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How to make spinal motor neurons

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ABSTRACT

All muscle movements, including breathing, walking, and fine motor skills rely on the function of the spinal motor neuron to transmit signals from the brain to individual muscle groups. Loss of spinal motor neuron function underlies several neurological disorders for which treatment has been hampered by the inability to obtain sufficient quantities of primary motor neurons to perform mechanistic studies or drug screens. Progress towards overcoming this challenge has been achieved through the synthesis of developmental biology paradigms and advances in stem cell and reprogramming technology, which allow the production of motor neurons *in vitro*. In this Primer, we discuss how the logic of spinal motor neuron development has been applied to allow generation of motor neurons either from pluripotent stem cells by directed differentiation and transcriptional programming, or from somatic cells by direct lineage conversion. Finally, we discuss methods to evaluate the molecular and functional properties of motor neurons generated through each of these techniques.

KEY WORDS: Motor neuron, Pluripotency, Stem cell

Introduction

In his memoir, Jean-Dominique Bauby lamented 'Other than my eye, two things are not paralyzed, my imagination and my memory' (Bauby, 1998). Such is the plight of those experiencing locked-in syndrome – a condition in which the brain remains relatively intact, but the terminal neurons that connect to all muscles except those servicing the eye are rendered non-functional. As a result, locked-in individuals are left with only their ability to take in visual stimuli and to have thoughts upon which they cannot act.

Two broadly defined neuronal types provide the connection between the brain and our musculature: the upper, or cortical spinal motor neurons (CSMNs) and the lower spinal motor neurons. As their name implies, the cell bodies of CSMNs reside in the cortex and transmit motor information down long axons into the spinal cord. Spinal motor neurons receive this information and through axons that project out of the spinal cord to the musculature, actuate muscle contraction through a specialized synapse, the neuromuscular junction (NMJ).

Significant injury to the descending spinal cord axons after physical trauma or stroke can result in complete paralysis. Although localized peripheral nerve injury to spinal motor neurons may only result in partial paralysis, conditions such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) can cause a more global degeneration of motor neurons and, in turn, a locked-in condition (Box 1).

Curiosity concerning how the terminal motor circuitry develops and is wired has inspired numerous studies, making spinal motor neurons one of the best-understood neuronal types. A desire to protect and eventually regenerate motor circuitry in the contexts of motor neuron disease and spinal cord injury has motivated attempts to utilize stem cell and reprogramming technologies to produce motor neurons for translational applications, including the modeling of these conditions.

In this Primer, we will first review the processes and events that control the specification and differentiation of spinal motor neurons during embryogenesis. We will then discuss how the emerging understanding of motor neuron development has led to methods for directed differentiation of mouse and human pluripotent stem cells (PSCs) into motor neurons. More recent efforts to obtain motor neurons directly from fibroblasts by forced expression of transcription factors important for motor neuron identity will also be outlined. Finally, we consider methods to validate the equivalency of *in vitro*-derived motor neurons to their bona fide counterparts. In this Primer, we focus our attention exclusively on spinal motor neurons (referred to hereafter simply as MNs) and direct readers interested in CSMN development and reprogramming to recent publications of note (Shoemaker and Arlotta, 2010; Woodworth et al., 2012; Greig et al., 2013).

MN development

Decades of embryological studies and genetic analyses in model organisms have illuminated the molecular basis of neural induction as well as further differentiation and specification of MNs during

Box 1. Motor neuron degeneration in disease

Motor neuron diseases (MNDs) result from the progressive degeneration and death of motor neurons (MNs). The two most studied MNDs are the childhood genetic disease spinal muscular atrophy (SMA) and the adult-onset neurodegenerative disease amyotrophic lateral sclerosis (ALS) (Burghes and Beattie, 2009; Ling et al., 2013). Both diseases involve neuromuscular dysfunction progressively leading to fatal paralysis.

SMA is an autosomal-recessive disease characterized by the selective loss of spinal MNs. The vast majority of SMA cases are caused by mutations in the ubiquitously expressed survival of motor neuron-1 (*SMN1*) gene. These mutations lead to the severe reduction in SMN levels, which is thought to affect small nuclear ribonucleoprotein biogenesis, as well as RNA transport in neurons (Burghes and Beattie, 2009). A growing collection of evidence indicates that therapeutics capable of elevating SMN levels could be effective in treating SMA (Passini and Cheng, 2011). Still, precisely how a deficiency in SMN, a ubiquitously expressed protein, causes selective loss of MNs remains unclear.

In contrast to SMA, ALS affects both the cortical and spinal MNs. Approximately 10% of ALS cases are classified as familial, leaving the majority of ALS cases to be considered sporadic in origin. Several themes are emerging in the molecular pathologies of ALS (reviewed by Ling et al., 2013). These include dysfunctions in RNA processing and protein homeostasis as well as endoplasmic reticulum stress and problems in axonal transport. Interestingly, in SMA and ALS distinct subtypes of lower MNs are thought to be initially vulnerable to degeneration (Kanning et al., 2010). Thus, studying diverse populations of MNs *in vitro* could illuminate differential responses and guide the development of new therapeutics.

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development. In the early 20th century, Spemann and Mangold's work with amphibian embryos revealed that signals emanating from the dorsal lip of the blastopore, now termed the Spemann–Mangold 'organizer', were required for the induction of neural fate during gastrulation (Hamburger, 1988). Further studies demonstrated that rather than providing positive signals to induce neural cell fate, the organizer was the source of factors that inhibit bone morphogenetic protein (BMP) signaling, including Chordin, Follistatin and Noggin (De Robertis, 2006). Although the requirement to inhibit BMP signaling is conserved in higher organisms, additional inductive signals, including fibroblast growth factors (FGFs), epidermal growth factors (EGFs) and Wnts have been identified (Stern, 2005) (Fig. 1A).

Following their initial generation, the cells of the neural tube are specified along both the rostral-caudal and the dorso-ventral axes. Gradients of signaling molecules along each axis provide a roadmap to guide the differentiation of the emerging neuronal types of each region. Progenitor domains are first specified and then refined through the cooperative action of external signals and downstream transcription factors (Jessell, 2000; Alaynick et al., 2011). Along the rostral-caudal axis, the neural tube is specified into the major components of the CNS, including the brain, midbrain, hindbrain and spinal cord (Fig. 1A). Although multiple signals have been proposed to contribute to the caudalization of the neurons in the spinal cord, chief among them is retinoic acid (RA). Early in development, RA, produced through the activity of retinaldehyde dehydrogenase 2 (RALDH-2; also known as ALDH1A2), emanates from the caudal paraxial mesoderm and is crucial for the initial distinction of neurons of the hindbrain and spinal cord from those in the forebrain and midbrain (Maden, 2007) (Fig. 1A).

Specification of MN fate

Within the dorso-ventral axis of the neural tube, progenitor cells are divided into five ventral progenitor domains termed p0, p1, p2, pMN and p3, which in turn give rise to interneuron subtypes V0-3 and motor neurons (Fig. 1B). A gradient of sonic hedgehog (Shh),

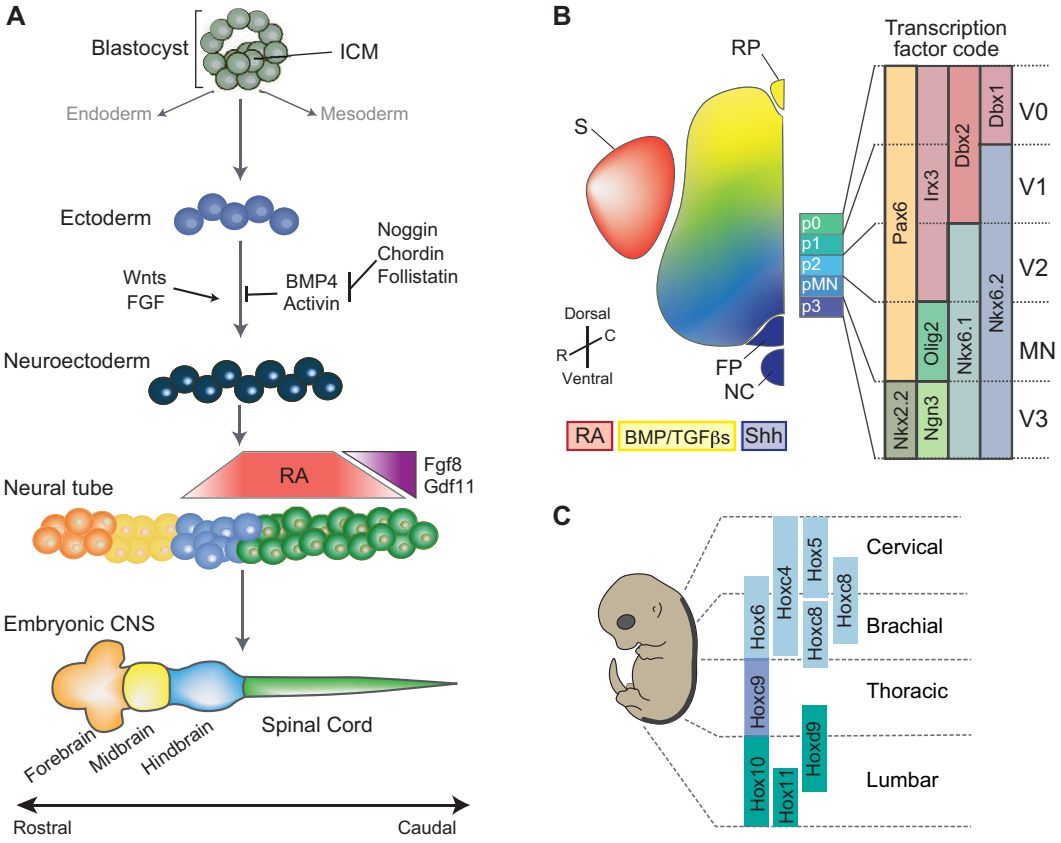

Fig. 1. Spinal cord development and motor neuron specification. (A) In early development, gastrulation results in the specification of the cells of the inner cell mass (ICM) into the three germ layers: ectoderm, endoderm and mesoderm. The dorsal region of the ectoderm is further specified into the neuroectoderm through the inhibition of BMP and activin signaling, and, in higher organisms, enhanced FGF and Wnt signaling. Neuralization proceeds through the formation of a neural plate and subsequent generation of neural folds, which in turn fuse to give rise to the neural tube. The neural tube is then patterned along the rostro-caudal axis (anterior-posterior) by a gradient of retinoic acid (RA) generated primarily by the action of Raldh2. In particular, a high level of RA allows the initial boundary of the spinal cord and hindbrain versus forebrain and hindbrain to be delineated. Fgfs and Gdf11 oppose the activity of RA and allow specification of more caudal spinal cord cell types. (B) Once the spinal cord is specified, continued release of RA (shaded red) from the somites (S) acts to refine the positional character of neurons along the rostral-caudal axis. The spinal cord is also patterned along the dorso-ventral axis through the combined action of sonic hedgehog (Shh; shaded blue) emanating from the notochord (NC) and floor plate (FP) and BMP/TGFβ signaling (shaded yellow) from the roof plate (RP). The ventral spinal cord can be divided into five progenitor domains (p0-p3 and pMN), which give rise to V0-V3 interneurons and motor neurons. The borders of progenitor domains are established through the cross-repressive action of pairs of transcription factors that are induced by Shh (Class II, in green and blue) or those that are repressed by Shh (Class I, in yellow and red). The combinatorial action of transcription factors allows the specification of each cell type. For example, pMNs (MN progenitors) express Pax6, Olig2, Nkx6.1 and Nkx6.2. (C) The Hox genes play a crucial role in the specification of MNs along the rostral-caudal axis. Similar to patterning along the ventral-dorsal axis, the coordinated interactions between Hox family members allows regional boundaries to be delineated. Specific expression of Hox accessory factors, such as FoxP1, can further specify MN subtypes.

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secreted from the notochord and cells of the floor plate provides ventral topographic information by regulating the expression of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors (Alaynick et al., 2011). Transcription factors downstream of Shh can be roughly divided into two classes based on their regulation in response to Shh signaling. Class II proteins, including Nkx6.1, Olig2 and Nkx2.2, are activated by Shh and in turn repress the expression of class I proteins, including Pax6, Irx3, Dbx1 and Dbx2 (Briscoe et al., 2000; Jessell, 2000; Alaynick et al., 2011) (Fig. 1B).

The cross-repressive activity between class II and class I proteins allows the consolidation of progenitor identity as well as the generation of sharp boundaries between adjacent domains (Briscoe et al., 2000). For example, the boundary between p3 and pMN is delimited by the activity of Pax6 and Nkx2.2. Indeed, in the mouse embryo, mutation of Pax6 results in a dorsal expansion of the Nkx2.2 expression boundary (Ericson et al., 1997). Similarly, the dorsal boundary between pMN and p2 is defined by the mutually repressive activities of Irx3 and Olig2 whereas the ventral boundary is delimited by the expression of Ngn3 (Neurog3) (Novitch et al., 2001; Sugimori et al., 2007). MN progenitors also express the HD transcription factors Nkx6.1 and Nkx6.2, which act to repress the other progenitor domains (Briscoe et al., 2000) (Fig. 1B). Expression of Olig2 within the pMN domain promotes expression of Ngn2, which is important for cell cycle exit as well as for induction of terminal MN transcription factors including Hb9 (Mnx1), Isl1, Isl2 and Lhx3 (Novitch et al., 2001). More recently, additional molecular mechanisms, including microRNA pathways, have also been shown to regulate the boundary between some progenitor domains (Chen et al., 2011).

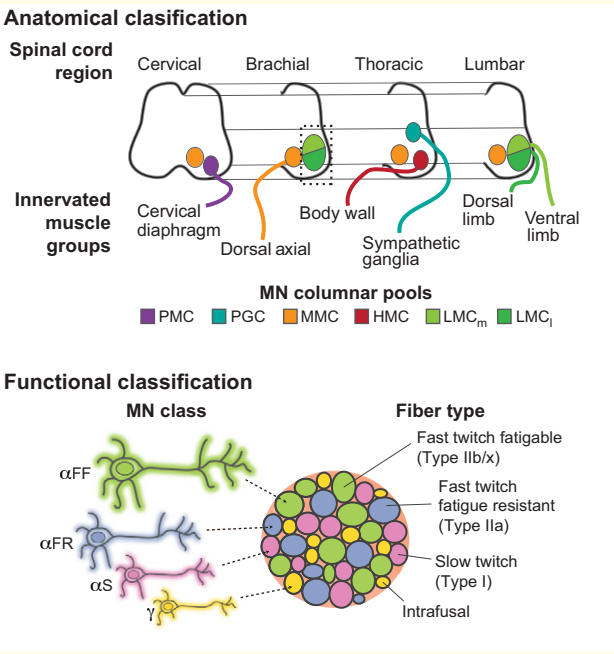
MN subtype specification

Although all MNs derive from a single ventral progenitor domain, further specification of MNs allows the coordinated movement of hundreds of distinct muscle groups. MNs can be further classified based on their anatomical and functional properties (Box 2). The positional identity of MNs along the rostro-caudal axis is determined by the coordinated action of multiple signaling molecules. High levels of RA promote rostral (e.g. cervical and brachial) identity whereas FGFs and Gdf11 activity give rise to more caudal (e.g. thoracic and lumbar) MNs (Liu et al., 2001). The combined signals from RA, FGFs, Wnts and TGFβ family members are integrated primarily by the Hox transcription factors to specify MN rostro-caudal subtype identity (Dasen and Jessell, 2009). Mouse and Human Hox genes are arrayed in four chromosomal clusters (*HoxA*, *HoxB*, *HoxC* and *HoxD*), each of which harbors a subset of 13 paralogous Hox genes (*Hox1*-*Hox13*). Within each cluster, the expression pattern of the Hox genes is spatially and temporally collinear with their chromosomal organization, such that *Hox1* genes are expressed in the rostral region of the organism and *Hox13* genes are expressed caudally (Pearson et al., 2005). Consistent with their conserved role in body patterning, Hox gene expression within the spinal cord determines MN columnar identity and selective muscle innervation, with *Hox4*-*8* genes expressed at brachial levels, *Hox8* and *Hox9* at the thoracic level and *Hox10* and *Hox11* in the lumbar region (Dasen et al., 2005; Dasen and Jessell, 2009) (Fig. 1C). Further specification of individual MN subtypes is provided by fine-tuning the Hox protein expression pattern both spatially and temporally (Philippidou and Dasen, 2013).

Experimental manipulation of the Hox code in mouse and chick embryos can alter MN subtype and projection pattern (Tiret et al., 1998; Wahba et al., 2001; Vermot et al., 2005; Wu et al., 2008; Misra et al., 2009; Jung et al., 2010; Philippidou et al., 2012; Lacombe et al., 2013). As a particularly dramatic example, mutation of *Hoxc9* in the mouse causes loss of thoracic preganglionic column (PGC) and hypaxial motor column (HMC) MNs and expansion of the brachial lateral motor column (LMC) domain (Jung et al., 2010). ChIP-Seq analysis of *Hoxc9* revealed that this protein directly binds numerous regions within diverse Hox loci and its disruption altered expression of multiple Hox genes, suggesting that Hoxc9 may represent a master regulator of MN subtype identity (Jung et al., 2010).

The ability of different Hox genes to play such an important role in determination of MN identity is perhaps surprising given that the

Box 2. Subclassification of MNs



Spinal MNs can be classified based on both anatomical and functional properties. Anatomically, MNs are divided into five major MN columnar identities: the phrenic motor column (PMC), lateral motor column (LMC), preganglionic column (PGC), hypaxial motor column (HMC) and median motor column (MMC) (Dasen and Jessell, 2009; Kanning et al., 2010; Philippidou and Dasen, 2013). MNs of each column reside in stereotypical regions along both the rostro-caudal and dorso-ventral axes. The MNs of the LMC can be further subdivided into MNs projecting either ventrally or dorsally within the limb, designated as medial (LMC_m) or lateral (LMC_l) groups, respectively. MN columnar pools can be further organized according to their innervation of particular muscle group targets.

The MNs of each pool can be further classified based on functional properties. These are defined by the type of muscle fiber innervated and are associated with morphological differences between MN classes. Individual muscles are composed of a mixture of fiber types, which can be grouped into two major categories: extrafusal and intrafusal. Whereas intrafusal fibers modulate the sensitivity of muscle to stretch, extrafusal fibers are primarily responsible for skeletal movement. Extrafusal muscle fibers can be further classified into fast twitch fatigable (FF, Type IIb/x),fast twitch fatigue resistant (FR, Type IIa) and slow twitch (S, Type I). α-MNs innervate these three extrafusal fiber types, whereas γ-MNs innervate intrafusal fibers. Both types of fibers can also be innervated by β-MNs (not shown). In addition to differences in the type of muscle fiber innervated, MN classes exhibit morphological differences with α-FF MNs typically representing the largest neurons and γ- and β-MNs being smaller (Kanning et al., 2010).

homeodomains of Hox genes are largely conserved between paralogs. However, there is accumulating evidence that further specificity of Hox function can be provided by accessory factors, including downstream effectors such as the Forkhead box (Fox) protein P1 and the HD protein Nkx6.1 (Philippidou and Dasen, 2013). FoxP1 is expressed at high levels in LMC MNs and *FoxP1*$^{-/-}$ mouse embryos exhibit disrupted columnar MN identities and alterations in MN cell body position and axonal wiring (Dasen et al., 2008; Rousso et al., 2008).

As discussed further below, the body of work describing the molecular underpinnings of MN specification during development has enabled recapitulation of these signals *in vitro* for the *ex vivo* generation of MNs. An exciting consequence of this progress is that it allows sufficient quantities of MNs to be derived in a controlled environment to interrogate further the detailed mechanism of MN development and specification. For example, Mazzoni and colleagues performed a series of chromatin immunoprecipitation assays during MN differentiation *in vitro* to investigate the molecular details regulating Hox gene expression (Mazzoni et al., 2013b). The authors found that addition of RA during MN differentiation led to recruitment of RA receptors to the *Hox1-5* chromatin domain that was followed by a rapid domain-wide removal of H3K27me3 and acquisition of cervical MN identity. Moreover, Cdx2, a transcription factor induced by Wnt and FGF, regulated the clearance of H3K27me3 from the *Hox1-9* chromatin domains, resulting in brachial or thoracic MN specification (Mazzoni et al., 2013b). Thus, the early modification of chromatin by patterning factors contributes to the specification of the rostro-caudal identity of MNs. This type of mechanistic, genome-wide study would surely be impossible with the limited quantities and mixed populations of cells that can be purified from the early embryo. Continued integration of findings from developmental studies in model organisms and *in vitro*-derived MNs will allow a greater understanding of MN specification, and as a consequence further improve strategies to recapitulate MN development *in vitro*.

Directed differentiation of PSCs into MNs

PSCs, either embryonic stem cells (ESCs) derived from pre-implantation blastocysts (Evans and Kaufman, 1981; Thomson et al., 1998), or induced pluripotent stem cells (iPSCs) obtained by reprogramming of somatic cells with defined transcription factors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), are characterized by their ability to proliferate indefinitely in culture while preserving their developmental potential to differentiate into derivatives of all three embryonic germ layers. By leveraging knowledge of developmental pathways that allow neural induction and further specification of MNs, stem cell biologists have designed multiple approaches to direct the differentiation of mouse and human PSCs to MNs (Wichterle et al., 2002; Li et al., 2005; Singh Roy et al., 2005; Di Giorgio et al., 2007; Chambers et al., 2009; Hu and Zhang, 2009; Karumbayaram et al., 2009; Boulting et al., 2011; Patani et al., 2011; Amoroso et al., 2013). MNs obtained through these methods have been shown to possess numerous characteristics of bona fide MNs, including distinctive electrophysiological responses, the ability to form functional NMJs and the capacity to engraft into the developing spinal cord. Just as the *in vivo* embryonic development of spinal MNs can be broken down into distinct stages, so too can the *in vitro* specification of MNs from PSCs. Specifically, the steps of neural induction followed by caudal and ventral patterning must all be appropriately executed for MNs to be produced.

Neural induction

In the absence of factors that promote pluripotency, such as leukemia inhibitory factor (LIF) and FGF, PSCs spontaneously differentiate into diverse lineages and lose the ability to self-renew or generate chimeric mice (Evans, 2011). Although spontaneous differentiation represents a hurdle for the maintenance of pluripotency, it can be exploited to give rise to differentiated cell types. Spontaneous differentiation into multiple lineages, including neurons, can be enhanced by inducing PSCs under non-adherent culture conditions to form multicellular aggregates, termed embryoid bodies (EBs) (Odorico et al., 2001). However, the efficiency of neural induction using these spontaneous approaches is low, and significant cellular heterogeneity within EBs hinders further mechanistic studies (Bain et al., 1995). Multiple strategies have been proposed to improve the production of neural precursors and cells with neuronal phenotypes from differentiating PSC populations. These approaches include treatment of EB cultures with RA (Bain et al., 1995), adherent co-culture of PSCs with PA6 or MS-5 stromal feeder cell lines (Kawasaki et al., 2000; Lee et al., 2007), continued propagation of nestin^{+} proliferating cells in defined media containing mitogens such as FGF2 and EGF (Okabe et al., 1996; Reubinoff et al., 2001; Joannides et al., 2007), lineage selection using genetic reporters (Li et al., 1998), and selective enzymatic digestion or manual selection of neural tube-like rosette structures (Zhang et al., 2001; Hu and Zhang, 2009).

More recently, it has been demonstrated that simultaneous inhibition of the TGFβ/Activin/Nodal and BMP signaling pathways, through the use of either small molecule antagonists or recombinant inhibitors, can induce a rapid and very efficient (>80%) neural conversion of human PSCs (Smith et al., 2008; Chambers et al., 2009; Zhou et al., 2010; Chambers et al., 2012) (Fig. 2A). Similar to the processes that occur during early development, inhibition of the TGFβ and BMP pathways is thought to promote differentiation of PSCs along the neuronal lineage primarily through inhibition of self-renewal as well as blocking differentiation towards alternative lineages (Chambers et al., 2009). Several other pathways, involving EGFs, FGFs and Wnts, have been described to regulate neuronal differentiation of human and mouse stem cells. In particular, FGF2 has been shown to promote induction and survival of neural progenitors (Streit et al., 2000; Wilson et al., 2000; Joannides et al., 2007). Therefore, enhancing FGF2 signaling during the neural induction phase of differentiation can increase the number of neural progenitors, whereas inhibiting it at subsequent stages promotes their transition into differentiated neurons (Joannides et al., 2007; Chambers et al., 2012).

Caudal and ventral patterning

Following neural induction of PSCs, neural progenitor cells can be patterned according to developmental principles. Treatment with RA promotes caudal (spinal cord) identity, while addition of either recombinant Shh or small molecule agonists of the Shh signaling pathway, such as smoothened agonist (SAG) or purmorphamine (PUR), promotes ventralization (Wichterle et al., 2002) (Fig. 2A). Time course studies reveal that differentiation *in vitro* proceeds with the same temporal regulation of transcription factors as is observed *in vivo*, with Sox1^{+} neural progenitors giving rise to Olig2^{+} MN progenitors, which then in turn begin to express Hb9 and Isl1 (Fig. 2B) (Wichterle et al., 2002). In the mouse, Hb9^{+} MNs begin to appear 3-5 days after addition of patterning factors (Wichterle et al., 2002; Di Giorgio et al., 2007). The timing of MN differentiation from human PSCs is more protracted and it can, depending on the specific protocol utilized, require an additional 2-4 weeks after

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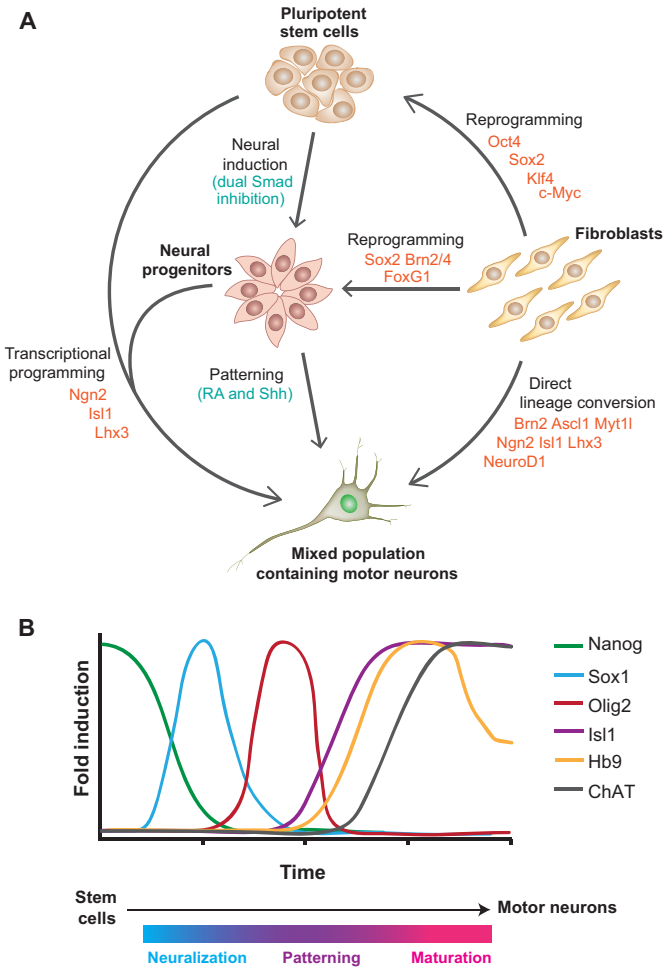

Fig. 2. Motor neurons (MNs) can be generated *in vitro* through the recapitulation of developmental principles. (A) Pluripotent stem cells (PSCs), either embryonic stem cells (ESCs) derived from the inner cell mass (ICM) of blastocysts, or induced pluripotent stem cells (iPSCs) obtained through reprogramming of somatic cells, can be efficiently directed to differentiate into neuronal progenitors through inhibition of BMP and TGFβ signaling cascades (dual Smad inhibition). Additional signals, such RA, Wnts and FGFs, also promote neuronal induction (not shown). Fibroblasts can alternatively be reprogrammed to neural progenitors through addition of Sox2, Brn2/4 and FoxG1. Neural progenitors are then patterned into motor neurons using RA to promote caudalization and either recombinant sonic hedgehog (Shh) or agonists of Shh signaling, such as SAG and PUR. Alternatively, stem cells or neural progenitors can be directly programmed into MNs using a combination of transcription factors (transcriptional programming). Finally, somatic cell types, including fibroblasts, can be directly converted into induced MNs (iMNs) through a combination of the BAM factors (Brn2, Ascl1 and Myt1l), which promote a general neuronal phenotype, and the MN-specific factors Ngn2, Isl1 and Lhx3. Although not required for generation of mouse iMNs, addition of NeuroD1 enhances the efficiency of conversion from fibroblasts into human iMNs. (B) Expression of transcriptional markers of motor neuron differentiation during development is recapitulated during directed differentiation of PSCs. The precise timing of each event varies between mouse and human cells and can be influenced by timing of small molecules and culture conditions. Neural induction involves repression of pluripotent marker genes, including Nanog, and induction of Sox1. Expression of the MN progenitor marker Olig2 precedes the induction of terminal MN markers, including Isl1, Hb9 and ChAT.

neural induction before electrophysiologically active, HB9+ISL1+ neurons are present (Chambers et al., 2009; Hu and Zhang, 2009; Boulting et al., 2011; Amoroso et al., 2013).

In contrast to the well-understood developmental mechanisms that allow the specification of generic MNs, how individual MN subtypes are generated *in vivo* is relatively less well understood. Despite this, several groups have made progress in manipulating MN subtypes generated by directed differentiation. Whereas early in development RA promotes spinal cord identity, continued exposure to RA establishes a rostral MN identity (Maden, 2007; Jessell, 2000). In the context of murine ESC differentiation, protocols that include treatment of cells with RA typically result in MNs with a cervical character, as judged by expression of Hoxc4 and Hoxa5 and lack of expression of Foxp1 or Hox8-11 (Peljto et al., 2010). Although MN differentiation is typically less efficient in the absence of RA, mouse and human studies have both shown that either endogenous Wnt and FGF signaling or inhibition of BMP/Activin/Nodal signaling cascades allow generation of MNs with caudal positional identity (Patani et al., 2009; Peljto et al., 2010; Patani et al., 2011). Moreover, in the context of human MN differentiation, Amoroso and colleagues recently reported a shift in the proportion of MNs expressing the median motor column (MMC) marker LHX3 or the LMC marker FOXP1 when SHH signaling was activated via a combination of SAG and PUR instead of recombinant SHH (Amoroso et al., 2013). This exquisite sensitivity of differentiating MNs to alterations in experimental protocol emphasizes the need for continual evaluation of MN differentiation protocols and also presents a new avenue for the optimization of further MN subtype specification.

Transcriptional programming of PSCs into MNs

Early methods for MN differentiation from human PSCs were relatively inefficient and required extended periods of time (40-60 days) before resulting cells exhibited electrophysiological characteristics of mature MNs. In order to improve the efficiency and timing of this process, Hester and colleagues coupled directed differentiation with a transcriptional programming approach (Fig. 2A) (Hester et al., 2011). After induction of neural progenitors from human PSCs, adenoviral delivery of *LHX3*, *ISL1* and *NGN2* (*NEUROG2*) (referred to as LIN factors), in combination with exogenous RA and SHH signaling, enabled rapid and efficient (>60-70%) acquisition of mature MN phenotypes, including electrophysiological properties, within 11 days (Hester et al., 2011).

Additional insight into transcriptional programming of PSCs into MNs has been gained using mouse ESCs genetically engineered to express the LIN factors in response to doxycycline. Within 24 hours of LIN activation, these cells exhibited widespread gene expression changes, with induction of the MN markers Hb9 and choline acetyltransferase (ChAT) occurring within two days (Mazzoni et al., 2013a). Unlike in the human system, addition of patterning factors was not required for MN generation; however, comparison with MNs differentiated from mouse ESCs under RA and Shh signaling conditions indicated that retinoid activity influenced the expression of genes controlling the rostral-caudal identity (Mazzoni et al., 2013a).

The combined approach of directed differentiation and transcriptional programming might allow a greater diversity of MN types to be specified *in vitro*. For example, whereas introduction of *Lhx3*, *Isl1* and *Ngn2* into mouse ESCs resulted in MNs with a spinal identity, replacement of *Lhx3* with *Phox2a* gave rise to MNs with cranial character (Mazzoni et al., 2013a). Further evidence that the precise outcome of transcriptional programming strategies can be manipulated comes from the observation that titration of the relative proportions of Lhx3 and Isl1 can influence the specification of MNs

versus V2 interneurons. *In vivo*, Lhx3 and Isl1 form a complex with the nuclear LIM interactor protein NLI (Ldb1) to specify MNs, whereas in the absence of Isl1, Lhx3, in complex with NLI, specifies V2 interneurons (Thaler et al., 2002). Consistent with this molecular mechanism, equimolar amounts of Lhx3 and Isl1 promoted MN generation from mouse ESCs, whereas excess Lhx3 expression gave rise to V2 interneurons. The use of an Isl1-Lhx3 fusion protein could also enhance the shift of the differentiating cells towards MNs (Lee et al., 2012). As the mechanisms which lead to the cooperative action of transcription factors during MN specification continue to be defined, it will be interesting to determine if similar fusion proteins may be utilized to guide cell fate more specifically or efficiently.

Direct lineage conversion of somatic cells into MNs

Recent success using defined factors to reprogram somatic cells to pluripotency, along with much earlier studies demonstrating the ability of a single factor, MyoD (Myod1), to convert fibroblasts into muscle cells, has led many researchers to explore further the ability of lineage-specific transcription factors to induce the conversion of specific cell types from unrelated somatic cells (reviewed by Graf, 2011). Following a similar approach to the one used to identify iPSC reprogramming factors, Vierbuchen and colleagues demonstrated that a set of three neural lineage-specific transcription factors, referred to as BAM factors [Brn2 (Pou3f2), Ascl1 and Myt1l] was sufficient to directly convert mouse fibroblasts into induced neuronal (iN) cells. Gene expression profiling and electrophysiological recordings revealed that these iN cells have properties of generic excitatory neurons (Vierbuchen et al., 2010; Marro et al., 2011). Following this initial report, microRNAs and additional proneuronal factors, including NeuroD1, were shown to cooperate with or replace the BAM factors during conversion of human fibroblasts into iNs (Ambasudhan et al., 2011; Pang et al., 2011; Yoo et al., 2011).

A crucial issue for the application of iN approaches in developmental and translational studies is the specification of precise neuronal subtypes. Our group demonstrated that BAM factor expression, in combination with four transcription factors (Lhx3, Isl1, Ngn2 and Hb9) was sufficient to convert mouse fibroblasts into cells with a MN phenotype, termed induced MNs or iMNs (Son et al., 2011) (Fig. 2A). iMNs were identified based on the expression of a transgenic *Hb9::*GFP reporter and exhibited molecular and functional properties of embryo-derived MNs, including gene expression profile, electrophysiological activity, formation of neuromuscular junctions and ability to integrate into the developing chick spinal cord. Interestingly, this study revealed that upon introduction of MN factors, fibroblasts, unlike PSCs, do not transition through an intermediate nestin^{+} neural progenitor state before becoming iMNs (Son et al., 2011). Similar to the case of iN cells, human fibroblasts could be converted to cells with a MN phenotype by the addition of NeuroD1 to the seven-factor iMN cocktail (Son et al., 2011). It has recently been shown that direct lineage conversion can be performed *in vivo*. For example, cardiomyocytes generated by direct conversion from cardiac fibroblasts can improve cardiovascular function in the mouse (Song et al., 2012). It will be exciting to determine if a similar approach can be adopted in the nervous system to repair injuries and/or reverse neurodegenerative disease.

Evaluation of MNs produced *in vitro*

Although the basic principles of MN specification are well established, many groups have reported differences in the timing and efficiency of differentiation, as well as the identity of the resulting PSC- or somatic cell-derived MNs. This variability may arise from overt differences in protocols, such as the combination, concentration and timing of addition of specific growth factors, as well as less transparent differences, such as the cellular density or precise media composition used to culture cells following specification. Concern about the extent to which minor modifications in protocols alter the identity of the resulting MNs is amplified because most groups rely on expression of transgenic reporters or a small handful of canonical marker genes, which may not be able to report on subtle MN subtype specific differences. Given the potential impact of these modifications on downstream studies such as *in vitro* disease modeling of MN disorders, continued effort both to carefully interrogate the effects of altering differentiation protocols and to standardize methods and analyses across multiple labs is crucial.

MNs can be assessed according to four primary characteristics that provide insights into the equivalency of *in vitro*-derived MNs to bona fide cells: (1) neuronal morphology and expression of characteristic MN marker genes, (2) characteristic electrophysiological activity and response to stimuli, (3) formation of functional neuromuscular junctions and (4) engraftment into the spinal cord *in vivo* (Fig. 3).

Morphology and marker analyses

When cultured in isolation, MNs exhibit unipolar morphology with a single axon extending from the soma, which is elaborated with dendrites. Although in dense cultures distinguishing the axon from surrounding dendrites can be difficult, this is aided by immunostaining of microtubule associated protein 2 (Map2), which marks proximal dendrites (Fig. 3A). Additional immunostaining of neuronal cytoskeletal proteins, such as β-III tubulin (Tuj1; Tubb3) as well as evaluation of the phosphorylation status of neurofilaments using SMI antibodies allows full appreciation of the complicated neuronal morphology typically present in MNs.

A crucial step towards the development of methods to generate specific cell types *in vitro* is the selection of appropriate cell type-specific markers that can allow the identification of differentiated cell types without the anatomical information provided *in vivo*. Like all other neurons, MNs are postmitotic, a property that can be determined by lack of bromodeoxyuridine (BrdU) incorporation into cellular DNA or by lack of immunoreactivity for cell proliferation proteins such as Ki67 (Mki67). MNs can be further distinguished from other neurons based on expression of canonical MN identity transcription factors, including Isl1 and Hb9, as well as markers of a more mature and cholinergic MN phenotype, such as the biosynthetic enzyme ChAT and the vesicular acetylcholine neurotransmitter transporter (vAChT) (Wichterle et al., 2002; Soundararajan et al., 2006; Karumbayaram et al., 2009; Boulting et al., 2011; Son et al., 2011; Amoroso et al., 2013). Expression of these transcription factors is thought to be common to the majority of MNs; however, as development proceeds, it is clear that some MNs express different subsets of these proteins (Vult von Steyern et al., 1999; Amoroso et al., 2013). The use of a single marker of MNs is further complicated by the observation that some canonical MN markers, such as Isl1, are also expressed in other neuronal cell types (Sun et al., 2008).

A basic appreciation of MN subtype can be gained by determining the expression profile of Hox genes and accessory factors such as FoxP1 (Amoroso et al., 2013). Alternative methods to immunohistochemistry include *in situ* hybridization or single-cell qRT-PCR, which may allow determination of mRNA expression of

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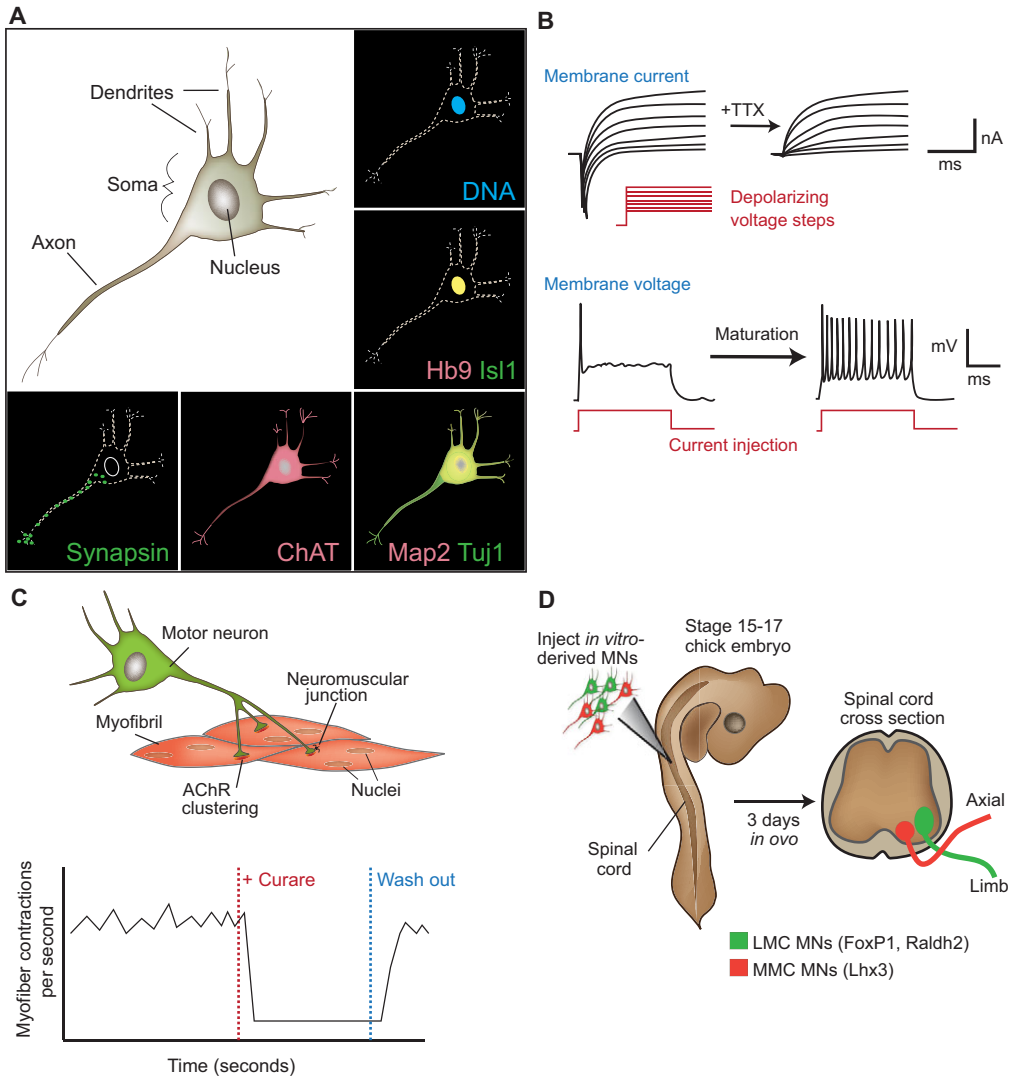

Fig. 3. Strategies to evaluate motor neurons (MNs) generated *in vitro*. (A) Unipolar neuronal morphology as well as expression of at least a subset of MN markers represents a minimal suggested requirement towards classification of *in vitro*-derived cells as MNs. Neuronal morphology can be additionally assessed by immunostaining for cytoskeletal proteins, such as Tuj1 and Map2. Whereas Tuj1 is present in both axons and dendrites, Map2 expression is limited to dendrites. MN identity can be evaluated based on the expression of transcription factors, such as Hb9 and Isl1, which are frequently co-expressed. However, subsets of MNs may express only one of these markers. Although typically not present immediately following differentiation, further maturation in culture of differentiated MNs results in expression of synaptic markers, such as synapsin, and biosynthetic enzymes required for the cholinergic activity of MNs, such as ChAT. Additional staining for column-specific markers, such as FoxP1 (not shown), can further illuminate the specific subtypes of MNs generated. (B) Electrophysiological activity of MNs. Top: voltage clamp recordings of membrane current in response to depolarizing voltage steps show fast inactivating outward currents that are eliminated following addition of tetrodotoxin (TTX). Bottom: relatively immature MNs exhibit single action potentials in response to current injection, whereas repetitive action potential firing is characteristic of functional maturation. (C) Co-culture of MNs with multi-nucleated myofibrils allows interrogation of neuromuscular junction (NMJ) formation. Functional NMJs elicit myofiber contraction, which can be blocked with curare, a reversible inhibitor of acetylcholine receptors (AchRs). Following removal of curare (washout) myofibers innervated by functional NMJs will resume contraction. (D) Engraftment of *in vitro*-derived MNs into the developing spinal cord represents a particularly stringent method of evaluating MN phenotype. Both the cell body position and axon projection pattern can be influenced by intrinsic MN subtype properties. The extent to which appropriate engraftment in the chick embryo mimics human or mouse motor neuron physiology remains an open question.

MN markers in single cells. It should be emphasized that although expression of canonical MNs markers has been shown to be a reliable indicator of other MN characteristics, expression of a small number of markers alone is an insufficient metric to evaluate the success of differentiation strategies. For this reason, genome-wide analysis of transcriptional profiles of purified MNs by RNA sequencing or microarrays represents a more effective strategy for evaluating MNs obtained through different methods, and their equivalency to bona fide MNs.

Electrophysiological activity

The primary function of all neurons is transmission of electrochemical signals, a property that requires the maintenance of voltage gradients across the cell membrane. The precise combination of ion pumps, ion channels and receptors embedded within the membrane allow different types of neurons to interpret and respond uniquely to stimuli. Physiological recordings have demonstrated that MNs derived *in vitro* using multiple strategies exhibit many of the electrophysiological characteristics relevant to

motor neuron function and circuitry (Fig. 3B). Both stem cell-derived MNs as well as iMNs respond to applications of GABA, glutamate, and glycine with increased inward currents, indicating that they express the proper receptors and can elicit the correct response to these stimuli (Miles et al., 2004; Boulting et al., 2011; Son et al., 2011; Amoroso et al., 2013). MNs produced *in vitro* also display spontaneous activity, express a range of voltage-activated channels and can fire action potentials in response to short current injections, as well as generate calcium transients in response to kainate (Boulting et al., 2011; Son et al., 2011; Amoroso et al., 2013). Some electrophysiological phenotypes of *in vitro*-derived MNs are dependent on the time spent in culture, including decrease in input resistance, spike frequency adaptation and rebound action potential firing (Miles et al., 2004; Takazawa et al., 2012).

As in the case of molecular markers of MNs, stereotypical electrophysiological response alone is insufficient to categorize cells as MNs. It is also important to note that different types of MNs exhibit differences in excitability and firing patterns, and that these responses evolve as MNs mature both *in vitro* and *in vivo* (Kanning et al., 2010). For example, rat embryonic and postnatal MNs exhibit marked differences in amplitude and rate of action potentials as well as differing responsiveness to neurotransmitters (Gao and Ziskind-Conhaim, 1998). Because patch clamping individual cells represents a technically challenging and low throughput method, it is frequently difficult to determine the extent to which electrophysiological characteristics vary between cells, even among cells of the same culture. New advances such as multi-electrode arrays may allow more global evaluation of many cells in parallel and offer the exciting opportunity to screen multiple genetic backgrounds or drug treatments for altered electrophysiological activity.

Formation of NMJs and *in vivo* engraftment

The main function of MNs *in vivo* is to innervate target muscles and integrate signals from the CNS to allow coordinated muscle contraction and body movement. Thus, the ultimate evidence that *in vitro*-derived MNs recapitulate their *in vivo* counterparts is the ability to reproducibly engraft into the adult spinal cord, to project to appropriate targets and to restore connectivity of a damaged CNS through formation of NMJs.

At the most basic level, it is clear that both mouse and human PSC-derived MNs as well as iMNs can form functional NMJs *in vitro* when co-cultured with muscle fibers (Miles et al., 2004; Son et al., 2011) (Fig. 3C). Clustering of acetylcholine receptors on myotubes adjacent to developing axons is observed within one day of co-culture with MNs differentiated from mouse ESCs. As early as two days after the initiation of co-culture, small endplate potentials can be detected by patch clamping innervated myotubes (Miles et al., 2004). In addition to the electrophysiological response to PSC-derived MNs or iMNs, innervated myotubes begin to exhibit coordinated contractions, which can be abrogated with the reversible acetylcholine receptor inhibitor curare (Miles et al., 2004; Son et al., 2011) (Fig. 3C). Although clustering of myotube acetylcholine receptors is a hallmark of NMJ formation, it is worth noting that different substrates can promote acetylcholine receptor clustering even in the absence of MNs (Peng and Cheng, 1982; Gingras et al., 2009). Thus, it is essential to also evaluate the functionality of NMJs. Recent advances in the optogenetics field have presented the possibility of engineering MNs that express light-activated channel rhodopsins such that neuronal activity, and as a consequence, myotube contraction, can be induced simply by shining a specific wavelength light on co-cultures (Weick et al., 2010; Tye and Deisseroth, 2012). The ability to tightly control MN activation should allow more in-depth studies of the synaptic junction formed *in vitro* and could provide a method to control MN activity when implanted *in vivo*. Moreover, as NMJs have been shown to undergo age-related degeneration both in wild-type mice and in transgenic models of ALS, it will be particularly interesting to determine if these events can be recapitulated and manipulated *in vitro* (Valdez et al., 2010; Valdez et al., 2012).

In addition to forming functional neuromuscular junctions with co-cultured myotubes, transplantation experiments have shown that *in vitro*-derived MNs are capable of integrating into the developing spinal cord. Perhaps owing to the ease of manipulation, most studies have been performed using chick embryos (Wichterle et al., 2002; Soundararajan et al., 2006; Son et al., 2011; Amoroso et al., 2013). For instance, transplantation of differentiated MNs and interneurons from an *Hb9::*GFP reporter ESC line into the chick neural tube demonstrated survival of many GFP^+ MNs after transplantation. Importantly, although the dorso-ventral position of the graft was not controlled, GFP^+ MNs settled in the ventral-lateral domain, whereas interneurons (marked by a rodent-specific antibody to Lim2) were observed in the dorsoventral domain (Wichterle et al., 2002). In addition, grafted *Hb9::*GFP^+ MNs differentiated from mouse ESCs have been shown to project axons into the periphery where they reached muscle targets and displayed elaborated terminals expressing multiple synaptic markers (Wichterle et al., 2002; Soundararajan et al., 2006). Similar results have been obtained using human ESC-derived MNs as well as mouse iMNs (Son et al., 2011; Amoroso et al., 2013).

Evidence that *in vitro*-derived MNs can correctly engraft into the developing spinal cord is further supported by an elegant study using different small molecules to direct the differentiation of mouse ESCs into two specific MN subtypes: cervical MMC MNs and brachial LMC MNs. When GFP-labeled LMC MNs were mixed with RFP-labeled MMC MNs and injected into the chick neural tube, the cell bodies of these neurons settled in the appropriate domains within the spinal cord and their axons projected to targets predicted by their transcription factor expression (Peljto et al., 2010) (Fig. 3D). More recently, work by Corti and colleagues demonstrated that MNs differentiated from genetically corrected SMA iPSCs could survive transplantation and correctly engraft in the spinal cord of a one-day-old mouse model of SMA (Corti et al., 2012). Furthermore, these cells could ameliorate disease phenotypes and extend the life span of SMN mutant mice, at least in part by providing neurotrophic support (Corti et al., 2012). It has also been shown that ESC-derived MNs, when transplanted into the tibial nerve of adult animals can form functional NMJs and ameliorate muscle atrophy caused by nerve transection (Yohn, et. al 2008).

In general, transplantation studies provide strong evidence that PSC-derived MNs and iMNs bear strong resemblance to their *in vivo* counterparts. However, these studies are technically challenging, largely qualitative and provide limited opportunities for mechanistic study. As the ability to generate MNs *in vitro* through diverse methods continues to progress it will be crucial to reach a consensus of best practices for evaluating MNs. Taken in isolation, gene expression, electrophysiological response and NMJ formation *in vitro* are, in our view, insufficient indicators of the success of MN differentiation strategies. Instead, we propose a multi-tiered approach that combines each of these metrics to allow diverse aspects of MN biology to be evaluated.

Concluding remarks and perspectives

Decades of research into CNS development and spinal MN specification have been synthesized to allow the *in vitro* generation of MNs through diverse methods. These cells can provide additional mechanistic insights into developmental principles and MN biology, though perhaps their most exciting application is the prospect of modeling human MN diseases. Several studies have shown the usefulness of these cells in studying the mechanisms of neural degeneration. For example, human MNs have been shown to exhibit selective sensitivity to glia cells expressing a mutant gene linked to ALS (Di Giorgio et al., 2008; Marchetto et al., 2008). Additionally, multiple groups have reported disease-specific phenotypes in differentiated iPSC-derived MNs from patients with SMA (Ebert et al., 2009; Chang et al., 2011; Corti et al., 2012; Wang et al., 2013) or ALS (Bilican et al., 2012; Egawa et al., 2012; Donnelly et al., 2013; Sareen et al., 2013). Building on this, the *in vitro* generation of MNs via direct conversion of fibroblasts may additionally accelerate disease-modeling studies with patient cells, as it does not require the time-consuming step of iPSC generation and characterization. iMNs could be utilized to provide a snapshot of disease processes from a large cohort of patient fibroblasts, and cell lines showing particularly interesting phenotypes could be then reprogrammed into iPSCs to allow the production of differentiated MNs in large quantities for further studies.

The progress in producing and understanding MNs has been remarkable; however, substantial challenges still remain. The molecular diversity of MN subtypes *in vivo* is only partially understood. Perhaps as a consequence of this, only a small number of markers are currently used to evaluate MN subtypes generated *in vitro*, and the extent to which altering methods of MN production may influence the resulting subtypes is largely unknown. The application of RNA sequencing, single-cell qRT-PCR and proteomics approaches will allow comprehensive comparison of MNs generated via different strategies. Moreover, as genome-wide interrogation of bona fide human MNs is only possible through laser-capture of post-mortem samples (Ravits et al., 2005), high-throughput analysis of human PSC-derived MNs as well as human iMNs may provide additional insights into the biology of human spinal MNs.

Regardless of the strategy used to generate MNs, downstream applications warrant careful consideration of the experimental conditions used for plating and culturing these cells. These include variations in the methods of dissociation, cell density, substrate, media composition and cellular heterogeneity of the cultures (Sandoe and Eggan, 2013). Without controlling for and understanding these variables, we run into the risk of comparing the MN 'apples' generated and cultured by one protocol or in one laboratory to the MN 'oranges' generated by another.

Spinal MNs were among the first specific neuronal cell types to be derived from PSCs and the rapid progress made towards making MNs *in vitro* might have provided inspiration to the locked-in Bauby who wondered, 'Does the cosmos contain keys for opening my diving bell? A subway line with no terminus? A currency strong enough to buy my freedom back?' (Bauby, 1998). We are optimistic that continued efforts to collaboratively establish best practices for MN production, culture and evaluation *in vitro* will provide the keys to unlock novel therapeutic strategies for devastating neurological disorders, including ALS.

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