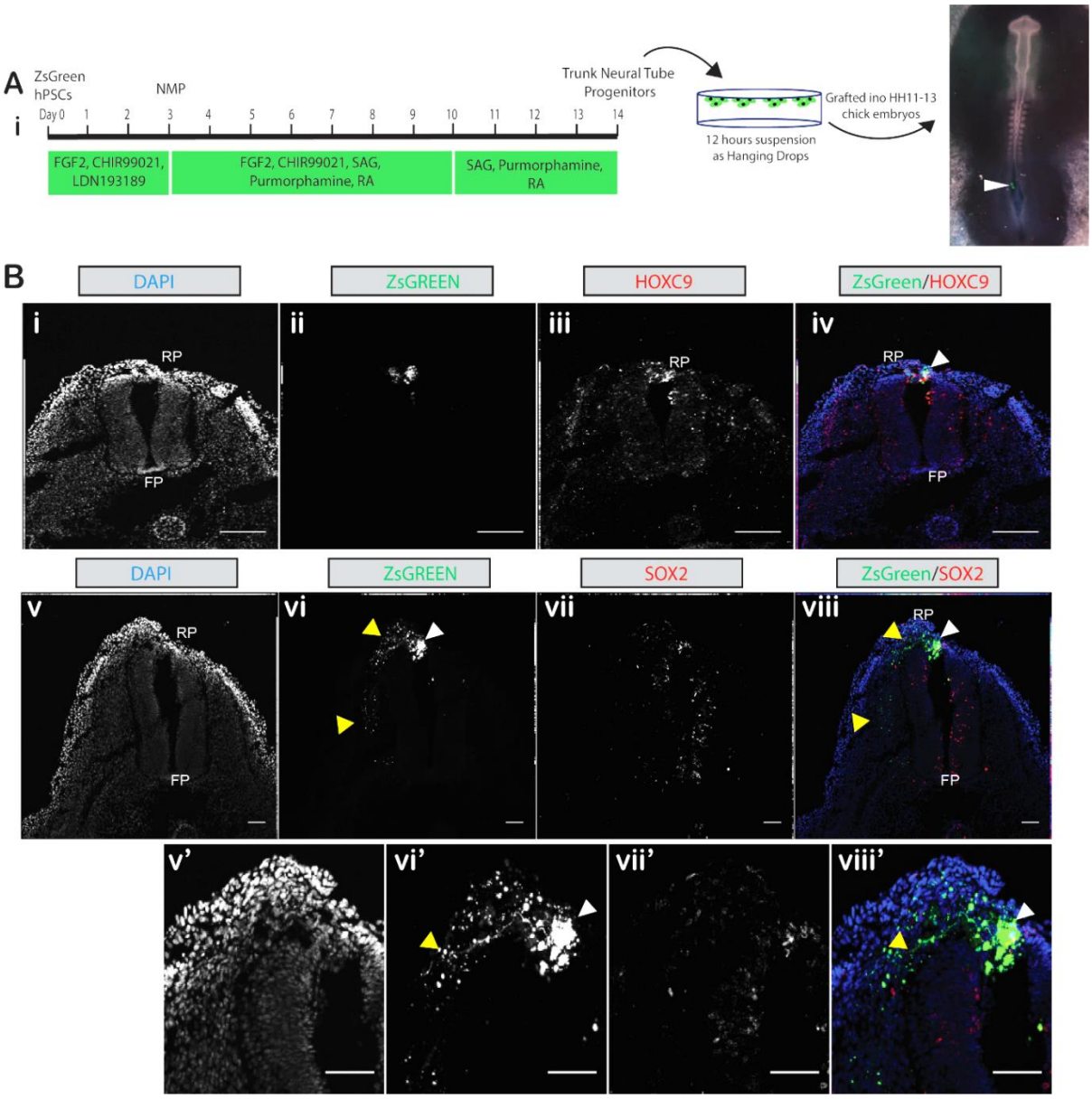
On Day 24 of differentiation immunofluorescence was used to observe the protein expression for markers of motor neuron identity within the SFCi55-ZsGr derived cells. SOX1, which is predominantly a marker of neurogenesis and early neural states, was widely expressed at Day 24 with expression in an average of 77% of cells in culture (**Figures 3.9Bi, Ci**). Average expression of OLIG2, a motor neuron progenitor marker, was observed in only 17% of cells (**Figures 3.9Bii, Cii**) whilst HB9, a marker of mature motor neurons, was not expressed by any of the cells in culture (**Figures 3.9Biii, Ciii**). These data suggest that at Day 24 of differentiation the cultures still contain a high percentage of neural progenitors and very few motor neuron progenitors. Therefore, the differentiation protocol was prolonged for a further 12 days to identify if a prolonged period of exposure to Shh agonists and neurotrophins could promote a higher induction of motor neurons. Suitably, at Day 36 an average of 38% of cells expressed SOX1, which was a reduction of 39% compared to Day 24

(**Figures 3.9Bi, Ci**). However, the reduction in neural progenitor marker expression was not reflected by an upregulation in motor neuron identity proteins. OLIG2 was only expressed in an average of 14% of cells in culture (**Figures 3.9Bii, Cii**) whilst HB9 was only expressed by 1% of cells (**Figures 3.9Biii, Ciii**). At both Day 24 and Day 36 of differentiation HOXC9 was expressed in an average of 85% and 84% of cells respectively (**Figures 3.9Biv, Civ**) showing that the cultures have obtained a robust thoracic HOX identity.



3.9 FGF, WNT & RA protocol fails to induce thoracic identity MNs. A. A Schematic showing the differentiation protocol followed. **B.** Immunofluorescence showing the expression of (i) SOX1, (ii) OLIG2, (iii) HB9 and (iv) HOXC9 at Day 24 and Day 36 of differentiation with the SFCi55-ZsGr cell line. Scale bar, 50µm. **C.** Quantification of the Day 24 and Day 36 expression (n=3).

To further define the potential of the cells generated from the FGF, WNT & RA differentiation protocol, cells were grafted into chick embryo neural tubes to examine their ability to integrate within the host tissue. A technique commonly manipulated within the literature to observe if *in vitro*-derived cells have the potential to function characteristically in an *in vivo* environment (H Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006a; Peljto *et al.*, 2010b; Son *et al.*, 2011). SFCi55-ZsGr hiPSCs, a cell line which constitutively expresses ZsGREEN (Lopez-Yrigoyen *et al.*, 2018), were differentiated under the FGF, WNT & RA protocol conditions until Day 14, by which time the cells were believed to have acquired a thoracic ventral neural tube progenitor identity (**Figure 3.10A**). At Day 14 of differentiation the cells were suspended, for 12 hours, in hanging drops. Cell clumps were selected and grafted into thoracic neural tube locations in HH Stage 10-11 embryos (as shown in **Figure 3.10A**). Embryos were incubated for a further 3 days post-grafting and then transverse sections of the neural tubes were collected to observe the integration of the ZsGREEN expressing cells. A total of 20 embryos received a thoracic neural tube graft. Of those 20 embryos, 11 embryos survived until 3 days post-grafting (11/20, 55%). Within these 11 embryos, under whole mount observations, it was identified that 8 embryos contained ZsGREEN expressing cells (8/11, 72.72% of those that survived). After collecting neural tube transverse sections, it was found that in all 8 of these embryos observed (8/8, 100%) the ZsGREEN expressing neural tube progenitors had integrated into the dorsal neural tube and roof plate regions (as shown in **Figure 3.10B**). In 3 embryos (3/8, 37.5% of those containing ZSGREEN+ cells) the ZsGREEN+ cells appeared to contribute to a dorsal migratory stream (**Figure 3.10B**) which is more characteristic of neural crest than neural tube progenitors (Bronner-Fraser and Fraser, 1988; Collazo, Bronner-Fraser and Fraser S E., 1993). All grafted cells retained a thoracic identity which can be observed by the continued expression of HOXC9 within the ZsGREEN expressing cells (**Figure 3.10Biii**). This thoracic identity was matched by the surrounding host tissue which also expressed HOXC9.



3.10 Neural Progenitors incorporate into the dorsal neural tube and display dorsal character. A. A Schematic showing the protocol followed to generate neural progenitors from the FGF, WNT & RA protocol at Day 14**.** SFCi55-ZsGr hiPSCs were used which constituently expresses ZsGREEN. **B.** Transverse cross sections of HH Stage 22 embryos. **(i, v)** DAPI labels all cell nuclei. **(ii, vi)** hPSC- derived grafted cells are ZsGREEN+. Immunofluorescence showing the expression of HOXC9 **(Biii)** and SOX2 **(Bvii). (iv, viii)** Neural Tube Progenitors integrated within the Roof Plate and Dorsal Neural Tube, shown by white arrowhead, and also contributed to a migratory stream; shown by yellow arrowhead. (**v'-viii'**) Higher magnification images shown. (Scale 100 µm, n=11) Abbreviations; RP= Roof Plate, FP= Floor Plate, DRG= Dorsal Root Ganglia.

3.3.3.2 Discussion

Overall, these results demonstrate that the presence of FGF, WNT and Retinoic Acid signals are compatible with the formation of high yield populations of $SOX1^+$ neural progenitors downstream of NMPs. Further displaying that WNT signals are compatible with a transition to a neural fate (Takemoto *et al.*, 2006; R. J. Garriock *et al.*, 2015; Edri *et al.*, 2019). Importantly, the expression of HOXC9 is maintained throughout the differentiation until Day 36. This shows that, as shown in **Section 3.3.2**, the presence of FGF and WNT signals are important for the maintenance of thoracic HOX identity. However, the presence of FGF, WNT and Retinoic Acid signals in the presence of Sonic Hedgehog agonists are not optimal for the generation of motor neuron identity downstream of NMPs. This protocol results in very low levels of $OLIG2^+$ motor neuron progenitors by Day 36 of differentiation with no presence of mature motor neurons.

Furthermore, engraftment of Day 14 neural tube progenitors into chick spinal cords resulted in dorsal neural tube integration and contribution to migratory streams similar to that of neural crest (Bronner-Fraser and Fraser, 1988; Collazo, Bronner-Fraser and Fraser S E., 1993). Recently, we have shown that *in vitro*-derived NMPs are capable of generating trunk neural crest (Frith *et al.*, 2018). This suggests that the conditions within the FGF, WNT and Retinoic Acid protocol are not capable of promoting an efficient neural conversion like that observed under the presence of TGF-β and BMP inhibition within the dual SMADi protocol. These data suggest that at Day 14 of differentiation, due to the failure to efficiently promote neuralisation of the NMP population, there is the production of a mixed population of neural crest and neural progenitors.

The FGF, WNT and RA protocol generates low yield induction of OLIG2+ motor neuron progenitors and integration of the Day 14 progenitors within the dorsal neural tube. These data suggest that the neural progenitors obtained have a strong dorsal neural tube character. This is fitting with previous work which has shown that neural patterning of NMPs results in large scale upregulation of dorsal neural tube genes and a dorsal default character (Denham *et al.*, 2015; Frith *et al.*, 2018a; Verrier, Davidson, Gierlin, *et al.*, 2018; Hackland *et al.*, 2019). Patterning of the dorsal neural tube is dependent upon dorsal-emanating BMP signals (Liem, Tremml and Jessell, 1997; Lee, Mendelsohn and Jessell, 1998). Therefore,

inhibition of BMP signals may be required to prevent induction of dorsal contaminating phenotypes and promote a ventral neural tube identity during future motor neuron differentiation protocols.

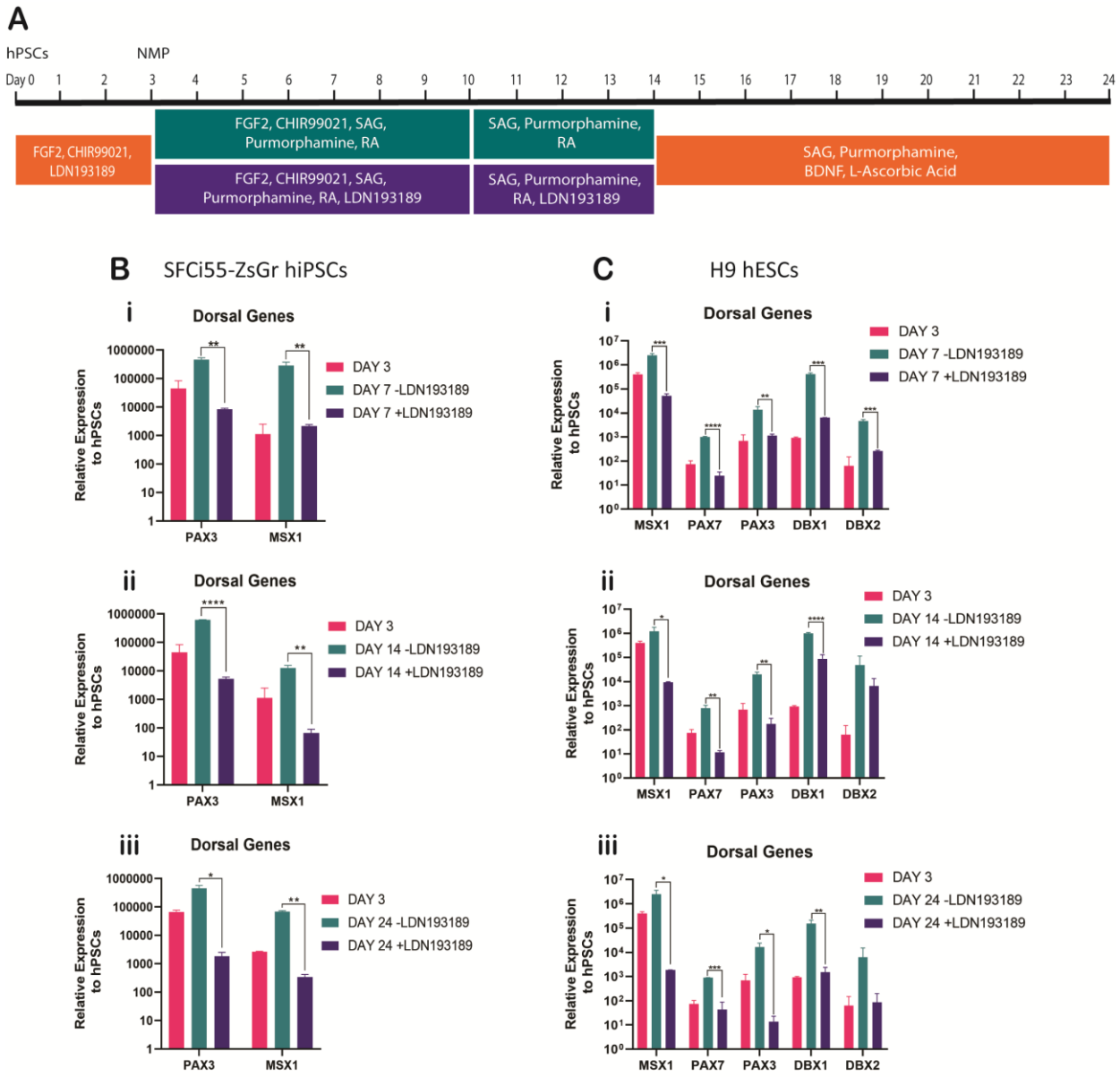
3.3.4 Investigating the effects of BMP signals on posterior motor neuron differentiation

3.3.4.1 BMP inhibition attenuates a dorsal identity during neural differentiation of NMPs

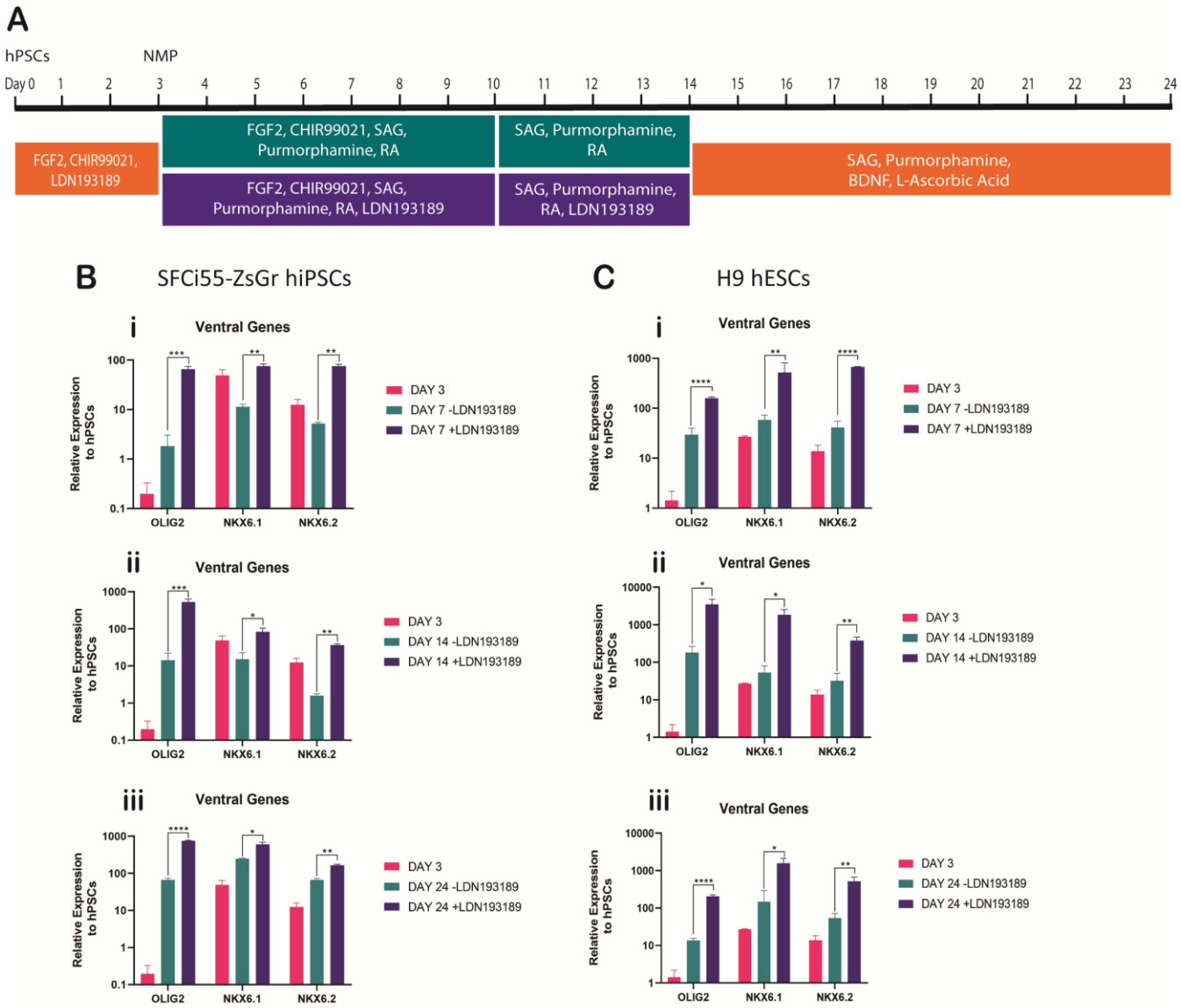
As shown previously, in **Section 3.3.3**, the FGF, WNT and Retinoic Acid protocol are capable of directing the generation of HOXC9^{+}/SOX1^{+} thoracic neural tube progenitors from NMPs. However, these progenitors appear to display a dorsal neural tube character and integrate within chick dorsal neural tubes. Therefore, there is a need to optimise the FGF, WNT and Retinoic Acid protocol to prevent dorsal neural tube contaminants, promote ventral neural tube identity and increase the yield of thoracic identity motor neurons. A key signalling pathway which I aimed to manipulate was the BMP pathway. BMP transcripts are highly expressed within the dorsal neural tube and roof plate (Basler *et al.*, 1993; Liem *et al.*, 1995; Lee, Mendelsohn and Jessell, 1998; Lee and Jessell, 1999) where they have been shown to be important for the induction of dorsal character and neuronal progenitors (Liem, Tremml and Jessell, 1997; Lee, Mendelsohn and Jessell, 1998; Pierani *et al.*, 1999; Timmer, Wang and Niswander, 2002). Therefore, I aimed to inhibit BMP signalling, using LDN193189 (a BMP pathway inhibitor), to test if this could result in reduced dorsal contaminants and enhance motor neuron progenitor induction in our cultures. Initially, the effects of BMP inhibition were tested in two different hPSC lines (SFCi55-ZsGr hiPSCs and H9 hESCs).

The BMP inhibitor, LDN193189, was continuously supplemented into the FGF, WNT and Retinoic Acid differentiation protocol media from the Day 3 NMP state until Day 14 of differentiation (**Figure 3.11**, green condition). Cells were isolated on Day 3, Day 7, Day 14 and Day 24 of differentiation to observe gene expression changes. In the SFCi55-ZsGr cell line, on Day 7 and Day 14 of differentiation there was a >10-fold significant reduction in *PAX3* and a >100-fold significant reduction in *MSX1* expression in the presence of BMP inhibition (**Figure 3.11Bi, 3.11Bii**). At Day 24 of differentiation the presence of BMP inhibition resulted in a >100-fold significant reduction in *PAX3* and *MSX1* expression (**Figure 3.11Biii**). Similarly, in the H9 cell line the presence of BMP inhibition resulted in a reduction of dorsal gene expression. On Day 7 of differentiation the transcription of *MSX1*, *PAX7*, *PAX3*, *DBX1* and *DBX2* was >10-fold significantly reduced, for all genes, in the presence of BMP inhibition (**Figure 3.11Ci**). At Day 14 of differentiation BMP inhibition resulted in the; >10-fold reduction in *PAX7* and *DBX1* and >100-fold reduction in *MSX1* and *PAX3* expression

(**Figure 3.11Cii**). Gene expression reduction was even greater at Day 24 with a >1000-fold reduction in *MSX1* and *PAX3*, >100-fold reduction in *DBX1* and a >10-fold significant reduction in *PAX7* (**Figure 3.11Ciii**). This demonstrates that the FGF, WNT and Retinoic Acid protocol, without the presence of BMP inhibition, was producing high levels of dorsal gene expressing contaminants which can be reduced via the exposure to BMP inhibition.



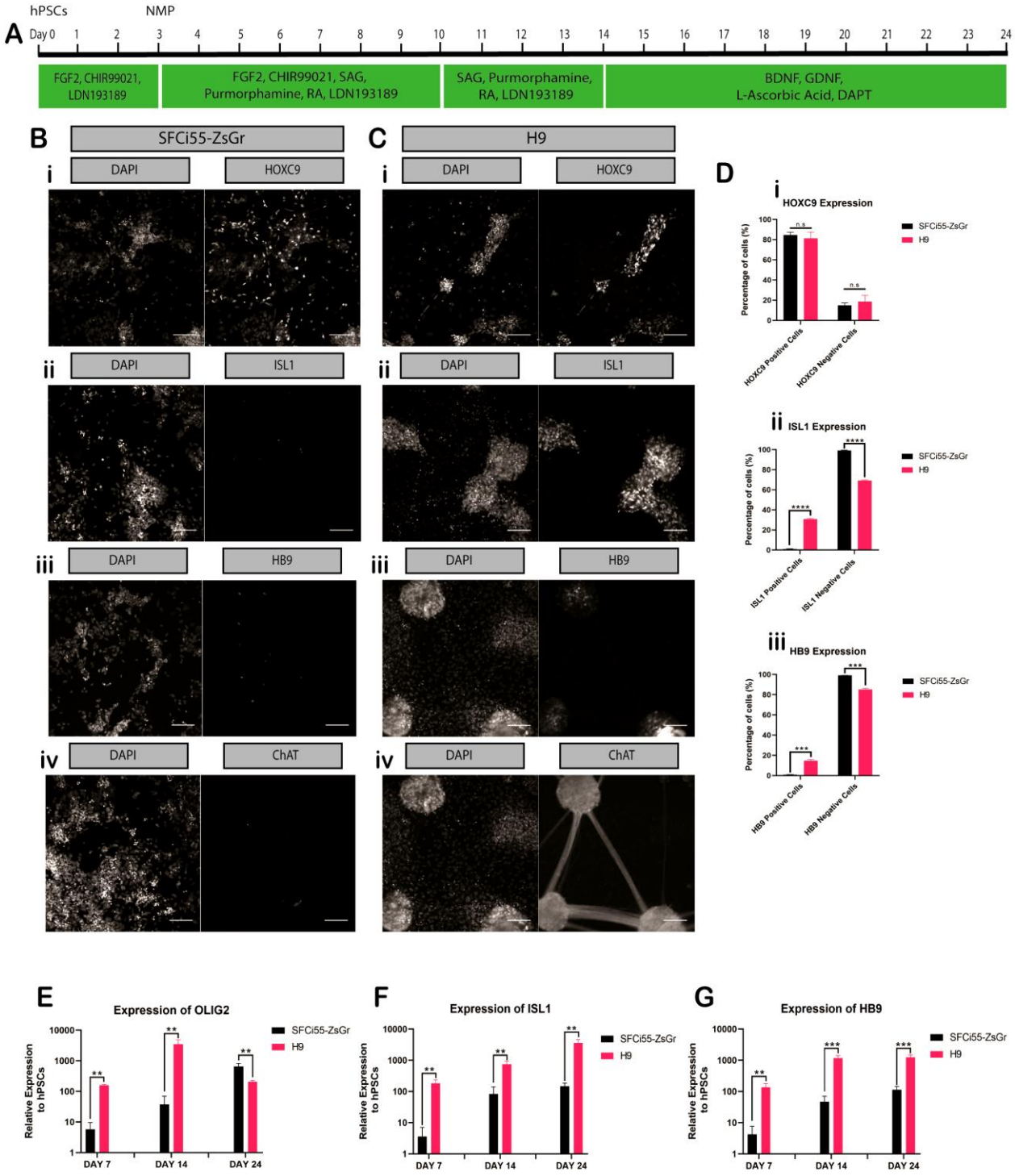
3.11 BMP inhibition inhibits dorsal character in neural progenitors A. A Schematic showing the differentiation protocol followed. **B & C.** quantitative PCR data from SFCi55-ZsGr **(B)** and H9 **(C)** differentiated neural progenitors in the absence or presence of a BMP inhibitor. Total RNA was extracted from the cells at DAY 7 **(i)**, DAY 14 **(ii)** and DAY 24 **(iii)** of differentiation. Data is shown as log fold expression changes which are calculated relative to GAPDH and significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. Unpaired t-test *=P<0.05; **=P<0.01, ***=P<0.001, ****=P<0.0001.)



3.12 BMP inhibition promotes ventral character in neural progenitors A. A Schematic showing the differentiation protocol followed. **B & C.** quantitative PCR data from SFCi55-ZsGr **(B)** and H9 **(C)** differentiated neural progenitors in the absence or presence of a BMP inhibitor. Total RNA was extracted from the cells at DAY 7 **(i)**, DAY 14 **(ii)** and DAY 24 **(iii)** of differentiation. Data is shown as log fold expression changes which are calculated relative to GAPDH and significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. Unpaired t-test *=P<0.05; **=P<0.01, ***=P<0.001, ****=P<0.0001.)

3.3.4.2 Addition of BMP inhibition to the FGF, WNT and Retinoic Acid protocol promotes ventral identity

Due to the presence of BMP inhibition resulting in a reduced gene expression for markers of dorsal neural tube identity, I next looked to see if this coincided with an upregulation in genes which are expressed in the ventral neural tube; predominantly genes of a motor neuron identity. SFCi55-ZsGr hiPSC and H9 hESC cell lines were both exposed to the FGF, WNT and Retinoic Acid differentiation protocol conditions with or without the presence of BMP inhibition, 100nM LDN193189, supplemented form Day 3 to Day 14 (**Figure 3.12A**). Within the SFCi55-ZsGr cell line, on day 7 of differentiation, there was a significant upregulation in the expression of *OLIG2*, *NKX6*.1 and *NKX6*.2 in response to BMP inhibition (**Figure 3.12bi**). Expression of both *OLIG2* and *NKX6.2* was increased by >10-fold. Again at Day 14 of differentiation both *OLIG2* and *NKX6*.2 were upregulated by a >10-fold increase whilst NKX6.1 was still significantly upregulated but experienced a <10-fold increase (**Figure 3.12bii**). At Day 24 of differentiation the presence of BMP inhibition still resulted in a significant upregulation of *OLIG2*, by a >10-fold increase, and *NKX6.1* and *NKX6.2*, by <10-fold increase (**Figure 3.12biii**). Differentiation of the H9 cell line in the presence of BMP inhibition also resulted in significant upregulation of ventral neural tube genes. BMP inhibition resulted in a <10-fold increase in induction of *OLIG2* and *NKX6.1* and a >10-fold increase in induction of *NKX6.2* at Day 7 of differentiation (**Figure 3.12Ci**). On Day 14 and Day 24 of differentiation OLIG2, *NKX6.1* and *NKX6.2* experienced a >10-fold significant increase in expression in response to BMP inhibition (**Figures 3.12Cii, 3.12Ciii**). Together, these data suggest that BMP inhibition promotes a ventral neural tube identity.



3.13 Addition of BMP inhibition is only efficient at inducing MNs in specific cell lines. A. Schematic showing the differentiation protocol followed. Immunofluorescence within the SFCi55-ZsGr **(B)** and H9 **(C)** cell lines showing the expression of (i) HOXC9, (ii) Islet1, (iii) HB9 and (iv) Cholinergic Acetyl Transferase at Day 24 of differentiation. Scale bar, 100µm. **D.** Comparison between the quantified IF expression between the two cell lines (n=4, Unpaired t-test *=P<0.05; **=P<0.01, ***=P<0.001, ****=P<0.0001). **E, F & G**. quantitative PCR data from H9 (red bars) and SFCi55-ZsGr (black bars) differentiated neural progenitors showing the relative expression of (E) OLIG2, (F) ISL1 and (G) HB9. Total RNA was extracted from the cells at DAY 7, DAY 14 and DAY 24 of differentiation. (Error bars indicate SEM around the mean. n=3, Unpaired t-test *=P<0.05; **=P<0.01, ***=P<0.001, ****=P<0.0001)

3.3.4.3 Variable effect of BMP inhibition on yields of NMP-derived motor neurons

The addition of a BMP inhibitor from Day 3 to Day 14 of differentiation, within the FGF, WNT and Retinoic Acid protocol reduced dorsal neural tube marker expression and enhanced the expression of ventral neural tube markers in multiple cell lines. I next wished to observe if this enhanced induction of ventral neural tube genes resulted in a higher yield of motor neurons. Therefore, I continued the differentiation protocol, with BMP inhibition until Day 24 and these culture conditions are termed the BMP inhibition protocol (**Figure 3.13A**). At Day 24 of differentiation immunofluorescence was used to observe the expression of specific proteins which are known to be markers of thoracic motor neuron identity. Differentiation of the SFCi55-Z hiPSC cell line resulted in 84% of the cells in culture expressing HOXC9 at Day 24 (**Figures 3.13Bi, 3.13Di**). Similarly, in the H9 hESC cell line 82% of cells expressed HOXC9 (**Figures 3.13Ci, 3.13Di**). This shows that both cell lines generated cultures of a thoracic identity at Day 24 of differentiation. Differentiation of the H9 cell line to Day 24 also resulted in expression of motor neuron maturity markers; an average of 30% of cells expressing ISLET1 (**Figures 3.13Cii, 3.13Dii**) and 14% expressing HB9 (**Figures 3.13Ciii, 3.13Diii**). Expression of Cholinergic Acetyltransferase was also strongly detected by immunofluorescence at Day 24 (**Figure 3.13Civ**). However, in SFCi55-ZsGr-derived cultures, at Day 24, an average of less than 1% of cells expressed ISLET1 (**Figures 3.13Bii, 3.13Dii**) and HB9 (**Figures 3.13Biii, 3.13Diii**) and expression of Cholinergic Acetyltransferase was not detected (**Figure 3.13Biv**). This significant difference in protein expression of post-mitotic motor neuron markers suggests that the protocol was efficient in generating motor neurons in the H9 cell line but was not effective in the SFCi55-ZsGr cell line.

To further observe the differences in differential capabilities of the different cell lines I also observed the changes in gene expression over the time-course of the differentiation. Expression of, the motor neuron progenitor marker, *OLIG2* was induced at a >10-fold higher induction and a >100-fold higher induction at Day 7 and Day 14 respectively in the H9 cell line compared to SFCi55-ZsGr (**Figure 3.13E**). In the H9 cell line there is a >10-fold downregulation of *OLIG2* from Day 14 to Day 24 of differentiation. This downregulation of *OLIG2* in the H9 cell line results in a significantly higher expression of *OLIG2* in the SFCi55-ZsGr cell line at Day 24 (**Figure 3.13E**). Reflecting this downregulation of *OLIG2* at Day 24, in the H9 cell line, there is a gradual increase of expression in the post-mitotic motor neuron

markers, *ISLET1* and *HB9* (**Figure 3.13F and Figure 3.13G** respectively). The expression of *ISLET1* and *HB9* is always significantly greater in the H9 cell line compared to SFCi55-ZsGr. At Day 24, the induction of *ISLET1* and *HB9* is >10-fold more highly expressed in the H9 cell line. This suggests that during differentiation the H9 cell line achieves a motor neuron progenitor state earlier, around Day 14, and then begins to generate post-mitotic motor neurons which the SFCi55-ZsGr cell line fail to generate by Day 24 of differentiation.

3.3.4.4 Discussion

Here, I have shown that the presence of BMP inhibition, during neural tube progenitor differentiation, is essential to decrease the levels of dorsal neural tube phenotypes. Previous findings have shown that the secretion of BMPs are essential for the induction of dorsal interneuron character (Basler *et al.*, 1993; Liem *et al.*, 1995; Liem, Tremml and Jessell, 1997; Lee, Mendelsohn and Jessell, 1998). Therefore, my findings further show the importance of BMP signalling in promoting dorsal character.

Furthermore, I have shown that the presence of BMP inhibition also results in enhanced levels of ventral neural tube progenitor identity with increased induction of *OLIG2*, *NKX6.1* and *NKX6.2*. Ventral neural tube identity has clearly been shown to be induced in response to Shh activity (Liem *et al.*, 1995; Ericson *et al.*, 1996; Briscoe *et al.*, 2000). During the patterning of the dorso-ventral axis of the neural tube it has been shown that BMP and Shh signals act antagonistically. With BMP signals inhibiting ventral neural tube progenitor induction (Basler *et al.*, 1993) and Shh signals inhibiting dorsal interneuron progenitor induction (Yamada *et al.*, 1991; Liem *et al.*, 1995). Furthermore, it has also been suggested that the actions of endogenous BMP inhibitors which are expressed by adjacent mesoderm to the neural tube (Graham and Lumsden, 1996; Connolly, Patel and Cooke, 1997; K Dale *et al.*, 1999) may play an important role in reducing BMP signals and enhancing the response of ventral neural cells to Shh signals to promote ventral identity (Liem, Jessell and Briscoe, 2000). This is further displayed as Shh signals are required to repress BMP-dependent Class I proteins, such as *MSX1*, *PAX3* and *PAX7*, in order to allow correct ventral neural tube patterning by the Class II proteins (Price *et al.*, 1992; Ericson, Rashbass, *et al.*, 1997; Qiu *et al.*, 1998; Briscoe *et al.*, 1999). Together, my results display the requirement for BMP

inhibition in the differentiation of an *in vitro* NMP-derived neural progenitor identity in order to allow the repression of dorsal character and induction of ventral identity and more specifically motor neuron identity. These results also suggest a role for BMP inhibitors in the repression of Class II proteins alongside the repression exerted by Shh signals.

Here, I have shown that the BMP inhibition protocol resulted in cell line specific induction yields of thoracic motor neurons from hPSC-derived NMPs. Within the H9 hESC cell line over 30% of cells express ISLET1 and 14% express HB9, demonstrating a population of mature motor neurons. However, in the SFCi55-ZsGr there is less than 1% expression of these post-mitotic motor neuron markers showing a failure to induce motor neuron identity in this cell line. Multiple studies have recently shown that variations between developmental fate are often observed during the differentiation of multiple PSC cell lines (Hu *et al.*, 2010; Kim *et al.*, 2010, 2011; Polo *et al.*, 2010; Bar-Nur *et al.*, 2011; Kajiwara *et al.*, 2012; Volpato *et al.*, 2018). Variability in differentiation yields have been associated with unique iPSC cell line specific epigenetic differences, acquired from the donor cell, which can hinder or predispose differentiation to a specific sub-lineage (Hu *et al.*, 2010; Kim *et al.*, 2010, 2011; Polo *et al.*, 2010; Bar-Nur *et al.*, 2011; Kajiwara *et al.*, 2012). Similarly, a recent study suggested that differential gene expression patterns between different iPSC differentiated cell lines was the underlying component for generation of differing subpopulations during neuronal differentiation (Volpato *et al.*, 2018). This could suggest that the lower yields of motor neuron induction, observed by the SFCi55-ZsGr hiPSC cell line, are due to retained donor cell epigenetic modifications which hinder differentiation towards a motor neuron identity which are not present within the H9 hESC cell line. Other studies have also shown that hiPSCs and hESCs exhibit different levels of endogenously expressed signalling factors and that correct differentiation can require cell line-specific antagonism or activation to counter these endogenous signalling levels (Kattman *et al.*, 2011; Nostro *et al.*, 2011; Nazareth *et al.*, 2013). Some cell lines have been shown to contain specific karyotypic abnormalities which result in differentially expressed genes which can impair the cells ability to differentiate. For example, one the most common chromosomal abnormalities found in hPSCs, 20q11.21, has been shown to impair SMAD-mediated signalling and hinder neural induction (Markouli *et al.*, 2019) Interestingly, endogenous TGF-β and BMP signals have been shown to be the most disparately expressed and those which are required to be regulated in cell line specific

manners to allow correct differentiation (Kattman *et al.*, 2011; Nostro *et al.*, 2011). Further suggesting that the different yields observed within this differentiation protocol could be due to the SFCi55-ZsGr hiPSC cell line containing higher levels of endogenous BMP signals which require higher levels of BMP antagonism to promote ventral identity and motor neuron induction.

3.3.5 Synergistic BMP & TGF-β inhibition alongside FGF-WNT & RA signals promote thoracic motor neuron identity downstream of NMPs

3.3.5.1 Increased levels of SMAD inhibition within the Prolonged FGF, WNT & Enhanced SMADi Protocol results in robust induction of thoracic identity motor neurons

As shown, in the previous section, the BMP inhibition protocol resulted in the generation of thoracic identity motor neurons within the H9 hESC cell line. However, there was no induction of thoracic motor neurons within the SFCi55-ZsGr hiPSC cell line. As suggested previously these cell line specific differences could potentially be attributed to the endogenous levels of TGF-β and BMP signals present within specific cell lines (Kattman *et al.*, 2011; Nostro *et al.*, 2011) which prevent both neural induction and ventralisation. As I have shown previously, in **Section 3.3.1**, and within existing literature the presence of dual SMAD inhibition is important to promote robust neuralisation (Chambers *et al.*, 2009c; Verrier, Davidson, Gierli, *et al.*, 2018) whilst extended presence of the SMAD inhibitors can prevent dorsal neural tube identity (**Section 3.3.4)**. Therefore, I examined if the presence of enhanced SMAD inhibition by supplementation of BMP signalling antagonists and TGF-β signalling antagonist from Day 3 to Day 10 of differentiation, alongside the presence of FGF and WNT signals to promote thoracic identity, resulted in the robust generation of thoracic identity motor neurons. These conditions are denoted as the Prolonged FGF, WNT & Enhanced SMADi conditions (**Figure 3.14A**). The differentiation was conducted in 4 hPSC cell lines (2 hiPSC lines; SFCi55-ZsGr and MIF1 and 2 hESC lines H7S14 and H9).

At Day 24 of differentiation immunofluorescence was used to observe protein expression within the cell cultures derived from multiple cell lines. Expression of OLIG2 was observed to various extents depending on the cell line (~10-60% of total cells) (**Figure 3.14B**). Importantly, post-mitotic motor neuron markers were highly expressed compared to all previous protocols. ISLET1 was expressed in an average of ~35-60% of total cells (**Figure 3.14C**) and HB9 was expressed in ~25-35% of cells depending on the cell line (**Figure 3.14D**). Expression of Neurofilament clearly marked the neuronal projections in all of the cell lines (**Figure 3.14C**) and Cholinergic Acetyltransferase was expressed highly within axons and synapses (**Figure 3.14D**). Importantly, the expression of HOXC9 was observed in an average of ~68-95% of total cells dependent on the cell line (**Figure 3.14B**). These findings suggest

the presence of enhanced SMAD inhibition, downstream of NMP identity, is essential to allow efficient neural induction for the promotion of thoracic identity motor neurons.

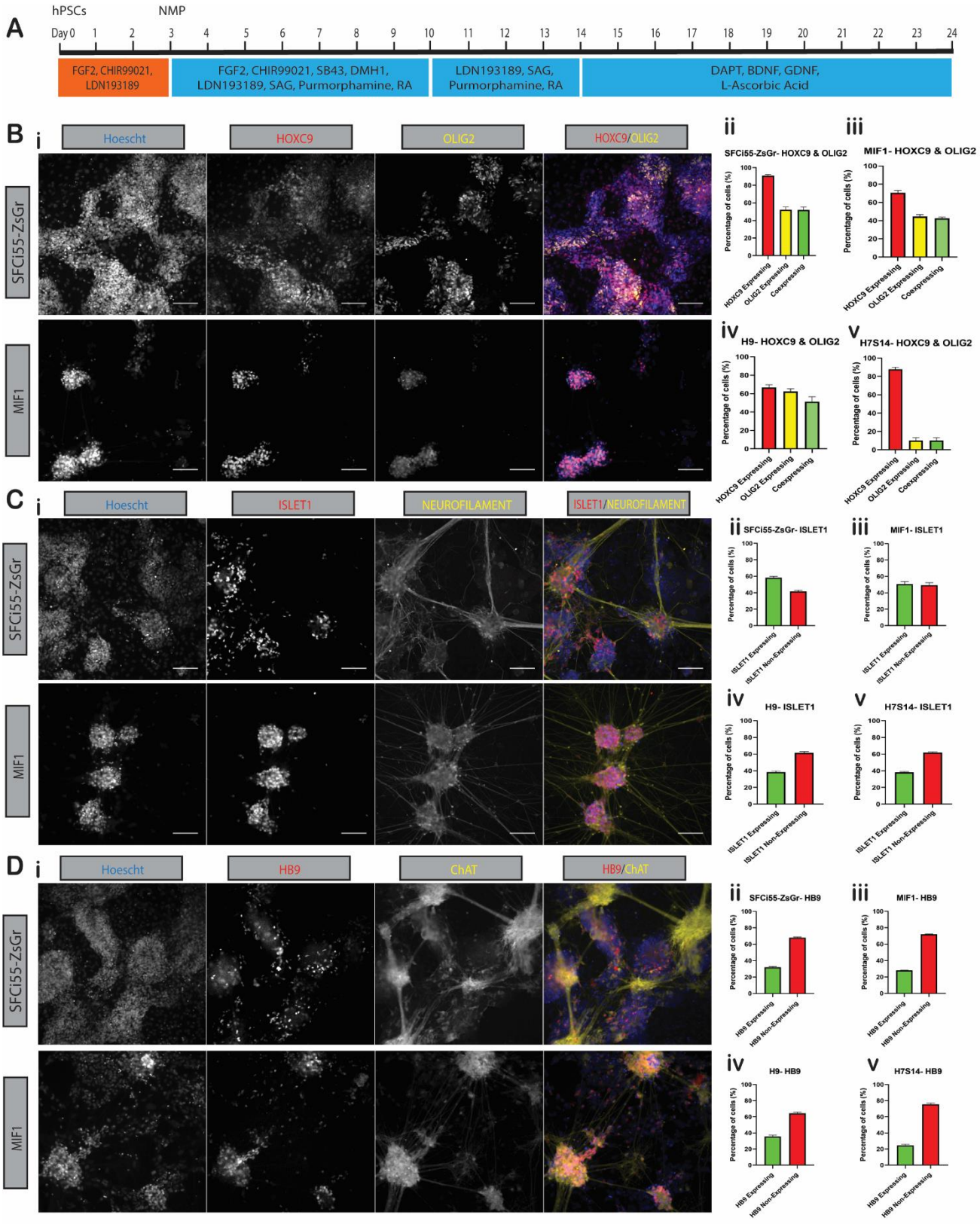


Figure 3.14. Prolonged FGF, WNT & Enhanced SMADi protocol produces mature motor neurons at Day 24. (A) A Schematic showing the differentiation protocol followed. **(i)**Immunofluorescence showing the expression of **(B)** HOXC9 and OLIG2 **(C)** ISL1 and Neurofilament-heavy chain and **(D)** HB9 and Cholinergic Acetyltransferase at Day 24 of differentiation, within the ZIPS and MIFF1 cell lines. Scale bar, 100µm. Immunofluorescence quantification of the DAY 24 expressions for the **(ii)** ZIPS, **(iii)** MIF1, **(iv)** H9 and **(v)** H7S14 cell lines. (n=3, Error bars indicate SEM around the mean).

Next, I used qPCR to identify the changes in gene expression which occurred over the time-course of the Prolonged FGF, WNT & Enhanced SMADi differentiation. To do this I extracted RNA from cells at Day 0, Day 3, Day 7, Day 14 and Day 24 of differentiation. SFCi55-ZsGr and MIF1 hiPSC and H9 and H7S14 hESC cell lines were used. Initially I observed the changes of gene expression in the neural tube progenitor marker, *PAX6*, and motor neuron progenitor markers, *NKX6.1*, *NKX6.2* and *OLIG2*. *PAX6* was the earliest gene to be upregulated, within all four cell lines, and reached peak expression around Day 7 of differentiation (**Figure 3.15A**). After Day 7 of differentiation the expression of *PAX6* is reduced and shows very low levels of expression by Day 24 of differentiation. This is representative of the cultures obtaining an early pan-neural tube progenitor identity similar to that shown in **Section 3.3.1** (**Figure 3.15C**). Expressions of *NKX6*.1 and *NKX6*.2 showed similar trends within the four cell lines with peak expressions around Day 7 to Day 14 of differentiation (**Figure 3.15Ai**). After Day 14 the expression of *NKX6*.1 and *NKX6*.2 showed a reduction in expression to Day 24 of differentiation in all cell lines. Similarly, *OLIG2* showed peak levels of expression at Day 14 of differentiation in all cell lines with >1000-fold higher induction at Day 14 compared to Day 3. Suggesting that Day 14 is representative of a motor neuron progenitor identity as it displays the highest levels of *NKX6.1*, *NKX6.2* and *OLIG2* expression.

Expression of genes which mark a mature motor neuron identity exhibited peak expression levels at Day 24 of differentiation (**Figure 3.15B**). In all cell lines, the expression of both *ISLET1* and *HB9* was >100-fold more highly induced at Day 24 of differentiation compared to Day 3 (**Figure 3.15B**). Both genes show lower levels of expression at earlier time-points within the differentiation which is reflective of their gene activation upon transition into a post-mitotic motor neuron identity (Thor *et al.*, 1991; Ericson *et al.*, 1992; Pfaff *et al.*, 1996; Tanabe, William and Jessell, 1998; Arber *et al.*, 1999; Thaler *et al.*, 1999, 2002). Notably, the

increase of expression within *ISLET1* and *HB9* was superseded by the increased expression of *NGN2*. Expression of *NGN2* is also upregulated from Day 7 of differentiation and remains relatively highly expressed until Day 24 (**Figure 3.15B**). NGN2 is known to accumulate in motor neuron progenitors, working antagonistically to *OLIG2*, to promote conversion of progenitors to post-mitotic motor neurons (Lee *et al.*, 2005). Interestingly, high expression levels of *LHX3* are also detected at the later time-points within the differentiation and in all cell lines there is a >1000-fold increased induction at Day 24 compared to Day 3 (**Figure 3.15B**). LHX3 is known to interact with ISLET1 to promote motor neuron maturation whilst continued expression of LHX3 at later time-points is a marker of motor neurons which contribute to the Median Motor Column for innervation of axial muscles (Sharma *et al.*, 1998; Thaler *et al.*, 2002). Expression of *LHX3* does show downregulation at later time points of differentiation within the SFCi55-ZsGr, H9 and MIF1 cell lines (**Figure 3.15B)** whilst the expression of *ISLET1* and *HOXC9* remains high (**Figures 3.15B, 3.15C**). This suggests that these culture conditions are generating populations of thoracic motor neurons which are characteristic of Hypaxial motor column identity.

HOX gene expressions were also tested to determine positional identity of the obtained motor neuron cultures. Within the cell lines tested there was a continued expression of thoracic identity HOX genes throughout the time-course of the experiment (**Figure 3.15C**). The thoracic identity HOX paralogs, *HOX8-9*, show upregulation by the Day 3 NMP state and their expression remains relatively constant throughout the different time points to Day 24. This further confirms the generation of populations of thoracic identity motor neurons.

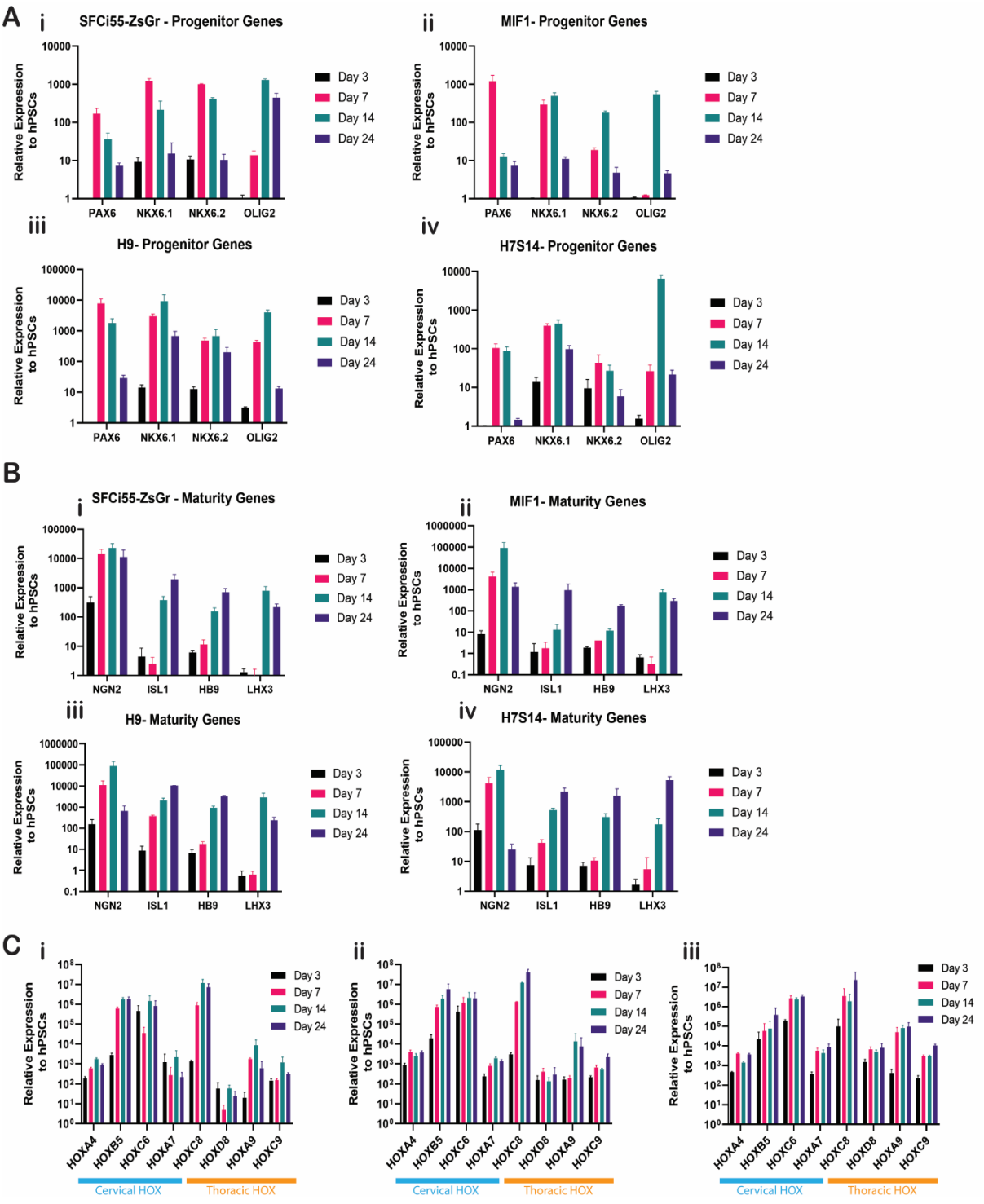
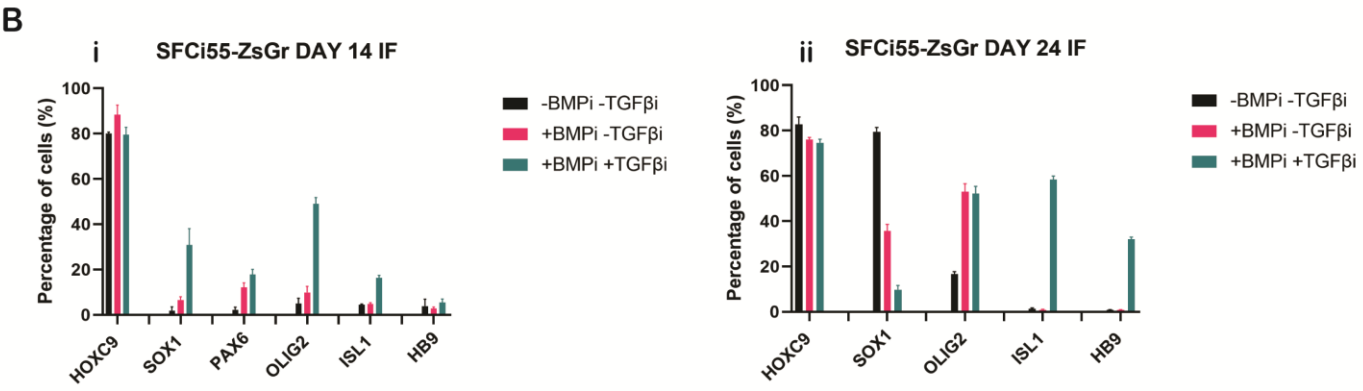
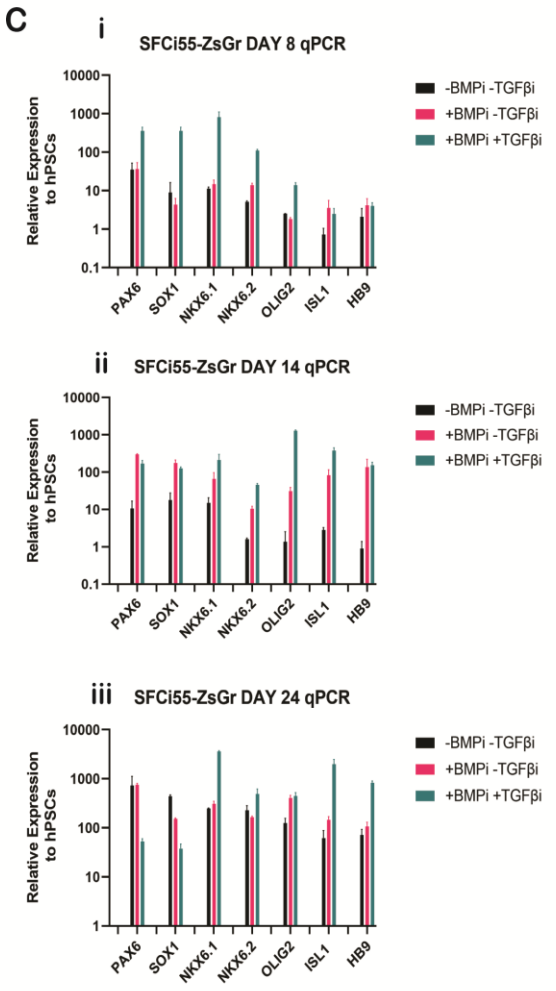


Figure 3.15. Characteristic Thoracic Motor Neuron Gene expression is observed over the differentiation time-course. quantitative PCR data showing relative gene expressions of **(A)** motor neuron progenitor markers and **(B)** motor neuron markers within **(i)** SFCi55-ZsGr, **(ii)** MIF1, **(iii)** H9 and **(iv)** H7S14 differentiated cells. **(C)** quantitative PCR data for HOX gene expression within **(i)** SFCi55-ZsGr, **(ii)** H7S14 and **(iii)** H9 differentiated cells. Cervical-identiy HOX genes are underlined by a blue line whilst Thoracic-identity Hox are underlined by an orange. In all cases Total RNA was extracted from the cells at Day 3, Day 7, Day 14 and Day 24 of differentiation and the data is shown as log expression changes which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3).


A
hPSCs
NMP
Day 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Protocols
FGF, WNT & RA
FGF2, CHIR99021, LDN193189
FGF2, CHIR99021, SAG, Purmorphamine, RA
SAG, Purmorphamine, RA
BDNF, GDNF L-Ascorbic Acid, DAPT
BMP inhibtion
+ LDN193189
+ LDN193189
Prolonged FGF, WNT & Enhanced DSMADi
+ LDN193189, SB43 & DMH1
+ LDN193189


B
i
SFCi55-ZsGr DAY 14 IF
Percentage of cells (%)
HOXC9
SOX1
PAX6
OLIG2
ISL1
HB9
-BMPi -TGFβi
+BMPi -TGFβi
+BMPi +TGFβi
ii
SFCi55-ZsGr DAY 24 IF


C
i
SFCi55-ZsGr DAY 8 qPCR
Relative Expression to hPSCs
PAX6
SOX1
NKX6.1
NKX6.2
OLIG2
ISL1
HB9
-BMPi -TGFβi
+BMPi -TGFβi
+BMPi +TGFβi
ii
SFCi55-ZsGr DAY 14 qPCR
iii
SFCi55-ZsGr DAY 24 qPCR

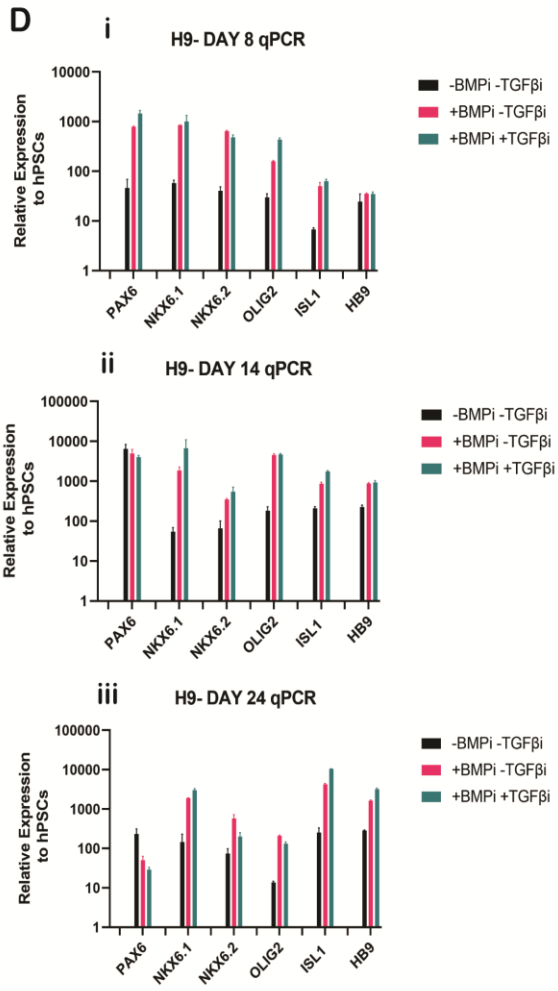

D
i
H9- DAY 8 qPCR
Relative Expression to hPSCs
PAX6
NKX6.1
NKX6.2
OLIG2
ISL1
HB9
-BMPi -TGFβi
+BMPi -TGFβi
+BMPi +TGFβi
ii
H9- DAY 14 qPCR
iii
H9- DAY 24 qPCR

Figure 3.16. Prolonged FGF, WNT & Enhanced SMADi Protocol is the most optimum for thoracic MN induction. (A) A schematic showing the different culturing conditions of the discussed differentiation protocols. **(B&C)** Averages of the percentage of cells within SFCi55-ZsGr cultures which express specific motor neuron differentiation markers upon exposure to the different culture conditions at Day 14 **(i)** and Day 24 **(ii)**. (Error bars indicate SEM around the mean. n=3). Quantitative PCR data showing relative gene expressions for motor neuron differentiation markers in SFCi55-ZsGr **(C)** and H9 **(D)** derived cultures. Total RNA was extracted from the cells at **(i)** Day 8, **(ii)** Day 14 and **(iii)** Day 24 of differentiation. In all cases the data is shown as log expression changes which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3.

3.3.5.2 Prolonged FGF, WNT & Enhanced SMADi Protocol promotes efficient thoracic MN induction

In order to assess which protocol provided the optimum conditions for the *in vitro* generation of thoracic identity motor neurons a direct comparison of expressions was made between the 3 separate protocols discussed previously in this chapter (**Figure 3.16A**). At Day 8 of differentiation under the Prolonged FGF, WNT & Enhanced SMADi protocol, within SFCi55-ZsGr- derived cultures, there is a clear upregulation of genes which mark early neural tube (*PAX6* and *SOX1*) and ventral neural tube identity (*NKX6.1*, *NKX6.2*, *OLIG2*) compared to the other protocols (**Figure 3.16Ci**). This shows that, alike in **Section 3.3.1**, the presence of both BMP and TGF-β inhibition promote a quicker induction of neural identity downstream of NMPs whilst also promoting ventral character. Within H9-derived cultures, at Day 8, the expression of early neural and early ventral neural tube genes (*PAX6*, *NKX6.1*, *NKX6.2*) show no differences in expression between the BMP inhibition protocol and the Prolonged FGF, WNT & Enhanced SMADi protocol (**Figure 3.16Di**). Only *OLIG2* shows enhanced expression within the Prolonged FGF, WNT & Enhanced SMADi protocol. However, both the BMP inhibition protocol and the Prolonged FGF, WNT & Enhanced SMADi protocol show much greater upregulation of the early neural genes compared to the WNT, FGF and Retinoic Acid protocol (**Figure 3.16Ci**). Again, this clearly demonstrates that BMP and TGF-β inhibition are required to promote neuralisation of NMPs whilst also displaying the cell line specific differences observed in **Section 3.3.4.2**.

At Day 14, when observing gene expression, there is a clear upregulation of motor neuron progenitor markers (*NKX6.1*, *NKX6.2* and *OLIG2*) within SFCi55-ZsGr- derived cultures upon

the exposure to BMP and TGF-β inhibition within the Prolonged FGF, WNT & Enhanced SMADi protocol (**Figure 3.16Cii**). Reflectively, by immunofluorescence, at Day 14 of differentiation an average of over 40% of cells in culture express OLIG2 within the Prolonged FGF, WNT & Enhanced SMADi protocol whilst under the other differentiation protocols less than 10% of cells express OLIG2 (**Figure 3.16Bi**). This displays that at day 14 of differentiation the Prolonged FGF, WNT & Enhanced SMADi protocol is the most efficient at generating a population of *NKX6.1*$^{+}$/*NKX6.2*$^{+}$/*OLIG2*$^{+}$ motor neuron progenitors.

Further comparison at Day 24 identified that the gene expression of motor neuron maturity markers (ISL1 and HB9) were highly expressed upon the exposure to BMP and TGF-β inhibition within the Prolonged FGF, WNT & Enhanced SMADi protocol compared to lower levels of expression within the other protocols (**Figure 3.16Ciii**). Higher yields of mature motor neurons were also shown to be induced from the Prolonged FGF, WNT & Enhanced SMADi protocol, detected by immunofluorescence, as on average >60% more cells express ISL1 and >20% more cells express HB9 than any other protocol (**Figure 3.16Bii**). This shows that the presence of both BMP and TGF-β inhibition during differentiation enhance the yield of mature motor neuron identity. Furthermore, the gene expression of both *PAX6* and *SOX1* were much higher within the WNT, FGF and Retinoic Acid protocol and BMP inhibition protocol compared to the Prolonged FGF, WNT & Enhanced SMADi protocol (**Figure 3.16Ciii**). This was reflected by an observed higher expression of SOX1, by immunofluorescence, within these conditions (**Figure 3.16Bii**). Taken together, this shows that neuralisation occurs at a slower rate within the WNT, FGF and Retinoic Acid and BMP inhibition protocols and early neural progenitor identity is still present at Day 24. This further shows the requirement of combined BMP and TGF-β inhibition to promote efficient neuralisation of NMPs and, in turn, allow more efficient motor neuron induction.

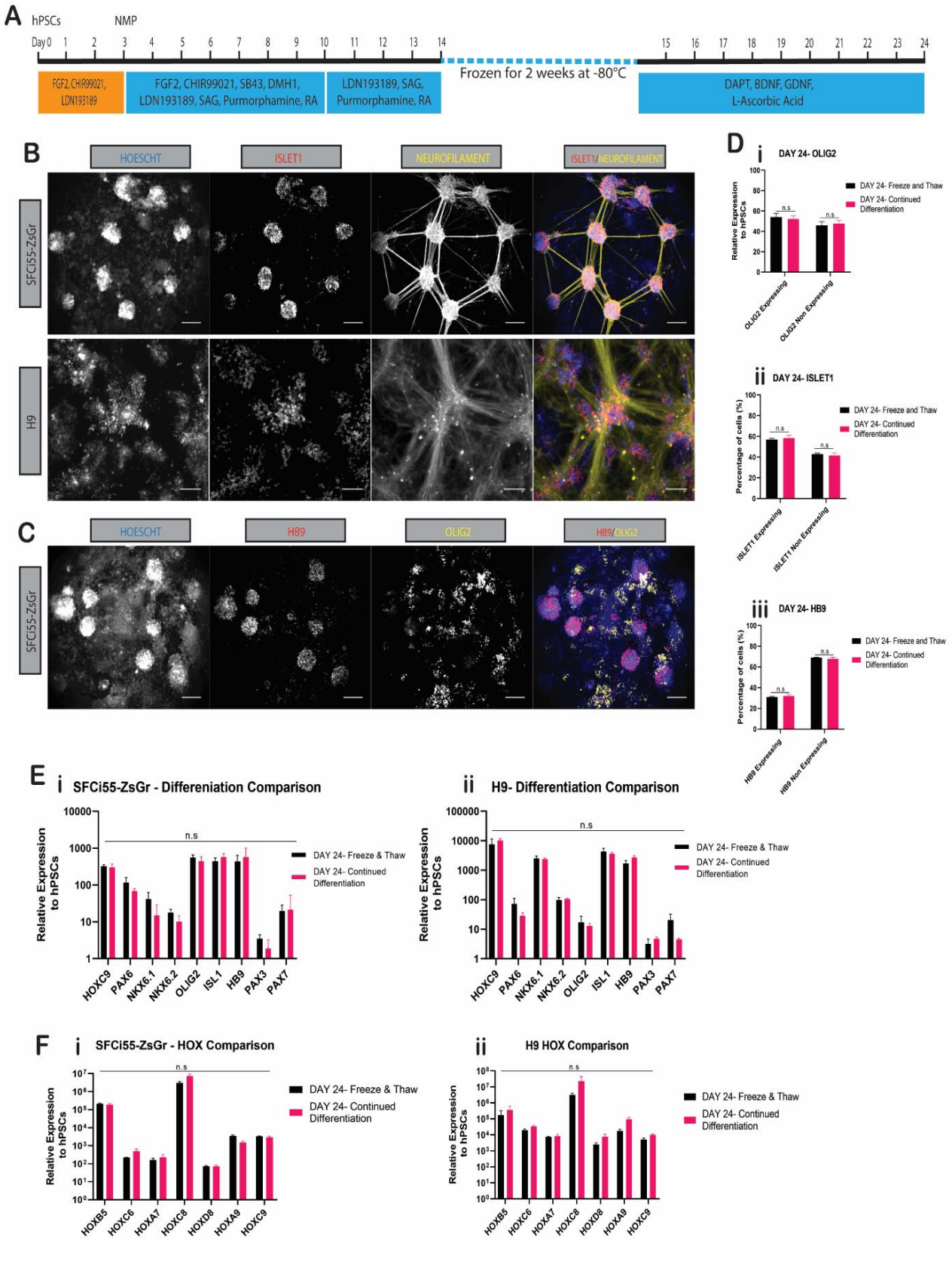
3.3.5.3 Thoracic motor neuron progenitors can be frozen and thawed with no impact on differential abilities

The ability to freeze and thaw cells during a differentiation protocol is a desirable characteristic for applications within regenerative medicine. As the Prolonged FGF, WNT & Enhanced SMADi protocol resulted in the highest yield of thoracic motor neurons I wished to observe if motor neuron progenitors of this protocol could be frozen and thawed at Day 14 of differentiation. Day 14 was selected as, as shown previously, these cell cultures represent a *NKX6.1*^{high}/*NKX6.2*^{high}/*OLIG2*^{high} motor neuron progenitor identity. Cells were frozen for two weeks at -80°C and then they were thawed and seeded into Day 14 Prolonged FGF, WNT & Enhanced SMADi culturing conditions (**Figure 3.17A**). At Day 24 differentiation was terminated and protein and gene expressions were tested to observe if the differential capability of the cells was affected during the freeze/thaw process.

Initially, I observed the protein expression, by immunofluorescence, at Day 24 of differentiation after freezing and thawing at Day 14. Post-mitotic motor neuron marker expression was shown through the expression of ISLET1 (**Figure 3.17B**) and HB9 (**Figure 3.17C**). Within SFCi55-ZsGr cultures an average of 57% of cells expressed ISLET1 whilst an average of 31% expressed HB9. Expression of Neurofilament was also shown to mark the neuronal projections (**Figure 3.17B**). Expression of the motor neuron progenitor marker, OLIG2, was retained in an average of 52% of cells in culture (**Figure 3.17C**). Importantly, when comparing the levels of expression within Day 24 cultures which had been frozen and thawed to those which had remained in continuous culturing there was no difference in percentage protein expression (**Figure 3.17D**).

In both the SFCi55-ZsGr and H9- derived cultures there was no statistical difference in gene expression within any of the neuronal and motor neuron genes (*PAX6*, *NKX6.1*, *NKX6.2* and *OLIG2*, *ISL1* and *HB9*) after freeze/thawing of cells compared to continual differentiation (**Figure 3.17E**). *PAX3* and *PAX7* expression was unaltered by Day 24 showing that there is a similar restriction in dorsal identity. In both the SFCi55-ZsGr and H9- derived cultures, after freezing and thawing compared to continual differentiation, there was no significant difference in expression within any of the HOX genes tested (**Figure 3.17F**). Showing that the freeze/thaw process had no impact on the cells ability to maintain thoracic HOX expression.

Figure 3.17. Motor Neuron Progenitors can be frozen & Thawed with no detrimental effects on differentiation. A. A Schematic showing the differentiation protocol followed. Immunofluorescence showing the expression of **(B)** ISLET1 and Neurofilament or **(C)** HB9 and OLIG2 at Day 24 of differentiation, within the ZIPS and H9 cell line. Scale bar, 100µm. **(D)** Immunofluorescence quantification of the DAY 24 expressions within the ZIPS cell line. Comparison is between cells which were frozen and thawed at Day 14 of differentiation or continuously differentiated. (n=3, Error bars indicate SEM around the mean). quantitative PCR data showing relative gene expression for **(E)** motor neuron differentiation markers and **(F)** HOX genes within **(i)** ZiPS and **(ii)** H9 differentiated cell. Total RNA was extracted from the cells at Day 24 of differentiation after they had been frozen at Day 14 of differentiation and thawed or continually differentiated. (Error bars indicate SEMaround the mean. n=3, Unpaired t-test; n.s= no significance, *=P<0.05; **=P<0.01, ***=P<0.001, ****=P<0.0001). In all cases the qPCR data is shown as 2-DDCT values which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs.


A
hPSCs
NMP
Day 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
FGF2, CHIR99021, LDN193189
FGF2, CHIR99021, SB43, DMH1, LDN193189, SAG, Purmorphamine, RA
LDN193189, SAG, Purmorphamine, RA
Frozen for 2 weeks at -80°C
DAPT, BDNF, GDNF, L-Ascorbic Acid
B
HOESCHT
ISLET1
NEUROFILAMENT
ISLET1/NEUROFILAMENT
SFCi55-ZsGr
H9
C
HOESCHT
HB9
OLIG2
HB9/OLIG2
SFCi55-ZsGr
D
i DAY 24- OLIG2
Relative Expression to hPSCs
DAY 24- Freeze and Thaw
DAY 24- Continued Differentiation
n.s
OLIG2 Expressing
OLIG2 Non Expressing
ii DAY 24- ISLET1
Percentage of cells (%)
ISLET1 Expressing
ISLET1 Non Expressing
iii DAY 24- HB9
HB9 Expressing
HB9 Non Expressing
E
i SFCi55-ZsGr - Differeniation Comparison
ii H9- Differentiation Comparison
HOXC9 PAX6 NKX6.1 NKX6.2 OLIG2 ISL1 HB9 PAX3 PAX7
F
i SFCi55-ZsGr - HOX Comparison
ii H9 HOX Comparison
HOXB5 HOXC6 HOXA7 HOXC8 HOXD8 HOXA9 HOXC9

3.3.5.4 Discussion

In this section I have clearly defined a protocol for the *in vitro* generation of thoracic identity HOXC9^{+} motor neurons from hPSCs. I have demonstrated that the initial differentiation is dependent on the rapid, high yield induction of a thoracic neurectoderm. Multiple existing protocols have explored the signals required to promote the generation of a posterior identity neurectoderm downstream of hPSC-derived NMPs (Lippmann *et al.*, 2015b; Verrier, Davidson, Gierlin, *et al.*, 2018). In agreement with these protocols I have identified that the highest yields of mature motor neurons are generated upon the exposure of NMPs to combined BMP and TGF-β inhibition which allows for a quicker and more robust neural induction by day 8 of differentiation (Verrier, Davidson, Gierli, *et al.*, 2018). Most popular conventional protocols adopt similar conditions as Chambers *et al.*, (2009) to differentiate hPSC-derived thoracic neurectoderm into what they believe to be thoracic identity motor neurons. However, as I have shown, in **Section 3.3.1**, the continued differentiation of thoracic HOXC9^{+} neurectorderm under the exposure to BMP and TGF-β inhibition and Shh agonists alone results in the failure to retain thoracic identity and generate HOXC9+ thoracic identity motor neurons by day 24. Here, I have clearly demonstrated the requirement for an extended period of FGF and Wnt signals, from Day 3 to Day 10, which allows for the retention of thoracic identity and the generation of HOXC9^{+} thoracic identity motor neurons. This finding has also been similarly demonstrated by a separate group in parallel whom have generated NMP-derived posterior motor neurons differently through 3D cultures (Mouilleau *et al.*, 2020). They demonstrate that exposure to Shh and Retinoic Acid after an NMP state results in brachial motor neurons. However, the generation of thoracic motor neurons is dependent upon the prolonged exposure to FGF signals (Mouilleau *et al.*, 2020). Coupled with our data, this suggests that FGF signals have an important role in stabilising and inducing thoracic HOX genes and thoracic identity. FGF signals have been shown to have a vital role in chromatin reorganisation to promote posterior neural differentiation and could be acting in a similar way to promote a stable thoracic identity (Patel *et al.*, 2013; Olivera-Martinez *et al.*, 2014). CDX2 has been shown to clear histone modifications from thoracic HOX chromatin domains (Mazzoni *et al.*, 2013b). Therefore, our data would suggest that continued presence of FGF2 signals, from day 3 to day 10, enhances CDX2 expression to promote chromatin remodelling and stabilisation of thoracic HOX

expressions to allow the induction of thoracic motor neurons. However, in the dual SMADi protocol (**Section 3.3.1**), the removal of FGF signals after day 3 of differentiation prevents the long term chromatin remodelling and stabilisation of thoracic HOX expression and results in the generation of more brachial identity motor neurons.

The ability to freeze and thaw progenitors of the Prolonged FGF, WNT & Enhanced SMADi protocol without any impact on differential capabilities provides great potential for uses in regenerative medicine. The robustness of the protocol to generate high yields of thoracic motor neurons across 4 separate cell lines is also of benefit for potential hiPSC cell-replacement therapies. Cell-line specific differences in differential capabilities have been associated with different genomic expressions and varying cell-line specific endogenous signalling levels (Kattman *et al.*, 2011; Nostro *et al.*, 2011; Nazareth *et al.*, 2013). This was similarly observed within the BMP inhibition protocol, described in **Section 3.3.4.2**. However, the Prolonged FGF, WNT & Enhanced SMADi protocol resulted in robust thoracic motor neuron induction in all cell lines tested suggesting that the strict modulation of BMP and TGF-β signals by high levels of antagonism is able to overcome cell-line specific elevated BMP and TGF-β signals.

Chapter 4- Comparing the influence of positional identity on the functionality of *in vitro* derived motor neurons

4.1- Introduction

Many techniques have been utilised to observe the functionality of PSC-derived motor neurons. Among the most commonly used are electrophysiology and spinal cord engraftment studies. Electrophysiology is advantageous as it identifies if *in vitro*-derived motor neurons have matured successfully to express the correct plasma membrane ion channels which allow the neurons to respond to endogenous stimuli and propagate action potentials. Patch clamp techniques have been widely used to observe if motor neurons can evoke action potentials in response to an electrical stimulus. Whilst others evoke action potentials through exposure to the endogenous stimulator, glutamate, or exogenous stimulants such as Kainite and KCl (Miles *et al.*, 2004; Boulting *et al.*, 2011; Son *et al.*, 2011; Mackenzie W. Amoroso *et al.*, 2013). Spinal cord engraftment studies allow for the validation of the ability of PSC-derived motor neurons to integrate within an *in vivo* environment and display correct neuronal character, such as projecting axons and innervating the correct target muscles. Multiple studies have utilised the chick embryonic spinal cord, likely due to its ease to manipulate, as a means to observe PSC-derived motor neuron integration (H Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006; Peljto *et al.*, 2010; Son *et al.*, 2011; Amoroso *et al.*, 2013).

Existing studies which have explored the functionality of PSC-derived motor neurons have done so in motor neurons which display a HOX negative Medial Motor Column (MMC) identity or a Hoxc4-Hoxc8 expressing brachial Lateral Motor Column (LMC) identity. Upon grafting these neurons into chick embryos they were shown to integrate within the ventral spinal cord and project stereotypical axons from the ventral root. Brachial LMC identity PSC-derived motor neurons were shown to integrate at brachial spinal cord levels and contribute to limb innervation (Mackenzie W. Amoroso *et al.*, 2013). Grafting the same identity neurons at thoracic levels still resulted in integration however the neurons retained their character and projected axons towards the limb and did not innervate thoracic targets (Peljto *et al.*, 2010). MMC Hox negative motor neurons, which are endogenously found at all

axial levels, were shown to be able to contribute to all axial levels (Hynek Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006; Son *et al.*, 2011). These preliminary findings would suggest that the positional identity acquired by PSC-derived motor neurons, *in vitro*, is fixed and dictates the functionality of these neurons upon grafting. Therefore, there is a need to further explore this before PSC-derived motor neurons can be used in potential cell replacement therapies. Furthermore, many of the existing grafting experiments have been conducted with mouse PSC-derived motor neurons and few findings have demonstrated the ability of human PSC-derived motor neurons to integrate within chick embryos.

Due to the inability of existing PSC differentiation protocols to reproducibly generate high yields of Hoxc9+ thoracic and more posterior identity motor neurons little has been shown about their electrophysiological function or their ability to integrate *in vivo*. Reflectively, it has not been observed if the thoracic identity obtained by PSC-derived motor neurons *in vitro* is fixed upon grafting or can be altered upon grafting into brachial or lumbosacral regions.

4.2- Aims

I have previously shown that human PSC-derived thoracic identity motor neurons can be generated by differentiating via an NMP-like state, see **Chapter 3**. We next wished to test if these motor neurons were functional. As, to the best of our knowledge, we have described the best existing protocol to generate high yields of thoracic motor neurons robustly we wished to compare the function of our thoracic identity motor neurons to those motor neurons generated by existing protocols which are of a predominantly anterior/brachial identity. Therefore, our aims were;

1) Compare the electrophysiological properties of cervical and thoracic identity hPSC-derived motor neurons
2) Compare the *in vivo* behaviours of cervical and thoracic identity hPSC-derived motor neuron progenitors following chick embryo grafting.

4.3- Results

4.3.1 Optimising a protocol for the hPSC-derivation of hindbrain/cervical motor neurons

4.3.1.1 Generation of PSC-derived hindbrain/cervical motor neurons

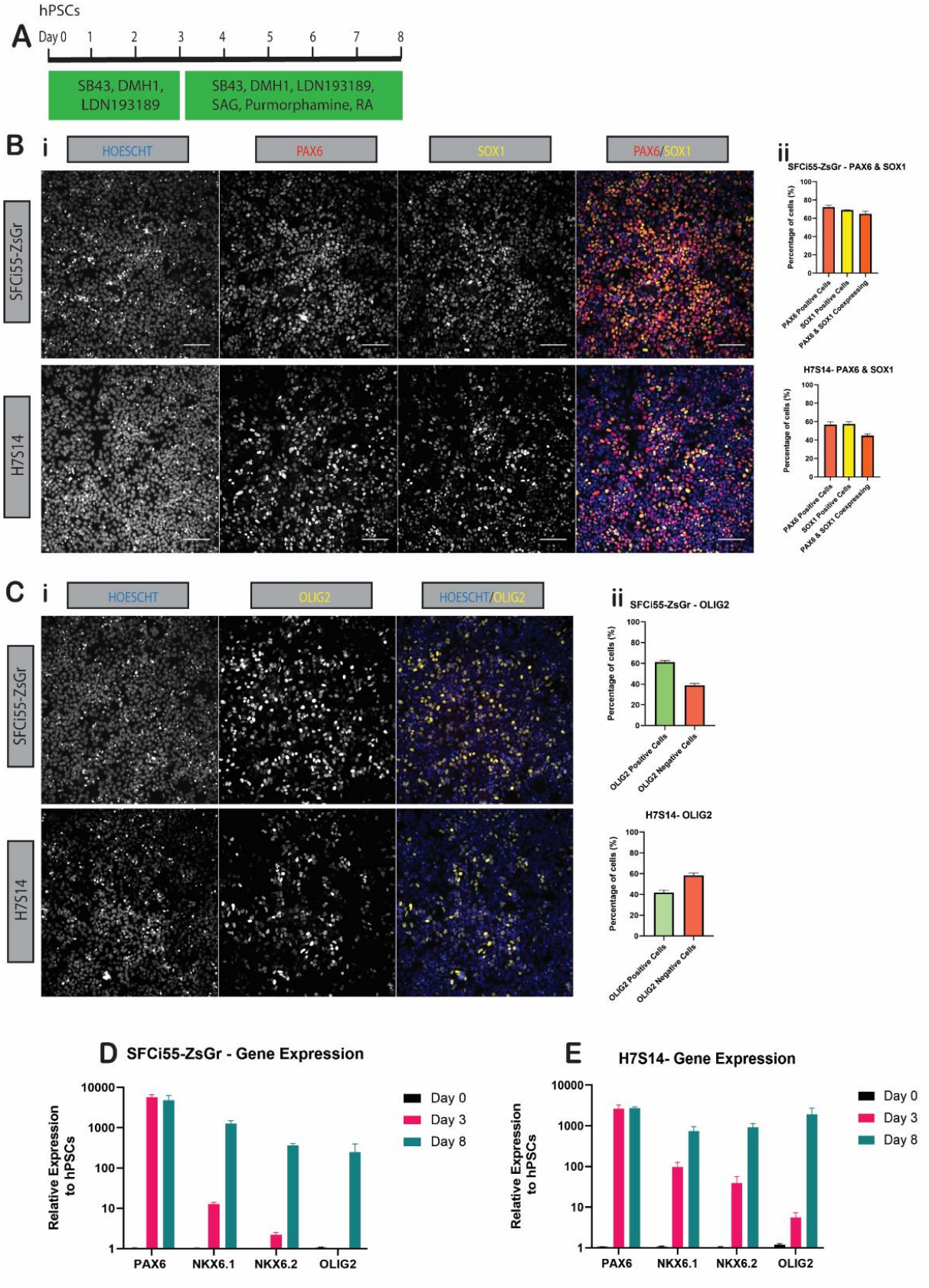
In order to compare thoracic identity motor neurons, derived in **Section 3.3.5**, with hindbrain/cervical motor neurons there was a requirement to produce a protocol for the in vitro generation of hPSC-derived hindbrain/cervical identity motor neurons. Using previously established protocols, I devised a protocol which utilised an initial exposure of hPSCs, from Day 0 to Day 10, to TGF-β and BMP family inhibitors to promote an anterior forebrain-identity neuroepithelium-like state (Lee *et al.*, 2007; Li *et al.*, 2008; Peljto *et al.*, 2010; M. W. Amoroso *et al.*, 2013). Subsequently, from Day 3 to Day 14, Retinoic Acid was used to promote hindbrain and cervical identity alongside the exposure to Sonic hedgehog (Shh) agonists which promote ventral neural tube character (Lee *et al.*, 2007; Li *et al.*, 2008). (**Figure 4.1A**).

The differentiation was conducted in both the hESC cell line, H7S14 and the hiPSC cell line, SFCi55-ZsGr. At Day 8 of differentiation the molecular profile of the cells in culture was tested to ensure that the culturing conditions were imposing a motor neuron progenitor identity. Initially, immunofluorescence was used to observe the expression of proteins which are known to mark both a neural tube and a motor neuron progenitor identity. Within SFCi55-ZsGr -derived cultures the expression of the early neural markers SOX1 and PAX6 were expressed by an average of 54% and 67% of cells in culture, respectively. (**Figure 4.1B**). H7S14-derived cultures demonstrated similar expression levels with an average of 57% of cells expressing SOX1 and 58% expressing PAX6 (**Figure 4.1B**). Expression of the motor neuron progenitor marker OLIG2 was also detected in an average of ~45-65% of total cells depending on the cell line. (**Figure 4.1C**).

I next observed the relative gene expressions within the derived cultures at both Day 3 and Day 8 of differentiation. At Day 3 of differentiation, for both cell lines, the expression of *PAX6* is the most largely upregulated gene (**Figures 4.1D, 4.1E**). *PAX6* remains highly upregulated, at >1000-fold induction relative to hPSCs, until Day 8 of differentiation which is

reflective of *PAX6* protein expression (**Figure 4.1B**). This suggests that an early neural identity is initiated as early as Day 3 which is retained until Day 8. Expression of the motor neuron progenitor domain markers *NKX6.1*, *NKX6.2* and *OLIG2* show sharp increases in expression at Day 8 of differentiation compared to Day 0 and Day 3 (**Figure 4.1D, 4.1E**). For example, *OLIG2* displays an average >100-fold increase in induction at Day 8 compared to Day 3. Again, this high induction of *OLIG2* expression is reflective of the high levels of protein expression (**Figure 4.1A**), suggesting that a large proportion of cells in culture at Day 8 of differentiation are reflective of a motor neuron progenitor identity.

Figure 4.1. Anterior protocol produces MN progenitors by Day 8 of differentiation. A. A Schematic showing the differentiation protocol followed. **(B &C) (i)** Immunofluorescence showing the expression of **(B)** PAX6 and SOX1 and **(C)** OLIG2 at Day 8 of differentiation within the SFCi55-ZsGr & H7S14 cell lines. Scale bar, 100µm. **(ii)** Quantification of these expressions n=3. Quantitative PCR data from **(D)** SFCi55-ZsGr and **(E)** H7S14 differentiated neural progenitors showing the relative expression of Motor Neuron progenitor genes. Total RNA was extracted from the cells at DAY 0, DAY 3 and DAY 8 of differentiation. Data is shown as log expression changes which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3)


hPSCs
A
Day 0 1 2 3 4 5 6 7 8
SB43, DMH1, LDN193189
SB43, DMH1, LDN193189, SAG, Purmorphamine, RA
B i
HOESCHT
PAX6
SOX1
PAX6/SOX1
SFCi55-ZsGr
H7S14
ii
SFCi55-ZsGr - PAX6 & SOX1
Percentage of cells (%)
PAX6 Positive Cells
SOX1 Positive Cells
PAX6 & SOX1 Coexpressing
H7S14- PAX6 & SOX1
C i
HOESCHT
OLIG2
HOESCHT/OLIG2
ii
SFCi55-ZsGr - OLIG2
OLIG2 Positive Cells
OLIG2 Negative Cells
H7S14- OLIG2
D SFCi55-ZsGr - Gene Expression
Relative Expression to hPSCs
Day 0
Day 3
Day 8
PAX6
NKX6.1
NKX6.2
OLIG2
E H7S14- Gene Expression

As a reproducible motor neuron progenitor identity was achieved by Day 8 of differentiation the differentiation protocol was continued downstream to try and generate a mature hindbrain/cervical motor neuron identity. Continued differentiation in the presence of neurotrophins (BDNF, GDNF and L-Ascorbic Acid) were used to promote survival and axonal outgrowth whilst the supplementation of a Notch/ γ-secretase inhibitor (DAPT) promoted motor neuron maturation (Ben-Shushan, Feldman and Reubinoff, 2015) (**Figure 4.2A**). To define the identity of the cells obtained at Day 24 of differentiation immunofluorescence and qPCR were utilised. Using immunofluorescence, it was detected that an average of 25% of cells in SFCi55-ZsGr derived cultures and 42% of cells in H7S14-derived cultures expressed OLIG2 (**Figure 4.2D**). Suggesting that even at Day 24 there is a residual progenitor population. Expression of the post mitotic motor neuron marker Islet1 is highly upregulated, by Day 24, being expressed in an average of 57% of cells in SFCi55-ZsGr cultures and 56% of cells in H7S14-derived cultures (**Figure 4.2B**). Further supporting the presence of a mature motor neuron population, there is also clear presence of Neurofilament-heavy chain expressing axonal projections (**Figure 4.2B**) which are also highly expressing, the motor neuron specific marker, Cholinergic Acetyltransferase (**Figure 4.2C**). At Day 24 there is no detected expression of HB9 in either SFCi55-ZsGr or H7S14 derived cultures (**Figure 4.2B**). Absence of HB9 expression is a marker of cervical and brachial identity motor neurons suggesting a potential restriction to this identity (Kania and Jessell, 2003; Rousso *et al.*, 2008).

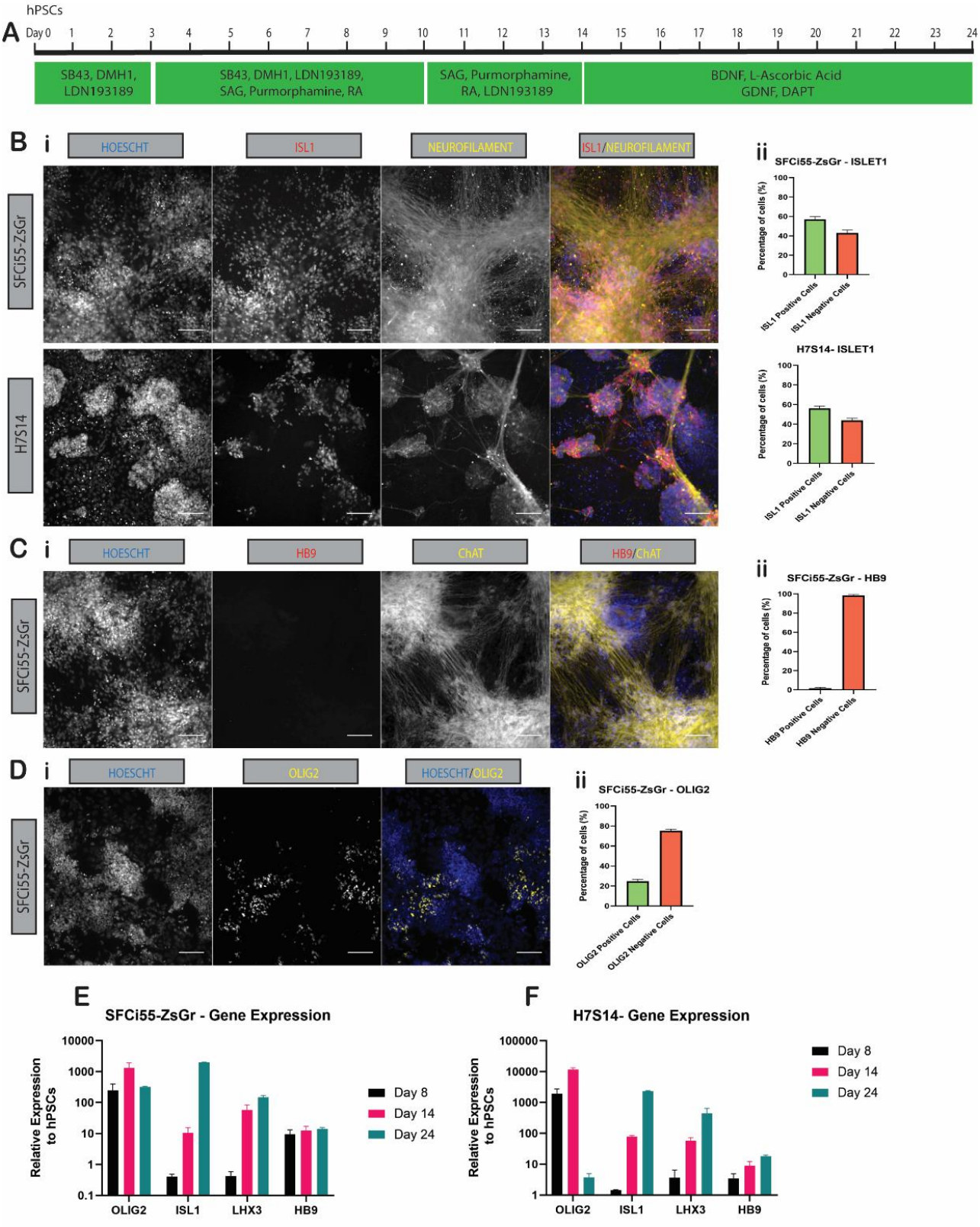

hPSCs
A
Day 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
SB43, DMH1, LDN193189
SB43, DMH1, LDN193189, SAG, Purmorphamine, RA
SAG, Purmorphamine, RA, LDN193189
BDNF, L-Ascorbic Acid GDNF, DAPT
B i
HOESCHT
ISL1
NEUROFILAMENT
ISL1/NEUROFILAMENT
SFCi55-ZsGr
H7S14
ii
SFCi55-ZsGr - ISLET1
Percentage of cells (%)
ISL1 Positive Cells
ISL1 Negative Cells
H7S14- ISLET1
C i
HOESCHT
HB9
ChAT
HB9/ChAT
SFCi55-ZsGr
ii
SFCi55-ZsGr - HB9
HB9 Positive Cells
HB9 Negative Cells
D i
HOESCHT
OLIG2
HOESCHT/OLIG2
SFCi55-ZsGr
ii
SFCi55-ZsGr - OLIG2
OLIG2 Positive Cells
OLIG2 Negative Cells
E
SFCi55-ZsGr - Gene Expression
Relative Expression to hPSCs
OLIG2
ISL1
LHX3
HB9
Day 8
Day 14
Day 24
F
H7S14- Gene Expression

Figure 4.2. Anterior protocol produces MN progenitors by Day 24 of differentiation. A. A Schematic showing the differentiation protocol followed. **(B, C & D) (i)** Immunofluorescence showing the expression of **(B)** ISL1 and Neurofilament Heavy Chain, **(C)** HB9 and ChAT and **(D)** OLIG2 at Day 24 of differentiation within the SFCi55-ZsGr & H7S14 cell lines. Scale bar, 100µm. **(ii)** Quantification of these expressions (n=3). quantitative PCR data from **(E)** SFCi55-ZsGr and **(F)** H7S14 differentiated neural progenitors showing the relative expression of Motor Neuron specific genes. Total RNA was extracted from the cells at DAY 8, DAY 14 and DAY 24 of differentiation. Data is shown as log expression changes which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3)

Gene expression analysis was conducted at Day 8, Day 14 and Day 24 to allow the observation of transcriptional changes over the differentiation time-course. *OLIG2* expression is already highly induced by Day 8 of differentiation, in both the SFCi55-ZsGr and H7S14-derived cell cultures. However, in both cell lines the expression of *OLIG2* increases from Day 8 to Day 14 and then demonstrates a downregulation in expression towards Day 24 (**Figures 4.2E, F**). Fitting with this downregulation in *OLIG2* there is an observed upregulation in both *ISLET1* and *LHX3* transcriptional expressions. *ISLET1* and *LHX3* both demonstrate a >100-fold increased induction at Day 24 compared to Day 8 in both SFCi55-ZsGr -derived and H7S14-derived cultures (**Figures 4.2E, F**). ISLET and LHX3 have both been demonstrated to be essential in the formation of the ISL1-LHX3 complex to promote motor neuron maturation (Thaler *et al.*, 2002; Song *et al.*, 2009; Cho *et al.*, 2014). This is strong evidence for a mature motor neuron population by Day 24. Similar to the inability to detect HB9 by immunofluorescence, the gene expression of *HB9* is not greatly altered over the time-course of the differentiation and never shows a greater than 10-fold increase in expression compared to that in hPSCs where it is known to be negligible (**Figures 4.2E, F**).

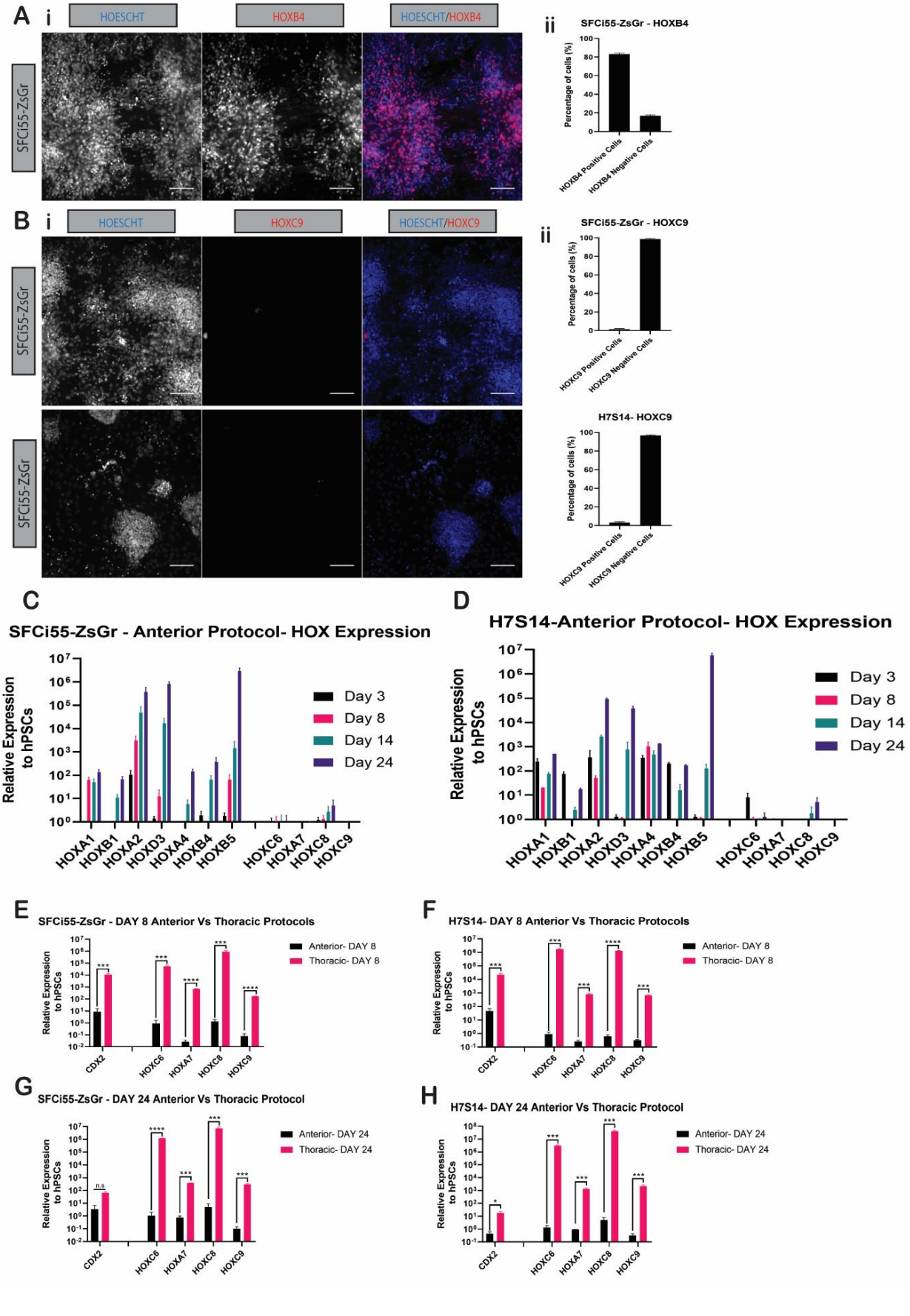
Immunofluorescence was initially used to confirm the positional identity of the in vitro-derived motor neurons. An average of 82% of cells in SFCi55-ZsGr -derived cultures expressed HOXB4 (**Figure 4.3A**). Whilst, expression of HOXC9 was detected in less than 1% and 3% of cells in SFCi55-ZsGr and H7S14-derived cultures, respectively (**Figure 4.3B**). When testing expression of HOX genes by qPCR, it was observed that there is clear expression of the HOX1-5 paralogs between Day 8 and Day 24 of the anterior differentiation protocol in SFCi55-ZsGr and H7S14- derived cultures (**Figures 4.3C, D**). However, there is a failure to induce the expression of the *HOX6-9* paralogs. Furthermore, when comparing the

expression of the *HOX6-9* paralogs under the anterior protocol to those levels induced under the Prolonged, FGF, WNT & Enhanced SMADi protocol at a stage matched time in the differentiation; the *HOX6-9* paralogs and *CDX2* are significantly induced to much higher expression levels in the Prolonged, FGF, WNT & Enhanced SMADi Protocol (**Figures 4.3E, F)** reflective of the thoracic identity. This clearly shows that the motor neurons derived from the anterior protocol are of a hindbrain/cervical/anterior brachial identity.

4.3.1.2 Discussion

Here I have shown that we can recapitulate current findings and generate hPSC-derived cervical/brachial identity motor neurons (Li *et al.*, 2008; Chambers *et al.*, 2009a; Mackenzie W. Amoroso *et al.*, 2013). I have previously shown that the generation of thoracic identity HOXC9+ motor neurons are dependent upon the differentiation of hPSCs via a NMP intermediary state (**Section 3.3.5**). Fittingly, the cervical/brachial motor neurons are HOX1-5 expressing and are generated by immediate exposure of hPSCs to BMP and TGF-β inhibition to promote neuralisation. This provides further evidence and support to the existing understanding that there are two sources of neural progenitors during axis elongation (N. Cambray and Wilson, 2007; Tzouanacou *et al.*, 2009; Gouti *et al.*, 2014).

Figure 4.3. Anterior motor neurons express HOX genes up to HOX5 paralogs. (A&B) (i) Immunofluorescence showing the expression of **(A)** HOXB4 and **(B)** HOXC9 at Day 24 of differentiation within the SFCi55-ZsGr & H7S14 cell lines. Scale bar, 100µm. **(ii)** Quantification of these expressions (n=3). Quantitative PCR data from **(C)** SFCi55-ZsGr and **(D)** H7S14 differentiated cells showing the relative expression of HOX genes. **(E, F, G, H)** Quantitative PCR data from **(E & G)** SFCi55-ZsGr and **(F & H)** H7S14 differentiated cells comparing the relative gene expression of CDX2 and posterior HOX genes at Day 8 **(E & F)** and Day 24 **(G & H)** of differentiation. Total RNA was extracted from the cells at DAY3, DAY 8, DAY 14 and DAY 24 of differentiation. Data is shown as log expression changes which are calculated relative to GAPDH and are significant compared to expression relative to hPSCs. (Error bars indicate SEM around the mean. n=3)


A
i
HOESCHT
HOXB4
HOESCHT/HOXB4
SFCi55-ZsGr
ii
SFCi55-ZsGr - HOXB4
Percentage of cells (%)
HOXB4 Positive Cells
HOXB4 Negative Cells
B
i
HOESCHT
HOXC9
HOESCHT/HOXC9
SFCi55-ZsGr
SFCi55-ZsGr
ii
SFCi55-ZsGr - HOXC9
H7S14- HOXC9
Percentage of cells (%)
HOXC9 Positive Cells
HOXC9 Negative Cells
C
SFCi55-ZsGr - Anterior Protocol- HOX Expression
Relative Expression to hPSCs
Day 3
Day 8
Day 14
Day 24
HOXA1
HOXB1
HOXA2
HOXD3
HOXA4
HOXB4
HOXB5
HOXC6
HOXA7
HOXC8
HOXC9
D
H7S14-Anterior Protocol- HOX Expression
Relative Expression to hPSCs
Day 3
Day 8
Day 14
Day 24
HOXA1
HOXB1
HOXA2
HOXD3
HOXA4
HOXB4
HOXB5
HOXC6
HOXA7
HOXC8
HOXC9
E
SFCi55-ZsGr - DAY 8 Anterior Vs Thoracic Protocols
Relative Expression to hPSCs
Anterior- DAY 8
Thoracic- DAY 8
CDX2
HOXC6
HOXA7
HOXC8
HOXC9
F
H7S14- DAY 8 Anterior Vs Thoracic Protocols
Relative Expression to hPSCs
Anterior- DAY 8
Thoracic- DAY 8
CDX2
HOXC6
HOXA7
HOXC8
HOXC9
G
SFCi55-ZsGr - DAY 24 Anterior Vs Thoracic Protocol
Relative Expression to hPSCs
Anterior- DAY 24
Thoracic- DAY 24
n.s
CDX2
HOXC6
HOXA7
HOXC8
HOXC9
H
H7S14- DAY 24 Anterior Vs Thoracic Protocol
Relative Expression to hPSCs
Anterior- DAY 24
Thoracic- DAY 24
CDX2
HOXC6
HOXA7
HOXC8
HOXC9

4.3.2 Examining the effect of axial identity on hPSC derived motor neurons

4.3.2.1 Comparing the electrophysiological functionality of hPSC derived anterior vs posterior motor neurons

Whole cell patch current-clamp recordings are largely used to observe the ability of PSC-derived motor neurons to fire action potentials (Soundararajan *et al.*, 2006a; Son *et al.*, 2011; M. W. Amoroso *et al.*, 2013). Therefore, I similarly utilised this technique to observe if the motor neurons generated from both the Prolonged FGF, WNT & Enhanced SMADi protocol and the Anterior protocol were capable of evoking action potentials. All Patch Clamp recordings were conducted by Dr Ke Ning using neurons which I prepared. At Day 36 of differentiation cells from both protocols were seeded at 50,000 cells per 13mm coverslip and used in whole-cell current clamp recordings. Anterior and thoracic PSC-derived motor neurons were both capable of evoking trains of action potentials in response to current pulses (**Figure 4.4A**). Interestingly, the PSC- derived motor neurons generated from both protocols showed equivalent action potential firing numbers under exposure to sustained current pulses (**Figure 4.4A, B**). Importantly the average action potential firing rate is between 10-15 Hz which is reflective of the firing rate within previously reported ES-derived motor neurons (Soundararajan *et al.*, 2006a; Son *et al.*, 2011; M. W. Amoroso *et al.*, 2013) and isolated embryonic motor neurons (Alessandri-Haber *et al.*, 1999).

I next observed the ability of motor neurons derived from the Prolonged FGF, WNT & Enhanced SMADi protocol, which have been frozen and thawed at day 14 of differentiation to evoke action potentials. Again, upon Day 36 of differentiation cells were seeded at 50,000 cells per 13mm coverslips and used in whole cell patch current-clamp recordings. Injections of incrementally staged current resulted in evoked action potential responses within the PSC-derived motor neurons (**Figure 4.4C**). Exposure to sustained current resulted in the firing of trains of action potentials with an average action potential firing rate of 20Hz (**Figure 4.4D**). This suggests that freezing and thawing of cells at day 14 of the Prolonged FGF, WNT & Enhanced SMADi protocol did not impact their electrophysiological function by Day 36 of differentiation. These data display the potential that thoracic motor neuron progenitors could be derived, frozen and then thawed at a later time point which may be of great benefit to international collaborations or potential cell therapy treatments.

Here, we have shown that the thoracic HOXC9^{+} motor neurons are electrophysiologically functional and are capable of evoking action potentials during whole cell patch current-clamp recordings. Importantly, the thoracic motor neurons display similar firing rates to the cervical/brachial PSC-derived motor neuron counterparts, PSC-derived motor neurons from the existing literature (Soundararajan *et al.*, 2006a; Son *et al.*, 2011) and tissue extracted motor neurons (Alessandri-Haber *et al.*, 1999). Firing rate is widely measured as in motor neurons the firing rate is responsible for the regulation of muscle contractility. Therefore, since our derived neurons fire action potentials at a similar rate to other published neurons and tissue extracted neurons of a similar age/stage this suggests our neurons are capable of displaying maturation in vitro. However, further sophisticated experiments, such as exploring susceptibility to Tetrodotoxin, will be required which can determine motor neuron-specific excitability and ion channel composition (Hynek Wichterle, Lieberam, *et al.*, 2002; Miles *et al.*, 2004; Boulting *et al.*, 2011; M. W. Amoroso *et al.*, 2013; Faustino Martins *et al.*, 2020). Ultimately, maturation of neurons is dependent upon synapse formation and interplay between stimuli and target which are not reproducible within our current *in vitro* culturing conditions. Therefore, co-culturing neurons with myoblasts to observe neuromuscular junction formations, will be required to confirm that the motor neurons display a mature electrophysiological functionality.

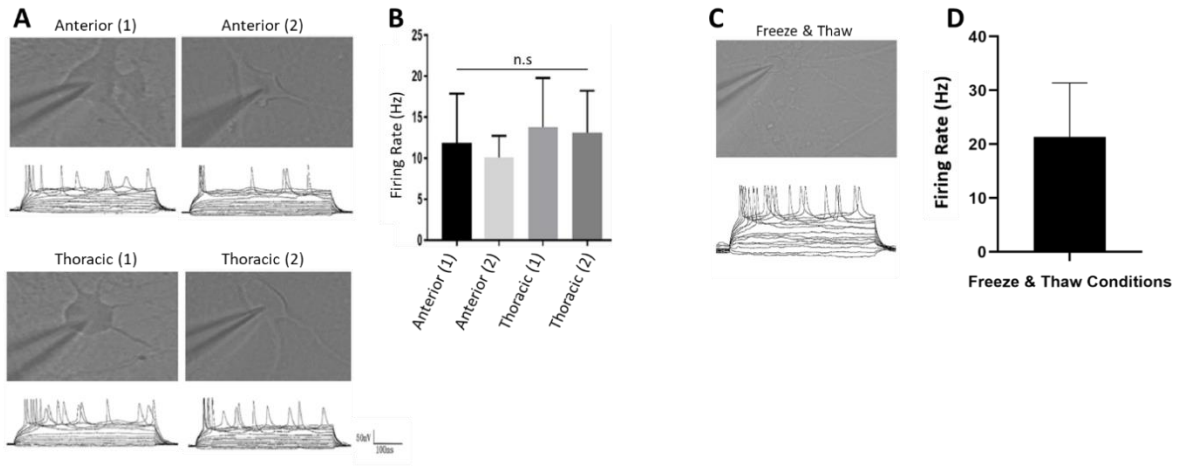


Figure 4.4. Action Potential Firing Rate of PSC-derived motor neurons. Whole-cell current clamp recordings were performed at room temperature and injected with 15 steps of currents from -80pA to +60pA (10pA increase for each step). **(A & C)** Representative traces of each group.

(B & D) Total firing number with magnitude of 20mV or higher were counted for analysis. (n=14, mean ± SEM; one-way ANOVA Tukey's multiple comparison test, no significance among groups).

4.3.2.2 Comparing the *in vivo* behaviours of hPSC derived anterior vs posterior motor neuron progenitors upon engraftment into chick embryos

Multiple studies have utilised chick embryo transplantation as a means to observe the ability of PSC-derived motor neurons to integrate and function correctly within an *in vivo* environment (H Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006; Peljto *et al.*, 2010; Son *et al.*, 2011; Amoroso *et al.*, 2013). However, these studies largely consist of the engraftment of brachial LMC identity or HOX negative MMC identity motor neurons. As I have generated a protocol for the efficient generation of HOXC9+ thoracic identity motor neurons, **Section 3.3.5**, I wished to assess if the PSC-derived thoracic motor neurons are similarly capable of integrating and functioning *in vivo* upon grafting into a thoracic chick embryo spinal cord. Thoracic identity motor neuron progenitors, which express high levels of OLIG2, ISL1 and HOXC9, were generated by Day 14 of differentiation using the protocol described in **Section 3.3.5**. The SFCi55-ZsGr cell line was used as it constituently expresses ZSGREEN (Lopez-Yrigoyen *et al.*, 2018) which can allow the tracking of cells upon engraftment. Day 14 PSC-derived thoracic motor neuron progenitors were grafted into HH11-13 stage chick embryo spinal cords at the level of the last forming somites, a level which will give rise to thoracic axial-level matched identity (see **Figure 4.5**). After a further 4-5 days of incubation the embryos, at HH Stage 26-30, were harvested and whole-mount embryos were screened to select those which presented with no developmental abnormalities and contained ZSGREEN+ grafts within thoracic neural tube locations (as in **Figure 4.5**).

A total of 30 embryos received a graft, of which 22 embryos survived to the full term of incubation and presented with no developmental abnormalities. Within these 22 embryos, 11 embryos contained ZSGREEN+ cells within thoracic spinal cord locations, within similar locations to that observed in **Figure 4.5**. 8 of these embryos were selected for cryosectioning. Transverse sections were collected within the thoracic spinal cord regions, containing the ZSGREEN+ cells, to assess the ability of the engrafted PSC-derived thoracic motor neuron progenitors to integrate within the thoracic spinal cord. A high percentage of these embryos (7/8, 87.5%) displayed the presence of ZSGREEN+ hPSC-derived motor

neuron progenitors integrated within the ventral spinal cord motor neuron domain (**Figure 4.6A**).

In the embryo, at thoracic spinal cord levels, motor neurons are responsible for innervating the dorsal axial mesoderm, the ventral axial mesenchyme and the sympathetic ganglion (seen as green, red and purple columns respectively in **Figure 4.6F**). Upon engraftment, the hPSC-derived thoracic motor neuron progenitors were shown to display correct axonal projections towards the axial muscles within half of the embryos observed (4/8, 50%, **Figure 4.6B**). Within a lower percentage of embryos (2/8, 25%) there were also observed projections towards the sympathetic ganglion. Due to the ability of the hPSC-derived thoracic motor neuron progenitors to integrate correctly within the endogenous chick motor neuron domain and to project axons in a manner reflective of endogenous thoracic motor neurons it would suggest that the *in vitro* protocol is efficient at deriving functional and positionally defined thoracic motor neurons.

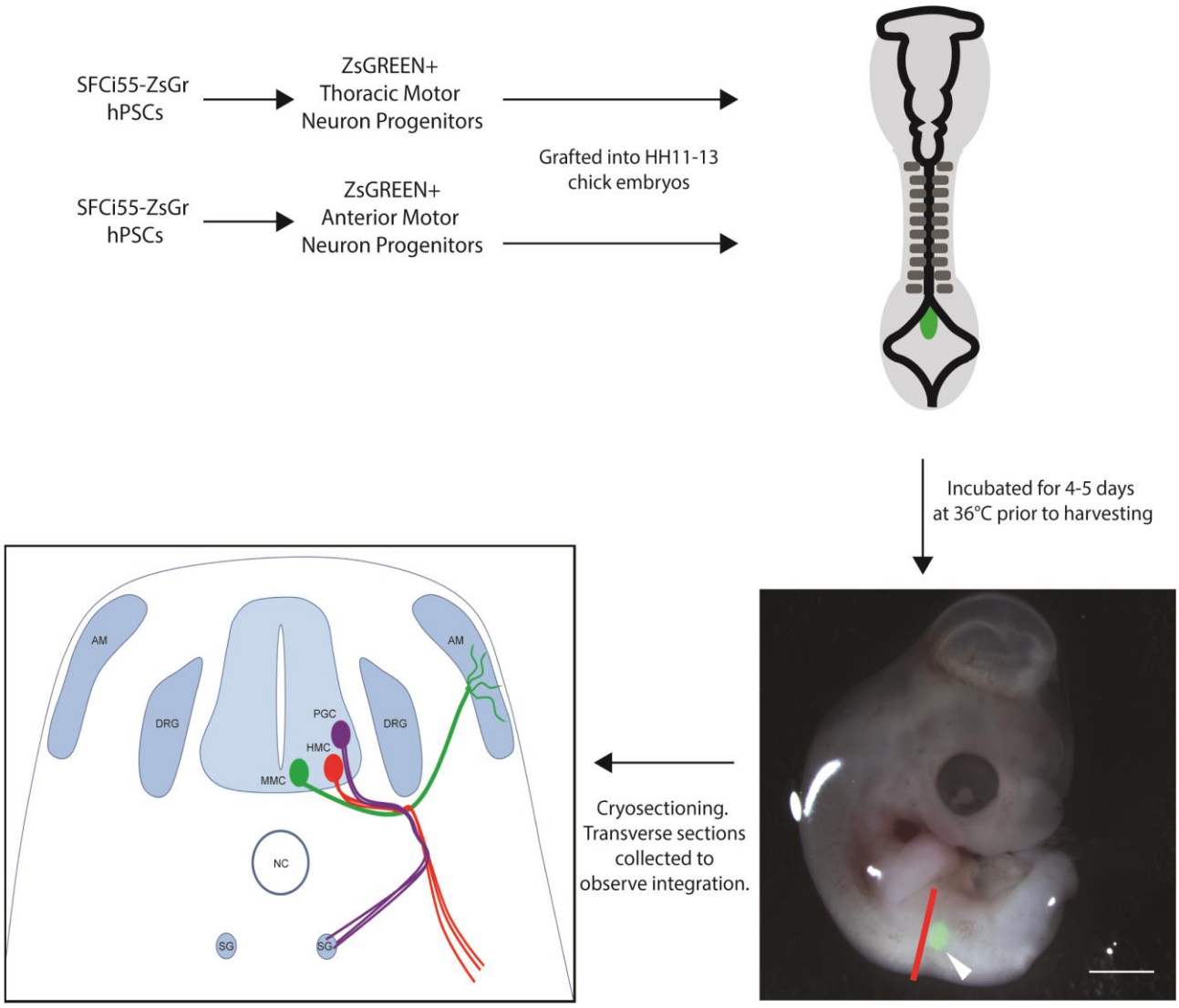
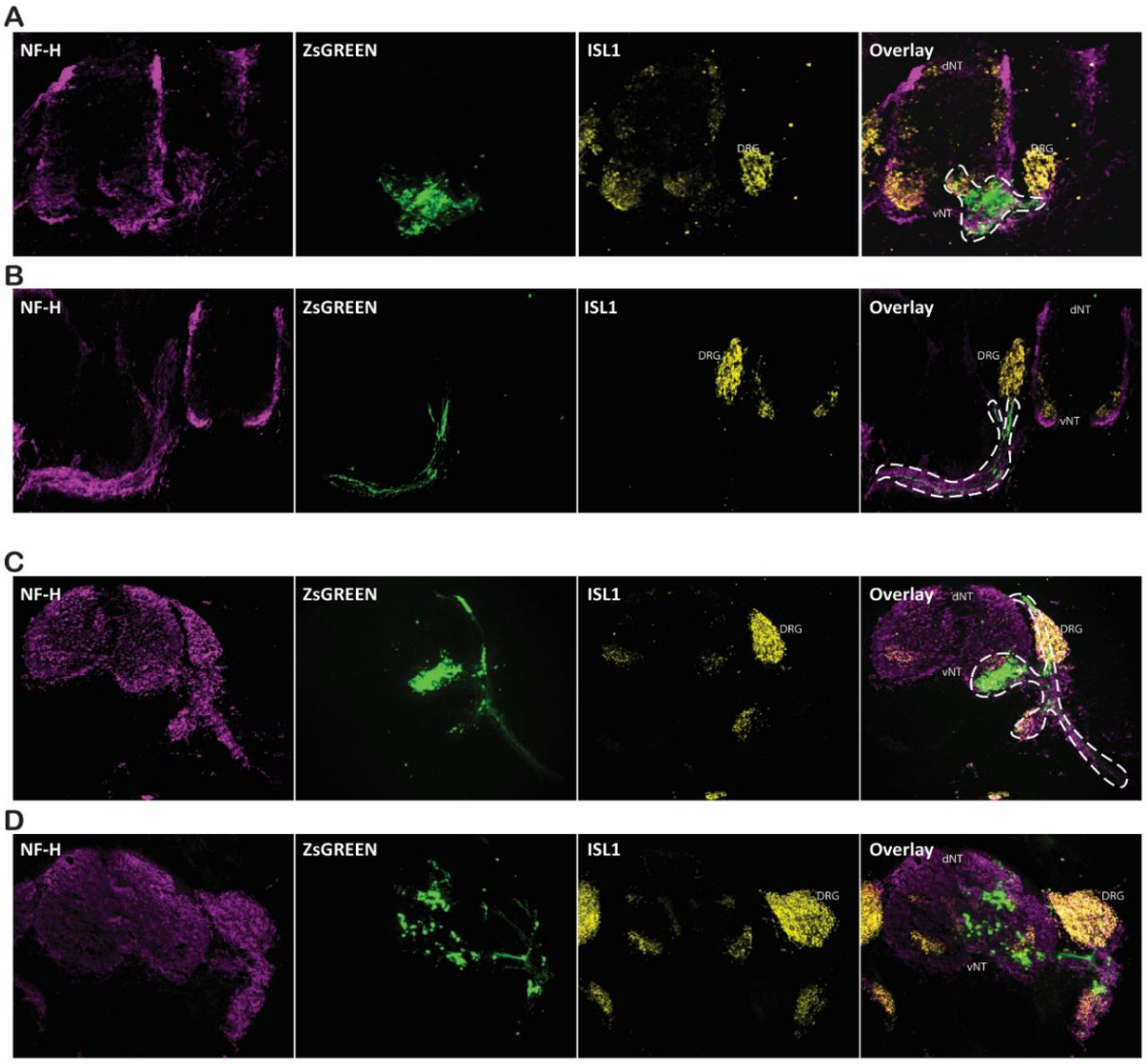


Figure 4.5. Chick Grafting Procedure. A Schematic showing the process followed. SFCi55-ZsGr hPSCs were used, as they contain a constituent ZSGREEN reporter, to generate anterior or thoracic motor neuron progenitors. ZSGREEN+ motor neuron progenitors were grafted into HH11-13 stage chick embryo spinal cords at the level of the last forming somite (yellow arrowhead). After 4-5 days incubation at 36°C the embryos were harvested and screened for the presence of ZSGREEN-positive cells within thoracic regions (shown by a white arrowhead). Scale bar, 2mm.Transverse sections were collected. ZSGreen+ Motor neuron progenitors were observed for their integration into the ventral neural tube and contributions of projections into the typical MN streams; PGC-Preganglionic Motor Column (purple), HMC- Hypaxial Motor Column (red) and MMC-Median Motor Column (green). Abbreviations; AM Axial Muscle, DRG Dorsal Root Ganglia, NC Notochord, SG Sympathetic Ganglion.



E

	Anterior	Thoracic
Integrated in vNT	5/8 (62.5%)	7/8 (87.5%)
Dispersion within dNT	7/8 (87.5%)	0/8 (0%)
Ectopic dorsal/DRG projections	7/8 (87.5%)	1/8 (12.5%)
Projections towards axial muscles	2/8 (25%)	4/8 (50%)
Projections to sympathetic ganglion	5/8 (62.5%)	2/8 (25%)

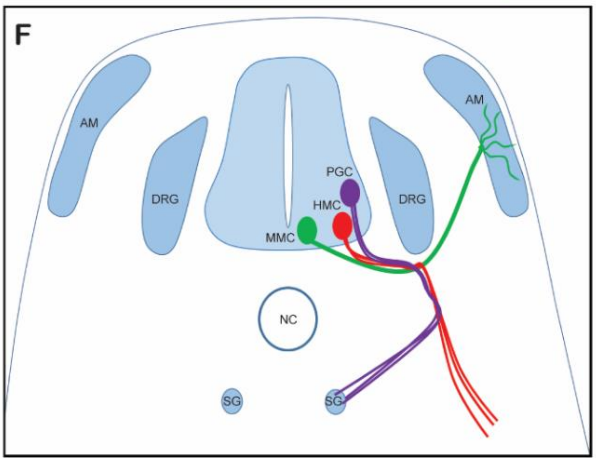


Figure 4.6. Integration of hPSC-derived anterior and thoracic motor neurons in chick spinal cords. (A & B) Transverse cross-sections showing the integration of ZSGREEN+ hPSC-derived **(A & B)** thoracic identity or **(C & D)** hindbrain/cervical motor neuron progenitors within HH26-30 chick embryo thoracic spinal cords. **(E)** Tabulated quantification of the representative scoring for the integration of the engrafted motor neuron progenitors. **(F)** A Schematic showing the typical MN streams found at thoracic axial levels; PGC- Preganglionic Motor Column (purple), HMC- Hypaxial Motor Column (red) and MMC-Median Motor Column (green).

Furthermore, I wished to observe if acquired positional identity of *in vitro* PSC-derived motor neurons remains fixed upon engraftment or if positional identity can display plasticity and be altered upon engraftment depending on the host environment. As a means to test this I looked at the ability of the PSC-derived hindbrain/cervical identity motor neurons, generated by the protocol in Section 3.3.1, to integrate within thoracic regions of chick embryo spinal cords. PSC-derived hindbrain/cervical identity motor neuron progenitors were generated using the SFCi55-ZsGr cell line. Again, cells were selected at Day 14 of differentiation at a time point where they express high levels of OLIG2 and ISL1 but within this case lack the expression of HOXC9. Cells were grafted into prospective thoracic regions of HH11-13 stage chick spinal cords at the level of the last forming somite (as shown in **Figure 4.5**). After 4-5 days of incubation the embryos were harvested and screened to select those which contained ZSGREEN+ cells within thoracic spinal cord locations and had no developmental defects.

28 embryos received a graft of PSC-derived hindbrain/cervical identity motor neuron progenitors within a thoracic location, of which 20 embryos survived a full term of incubation to HH26-30 with no developmental abnormalities. Within these 20 embryos, 11 embryos presented with ZSGREEN+ cells within spinal cord locations. 8 of these embryos were sectioned transversely to observe the integration of the PSC-derived ZSGREEN+ cells. 5 of the observed embryos (5/8, 62.5%) had the presence of motor neuron progenitors located within ventral/motor neuron spinal cord domains (**Figure 4.6C**). Within these embryos, there was also a large percentage (7/8, 87.5%) which displayed the presence of ZSGREEN+ cells dispersed across the dorso-ventral neural tube axis (**Figure 4.6D**). When observing the axonal projections of the ZSGREEN+ motor neuron progenitors there were

contributions to axial muscle innervating columns (2/8, 25%) and projections towards the sympathetic ganglion (5/8, 62.5%) (**Figure 4.6C**). However, there were also a large percentage of embryos which displayed ectopic neuronal projections (7/8, 87.5%) (**Figure 4.6C, D**). These projections were often observed to project into the dorsal neural tube and exit the neural tube similar to that of dorsolateral projecting cranial motor neurons (Krammer, 1987; Snider and Palavali, 1990; Ericson, Briscoe, *et al.*, 1997; Liinamaa, Keane and Richmond, 1997). These findings would suggest that upon grafting into a thoracic region the hPSC-derived hindbrain/cervical identity motor neuron progenitors retain a degree of intrinsic *in vitro*-derived behaviour and do not display plasticity in adopting behaviours of their surrounding environment.

4.3.2.3 Discussion

A large degree of the thoracic motor neuron progenitors integrated within the ventral motor neuron domain of the thoracic neural tube. These progenitors were capable of maturing and projecting axons within a stereotypical manner characteristic of their *in vivo* counterparts with very few ectopic behaviours observed. This suggests great potential for the use of thoracic-derived motor neuron progenitors within cell replacement therapies and regenerative medicine. 25% of embryos displayed hPSC-derived thoracic motor neuron projections towards the sympathetic ganglion characteristic of preganglionic motor column (PGC) contribution (Dasen, Liu and Jessell, 2003a; Dasen *et al.*, 2008; Rousso *et al.*, 2008) whilst 50% of embryos displayed contribution to projections characteristic of the hypaxial motor column (HMC) (Prasad and Hollyday, 1991; Tsuchida *et al.*, 1994). This is reflective of the molecular profiles of the Day 24 mature motor neurons, **Section 3.3.3**, which retain HOXC9 expression suggesting a PGC and HMC motor neurons are present at Day 24 of differentiation. Whilst the motor neurons which occupy the preganglionic motor column and hypaxial motor column express different molecular markers and project axons differently they are both specific to the thoracic region (Dasen, Liu and Jessell, 2003a; Thaler *et al.*, 2004; Dasen *et al.*, 2008; Rousso *et al.*, 2008). *In vivo*, the motor neurons contributing to both of these motor columns arise from the same population of ISLET1/2 expressing motor neuron progenitors (Thaler *et al.*, 2004). This suggests that the thoracic HOXC9^{+}

motor neuron progenitors obtained by Day 14 of differentiation, at the point of grafting, likely represent a population of motor neuron progenitors which are capable of contributing to both the preganglionic motor column and hypaxial motor columns.

Transplantation of hPSC-derived brachial/cervical identity motor neuron progenitors into the thoracic neural tube resulted in a large degree of ectopic behaviours which were not observed upon engraftment of the thoracic motor neuron progenitors within the same region. 87.5% of embryos displayed dorsal displacement of the brachial/cervical identity motor neuron progenitors across the neural tube. In multiple embryos ectopic dorsolateral projecting axons characteristic of cranial motor neurons were also observed (Krammer, 1987; Snider and Palavali, 1990; Ericson, Briscoe, *et al.*, 1997; Liinamaa, Keane and Richmond, 1997). This would suggest that upon engraftment into the thoracic region the motor neuron progenitors retain their *in vitro* obtained positional identity and display behaviours characteristic of their brachial/cervical identity. Although we do observe large degrees of ectopic projections upon engraftment of the anterior motor neuron progenitors there are some projections which appear to adopt projection pathways similar to the thoracic engrafted domain counterparts (with 62.5% of embryos displaying projections to the sympathetic ganglion). Therefore, suggesting that some of the engrafted progenitors retain a degree of plasticity and are capable to be patterned and change positional fate reflective of their engrafted environment. Multiple studies have demonstrated that transplanted tissue can alter positional identity upon engraftment suggesting that positional identity can display a degree of plasticity (Le Douarin and Teillet, 1974; Le Douarin *et al.*, 1975; Matise and Lance Jones, 1996). However, these studies are often carried out with tissue at early progenitor stages which have larger degrees of intrinsic plasticity. Transplantation of more mature tissue often results in ectopic behaviours upon engraftment and an inability to alter positional identity (Lance-Jones and Landmesser, 1980; Peljto *et al.*, 2010a). Taken together, our results would suggest that the brachial/cervical motor neuron progenitors display both ectopic behaviours and a degree of plasticity upon engraftment into the thoracic spinal cord. Further experiments will be required to explore the positional plasticity of these hPSC-derived progenitors. Grafting of cells into similar thoracic regions at earlier and later time-points of differentiation will allow us to see if the plasticity of cells is restricted by the developmental stage of the motor neurons.

Reciprocally, grafting the same motor neuron progenitors within the same region of the embryo but at later developmental stages will identify if the plasticity of the cells is an artefact of the endogenous environmental signals which are present during early development. Lastly, engraftment of co-cultures of differentially labelled anterior and thoracic motor neuron progenitors within the thoracic regions will be an extremely useful tool to identify the ability of the different motor neuron subtypes to integrate and function within the host environment whilst overcoming the limitations of potential engraftment differences.

Due to our ability to now efficiently generate thoracic identity motor neurons we can now further explore the plasticity of thoracic identity tissue by engraftment into brachial/cervical and lumbosacral regions. Engraftment of hPSC-derived motor neurons of differing developmental time points will also allow for the elucidation of the time-point at which positional identity become fixed. These experiments will be beneficial for potential cell-replacement therapies as transplantation of correct positionally defined neural progenitors has already been shown to be required to allow for correct regeneration of the spinal cord (Kadoya *et al.*, 2016; Rosenzweig *et al.*, 2018b).

Chapter 5 Summary of key finding and Future Works

Overall, I have characterised the signals which are important for the promotion of a pre-neural and neural tube identity downstream of hPSC-derived neuromesdoermal progenitors. Of note, I demonstrate that WNT signals, alongside FGF signals, are conducive with the production of a pre-neural identity and are not detrimental and specific to mesodermal specification as is commonly shown. In agreement with parallel publications I have also demonstrated the requirement for BMP and TGFβ antagonism, alongside Retinoic Acid, to increase the rate of neural induction from PSC-derived NMPs. Continued presence of BMP and TGFβ antagonism are also required to repress dorsal neural tube identity and promote ventral identities. Furthermore, I have demonstrated that the presence of extended FGF and WNT signals are essential for the maintenance of CDX2 and thoracic HOX expression during neural restriction of NMPs. Fundamentally, suggesting that during neural restriction the FGF-CDX relationship has a greater importance on the maintenance of thoracic HOX than the fully defined WNT-CDX-thoracic HOX relationship.

Uniquely, combining the presence of BMP and TGFβ antagonism alongside extended durations of FGF and Wnt signals, after an NMP intermediate state, resulted in high yields of mature motor neurons. To the best of my knowledge, this characterised protocol provides the highest yields of thoracic motor neurons of any existing protocol and is reproducible within multiple hPSC cell lines. Furthermore, the hPSC-derived thoracic identity motor neurons are electrophysiologically functional and demonstrate the ability to integrate within the neural tube of chick embryos which shows great promise for regenerative medicine.

I have shown that thoracic hPSC-derived motor neuron progenitors can integrate within thoracic chick embryo neural tubes whilst hindbrain/cervical hPSC-derived motor neuron progenitors display a higher degree of ectopic activities. Suggesting that acquired positional identity plays a large role on the behaviours of engrafted cells which demonstrate limited levels of plasticity within the host environment. This is of vital importance when considering the transplantation of tissue during cell replacement therapies.

Ultimately, the ability to now generate high yields of thoracic motor neurons will be of great benefit to further explore disease vulnerabilities within these cell types and more in depth

analysis of positional identity plasticity. In future works it will be important to fully characterise the positional identity of the mature posterior motor neurons obtained. Further exploration of the expression profiles of the motor neurons at day 24 will allow me to identify the specific columnar identity of the hPSC-derived motor neurons. This will be important as this will inform on which muscular targets these neurons are likely to innervate for cell replacement therapies. As previously discussed, there is also a need to extend the scope of the grafting experiments to explore different positional engraftment sites and grafting cells at different times of differentiation. These experiments will have great impact on potential cell replacement therapies as it will allow me to identify if hPSC-derived motor neurons display plasticity upon integration or if the *in vitro* derived positional identity remains fixed and robust. Finally, it would be interesting to observe if these in vitro-derived motor neurons display any characteristic and functional abnormalities when derived from Amyotrophic Lateral Sclerosis (ALS) patient derived and C9orf mutant hES cells. Exploring the capabilities of these cells to differentiate, conduct action potentials and engraft into host tissue could provide a useful tool within disease modelling.

Chapter 6 References

Aires, R. *et al.* (2019) 'Tail Bud Progenitor Activity Relies on a Network Comprising Gdf11, Lin28, and Hox13 Genes', *Developmental Cell*. Cell Press, 48(3), pp. 383-395.e8. doi: 10.1016/j.devcel.2018.12.004.

van den Akker, E. *et al.* (2002) 'Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation', *Development*, pp. 2181–2193.

Albors, A. R., Halley, P. A. and Storey, K. G. (2018) 'Lineage tracing of axial progenitors using nkx1-2creert2 mice defines their trunk and tail contributions', *Development (Cambridge)*. Company of Biologists Ltd, 145(19). doi: 10.1242/dev.164319.

Alessandri-Haber, N. *et al.* (1999) 'Specific distribution of sodium channels in axons of rat embryo spinal motoneurones', *Journal of Physiology*. Cambridge University Press, 518(1), pp. 203–214. doi: 10.1111/j.1469-7793.1999.0203r.x.

Allan, D. *et al.* (2001) 'RARγ and Cdx1 interactions in vertebral patterning', *Developmental Biology*. Academic Press Inc., 240(1), pp. 46–60. doi: 10.1006/dbio.2001.0455.

Amin, S. *et al.* (2016a) 'Cdx and T Brachyury Co-activate Growth Signaling in the Embryonic Axial Progenitor Niche', *Cell Reports*. Elsevier B.V., 17(12), pp. 3165–3177. doi: 10.1016/j.celrep.2016.11.069.

Amin, S. *et al.* (2016b) 'Cdx and T Brachyury Co-activate Growth Signaling in the Embryonic Axial Progenitor Niche', *Cell Reports*. ElsevierCompany., 17(12), pp. 3165–3177. doi: 10.1016/j.celrep.2016.11.069.

Amoroso, Mackenzie W. *et al.* (2013) 'Accelerated high-yield generation of limb-innervating motor neurons from human stem cells', *Journal of Neuroscience*, 33(2), pp. 574–586. doi: 10.1523/JNEUROSCI.0906-12.2013.

Amoroso, M. W. *et al.* (2013) 'Accelerated High-Yield Generation of Limb-Innervating Motor Neurons from Human Stem Cells', *Journal of Neuroscience*, 33(2), pp. 574–586. doi: 10.1523/JNEUROSCI.0906-12.2013.

Anderson, M. J. *et al.* (2020) 'Fgf4 is critical for maintaining Hes7 levels and Notch

oscillations in the somite segmentation clock', *bioRxiv*, p. 2020.02.12.945931. doi: 10.1101/2020.02.12.945931.

Arber, S. *et al.* (1999) 'Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity', *Neuron*. Cell Press, 23(4), pp. 659–674. doi: 10.1016/S0896-6273(01)80026-X.

Bachiller, D. *et al.* (2000) 'The organizer factors Chordin and Noggin are required for mouse forebrain development', *Nature*, 403(6770), pp. 658–661. doi: 10.1038/35001072.

Bar-Nur, O. *et al.* (2011) 'Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells', *Cell Stem Cell*. Cell Stem Cell, 9(1), pp. 17–23. doi: 10.1016/j.stem.2011.06.007.

Basler, K. *et al.* (1993) 'Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGF beta family member.', *Cell*, 73(4), pp. 687–702. doi: 10.1016/0092-8674(93)90249-p.

Beck, F. *et al.* (1995) 'Expression of Cdx-2 in the mouse embryo and placenta: Possible role in patterning of the extra-embryonic membranes', *Developmental Dynamics*, 204(3), pp. 219–227. doi: 10.1002/aja.1002040302.

Beddington, R. S. P. (1994) 'Induction of a second neural axis by the mouse node', *Development*, 120(3), pp. 613–620.

Bel-Vialar, S., Itasaki, N. and Krumlauf, R. (2002) 'Initiating Hox gene expression: In the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups', *Development*, pp. 5103–5115.

Ben-Shushan, E., Feldman, E. and Reubinoff, B. E. (2015) 'Notch signaling regulates motor neuron differentiation of human embryonic stem cells', *Stem Cells*, 33(2), pp. 403–415. doi: 10.1002/stem.1873.

Bertrand, N., Médevielle, F. and Pituello, F. (2000) 'FGF signalling controls the timing of Pax6 activation in the neural tube.', *Development (Cambridge, England)*, 127(22), pp. 4837–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11044398 (Accessed: 28 April 2020).

Blumberg, B. *et al.* (1997) 'An essential role for retinoid signaling in anteroposterior neural

patterning', *Development*, 124(2), pp. 373–379.

Boettger, T. *et al.* (2001) 'The avian organizer', *International Journal of Developmental Biology*. UPV/EHU Press, 45(1), pp. 281–287. doi: 10.1387/ijdb.11291858.

Bouldin, C. M. *et al.* (2015) 'Wnt signaling and tbx16 form a bistable switch to commit bipotential progenitors to mesoderm', *Development (Cambridge)*. Company of Biologists Ltd, 142(14), pp. 2499–2507. doi: 10.1242/dev.124024.

Boulet, A. M. and Capecchi, M. R. (2012) 'Signaling by FGF4 and FGF8 is required for axial elongation of the mouse embryo', *Developmental Biology*. Elsevier, 371(2), pp. 235–245. doi: 10.1016/j.ydbio.2012.08.017.

Boulting, G. L. *et al.* (2011) 'A functionally characterized test set of human induced pluripotent stem cells', *Nature Biotechnology*, 29(3), pp. 279–287. doi: 10.1038/nbt.1783.

Bouwmeester, T. *et al.* (1996) 'Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer', *Nature*, 382(6592), pp. 595–601. doi: 10.1038/382595a0.

Briscoe, J. *et al.* (1999) 'Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling', *Nature*, 398(6728), pp. 622–627. doi: 10.1038/19315.

Briscoe, J. *et al.* (2000) 'A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube', *Cell*. Cell Press, 101(4), pp. 435–445. doi: 10.1016/S0092-8674(00)80853-3.

Bronner-Fraser, M. and Fraser, S. E. (1988) 'Cell lineage analysis reveals multipotency of some avian neural crest cells', *Nature*. Nature, 335(6186), pp. 161–164. doi: 10.1038/335161a0.

Brown, J. M. and Storey, K. G. (2000) 'A region of the vertebrate neural plate in which neighbouring cells can adopt neural or epidermal fates', *Current Biology*, 10(14), pp. 869–872. doi: 10.1016/S0960-9822(00)00601-1.

Bueker, E. D. (1944) 'Differentiation of the lateral motor column in the avian spinal cord', *Science*, p. 169. doi: 10.1126/science.100.2591.169.

Bylund, M. *et al.* (2003) 'Vertebrate neurogenesis is counteracted by Sox1-3 activity', *Nature Neuroscience*, 6(11), pp. 1162–1168. doi: 10.1038/nn1131.

Cambray, N. and Wilson, V. (2002) 'Axial progenitors with extensive potency are localised to the mouse chordoneural hinge.', *Development (Cambridge, England)*, 129(20), pp. 4855–66. doi: 10.1016/s0925-4773(98)00015-x.

Cambray, Noemi and Wilson, V. (2007) 'Two distinct sources for a population of maturing axial progenitors', *Development*, 134(15), pp. 2829–2840. doi: 10.1242/dev.02877.

Cambray, N. and Wilson, V. (2007) 'Two distinct sources for a population of maturing axial progenitors', *Development*, 134(15), pp. 2829–2840. doi: 10.1242/dev.02877.

Carpenter EM *et al.* (1997) 'Targeted disruption of Hoxd-10 affects mouse hindlimb development.', *Development*, 124(22), pp. 4505–14.

Cauthen, C. A. *et al.* (2001) 'Comparative analysis of the expression patterns of Wnts and Frizzleds during early myogenesis in chick embryos', *Mechanisms of Development*, 104(1–2), pp. 133–138. doi: 10.1016/S0925-4773(01)00369-0.

Chalamalasetty, R. B. *et al.* (2014) 'Mesogenin 1 is a master regulator of paraxial presomitic mesoderm differentiation', *Development (Cambridge)*. Company of Biologists Ltd, 141(22), pp. 4285–4297. doi: 10.1242/dev.110908.

Chambers, S. M. *et al.* (2009a) 'Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling', *Nature Biotechnology*, 27(3), pp. 275–280. doi: 10.1038/nbt.1529.

Chambers, S. M. *et al.* (2009b) 'Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling', *Nature Biotechnology*. NIH Public Access, 27(3), pp. 275–280. doi: 10.1038/nbt.1529.

Chambers, S. M. *et al.* (2009c) 'Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling', *Nature Biotechnology*, 27(3), pp. 275–280. doi: 10.1038/nbt.1529.

Chapman, D. L. *et al.* (1996) 'Tbx6, a mouse T-box gene implicated in paraxial mesoderm formation at gastrulation', *Developmental Biology*. Academic Press Inc., 180(2), pp. 534–

542. doi: 10.1006/dbio.1996.0326.

Chapman, D. L. and Papaioannou, V. E. (1998) 'Three neural tubes in mouse embryos with mutations in T-box gene Tbx6', *Nature*, 391(6668), pp. 695–697. doi: 10.1038/35624.

Charité, J. *et al.* (1998) 'Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements.', *Development (Cambridge, England)*, 125(22), pp. 4349–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9778495 (Accessed: 20 December 2019).

Chen, J. A. *et al.* (2011) 'Mir-17-3p Controls Spinal Neural Progenitor Patterning by Regulating Olig2/Irx3 Cross-Repressive Loop', *Neuron*, 69(4), pp. 721–735. doi: 10.1016/j.neuron.2011.01.014.

Chesnutt, C. *et al.* (2004) 'Coordinate regulation of neural tube patterning and proliferation by TGFβ and WNT activity', *Developmental Biology*. Academic Press Inc., 274(2), pp. 334–347. doi: 10.1016/j.ydbio.2004.07.019.

Chiang, C. *et al.* (1996) 'Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function', *Nature*, 383(6599), pp. 407–413. doi: 10.1038/383407a0.

Cho, H. H. *et al.* (2014) 'Isl1 Directly Controls a Cholinergic Neuronal Identity in the Developing Forebrain and Spinal Cord by Forming Cell Type-Specific Complexes', *PLoS Genetics*. Public Library of Science, 10(4). doi: 10.1371/journal.pgen.1004280.

Colbert, M. C., Linney, E. and LaMantia, A. S. (1993) 'Local sources of retinoic acid coincide with retinoid-mediated transgene activity during embryonic development.', *Proceedings of the National Academy of Sciences of the United States of America*, 90(14), pp. 6572–6. doi: 10.1073/pnas.90.14.6572.

Collazo, A., Bronner-Fraser, M. . and Fraser S E. (1993) 'Vital dye labelling of Xenopus laevis trunk neural crest reveals multipotency and novel pathways of migration - PubMed', *Development*, 2(118), pp. 363–76. Available at: https://pubmed.ncbi.nlm.nih.gov/7693414/ (Accessed: 6 August 2020).

Conlon, R. A. and Rossant, J. (1992) 'Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo.', *Development (Cambridge, England)*, 116(2), pp.

357–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1363087 (Accessed: 20 December 2019).

Connolly, D. J., Patel, K. and Cooke, J. (1997) 'Chick noggin is expressed in the organizer and neural plate during axial development, but offers no evidence of involvement in primary axis formation - PubMed', *Int J Dev Biology*, 41(2), pp. 389–96. Available at: https://pubmed.ncbi.nlm.nih.gov/9184349/ (Accessed: 7 August 2020).

Del Corral, R. D. *et al.* (2003) 'Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension', *Neuron*. Cell Press, 40(1), pp. 65–79. doi: 10.1016/S0896-6273(03)00565-8.

Cortés, D. *et al.* (2017) 'The non-survival effects of Glial cell line-derived neurotrophic factor on neural cells', *Frontiers in Molecular Neuroscience*. Frontiers Media S.A. doi: 10.3389/fnmol.2017.00258.

Cox, W. G. and Hemmati-Brivanlou, A. (1995) 'Caudalization of neural fate by tissue recombination and bFGF', *Development*, 121(12), pp. 4349–4358.

Cunningham, T. J., Brade, T., *et al.* (2015) 'Retinoic acid activity in undifferentiated neural progenitors is sufficient to fulfill its role in restricting Fgf8 expression for somitogenesis', *PLoS ONE*, 10(9), pp. 1–15. doi: 10.1371/journal.pone.0137894.

Cunningham, T. J., Kumar, S., *et al.* (2015) 'Wnt8a and Wnt3a cooperate in the axial stem cell niche to promote mammalian body axis extension', *Developmental Dynamics*, 244(6), pp. 797–807. doi: 10.1002/dvdy.24275.

Cunningham, T. J., Colas, A. and Duester, G. (2016) 'Early molecular events during Retinoic acid induced differentiation of neuromesodermal progenitors', *Biology Open*. Company of Biologists Ltd, 5(12), pp. 1821–1833. doi: 10.1242/bio.020891.

Cunningham, T. J., Zhao, X. and Duester, G. (2011) 'Uncoupling of retinoic acid signaling from tailbud development before termination of body axis extension', *Genesis*, pp. 776–783. doi: 10.1002/dvg.20763.

Dasen, J. S. *et al.* (2005a) 'A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity', *Cell*, 123(3), pp. 477–491. doi: 10.1016/j.cell.2005.09.009.

Dasen, J. S. *et al.* (2005b) 'A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity', *Cell*, 123(3), pp. 477–491. doi: 10.1016/j.cell.2005.09.009.

Dasen, J. S. *et al.* (2008) 'Hox Repertoires for Motor Neuron Diversity and Connectivity Gated by a Single Accessory Factor, FoxP1', *Cell*, 134(2), pp. 304–316. doi: 10.1016/j.cell.2008.06.019.

Dasen, J. S., Liu, J. P. and Jessell, T. M. (2003a) 'Motor neuron columnar fate imposed by sequential phases of Hox-c activity', *Nature*, 425(6961), pp. 926–933. doi: 10.1038/nature02051.

Dasen, J. S., Liu, J. P. and Jessell, T. M. (2003b) 'Motor neuron columnar fate imposed by sequential phases of Hox-c activity', *Nature*, 425(6961), pp. 926–933. doi: 10.1038/nature02051.

Delfino-Machín, M., Lunn, J Simon, *et al.* (2005) 'Specification and maintenance of the spinal cord stem zone.', *Development (Cambridge, England)*, 132(19), pp. 4273–83. doi: 10.1242/dev.02009.

Delfino-Machín, M., Lunn, J. S., *et al.* (2005a) 'Specification and maintenance of the spinal cord stem zone', *Development*, 132(19), pp. 4273–4283. doi: 10.1242/dev.02009.

Delfino-Machín, M., Lunn, J. S., *et al.* (2005b) 'Specification and maintenance of the spinal cord stem zone', *Development*. The Company of Biologists Ltd, 132(19), pp. 4273–4283. doi: 10.1242/dev.02009.

Denham, M. *et al.* (2015) 'Multipotent caudal neural progenitors derived from human pluripotent stem cells that give rise to lineages of the central and peripheral nervous system', *Stem Cells*. Wiley-Blackwell, 33(6), pp. 1759–1770. doi: 10.1002/stem.1991.

Deschamps, J. *et al.* (1999) 'Initiation, establishment and maintenance of Hox gene expression patterns in the mouse', *International Journal of Developmental Biology*. UPV/EHU Press, pp. 635–650. doi: 10.1387/ijdb.10668974.

Diaz-Cuadros, M. *et al.* (2020) 'In vitro characterization of the human segmentation clock', *Nature*. Nature Research, 580(7801), pp. 113–118. doi: 10.1038/s41586-019-1885-9.

Diez del Corral, R., Breitkreuz, D. N. and Storey, K. G. (2002) 'Onset of neuronal

differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling.', *Development (Cambridge, England)*, 129(7), pp. 1681–91. Available at: papers2://publication/uuid/1A5A8EC4-D5BD-48C3-8395-69C8084E04A4%5Cnhttp://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=11923204&retmode=ref&cmd=prlinks%5Cnpapers2://publication/uuid/6B4994D1-C07D-4DB7-8173-147F90CCED61%0Ahttp://www.nc.

Le Douarin, N. M. *et al.* (1975) 'Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterotopic transplantations', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 72(2), pp. 728–732. doi: 10.1073/pnas.72.2.728.

Le Douarin, N. M. and Dupin, E. (2018) 'The "beginnings" of the neural crest', *Developmental Biology*. Elsevier Inc., pp. S3–S13. doi: 10.1016/j.ydbio.2018.07.019.

Le Douarin, N. M. and Teillet, M. A. M. (1974) 'Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique', *Developmental Biology*. Dev Biol, 41(1), pp. 162–184. doi: 10.1016/0012-1606(74)90291-7.

Du, Z.-W. *et al.* (2015) 'Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells', *Nature Communications*. Nature Publishing Group, 6, p. 6626. doi: 10.1038/ncomms7626.

Duboule, D. and Dollé, P. (1989) 'The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes.', *The EMBO Journal*. Wiley, 8(5), pp. 1497–1505. doi: 10.1002/j.1460-2075.1989.tb03534.x.

Dubrulle, J., McGrew, M. J. and Pourquié, O. (2001) 'FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation', *Cell*, 106(2), pp. 219–232. doi: 10.1016/S0092-8674(01)00437-8.

Dupé, V. *et al.* (1997) 'In vivo functional analysis of the Hoxa-1 3' retinoic acid response element (3'RARE)', *Development*, 124(2), pp. 399–410.

Dupé, V. and Lumsden, A. (2001) 'Hindbrain patterning involves graded responses to

retinoic acid signalling', *Development*, 128(12), pp. 2199–2208.

Duprey, P. *et al.* (1988) 'A mouse gene homologous to the Drosophila gene caudal is expressed in epithelial cells from the embryonic intestine.', *Genes & development*, 2(12 A), pp. 1647–1654. doi: 10.1101/gad.2.12a.1647.

Durston, A. J. *et al.* (1989) 'Retinoic acid causes an anteroposterior transformation in the developing central nervous system', *Nature*, 340(6229), pp. 140–144. doi: 10.1038/340140a0.

Economides, K. D., Zeltser, L. and Capecchi, M. R. (2003) 'Hoxb13 mutations cause overgrowth of caudal spinal cordand tail vertebrae', *Developmental Biology*. Academic Press Inc., 256(2), pp. 317–330. doi: 10.1016/S0012-1606(02)00137-9.

Edri, S. *et al.* (2019) 'An epiblast stem cell-derived multipotent progenitor population for axial extension', *Development (Cambridge)*. Company of Biologists Ltd, 146(10). doi: 10.1242/dev.168187.

Ensini, M. *et al.* (1998) 'The control of rostrocaudal pattern in the developing spinal cord: Specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm', *Development*, 125(6), pp. 969–982.

Ericson, J. *et al.* (1992) 'Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1', *Science*, 256(5063), pp. 1555–1560. doi: 10.1126/science.1350865.

Ericson, J. *et al.* (1996) 'Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity', *Cell*. Cell Press, 87(4), pp. 661–673. doi: 10.1016/S0092-8674(00)81386-0.

Ericson, J., Briscoe, J., *et al.* (1997) 'Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube', in *Cold Spring Harbor Symposia on Quantitative Biology*, pp. 451–466.

Ericson, J., Rashbass, P., *et al.* (1997) 'Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling', *Cell*. Cell Press, 90(1), pp. 169–180. doi: 10.1016/S0092-8674(00)80323-2.

Erskine, L., Patel, K. and Clarke, J. D. W. (1998) 'Progenitor dispersal and the origin of early neuronal phenotypes in the chick embryo spinal cord', *Developmental Biology*. Academic Press Inc., 199(1), pp. 26–41. doi: 10.1006/dbio.1998.8912.

Estevez-silva, M. C. *et al.* (2018a) 'Single-injection ex ovo transplantation method for broad spinal cord engraftment of human pluripotent stem cell-derived motor neurons', *Journal of Neuroscience Methods*. Elsevier B.V., 298, pp. 16–23. doi: 10.1016/j.jneumeth.2018.01.006.

Estevez-silva, M. C. *et al.* (2018b) 'Single-injection ex ovo transplantation method for broad spinal cord engraftment of human pluripotent stem cell-derived motor neurons', *Journal of Neuroscience Methods*. Elsevier B.V., 298, pp. 16–23. doi: 10.1016/j.jneumeth.2018.01.006.

Evans, M. J. and Kaufman, M. H. (1981) 'Establishment in culture of pluripotential cells from mouse embryos', *Nature*, 292(5819), pp. 154–156. doi: 10.1038/292154a0.

Faiella, A. *et al.* (1994) 'Inhibition of retinoic acid-induced activation of 3' human HOXB genes by antisense oligonucleotides affects sequential activation of genes located upstream in the four HOX clusters', *Proceedings of the National Academy of Sciences of the United States of America*, 91(12), pp. 5335–5339. doi: 10.1073/pnas.91.12.5335.

Fainsod, A. *et al.* (1997) 'The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4', *Mechanisms of Development*, 63(1), pp. 39–50. doi: 10.1016/S0925-4773(97)00673-4.

Faravelli, I. *et al.* (2014) 'Motor neuron derivation from human embryonic and induced pluripotent stem cells: Experimental approaches and clinical perspectives', *Stem Cell Research and Therapy*. BioMed Central Ltd., p. 87. doi: 10.1186/scrt476.

Faustino Martins, J. M. *et al.* (2020) 'Self-Organizing 3D Human Trunk Neuromuscular Organoids', *Cell Stem Cell*. Cell Press, 26(2), pp. 172-186.e6. doi: 10.1016/j.stem.2019.12.007.

Favier, B. (1997) 'Developmental functions of mammalian Hox genes', *Molecular Human Reproduction*. Oxford University Press (OUP), 3(2), pp. 115–131. doi: 10.1093/molehr/3.2.115.

Forlani, S. (2003) 'Acquisition of Hox codes during gastrulation and axial elongation in the

mouse embryo', *Development*, 130(16), pp. 3807–3819. doi: 10.1242/dev.00573.

Frasch, M., Chen, X. and Lufkin, T. (1995) 'Evolutionary-conserved enhancers direct region-specific expression of the murine Hoxa-1 and Hoxa-2 loci in both mice and Drosophila.', *Development (Cambridge, England)*, 121(4), pp. 957–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7743939 (Accessed: 20 December 2019).

Frith, T. J. R. *et al.* (2018a) 'Human axial progenitors generate trunk neural crest cells in vitro', *eLife*. eLife Sciences Publications Ltd, 7. doi: 10.7554/eLife.35786.

Frith, T. J. R. *et al.* (2018b) 'Human axial progenitors generate trunk neural crest cells in vitro', *eLife*. eLife Sciences Publications Ltd, 7. doi: 10.7554/eLife.35786.

Fujii, H. *et al.* (1997) 'Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos', *EMBO Journal*, 16(14), pp. 4163–4173. doi: 10.1093/emboj/16.14.4163.

Gamer, L. W. and Wright, C. V. E. (1993) 'Murine Cdx-4 bears striking similarities to the Drosophila caudal gene in its homeodomain sequence and early expression pattern', *Mechanisms of Development*, 43(1), pp. 71–81. doi: 10.1016/0925-4773(93)90024-R.

Garriock, Robert J. *et al.* (2015) 'Lineage tracing of neuromesodermal progenitors reveals novel wnt-dependent roles in trunk progenitor cell maintenance and differentiation', *Development (Cambridge)*. Company of Biologists Ltd, 142(9), pp. 1628–1638. doi: 10.1242/dev.111922.

Garriock, R. J. *et al.* (2015) 'Lineage tracing of neuromesodermal progenitors reveals novel Wnt-dependent roles in trunk progenitor cell maintenance and differentiation', *Development*, 142(9), pp. 1628–1638. doi: 10.1242/dev.111922.

Gaunt, S. J. (2018) 'Mouse embryo hox gene enhancers assayed in cell culture: Hoxb4, b8 and a7 are activated by Cdx1 protein', *International Journal of Developmental Biology*. University of the Basque Country Press, 62(11–12), pp. 717–722. doi: 10.1387/ijdb.180157sg.

Gaunt, S. J., George, M. and Paul, Y. L. (2013) 'Direct activation of a mouse Hoxd11 axial expression enhancer by Gdf11/Smad signalling', *Developmental Biology*. Academic Press

Inc., 383(1), pp. 52–60. doi: 10.1016/j.ydbio.2013.08.025.

Gaunt, S. J., Krumlauf, R. and Duboule, D. (1989) 'Mouse homeo-genes within a subfamily, Hox-1.4, -2.6 and -5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation.', *Development (Cambridge, England)*, 107(1), pp. 131–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2576400 (Accessed: 18 December 2017).

Gofflot, F., Hall, M. and Morriss-Kay, G. M. (1997) 'Genetic patterning of the developing mouse tail at the time of posterior neuropore closure.', *Developmental dynamics : an official publication of the American Association of Anatomists*, 210(4), pp. 431–45. doi: 10.1002/(SICI)1097-0177(199712)210:4<431::AID-AJA7>3.0.CO;2-H.

Goto, H. *et al.* (2017) 'FGF and canonical Wnt signaling cooperate to induce paraxial mesoderm from tailbud neuromesodermal progenitors through regulation of a two-step epithelial to mesenchymal transition', *Development*, 144(8), pp. 1412–1424. doi: 10.1242/dev.143578.

Gouti, M. *et al.* (2014a) 'In vitro generation of neuromesodermal progenitors reveals distinct roles for wnt signalling in the specification of spinal cord and paraxial mesoderm identity', *PLoS Biology*. Public Library of Science, 12(8). doi: 10.1371/journal.pbio.1001937.

Gouti, M. *et al.* (2014b) 'In vitro generation of neuromesodermal progenitors reveals distinct roles for wnt signalling in the specification of spinal cord and paraxial mesoderm identity', *PLoS Biology*. Public Library of Science, 12(8), p. e1001937. doi: 10.1371/journal.pbio.1001937.

Gouti, M. *et al.* (2014c) 'In vitro generation of neuromesodermal progenitors reveals distinct roles for wnt signalling in the specification of spinal cord and paraxial mesoderm identity', *PLoS Biology*, 12(8). doi: 10.1371/journal.pbio.1001937.

Gouti, M. *et al.* (2017) 'A Gene Regulatory Network Balances Neural and Mesoderm Specification during Vertebrate Trunk Development', *Developmental Cell*. Elsevier Inc., 41(3), pp. 243-261.e7. doi: 10.1016/j.devcel.2017.04.002.

Graham, A. and Lumsden, A. (1996) 'Interactions between rhombomeres modulate Krox-20

and follistatin expression in the chick embryo hindbrain | Development', *Development*, 122, pp. 473–80. Available at: https://dev.biologists.org/content/122/2/473 (Accessed: 7 August 2020).

Griffin, K. J. P. *et al.* (1998) 'Molecular identification of spadetail: Regulation of zebrafish trunk and tail mesoderm formation by T-box genes', *Development*, 125(17), pp. 3379–3388.

Gutman, C. R., Ajmera, M. K. and Hollyday, M. (1993) 'Organization of motor pools supplying axial muscles in the chicken', *Brain Research*, 609(1–2), pp. 129–136. doi: 10.1016/0006-8993(93)90865-K.

Hackland, J. O. S. *et al.* (2019) 'FGF Modulates the Axial Identity of Trunk hPSC-Derived Neural Crest but Not the Cranial-Trunk Decision', *Stem Cell Reports*. Cell Press, 12(5), pp. 920–933. doi: 10.1016/j.stemcr.2019.04.015.

Hawley, S. H. B. *et al.* (1995) 'Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction', *Genes and Development*. Cold Spring Harbor Laboratory Press, 9(23), pp. 2923–2935. doi: 10.1101/gad.9.23.2923.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994) 'Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity', *Cell*, 77(2), pp. 283–295. doi: 10.1016/0092-8674(94)90320-4.

Hemmati-Brivanlou, A. and Melton, D. A. (1994) 'Inhibition of activin receptor signaling promotes neuralization in Xenopus', *Cell*, 77(2), pp. 273–281. doi: 10.1016/0092-8674(94)90319-0.

Henderson, C. E. *et al.* (1994) 'GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle', *Science*, 266(5187), pp. 1062–1064. doi: 10.1126/science.7973664.

Hoey, T. *et al.* (1986) 'Homeo box gene expression in anterior and posterior regions of the Drosophila embryo', *Proceedings of the National Academy of Sciences of the United States of America*, 83(13), pp. 4809–4813. doi: 10.1073/pnas.83.13.4809.

Hollyday, M., Hamburger, V. and Farris, J. M. G. (1977) 'Localization of motor neuron pools supplying identified muscles in normal and supernumerary legs of chick embryo',

Proceedings of the National Academy of Sciences of the United States of America, 74(8), pp. 3582–3586. doi: 10.1073/pnas.74.8.3582.

Hollyday, M. and Jacobson, R. D. (1990) 'Location of motor pools innervating chick wing', *Journal of Comparative Neurology*, 302(3), pp. 575–588. doi: 10.1002/cne.903020313.

Hollyday, M., McMahon, J. A. and McMahon, A. P. (1995) 'Wnt expression patterns in chick embryo nervous system', *Mechanisms of Development*, 52(1), pp. 9–25. doi: 10.1016/0925-4773(95)00385-E.

Hooiveld, M. H. *et al.* (1999) 'Novel interactions between vertebrate Hox genes.', *The International journal of developmental biology*, 43(7), pp. 665–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10668976 (Accessed: 21 December 2019).

Houle, M. *et al.* (2000) 'Retinoic Acid Regulation of Cdx1: an Indirect Mechanism for Retinoids and Vertebral Specification', *Molecular and Cellular Biology*. American Society for Microbiology, 20(17), pp. 6579–6586. doi: 10.1128/mcb.20.17.6579-6586.2000.

Houle, M., Sylvestre, J. R. and Lohnes, D. (2003) 'Retinoic acid regulates a subset of Cdx1 funtion in vivo', *Development*, 130(26), pp. 6555–6567. doi: 10.1242/dev.00889.

Hu, B. Y. *et al.* (2010) 'Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(9), pp. 4335–4340. doi: 10.1073/pnas.0910012107.

Hu, B. Y. and Zhang, S. C. (2009) 'Differentiation of spinal motor neurons from pluripotent human stem cells', *Nature Protocols*, 4(9), pp. 1295–1304. doi: 10.1038/nprot.2009.127.

Hynes, M. *et al.* (1995) 'Control of neuronal diversity by the floor plate: Contact-mediated induction of midbrain dopaminergic neurons', *Cell*, 80(1), pp. 95–101. doi: 10.1016/0092-8674(95)90454-9.

Ikeya, M. and Takada, S. (2001) 'Wnt-3a is required for somite specification along the anteroposterior axis of the mouse embryo and for regulation of cdx-1 expression', *Mechanisms of Development*, 103(1–2), pp. 27–33. doi: 10.1016/S0925-4773(01)00338-0.

Ille, F. *et al.* (2007) 'Wnt/BMP signal integration regulates the balance between proliferation

and differentiation of neuroepithelial cells in the dorsal spinal cord', *Developmental Biology*. Academic Press Inc., 304(1), pp. 394–408. doi: 10.1016/j.ydbio.2006.12.045.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1998) 'Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3', *EMBO Journal*, 17(12), pp. 3413–3427. doi: 10.1093/emboj/17.12.3413.

Iulianella, A. *et al.* (1999) 'A molecular basis for retinoic acid-induced axial truncation', *Developmental Biology*. Academic Press Inc., 205(1), pp. 33–48. doi: 10.1006/dbio.1998.9110.

Jung, H. *et al.* (2010) 'Global Control of Motor Neuron Topography Mediated by the Repressive Actions of a Single Hox Gene', *Neuron*, 67(5), pp. 781–796. doi: 10.1016/j.neuron.2010.08.008.

Jung, H. *et al.* (2014) 'Evolving hox activity profiles govern diversity in locomotor systems', *Developmental Cell*. Cell Press, 29(2), pp. 171–187. doi: 10.1016/j.devcel.2014.03.008.

Jungbluth, S., Koentges, G. and Lumsden, A. (1997) 'Coordination of early neural tube development by BDNF/trkB.', *Development (Cambridge, England)*, 124(10), pp. 1877–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9169835 (Accessed: 21 April 2020).

K Dale, K. *et al.* (1999) 'Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin - PubMed', *Development*, 126(2), pp. 397–408. Available at: https://pubmed.ncbi.nlm.nih.gov/9847252/ (Accessed: 7 August 2020).

Kadoya, K. *et al.* (2016) 'Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration', *Nature Medicine*. Nature Publishing Group, 22(5), pp. 479–487. doi: 10.1038/nm.4066.

Kajiwara, M. *et al.* (2012) 'Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 109(31), pp. 12538–12543. doi: 10.1073/pnas.1209979109.

Kania, A. and Jessell, T. M. (2003) 'Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions.', *Neuron*, 38(4), pp.

581–96. doi: 10.1016/s0896-6273(03)00292-7.

Karabagli, H. *et al.* (2002) 'Comparison of the expression patterns of several fibroblast growth factors during chick gastrulation and neurulation', *Anatomy and Embryology*, 205(5–6), pp. 365–370. doi: 10.1007/s00429-002-0264-7.

Kattman, S. J. *et al.* (2011) 'Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines', *Cell Stem Cell*. Cell Stem Cell, 8(2), pp. 228–240. doi: 10.1016/j.stem.2010.12.008.

Keenan, I. D., Sharrard, R. M. and Isaacs, H. V. (2006) 'FGF signal transduction and the regulation of Cdx gene expression', *Developmental Biology*. Academic Press Inc., 299(2), pp. 478–488. doi: 10.1016/j.ydbio.2006.08.040.

Kelly GM *et al.* (1995) 'Zebrafish wnt8 and wnt8b share a common activity but are involved in distinct developmental pathways.', *Development*, 121(6), pp. 1787–99.

Kengaku, M. and Okamoto, H. (1995) 'bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in Xenopus', *Development*, 121(9), pp. 3121–3130.

Khokha, M. K. *et al.* (2005) 'Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures', *Developmental Cell*, 8(3), pp. 401–411. doi: 10.1016/j.devcel.2005.01.013.

Kim, K. *et al.* (2010) 'Epigenetic memory in induced pluripotent stem cells', *Nature*. Nature Publishing Group, 467(7313), pp. 285–290. doi: 10.1038/nature09342.

Kim, K. *et al.* (2011) 'Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells', *Nature Biotechnology*. NIH Public Access, 29(12), pp. 1117–1119. doi: 10.1038/nbt.2052.

Kimmel, C. B. *et al.* (1989) 'A mutation that changes cell movement and cell fate in the zebrafish embryo', *Nature*, 337(6205), pp. 358–362. doi: 10.1038/337358a0.

Koch, F. *et al.* (2017) 'Antagonistic Activities of Sox2 and Brachyury Control the Fate Choice of Neuro-Mesodermal Progenitors', *Developmental Cell*. Elsevier Inc., 42(5), pp. 514-526.e7. doi: 10.1016/j.devcel.2017.07.021.

Kong, J. H. *et al.* (2015) 'Notch Activity Modulates the Responsiveness of Neural Progenitors to Sonic Hedgehog Signaling', *Developmental Cell*. Cell Press, 33(4), pp. 373–387. doi: 10.1016/j.devcel.2015.03.005.

Koshida, S. *et al.* (1998) 'Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast', *Development*, 125(10), pp. 1957–1966.

Krammer, E. B. . B. M. F. . E. T. P. . R. M. . G. H. (1987) *The Motoneuronal Organization of the Spinal Accessory Nuclear Complex | Eva B. Krammer | Springer*. Available at: https://www.springer.com/gp/book/9783540174592 (Accessed: 19 August 2020).

Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002) 'Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm', *Development*, pp. 4335–4346.

Kumamaru, H. *et al.* (2018) 'Generation and post-injury integration of human spinal cord neural stem cells', *Nature Methods*. Nature Publishing Group, 15(9), pp. 723–731. doi: 10.1038/s41592-018-0074-3.

Kumar, S. and Duester, G. (2014) 'Retinoic acid controls body axis extension by directly repressing Fgf8 transcription', *Development (Cambridge)*. Company of Biologists Ltd, 141(15), pp. 2972–2977. doi: 10.1242/dev.112367.

Lamb, T. M. and Harland, R. M. (1995) 'Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern', *Development*, 121(11), pp. 3627–3636.

Lance-Jones, C. and Landmesser, L. (1981) 'Pathway selection by embryonic chick motoneurons in an experimentally altered environment', *Proceedings of the Royal Society of London. Series B. Biological Sciences*. The Royal SocietyLondon, 214(1194), pp. 19–52. doi: 10.1098/rspb.1981.0080.

Lance-Jones, C. and Landmesser, L. (1980) 'Motoneurone projection patterns in the chick hind limb following early partial reversals of the spinal cord.', *The Journal of Physiology*, 302(1), pp. 581–602. doi: 10.1113/jphysiol.1980.sp013262.

Landmesser, L. (1978) 'The distribution of motoneurones supplying chick hind limb

muscles.', *The Journal of Physiology*. Wiley-Blackwell, 284(1), pp. 371–389. doi: 10.1113/jphysiol.1978.sp012545.

Langston, A. W., Thompson, J. R. and Gudas, L. J. (1997) 'Retinoic acid-responsive enhancers located 3' of the Hox A and Hox B homeobox gene clusters. Functional analysis.', *The Journal of biological chemistry*, 272(4), pp. 2167–75. doi: 10.1074/jbc.272.4.2167.

Lee, H. *et al.* (2007) 'Directed Differentiation and Transplantation of Human Embryonic Stem Cell-Derived Motoneurons', *Stem Cells*, 25(8), pp. 1931–1939. doi: 10.1634/stemcells.2007-0097.

Lee, K. J., Dietrich, P. and Jessell, T. M. (2000) 'Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification', *Nature*, 403(6771), pp. 734–740. doi: 10.1038/35001507.

Lee, K. J. and Jessell, T. M. (1999) 'THE SPECIFICATION OF DORSAL CELL FATES IN THE VERTEBRATE CENTRAL NERVOUS SYSTEM', *Annual Review of Neuroscience*. Annual Reviews, 22(1), pp. 261–294. doi: 10.1146/annurev.neuro.22.1.261.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998) 'Neuronal patterning by BMPs: A requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord', *Genes and Development*. Cold Spring Harbor Laboratory Press, 12(21), pp. 3394–3407. doi: 10.1101/gad.12.21.3394.

Lee, S. *et al.* (2009) 'Retinoid Signaling and Neurogenin2 Function Are Coupled for the Specification of Spinal Motor Neurons through a Chromatin Modifier CBP', *Neuron*. NIH Public Access, 62(5), pp. 641–654. doi: 10.1016/j.neuron.2009.04.025.

Lee, S. K. *et al.* (2005) 'Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells', *Genes and Development*, 19(2), pp. 282–294. doi: 10.1101/gad.1257105.

Li, X. J. *et al.* (2008) 'Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules', *Stem Cells*, 26(4), pp. 886–893. doi: 10.1634/stemcells.2007-0620.Directed.

Lickert, H. *et al.* (2000) 'Wnt/β-catenin signaling regulates the expression of the homeobox

gene Cdx1 in embryonic intestine', *Development*, 127(17), pp. 3805–3813.

Liem, K. F. *et al.* (1995) 'Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm', *Cell*, 82(6), pp. 969–979. doi: 10.1016/0092-8674(95)90276-7.

Liem, K. F., Jessell, T. M. and Briscoe, J. (2000) 'Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites - PubMed', *Development*, pp. 4855–66. Available at: https://pubmed.ncbi.nlm.nih.gov/11044400/ (Accessed: 7 August 2020).

Liem, K. F., Tremml, G. and Jessell, T. M. (1997) 'A role for the roof plate and its resident TGFβ-related proteins in neuronal patterning in the dorsal spinal cord', *Cell*. Cell Press, 91(1), pp. 127–138. doi: 10.1016/S0092-8674(01)80015-5.

Liinamaa, T. L., Keane, J. and Richmond, F. J. R. (1997) 'Distribution of motoneurons supplying feline neck muscles taking origin from the shoulder girdle', *Journal of Comparative Neurology*. John Wiley & Sons, Ltd, 377(2), pp. 298–312. doi: 10.1002/(SICI)1096-9861(19970113)377:2<298::AID-CNE10>3.0.CO;2-N.

Lippmann, E. S. *et al.* (2015a) 'Deterministic HOX patterning in human pluripotent stem cell-derived neuroectoderm', *Stem Cell Reports*. The Authors, 4(4), pp. 632–644. doi: 10.1016/j.stemcr.2015.02.018.

Lippmann, E. S. *et al.* (2015b) 'Deterministic HOX patterning in human pluripotent stem cell-derived neuroectoderm', *Stem Cell Reports*. Cell Press, 4(4), pp. 632–644. doi: 10.1016/j.stemcr.2015.02.018.

Liu, P. *et al.* (1999) 'Requirement for Wnt3 in vertebrate axis formation', *Nature Genetics*, 22(4), pp. 361–365. doi: 10.1038/11932.

Lopez-Yrigoyen, M. *et al.* (2018) 'A human iPSC line capable of differentiating into functional macrophages expressing ZsGreen: A tool for the study and in vivo tracking of therapeutic cells', *Philosophical Transactions of the Royal Society B: Biological Sciences*. Royal Society Publishing, 373(1750). doi: 10.1098/rstb.2017.0219.

Lu, Q. R. *et al.* (2002) 'Common developmental requirement for Olig function indicates a

motor neuron/oligodendrocyte connection', *Cell*. Cell Press, 109(1), pp. 75–86. doi: 10.1016/S0092-8674(02)00678-5.

Maden, M. *et al.* (1998) 'The distribution of endogenous retinoic acid in the chick embryo: Implications for developmental mechanisms', *Development*, 125(21), pp. 4133–4144.

Marklund, U. *et al.* (2010) 'Domain-specific control of neurogenesis achieved through patterned regulation of Notch ligand expression', *Development*, 137(3), pp. 437–445. doi: 10.1242/dev.036806.

Markouli, C. *et al.* (2019) 'Gain of 20q11.21 in Human Pluripotent Stem Cells Impairs TGF-β-Dependent Neuroectodermal Commitment', *Stem Cell Reports*. Cell Press, 13(1), pp. 163–176. doi: 10.1016/j.stemcr.2019.05.005.

Marsh-Armstrong, N. *et al.* (1995) 'Retinoic acid in the anteroposterior patterning of the zebrafish trunk', *Roux's Archives of Developmental Biology*. Springer-Verlag, 205(3–4), pp. 103–113. doi: 10.1007/BF00357756.

Marshall, H. *et al.* (1992a) 'Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity', *Nature*, 360(6406), pp. 737–741. doi: 10.1038/360737a0.

Marshall, H. *et al.* (1992b) 'Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity', *Nature*, 360(6406), pp. 737–741. doi: 10.1038/360737a0.

Marshall, H. *et al.* (1994) 'A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1', *Nature*, 370(6490), pp. 567–571. doi: 10.1038/370567a0.

Marti, E. *et al.* (1995) 'Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants', *Nature*, 375(6529), pp. 322–325. doi: 10.1038/375322a0.

Martí, E. *et al.* (1995) 'Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo.', *Development (Cambridge, England)*, 121(8), pp. 2537–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7671817 (Accessed: 14 January 2020).

Martin, B. L. and Kimelman, D. (2008) 'Regulation of Canonical Wnt Signaling by Brachyury Is

Essential for Posterior Mesoderm Formation', *Developmental Cell*. Elsevier, 15(1), pp. 121–133. doi: 10.1016/j.devcel.2008.04.013.

Martin, G. R. (1981) 'Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 78(12 II), pp. 7634–7638. doi: 10.1073/pnas.78.12.7634.

Mastromina, I. *et al.* (2018) 'Myc activity is required for maintenance of the neuromesodermal progenitor signalling network and for segmentation clock gene oscillations in mouse', *Development (Cambridge)*. Company of Biologists Ltd, 145(14). doi: 10.1242/dev.161091.

Mathis, L. and Nicolas, J. F. (2000) 'Different clonal dispersion in the rostral and caudal mouse central nervous system.', *Development*, 127(6), pp. 1277–1290. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10683180&retmode=ref&cmd=prlinks%5Cnpapers2://publication/uuid/C0270027-AD12-4E66-805D-B51A34518C4D.

Matise, M. P. and Lance Jones, C. (1996) 'A critical period for the specification of motor pools in the chick lumbosacral spinal cord', *Development*, 122(2), pp. 659–669.

Matsubara, Y. *et al.* (2017) 'Anatomical integration of the sacral-hindlimb unit coordinated by GDF11 underlies variation in hindlimb positioning in tetrapods', *Nature Ecology and Evolution*. Nature Publishing Group, 1(9), pp. 1392–1399. doi: 10.1038/s41559-017-0247-y.

Maury, Y. *et al.* (2015) 'Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes', *Nature Biotechnology*, 33(1), pp. 89–96. doi: 10.1038/nbt.3049.

Mazzoni, E. O. *et al.* (2013a) 'Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals', *Nature Neuroscience*. Nat Neurosci, 16(9), pp. 1191–1198. doi: 10.1038/nn.3490.

Mazzoni, E. O. *et al.* (2013b) 'Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals', *Nature Neuroscience*, 16(9), pp. 1191–1198. doi:

10.1038/nn.3490.

McGinnis, W. and Krumlauf, R. (1992) 'Homeobox genes and axial patterning', *Cell*, pp. 283–302. doi: 10.1016/0092-8674(92)90471-N.

McGrew, L. L., Lai, C. J. and Moon, R. T. (1995) 'Specification of the Anteroposterior Neural Axis through Synergistic Interaction of the Wnt Signaling Cascade withnogginandfollistatin', *Developmental Biology*, 172(1), pp. 337–342. doi: 10.1006/dbio.1995.0027.

McMahon, J. A. *et al.* (1998) 'Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite', *Genes and Development*. Cold Spring Harbor Laboratory Press, 12(10), pp. 1438–1452. doi: 10.1101/gad.12.10.1438.

Megason, S. G. and McMahon, A. P. (2002) 'A mitogen gradient of dorsal midline Wnts organizes growth in the CNS', *Development*, pp. 2087–2098.

Metzis, V. *et al.* (2018) 'Nervous System Regionalization Entails Axial Allocation before Neural Differentiation', *Cell*. Cell Press, 175(4), pp. 1105-1118.e17. doi: 10.1016/j.cell.2018.09.040.

Meyer, B. I. and Gruss, P. (1993) 'Mouse Cdx-1 expression during gastrulation', *Development*, 117(1), pp. 191–203.

Miles, G. B. *et al.* (2004) 'Functional properties of motoneurons derived from mouse embryonic stem cells', *Journal of Neuroscience*, 24(36), pp. 7848–7858. doi: 10.1523/JNEUROSCI.1972-04.2004.

Miller, D. F. B. *et al.* (2001) 'Cross-regulation of Hox genes in the Drosophila melanogaster embryo', *Mechanisms of Development*, 102(1–2), pp. 3–16. doi: 10.1016/S0925-4773(01)00301-X.

Millonig, J. H., Millen, K. J. and Hatten, M. E. (2000) 'The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS', *Nature*, 403(6771), pp. 764–769. doi: 10.1038/35001573.

Mizuguchi, R. *et al.* (2001) 'Combinatorial roles of Olig2 and Neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons', *Neuron*. Cell Press, 31(5), pp. 757–771. doi: 10.1016/S0896-6273(01)00413-5.

Morrison, A. *et al.* (1996) 'In vitro and transgenic analysis of a human HOXD4 retinoid-responsive enhancer.', *Development (Cambridge, England)*, 122(6), pp. 1895–907. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8674428 (Accessed: 20 December 2019).

Mouilleau, V. *et al.* (2020) 'Dynamic extrinsic pacing of the HOX clock in human axial progenitors controls motor neuron subtype specification', *bioRxiv*, p. 2020.06.27.175646. doi: 10.1101/2020.06.27.175646.

Muroyama, Y. *et al.* (2002) 'Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord', *Genes and Development*, 16(5), pp. 548–553. doi: 10.1101/gad.937102.

Naiche, L. A., Holder, N. and Lewandoski, M. (2011) 'FGF4 and FGF8 comprise the wavefront activity that controls somitogenesis', *Proceedings of the National Academy of Sciences of the United States of America*, 108(10), pp. 4018–4023. doi: 10.1073/pnas.1007417108.

Nazareth, E. J. P. *et al.* (2013) 'High-throughput fingerprinting of human pluripotent stem cell fate responses and lineage bias', *Nature Methods*. PMC Canada manuscript submission, 10(12), pp. 1225–1231. doi: 10.1038/nmeth.2684.

Neely, M. D. *et al.* (2012) 'DMH1, a highly selective small molecule BMP inhibitor promotes neurogenesis of hiPSCs: Comparison of PAX6 and SOX1 expression during neural induction', *ACS Chemical Neuroscience*. ACS Chem Neurosci, 3(6), pp. 482–491. doi: 10.1021/cn300029t.

Niederreither, K. *et al.* (1997) 'Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development', *Mechanisms of Development*, 62(1), pp. 67–78. doi: 10.1016/S0925-4773(96)00653-3.

Niederreither, K. *et al.* (2000) 'Retinoic acid synthesis and hindbrain patterning in the mouse embryo', *Development*, 127(1), pp. 75–85.

Nieto, M. A. (2002) 'The snail superfamily of zinc-finger transcription factors', *Nature Reviews Molecular Cell Biology*, pp. 155–166. doi: 10.1038/nrm757.

Nieuwkoop, P. and Nigtevecht, G. (1954) 'Neural activation and tansformation in xxplants of

competent ectoderm under the influence of fragments of anterior notochord in Urodeles', *Journal of embryology and experimental morphology*, 2(3), pp. 175–193.

Nikolopoulou, E. *et al.* (2017) 'Neural tube closure: Cellular, molecular and biomechanical mechanisms', *Development (Cambridge)*. Company of Biologists Ltd, pp. 552–566. doi: 10.1242/dev.145904.

Nishimura, N. *et al.* (2012) 'A systematic survey and characterization of enhancers that regulate Sox3 in neuro-sensory development in comparison with Sox2 enhancers', *Biology*. MDPI AG, 1(3), pp. 714–735. doi: 10.3390/biology1030714.

Nordström, U., Maier, E., Jessell, Thomas M., *et al.* (2006) 'An early role for Wnt signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity', *PLoS Biology*, 4(8), pp. 1438–1452. doi: 10.1371/journal.pbio.0040252.

Nordström, U., Maier, E., Jessell, Thomas M, *et al.* (2006) 'An Early Role for Wnt Signaling in Specifying Neural Patterns of Cdx and Hox Gene Expression and Motor Neuron Subtype Identity', *PLoS Biology*. Edited by R. Nusse. Public Library of Science, 4(8), p. e252. doi: 10.1371/journal.pbio.0040252.

Nostro, M. C. *et al.* (2011) 'Stage-specific signaling through TGFβ family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells', *Development*. Company of Biologists, 138(5), pp. 861–871. doi: 10.1242/dev.055236.

Novitch, B. G., Chen, A. I. and Jessell, T. M. (2001) 'Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2', *Neuron*. Cell Press, 31(5), pp. 773–789. doi: 10.1016/S0896-6273(01)00407-X.

Nowotschin, S. *et al.* (2012) 'Interaction of Wnt3a, Msgn1 and Tbx6 in neural versus paraxial mesoderm lineage commitment and paraxial mesoderm differentiation in the mouse embryo', *Developmental Biology*. Academic Press Inc., 367(1), pp. 1–14. doi: 10.1016/j.ydbio.2012.04.012.

Olivera-Martinez, I. *et al.* (2012) 'Loss of FGF-Dependent Mesoderm Identity and Rise of Endogenous Retinoid Signalling Determine Cessation of Body Axis Elongation', *PLoS Biology*, 10(10). doi: 10.1371/journal.pbio.1001415.

Olivera-Martinez, I. *et al.* (2014) 'Major transcriptome re-organisation and abrupt changes in signalling, cell cycle and chromatin regulation at neural differentiation in vivo', *Development*, 141(16), pp. 3266–3276. doi: 10.1242/dev.112623.

Palomero, T. *et al.* (2006) 'NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 103(48), pp. 18261–18266. doi: 10.1073/pnas.0606108103.

Parr, B. A. *et al.* (1993) 'Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds', *Development*, 119(1), pp. 247–261.

Patel, N. S. *et al.* (2013) 'FGF Signalling Regulates Chromatin Organisation during Neural Differentiation via Mechanisms that Can Be Uncoupled from Transcription', *PLoS Genetics*. Public Library of Science (PLoS), 9(8), p. 1003614. doi: 10.1371/annotation/c066bb84-13ea-4b36-a481-f149df8ce929.

Peljto, M. *et al.* (2010a) 'Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation', *Cell Stem Cell*. NIH Public Access, 7(3), pp. 355–366. doi: 10.1016/j.stem.2010.07.013.

Peljto, M. *et al.* (2010b) 'Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation', *Cell Stem Cell*. Elsevier Ltd, 7(3), pp. 355–366. doi: 10.1016/j.stem.2010.07.013.

Peljto, M. *et al.* (2010c) 'Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation', *Cell Stem Cell*, 7(3), pp. 355–366. doi: 10.1016/j.stem.2010.07.013.

Pera, E. M. and Kessel, M. (1997) 'Patterning of the chick forebrain anlage by the prechordal plate', *Development*, 124(20), pp. 4153–4162.

Perea-Gomez, A. *et al.* (2002) 'Nodal antaginists in the anterior visceral endoderm prevent the formation of multiple primitive streaks', *Developmental Cell*. Cell Press, 3(5), pp. 745–756. doi: 10.1016/S1534-5807(02)00321-0.

Pevny, L. H. *et al.* (1998) 'A role for SOX1 in neural determination', *Development*, 125(10),

pp. 1967–1978.

Pfaff, S. L. *et al.* (1996) 'Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation', *Cell*. Cell Press, 84(2), pp. 309–320. doi: 10.1016/S0092-8674(00)80985-X.

Piccolo, S. *et al.* (1996) 'Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of chordin to BMP-4', *Cell*. Cell Press, 86(4), pp. 589–598. doi: 10.1016/S0092-8674(00)80132-4.

Pierani, A. *et al.* (1999) 'A Sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord', *Cell*. Cell Press, 97(7), pp. 903–915. doi: 10.1016/S0092-8674(00)80802-8.

Pillemer, G. *et al.* (1998) 'Nested expression and sequential downregulation of the Xenopus caudal genes along the anterior-posterior axis', *Mechanisms of Development*, 71(1–2), pp. 193–196. doi: 10.1016/S0925-4773(97)00193-7.

Placzek, M. *et al.* (1991) 'Control of dorsoventral pattern in vertebrate neural development: Induction and polarizing properties of the floor plate', in *Development*, pp. 105–122.

Plaza, S. *et al.* (2008) 'Cross-regulatory protein-protein interactions between Hox and Pax transcription factors', *Proceedings of the National Academy of Sciences of the United States of America*, 105(36), pp. 13439–13444. doi: 10.1073/pnas.0806106105.

Polo, J. M. *et al.* (2010) 'Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells', *Nature biotechnology*. 2010/07/19, 28(8), pp. 848–855. doi: 10.1038/nbt.1667.

Pownall, M. E. *et al.* (1996) 'eFGF, Xcad3 and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus', *Development*, 122(12), pp. 3881–3892.

Prasad, A. and Hollyday, M. (1991) 'Development and migration of avian sympathetic preganglionic neurons', *Journal of Comparative Neurology*, 307(2), pp. 237–258. doi: 10.1002/cne.903070207.

Price, M. *et al.* (1992) 'Regional expression of the homeobox gene Nkx-2.2 in the developing mammalian forebrain', *Neuron*, 8(2), pp. 241–255. doi: 10.1016/0896-6273(92)90291-K.

Prinos, P. *et al.* (2001) 'Multiple pathways governing Cdx1 expression during murine development', *Developmental Biology*. Academic Press Inc., 239(2), pp. 257–269. doi: 10.1006/dbio.2001.0446.

Psychoyos, D. and Stern, C. D. (1996) 'Fates and migratory routes of primitive streak cells in the chick embryo.', *Development (Cambridge, England)*, 122(5), pp. 1523–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8625839.

Qiu, M. *et al.* (1998) 'Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development.', *Mechanisms of development*, 72(1–2), pp. 77–88. doi: 10.1016/s0925-4773(98)00018-5.

Rabadán, M. A. *et al.* (2012) 'Jagged2 controls the generation of motor neuron and oligodendrocyte progenitors in the ventral spinal cord', *Cell Death and Differentiation*, 19(2), pp. 209–219. doi: 10.1038/cdd.2011.84.

Rayon, T. *et al.* (2019) 'Species-specific developmental timing is associated with global differences in protein stability in mouse and human', *bioRxiv*. Cold Spring Harbor Laboratory, p. 2019.12.29.889543. doi: 10.1101/2019.12.29.889543.

Reifers, F. *et al.* (2000) 'Overlapping and distinct functions provided by fgf17, a new zebrafish member of the Fgf8/17/18 subgroup of Fgfs', *Mechanisms of Development*, 99(1–2), pp. 39–49. doi: 10.1016/S0925-4773(00)00475-5.

Reubinoff, B. E. *et al.* (2000) 'Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro', *Nature Biotechnology*, 18(4), pp. 399–404. doi: 10.1038/74447.

Rijli, F. M. *et al.* (1993) 'A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene', *Cell*, 75(7), pp. 1333–1349. doi: 10.1016/0092-8674(93)90620-6.

Roelink, H. *et al.* (1994) 'Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord', *Cell*, 76(4), pp. 761–775. doi: 10.1016/0092-8674(94)90514-2.

Roelink, H. *et al.* (1995) 'Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis', *Cell*, pp. 445–

455. doi: 10.1016/0092-8674(95)90397-6.

Rosenzweig, E. S. *et al.* (2018a) 'Restorative effects of human neural stem cell grafts on the primate spinal cord', *Nature Publishing Group*. Nature Publishing Group, 24(4), pp. 484–490. doi: 10.1038/nm.4502.

Rosenzweig, E. S. *et al.* (2018b) 'Restorative effects of human neural stem cell grafts on the primate spinal cord', *Nature Publishing Group*. Nature Publishing Group, 24(4), pp. 484–490. doi: 10.1038/nm.4502.

Rossant, J. *et al.* (1991) 'Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis', *Genes and Development*, 5(8), pp. 1333–1344. doi: 10.1101/gad.5.8.1333.

Rousso, D. L. *et al.* (2008) 'Coordinated Actions of the Forkhead Protein Foxp1 and Hox Proteins in the Columnar Organization of Spinal Motor Neurons', *Neuron*. Cell Press, 59(2), pp. 226–240. doi: 10.1016/j.neuron.2008.06.025.

Rowitch, D. H. *et al.* (1999) 'Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells', *Journal of Neuroscience*. Society for Neuroscience, 19(20), pp. 8954–8965. doi: 10.1523/jneurosci.19-20-08954.1999.

Sakai, Y. *et al.* (2001) 'The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo', *Genes and Development*, 15(2), pp. 213–225. doi: 10.1101/gad.851501.

Sasai, N., Kutejova, E. and Briscoe, J. (2014) 'Integration of Signals along Orthogonal Axes of the Vertebrate Neural Tube Controls Progenitor Competence and Increases Cell Diversity', *PLoS Biology*. Public Library of Science, 12(7), pp. 1–20. doi: 10.1371/journal.pbio.1001907.

Sasai, Y. *et al.* (1994) 'Xenopus chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes', *Cell*, 79(5), pp. 779–790. doi: 10.1016/0092-8674(94)90068-X.

Savory, J. G. A. *et al.* (2009) 'Cdx2 regulation of posterior development through non-Hox targets', *Development*, 136(24), pp. 4099–4110. doi: 10.1242/dev.041582.

Scardigli, R. *et al.* (2001) 'Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord', *Neuron*. Cell Press, 31(2), pp. 203–217. doi:

10.1016/S0896-6273(01)00358-0.

Sefton, M., Sanchez, S. and Nieto, M. A. (1998) 'Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo', *Development*, 125(16), pp. 3111 LP – 3121. Available at: http://dev.biologists.org/content/125/16/3111.abstract.

Shah, V., Drill, E. and Lance-Jones, C. (2004) 'Ectopic expression of Hoxd10 in thoracic spinal segments induces motoneurons with a lumbosacral molecular profile and axon projections to the limb', in *Developmental Dynamics*, pp. 43–56. doi: 10.1002/dvdy.20103.

Sharma, K. *et al.* (1998) 'LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons', *Cell*. Cell Press, 95(6), pp. 817–828. doi: 10.1016/S0092-8674(00)81704-3.

Shih, J. and Fraser, S. E. (1996) 'Characterizing the zebrafish organizer: Microsurgical analysis at the early-shield stage', *Development*, 122(4), pp. 1313–1322.

Shum, A. S. W. *et al.* (1999) 'Retinoic acid induces down-regulation of Wnt-3a, apoptosis and diversion of tail bud cells to a neural fate in the mouse embryo', *Mechanisms of Development*, 84(1–2), pp. 17–30. doi: 10.1016/S0925-4773(99)00059-3.

Simeone, A. *et al.* (1993) 'A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo.', *The EMBO Journal*. Wiley, 12(7), pp. 2735–2747. doi: 10.1002/j.1460-2075.1993.tb05935.x.

Smith, J. R. *et al.* (2008) 'Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm', *Developmental Biology*. Academic Press Inc., 313(1), pp. 107–117. doi: 10.1016/j.ydbio.2007.10.003.

Snider, W. D. and Palavali, V. (1990) 'Early axon and dendritic outgrowth of spinal accessory motor neurons studied with dii in fixed tissues', *Journal of Comparative Neurology*. J Comp Neurol, 297(2), pp. 227–238. doi: 10.1002/cne.902970206.

Sockanathan, S. and Jessell, T. M. (1998) 'Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons', *Cell*, 94(4), pp. 503–514. doi: 10.1016/S0092-

8674(00)81591-3.

Son, E. Y. *et al.* (2011) 'Conversion of mouse and human fibroblasts into functional spinal motor neurons', *Cell Stem Cell*, 9(3), pp. 205–218. doi: 10.1016/j.stem.2011.07.014.

Song, M. R. *et al.* (2009) 'Islet-to-LMO stoichiometries control the function of transcription complexes that specify motor neuron and V2a interneuron identity', *Development*, 136(17), pp. 2923–2932. doi: 10.1242/dev.037986.

Soundararajan, P. *et al.* (2006a) 'Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons', *Journal of Neuroscience*. Society for Neuroscience, 26(12), pp. 3256–3268. doi: 10.1523/JNEUROSCI.5537-05.2006.

Soundararajan, P. *et al.* (2006b) 'Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons', *Journal of Neuroscience*, 26(12), pp. 3256–3268. doi: 10.1523/JNEUROSCI.5537-05.2006.

Spemann, H. and Mangold, H. (2001) 'Induction of embryonic primordia by implantation of organizers from a different species', *International Journal of Developmental Biology*, 45(1 SPEC. ISS. 1), pp. 13–38. doi: 10.1387/ijdb.11291841.

Stasiulewicz, M. *et al.* (2015) 'A conserved role for Notch signaling in priming the cellular response to Shh through ciliary localisation of the key Shh transducer Smo', *Development (Cambridge)*. Company of Biologists Ltd, 142(13), pp. 2291–2303. doi: 10.1242/dev.125237.

van Straaten, H. W. M. *et al.* (1988) 'Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo', *Anatomy and Embryology*. Springer-Verlag, 177(4), pp. 317–324. doi: 10.1007/BF00315839.

Struhl, G. and White, R. A. H. (1985) 'Regulation of the Ultrabithorax gene of drosophila by other bithorax complex genes', *Cell*, 43(2 PART 1), pp. 507–519. doi: 10.1016/0092-8674(85)90180-1.

Stuhlmiller, T. J. and García-Castro, M. I. (2012) 'Current perspectives of the signaling pathways directing neural crest induction', *Cellular and Molecular Life Sciences*. Springer,

pp. 3715–3737. doi: 10.1007/s00018-012-0991-8.

Subramanian, V., Meyer, B. I. and Gruss, P. (1995) 'Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes', *Cell*, 83(4), pp. 641–653. doi: 10.1016/0092-8674(95)90104-3.

Sugimori, M. *et al.* (2007) 'Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord', *Development*, 134(8), pp. 1617–1629. doi: 10.1242/dev.001255.

Suh, J. *et al.* (2019) 'Growth differentiation factor 11 locally controls anterior–posterior patterning of the axial skeleton', *Journal of Cellular Physiology*. Wiley-Liss Inc., 234(12), pp. 23360–23368. doi: 10.1002/jcp.28904.

Swindell, E. C. *et al.* (1999) 'Complementary domains of retinoic acid production and degradation in the early chick embryo', *Developmental Biology*. Academic Press Inc., 216(1), pp. 282–296. doi: 10.1006/dbio.1999.9487.

Takada, S. *et al.* (1994) 'Wnt-3a regulates somite and tailbud formation in the mouse embryo', *Genes and Development*. Cold Spring Harbor Laboratory Press, 8(2), pp. 174–189. doi: 10.1101/gad.8.2.174.

Takahashi, K. *et al.* (2007) 'Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors', *Cell*. Cell Press, 131(5), pp. 861–872. doi: 10.1016/J.CELL.2007.11.019.

Takahashi, K. and Yamanaka, S. (2006) 'Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors', *Cell*, 126(4), pp. 663–676. doi: 10.1016/j.cell.2006.07.024.

Takemoto, T. *et al.* (2006) 'Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1', *Development*, 133(2), pp. 297–306. doi: 10.1242/dev.02196.

Takemoto, T. *et al.* (2011a) 'Tbx6-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells', *Nature*, 470(7334), pp. 394–398. doi: 10.1038/nature09729.

Takemoto, T. *et al.* (2011b) 'Tbx6-dependent Sox2 regulation determines neural or

mesodermal fate in axial stem cells', *Nature*. Nature Publishing Group, 470(7334), pp. 394–398. doi: 10.1038/nature09729.

Tanabe, Y., William, C. and Jessell, T. M. (1998) 'Specification of motor neuron identity by the MNR2 homeodomain protein.', *Cell*, 95(1), pp. 67–80. doi: 10.1016/s0092-8674(00)81783-3.

Taylor, J. K. *et al.* (1997) 'Activation of enhancer elements by the homeobox gene Cdx2 is cell line specific', *Nucleic Acids Research*, 25(12), pp. 2293–2300. doi: 10.1093/nar/25.12.2293.

Thaler, J. *et al.* (1999) 'Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9', *Neuron*. Cell Press, 23(4), pp. 675–687. doi: 10.1016/S0896-6273(01)80027-1.

Thaler, J. P. *et al.* (2002) 'LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions', *Cell*. Cell Press, 110(2), pp. 237–249. doi: 10.1016/S0092-8674(02)00823-1.

Thaler, J. P. *et al.* (2004) 'A Postmitotic Role for Isl-Class LIM Homeodomain Proteins in the Assignment of Visceral Spinal Motor Neuron Identity', *Neuron*. Cell Press, 41(3), pp. 337–350. doi: 10.1016/S0896-6273(04)00011-X.

Thomas, P. and Beddington, R. (1996) 'Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo', *Current Biology*. Cell Press, 6(11), pp. 1487–1496. doi: 10.1016/S0960-9822(96)00753-1.

Thomson, J. A. *et al.* (1998) 'Embryonic stem cell lines derived from human blastocysts', *Science*. American Association for the Advancement of Science, 282(5391), pp. 1145–1147. doi: 10.1126/science.282.5391.1145.

Thor, S. *et al.* (1991) 'The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat.', *Neuron*, 7(6), pp. 881–9. doi: 10.1016/0896-6273(91)90334-v.

Timmer, J. R., Wang, C. and Niswander, L. (2002) 'BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription

factors', *Development*, 129(10), pp. 2459–2472.

Tonge, P. D. and Andrews, P. W. (2010) 'Retinoic acid directs neuronal differentiation of human pluripotent stem cell lines in a non-cell-autonomous manner', *Differentiation*, 80(1), pp. 20–30. doi: 10.1016/j.diff.2010.04.001.

Tosney, K. W., Hotary, K. B. and Lance-Jones, C. (1995) 'Specifying the target identity of motoneurons', *BioEssays*, 17(5), pp. 379–382. doi: 10.1002/bies.950170503.

Tsakiridis, A. *et al.* (2014) 'Distinct Wnt-driven primitive streak-like populations reflect in vivo lineage precursors', *Development (Cambridge)*, 141(6), pp. 1209–1221. doi: 10.1242/dev.101014.

Tsakiridis, A. and Wilson, V. (2015) 'Assessing the bipotency of in vitro-derived neuromesodermal progenitors', *F1000Research*. Faculty of 1000 Ltd, 4. doi: 10.12688/f1000research.6345.2.

Tsuchida, T. *et al.* (1994) 'Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes', *Cell*, 79(6), pp. 957–970. doi: 10.1016/0092-8674(94)90027-2.

Turner, D. A. *et al.* (2014) 'Wnt/ -catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells', *Development*, 141(22), pp. 4243–4253. doi: 10.1242/dev.112979.

Turner, David A *et al.* (2014) 'Wnt/β-catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells.', *Development (Cambridge, England)*. Company of Biologists, 141(22), pp. 4243–53. doi: 10.1242/dev.112979.

Turner, David A. *et al.* (2014a) 'Wnt/β-catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells', *Development (Cambridge)*. Company of Biologists Ltd, 141(22), pp. 4243–4253. doi: 10.1242/dev.112979.

Turner, David A. *et al.* (2014b) 'Wnt/β-catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic

stem cells', *Development (Cambridge)*. Company of Biologists Ltd, 141(22), pp. 4243–4253. doi: 10.1242/dev.112979.

Tzouanacou, E. *et al.* (2009a) 'Redefining the Progression of Lineage Segregations during Mammalian Embryogenesis by Clonal Analysis', *Developmental Cell*, 17(3), pp. 365–376. doi: 10.1016/j.devcel.2009.08.002.

Tzouanacou, E. *et al.* (2009b) 'Redefining the Progression of Lineage Segregations during Mammalian Embryogenesis by Clonal Analysis', *Developmental Cell*, 17(3), pp. 365–376. doi: 10.1016/j.devcel.2009.08.002.

Uchikawa, M. *et al.* (2003) 'Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals', *Developmental Cell*. Cell Press, 4(4), pp. 509–519. doi: 10.1016/S1534-5807(03)00088-1.

Uchiyama, H. *et al.* (2001) 'Cloning and characterization of the T-box gene Tbx6 in Xenopus laevis.', *Development, growth & differentiation*, 43(6), pp. 657–69. doi: 10.1046/j.1440-169x.2001.00606.x.

Vallstedt, A. *et al.* (2001) 'Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification', *Neuron*. Cell Press, 31(5), pp. 743–755. doi: 10.1016/S0896-6273(01)00412-3.

van de Ven, C. *et al.* (2011) 'Concerted involvement of Cdx/Hox genes and Wnt signaling in morphogenesis of the caudal neural tube and cloacal derivatives from the posterior growth zone', *Development*, 138(17), pp. 3859–3859. doi: 10.1242/dev.072462.

Verrier, L., Davidson, L., Gierliński, M., *et al.* (2018) 'Neural differentiation, selection and transcriptomic profiling of human neuromesodermal progenitor-like cells in vitro', *Development (Cambridge)*. Company of Biologists Ltd, 145(16 Special Issue). doi: 10.1242/dev.166215.

Verrier, L., Davidson, L., Gierlin, M., *et al.* (2018) 'Neural differentiation , selection and transcriptomic profiling of human neuromesodermal progenitor-like cells in vitro'. doi: 10.1242/dev.166215.

Verrier, L., Davidson, L., Gierli, M., *et al.* (2018) 'Neural differentiation , selection and

transcriptomic profiling of human neuromesodermal progenitors-like cells in vitro', (June). doi: 10.1242/dev.166215.

Volpato, V. *et al.* (2018) 'Reproducibility of Molecular Phenotypes after Long-Term Differentiation to Human iPSC-Derived Neurons: A Multi-Site Omics Study', *Stem Cell Reports*. Cell Press, 11(4), pp. 897–911. doi: 10.1016/j.stemcr.2018.08.013.

Wahl, M. B. *et al.* (2007) 'FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis', *Development*, 134(22), pp. 4033–4041. doi: 10.1242/dev.009167.

Walther, C. and Gruss, P. (1991) 'Pax-6, a murine paired box gene, is expressed in the developing CNS', *Development*, 113(4), pp. 1435–1450.

Wichterle, Hynek, Wichterle, H., *et al.* (2002) 'Directed differentiation of embryonic stem cells into motor neurons.', *Cell*, 110(3), pp. 385–97. doi: 10.1016/S0092-8674(02)00835-8.

Wichterle, H *et al.* (2002) 'Directed differentiation of embryonic stem cells into motor neurons', *Cell*, 110, pp. 385–397.

Wichterle, Hynek, Lieberam, I., *et al.* (2002) 'Directed differentiation of embryonic stem cells into motor neurons', *Cell*, 110(3), pp. 385–397. doi: 10.1016/S0092-8674(02)00835-8.

Wilkinson, D. G., Bhatt, S. and Herrmann, B. G. (1990) 'Expression pattern of the mouse T gene and its role in mesoderm formation', *Nature*. Nature Publishing Group, 343(6259), pp. 657–659. doi: 10.1038/343657a0.

Wilson, V. and Beddington, R. S. P. (1996) 'Cell fate and morphogenetic movement in the late mouse primitive streak', *Mechanisms of Development*, 55(1), pp. 79–89. doi: 10.1016/0925-4773(95)00493-9.

Wine-Lee, L. *et al.* (2004) 'Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord', *Development*, 131(21), pp. 5393–5403. doi: 10.1242/dev.01379.

Wittler, L. *et al.* (2007) 'Expression of Msgn1 in the presomitic mesoderm is controlled by synergism of WNT signalling and Tbx6', *EMBO Reports*, 8(8), pp. 784–789. doi: 10.1038/sj.embor.7401030.

Wood, T. R. *et al.* (2019) 'Neuromesodermal progenitors separate the axial stem zones while producing few single- and dual-fated descendants', *BioRxiv*. Cold Spring Harbor Laboratory, p. 622571. doi: 10.1101/622571.

Wymeersch, Filip J *et al.* (2016) 'Position-dependent plasticity of distinct progenitor types in the primitive streak.', *eLife*, 5, p. e10042. doi: 10.7554/eLife.10042.

Wymeersch, Filip J. *et al.* (2016) 'Position-dependent plasticity of distinct progenitor types in the primitive streak', *eLife*, 5(JANUARY2016), pp. 1–28. doi: 10.7554/eLife.10042.

Wymeersch, F. J. *et al.* (2019a) 'Transcriptionally dynamic progenitor populations organised around a stable niche drive axial patterning', *Development (Cambridge)*. Company of Biologists Ltd, 146(1). doi: 10.1242/dev.168161.

Wymeersch, F. J. *et al.* (2019b) 'Transcriptionally dynamic progenitor populations organised around a stable niche drive axial patterning', *Development (Cambridge)*. Company of Biologists Ltd, 146(1). doi: 10.1242/dev.168161.

Yamada, T. *et al.* (1991) 'Control of cell pattern in the developing nervous system: Polarizing activity of the floor plate and notochord', *Cell*, 64(3), pp. 635–647. doi: 10.1016/0092-8674(91)90247-V.

Yamaguchi, T. P. *et al.* (1999) 'T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification', *Genes and Development*, 13(24), pp. 3185–3190. doi: 10.1101/gad.13.24.3185.

Yamaguchi, T. P. (2008) 'Genetics of Wnt signaling during early mammalian development', *Methods in Molecular Biology*. Methods Mol Biol, 468, pp. 287–305. doi: 10.1007/978-1-59745-249-6_23.

Yamamoto, M. *et al.* (2004) 'Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo', *Nature*, 428(6981), pp. 387–392. doi: 10.1038/nature02418.

Yohn, D. C. *et al.* (2008) 'Transplanted mouse embryonic stem-cell-derived motoneurons form functional motor units and reduce muscle atrophy', *Journal of Neuroscience*. Society for Neuroscience, 28(47), pp. 12409–12418. doi: 10.1523/JNEUROSCI.1761-08.2008.

Yoshikawa, Y. *et al.* (1997) 'Evidence that absence of Wnt-3a signaling promotes

neuralization instead of paraxial mesoderm development in the mouse', *Developmental Biology*. Academic Press Inc., 183(2), pp. 234–242. doi: 10.1006/dbio.1997.8502.

Young, T. *et al.* (2009) 'Cdx and Hox Genes Differentially Regulate Posterior Axial Growth in Mammalian Embryos', *Developmental Cell*, 17(4), pp. 516–526. doi: 10.1016/j.devcel.2009.08.010.

Zechner, D. *et al.* (2007) 'Bmp and Wnt/β-catenin signals control expression of the transcription factor Olig3 and the specification of spinal cord neurons', *Developmental Biology*. Academic Press Inc., 303(1), pp. 181–190. doi: 10.1016/j.ydbio.2006.10.045.

Zhang, X. *et al.* (2010) 'Pax6 is a human neuroectoderm cell fate determinant', *Cell Stem Cell*. Cell Press, 7(1), pp. 90–100. doi: 10.1016/j.stem.2010.04.017.

Zhao, X. and Duester, G. (2009) 'Effect of retinoic acid signaling on Wnt/β-catenin and FGF signaling during body axis extension', *Gene Expression Patterns*. Gene Expr Patterns, 9(6), pp. 430–435. doi: 10.1016/j.gep.2009.06.003.

Zhou, J. *et al.* (2010) 'High-efficiency induction of neural conversion in human ESCs and human induced pluripotent stem cells with a single chemical inhibitor of transforming growth factor beta superfamily receptors', *Stem Cells*, 28(10), pp. 1741–1750. doi: 10.1002/stem.504.

Zhou, Q. and Anderson, D. J. (2002) 'The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification', *Cell*. Cell Press, 109(1), pp. 61–73. doi: 10.1016/S0092-8674(02)00677-3.

Zimmerman, L. B., De Jesús-Escobar, J. M. and Harland, R. M. (1996) 'The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4', *Cell*. Cell Press, 86(4), pp. 599–606. doi: 10.1016/S0092-8674(00)80133-6.

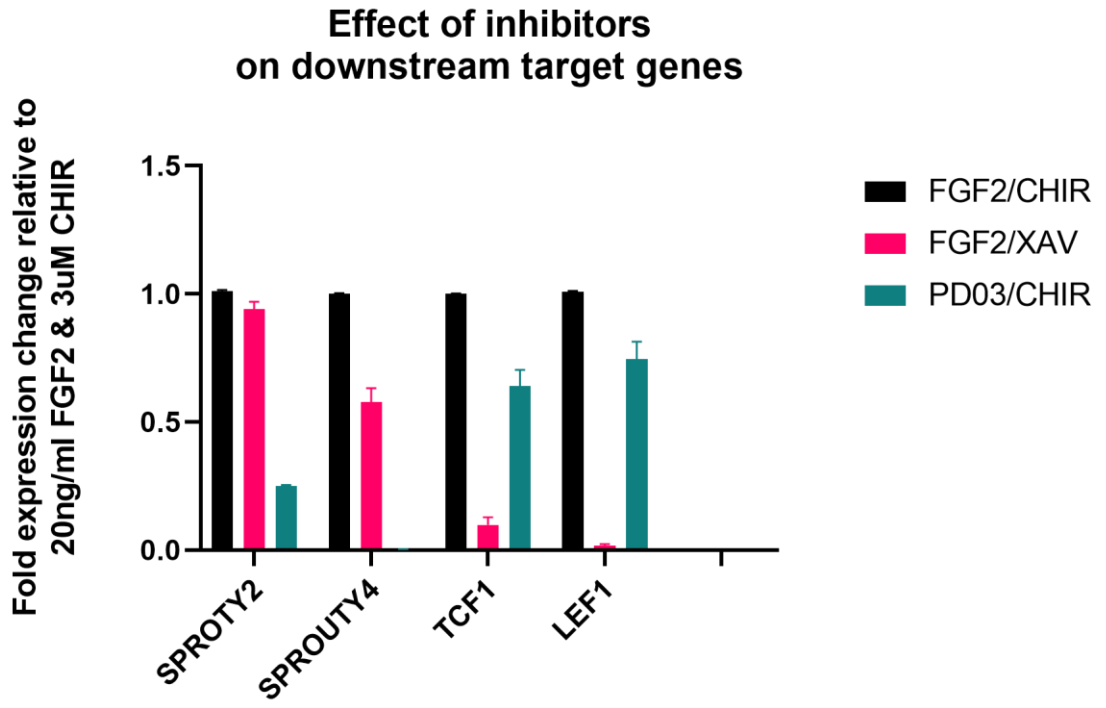


Figure 7.1 Efficacy of FGF and WNT inhibitors. Quantitative PCR data showing the relative gene expression for FGF (*SPROUTY2* and *SPROUTY4*) and WNT target genes (TCF and LEF1) after DAY 3 hPSC-derived NMPs were cultured for a further 4 days under the presence of FGF2 and CHIRON or with the addition of an inhibitor of either of the pathways. Upon exposure to PD03, an inhibitor of the FGF signalling pathway there is a clear reduction in SPROUTY2 and SPROUTY 4, with almost complete loss of *SPROUTY4* expression. Similarly, upon exposure to XAV, a WNT signalling pathway inhibitor there is reduction and almost abolition of *TCF1* and *LEF1* expression. Downregulation of *SPROUTY4* under the presence of WNT inhibition and *TCF1* and *LEF1* under the presence of FGF inhibition suggests a level of cross-talk between the signalling pathways which are known to exist.

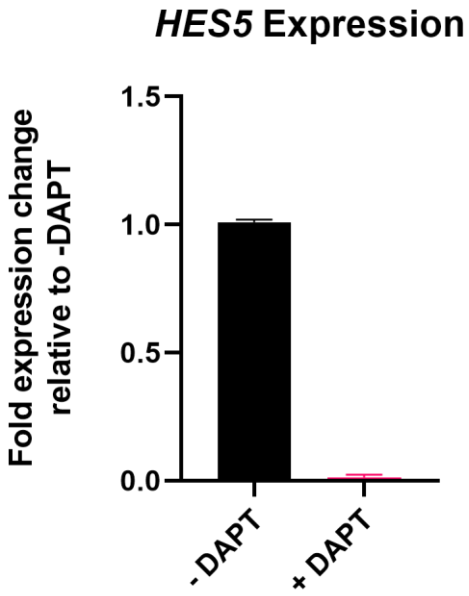


Figure 7.2 Efficacy of DAPT. Quantitative PCR data showing the relative gene expression of *HES5* after DAY 3 hPSC-derived NMPs were cultured for a further 4 days under the presence of FGF2 and CHIRON or with the addition of DAPT (a NOTCH signalling pathway inhibitor). Upon exposure to DAPT, there is a clear abolition of *HES5* (a NOTCH pathway target gene) expression. Suggesting that DAPT is a very efficient NOTCH signalling pathway inhibitor.