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Transcription Factor Mechanisms Guiding Motor Neuron Differentiation and Diversification

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Abstract

The embryonic generation of motor neurons is a complex process involving progenitor patterning, fate specification, differentiation, and maturation. Throughout this progression, the differential expression of transcription factors has served as our road map for the eventual cell fate of nascent motor neurons. Recent findings from in-vivo and in-vitro models of motor neuron development have expanded our understanding of how transcription factors govern motor neuron identity and their individual regulatory mechanisms. With the advent of next generation sequencing approaches, researchers now have unprecedented access to the gene regulatory dynamics involved in motor neuron development and are uncovering new connections linking neurodevelopment and neurodegenerative disease.

Introduction

Motor neurons (MNs) are a crucial neuronal subtype responsible for innervating musculature in the periphery and controlling both autonomic and volitional movement. During embryogenesis, combinatorial expression of transcription factors (TFs) guides MN differentiation and diversification [1]. In this review, we survey recent research elucidating the evolutionary origin and broad conservation of these TF programs as well as the DNA-binding mechanics of individual TFs and TF-complexes. We also highlight novel applications of next-generation sequencing technology that have provided valuable genomic and transcriptomic signatures to in-vivo and in-vitro derived MNs.

Motor Neuron Generation

MN generation begins in the embryonic neuroepithelium, wherein opposing gradients of diffusible morphogens (Shh, BMPs, Wnt, RA) pattern proliferating progenitors into discrete domains along the dorsal-ventral body axis. In the ventral spinal cord, MNs are generated

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Conflict of interest

The authors declare no conflicts of interest.

from the Olig2+ pMN domain (Figure 1A). Nascent MNs migrate away from the midline and assume positions in distinct motor columns along the rostral-caudal axis that are in register with their target tissues. Motor neurons located within the Medial Motor column (MMC) are found throughout the spinal cord and project to axial muscles. In contrast, Lateral Motor column (LMC) neurons are present at limb levels and innervate target muscles in the fore- and hindlimb, whereas at thoracic regions, Hypaxial Motor column neurons (HMC) and Preganglionic Motor column neurons (PGC) project to body wall muscles and the sympathetic chain ganglia respectively (Figure 1B). Columnar identity is largely defined by Hox proteins, a class of TFs whose clustered 5′ to 3′ chromosomal order maps to their topological expression in the rostral caudal axis [2]. Within a motor column, MNs are further segregated into divisions which delineate broad axonal trajectories. For example, the LMC is divided into medial (LMCm) and lateral (LMCl) divisions that target ventral and dorsal muscles in the limb. Located within each division are motor pools that project to discrete muscles within each area (Figure 1C). Divisional identities are defined in part by the expression of FoxP1 and LIM-homeodomain TFs, whereas motor pools can be distinguished by expression of ETS as well as Hox TFs [3]. Importantly, once generated, MNs themselves are also important players in sculpting the final complement of MNs. MNs within the LMCm are the source of local retinoid signaling via Raldh2 expression that stimulate LMCl generation [4]. Further, GDE2, a retinoid induced GPI-anchor cleaving enzyme expressed in LMC neurons, non-cell autonomously promotes the generation of specific late-born LMC motor pools [5].

The early stages of MN development can be effectively modeled in-vitro using either undifferentiated Embryonic Stem Cells (ESCs) or induced Pluripotent Stem Cells (iPSCs). iPSCs are differentiated cell types that have been reverted to an unspecified progenitor state. iPSCs afford researchers a large array of starting cell types and enable research on human cells without the ethical restraints of collecting fetal tissue [6–8]. These progenitor cells can be subsequently differentiated by applying exogenous factors that promote neuronal differentiation, including RA, Shh agonists, and Notch antagonists (Induced Differentiation) or by forcing expression of MN TFs such as Lhx1, Isl1, and Ngn2 (Direct Neuronal Programming) (Figure 2A). These reductionist in-vitro platforms allow for the analysis of MNs in an isolated, controlled condition; and they allow access for transcriptomic analysis at the single cell level.

Motor Neuron Differentiation: Insights from in-vitro platforms

The basic helix-loop-helix (bHLH) TF Olig2 is crucial for specifying MN progenitors in the pMN domain but whether Olig2 promotes neurogenesis or maintains progenitor character is indeterminate. Olig2 induces Ngn2, another bHLH TF required for neuronal differentiation but Olig2 has also been shown to repress terminal MN homeodomain TFs [9,10]. To gain insight into this question, Sagner and colleagues utilized single-cell transcriptomics to map the gene regulatory networks used by Olig2 during ESC-derived MN generation. Distinct transcriptomic profiles can effectively separate early progenitors, MN progenitors, early MNs, and late MNs. Interestingly, the transition into an early post-mitotic MN is accompanied by an increase in Olig2 expression. Chromatin immunoprecipitation sequencing (ChIPseq) revealed that Olig2 acts as a transcriptional repressor for *Hes1/Hes5*,

which are canonical Notch target genes that maintain progenitor character by inhibiting proneuronal TFs [11]. These observations lead to a biphasic model for Olig2 function. Initially, progenitors have low levels of Olig2 expression that permits partial *Hes* expression. Upon differentiation, Olig2 expression is significantly heightened, repressing *Hes1/Hes5* and disinhibiting the expression of proneuronal TFs [12]. Underscoring these changes, unbiased statistical analysis of single cell RNAseq data during the differentiation of murine ESCs into MNs also reveals distinct transcriptional states as cells transition through four phases: pluripotency, neural precursors, MN specified progenitors, and MNs [13]. MN differentiation in-vivo is an asynchronous process, and bulk profiling yields a mélange of cell types in different stages. The single cell resolution of these studies provides a much clearer window into the transcriptional states occupied by differentiating MNs.

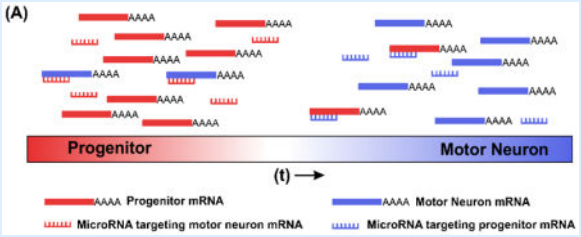
Once the correct TFs for terminal MN identity are induced, what are the mechanisms that ensure their expression? Newly born MNs express the LIM-homeodomain proteins Isl1 and Lhx3. They function within a TF complex with nuclear LIM interactor (NLI) to specify MN identity, and these interactions are dependent on key residues within the Lhx3 LIM domain [14,15]. Recent investigations have revealed that the Isl1-Lhx3 complex stabilizes its expression in an autoregulatory manner via binding to enhancers adjacent to the *Isl1* and *Lhx3* loci. Further, the Isl1-Lhx3 complex upregulates the expression of LIM only Protein 4 (LMO4). LMO4 works in parallel to block the assembly of an Lhx3-only TF complex, which would misdirect the cell towards an interneuron fate [16]. Importantly, sustained Isl1-Lhx3 expression is not a universal feature of all MNs. For example, HMC and LMC MNs lose expression of Lhx3 as they mature, raising the question how expression of terminal MN identity genes regulated by Isl1-Lhx3 is maintained. Rhee et al. performed ChIP-seq from acetylated histone H3 lysine 27 and ATACseq to map genomic regions with an open, accessible chromatin configuration over the course of HMC MN differentiation. They discovered that movement from early to late stages of differentiation is accompanied by highly dynamic changes in enhancer accessibility. Isl1 complexes with Lhx3 to engage the early enhancers, and following Lhx3 downregulation, partners with Onecut1 TFs at late stage enhancers [17]. These relay enhancers thereby ensure stable expression of effector genes over time. The presence of transient enhancers was also observed during the direct neuronal programing of ESCs into spinal MNs; however, contrasting TF-complex dynamics were found. In this paradigm, the same Isl1-Lhx3 TF complex was reported to navigate between early- and late-accessible enhancers without dissociating, and activity at early enhancers repressed progenitor genes rather than activating neuronal genes [18]. This research illustrates how TFs work to control neuronal differentiation amid a transforming chromatin environment in different settings. Importantly, effective TF function is also supported by a range of crucial cofactors working at both the DNA and RNA level (see Box 1).

Box 1

Spatiotemporal control of TF Function is Enabled by Nucleic Acid Regulators

Multiple DNA/RNA regulators have been recently reported that help TFs fine tune MN identity. At the level of chromatin, the Isl1-Lhx3 TF complexes include single stranded DNA-binding proteins Ssdp1 and Ssdp2. These proteins help recruit histone acetylases, which induce a more open chromatin configuration. In the developing chick spinal cord, genetic knockdown of Sspd1/2 prevents the generation of MNs [35]. At the level of RNA transcription, Topoisomerase IIβ (Top2β) controls the specification of the phrenic MNs by ensuring effective transcription of Pbx and Hox TFs [36]. Top2β null mice exhibit diminished expression of Hoxc6 and Hoxc8 as well as the obligate TALE co-factors Pbx1 and Pbx3. Top2β likely ensures effective transcription of Hox and Pbx transcripts by creating temporary DNA breaks which ameliorate physical stress during RNA synthesis.

At the level of protein synthesis, microRNAs (miRNA) prevent the translation of specific mRNA transcripts based on sequence homology. Researchers have identified *mir-27* as a novel regulator of HoxA5 translation. Using in-silico modeling and genetic approaches, Li et al. reveal how mir-27 dampens transcriptional noise by suppressing HoxA5 translation in MN progenitors. Furthermore, disruption of the miRNA processing machinery causes precocious protein expression of HoxA5 in ventricular zone (VZ) progenitors and newly differentiating intermediate zone (IZ) cells and blurs the transition from rostral HoxA5 to caudal HoxC8 domains [37]. Conversely, mir-375 is upregulated in newly differentiated MNs and dampens translation of progenitor proteins Pax6 and CCND2, a cyclin dependent kinase that promotes proliferation [34]. Thus, miRNAs can prevent the ectopic expression of proneuronal genes during proliferation and progenitor genes during differentiation, sharpening the transition between developmental stages (Panel A). These results illustrate how accessory proteins/molecules facilitate TF function during enhancer binding, RNA transcription, and protein translation.



In-vivo Mechanisms of Motor Neuron Diversification

LMCl versus LMCm axonal targeting is regulated by differential expression of Eph-Ephrin guidance cues whose expression is downstream of LIM-homeodomain TFs [19,20]. Do TFs directly influence the expression of axon guidance factors in other MN populations? New studies have uncovered a similar system regulating axon guidance in the hindbrain. In the absence of Islet1, branchiomotor neuron innervation in the face and jaw is disrupted due to diminished expression of the guidance factor Slit2. Importantly, ChIPseq shows that the Isl1-

Lhx3 and Isl1-Lhx4 TF-complexes can directly bind *Slit2* enhancers [21]. These results suggest that the same TFs that separate subtype identity can directly promote the expression of axon guidance cues instead of functioning through intermediate transcriptional networks.

Recent findings indicate that Hox proteins play an analogous role in determining MN peripheral connectivity. In the developing forelimb, Catela et al. elegantly describe how combinatorial expression of Hoxc6 and Hoxc8 TFs along with Meis and Pbx co-factors segregate brachial MN projection subtypes via regulation of Ret and Gfrα receptors [22]. Similarly, phrenic motor neurons fail to fully innervate diaphragm muscles in the absence of Hoxa5 expression [23]. Interestingly, strong Hoxa5 expression was found in the diaphragm itself. Does corresponding Hox gene expression in MNs and their respective target tissue aid in topographic axon targeting? In support of this idea, recent experiments in the zebrafish hindbrain reveal a gradient of Hox5 expression in cranial MNs along the anterior-posterior axis. Posterior MNs express high Hox5 and preferentially innervate posterior pharyngeal arches that also express high Hox5 [24]. Analogous mechanisms have also been described in the larval *Drosophila melanogaster*. In the fly system, somatic MNs expressing the Hox ortholog Deformed (Dfd) are specifically targeted to head muscles that also express Dfd, to create the motor circuits necessary to control feeding [25].

As illustrated by the bi-phasic activity of Olig2 in MN differentiation [12], the degree of TF expression within the same cell type can also influence aspects of MN identity. Mendelsohn and colleagues have recently identified a role for differential HoxC TF expression in separating digit-innervating motor neurons within the LMC. FoxP1+ LMC MNs express Raldh2 and innervate the forelimb; however, the authors find that MNs that project to the digits do not express Raldh2 [26]. They postulate that low (but not zero) levels of Hoxc9, which normally inhibits both FoxP1 and Raldh2, gives digit innervating MNs their unique phenotype that distinguishes them from LMC MNs.

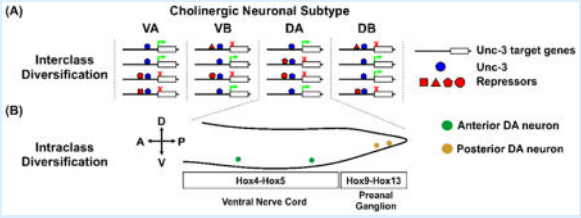
Positional coding by Hox genes is widely conserved among many organisms but the evolutionary origins of limb-innervating LMC neurons have been unclear. Jung and colleagues explore this question in the little skate *Leucorja erinacea,* which displays an ambulatory gate using its pectoral and pelvic fins. They find that the LMC Hox code is conserved in *L. erinacea.* Interestingly*,* these organisms lack a thoracic HoxC gene cluster creating a continuous FoxP1+ LMC, which conforms to the juxtaposition of pectoral and pelvic fins [27]. Their results illustrate the broad conservation of Hox genes and columnar identity, and also indicate that LMC Hox programs evolved far before tetrapods moved to land, in a common ancestor to all vertebrates with paired appendages.

Taken together, these in-vivo data reinforce a broad evolutionary conservation of Hox and LIM-homeodomain TFs in establishing and maintaining diverse MN subtypes. In these efforts, tractable model systems have proven invaluable in decoding MN identity—this is seen in the nematode worm *C. elegans*, where investigators have elucidated a nuanced combination of transcriptional repressors and positional Hox coding to diversify MNs (see Box 2). It is now apparent that TFs, once considered broad cell fate determinants, directly contribute to the diverse terminal phenotypes of MNs across the hindbrain and spinal cord.

Box 2

MN diversification in *Caenorhabditis elegans*

In contrast to vertebrates and insects, the motor system of the nematode *C. elegans* is highly defined with 75 discrete, identifiable MNs distributed throughout the ventral nerve cord. MNs are first grouped by their neurotransmitter system, either cholinergic or GABA-ergic. Within these groups, MNs are further delineated into 8 classes based on morphology, position, and the combinatorial expression of effector genes. This invariant topology allows researchers to precisely define the mechanisms of MN diversification. The terminal selector gene *Unc-3* encodes a COE-type transcription factor necessary for the cholinergic MN lineage. Using forward genetics, Kerk and colleagues demonstrate that cholinergic MN subtypes are diversified through the co-expression of selective repressor TFs that block subsets of UNC-3 enhancers (interclass diversification). They also determine that continued expression of the repressors is required to maintain individual MN identities after development [38] (Panel A). Within a given MN class, additional subtypes exist depending on their somal position along the anterior-posterior body axis. In contrast to the inter-class co-repressor scheme, this intra-class diversification is dependent on region-specific Hox gene expression [39] (Panel B). Importantly, several of the repressor TFs are evolutionarily conserved, raising the possibility that this nuanced combination of subtype repressors and positional Hox coding may operate in vertebrates.



Motor Neurons in Development and Disease

As more and more groups begin to utilize in-vitro differentiation platforms, it becomes critically important to evaluate whether the same types of MNs arise from separate induction protocols. For instance, the disparate results of the Isl1-Lhx3 transient enhancer experiments may arise from the inherent differences between the differentiation of MNs with standard exogenous factors [17] versus direct programming [18]. To address these questions, investigators have generated single cell transcriptomic profiles of ESCs undergoing standard or directed MN induction. Notably, they find that each programming method takes a unique transcriptomic pathway but produces comparable differentiated neurons [28]. These findings shed light on why divergent intermediate transcriptional states can yield similar end-products (Figure 2B). Additional experiments also show promising congruency between in-vitro derived MNs and primary MNs. Similar transcriptomic profiles have been generated comparing MNs derived from genetically unmatched human ESCs versus iPSCs. Here too, investigators find largely overlapping gene expression among the two conditions [29]. These

exciting results lend credence to in-vitro derived neurons as representative models of in-vivo MN generation.

MNs are particularly vulnerable in multiple degenerative diseases, and their loss and/or dysfunction produces a gamut of debilitating behavioral consequences [30,31]. Accordingly, a research niche has emerged to understand whether in-vitro derived MNs offer an appropriate model of disease and/or a suitable substrate for stem cell replacement therapy. iPSC induction and in-vitro MN differentiation can occur over the course of weeks while certain neurodegenerative conditions develop over the span of decades. A critical question becomes: do patient iPSC derived MNs resemble their in-vivo counterparts? To address this question, Ho et al. generated transcriptomic profiles of human iPSC differentiated MNs using exogenous factors. They compared these profiles to the transcriptome of in-vivo isolated fetal and adult stage MNs. They discovered that the iPSC MNs more closely resembled the transcriptional state of fetal MNs, suggesting that these neurons may be inappropriate to model late onset neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) [32]. However, hierarchical clustering identified gene modules that were associated with embryonic development and maturation, and gene expression within these modules was significantly disrupted in ALS derived MNs. Is this perturbation of development related genes merely the result of studying the effect of disease mutations on insufficiently mature MNs; or could the same pathways that ensure proper generation of neurons be subsequently repurposed to safeguard neuronal survival? In support of the latter, we have recently shown how GDE2, which promotes neuronal differentiation embryonically, is essential for MN survival postnatally [33]. In addition, expression of mir-375, which helps secure neuronal identity during differentiation (see Box 1), reduces apoptosis following DNA damage [34]. As we accrue more comprehensive pictures of the degenerating transcriptome and proteome, leveraging these possible connections to developmental programs may help identify new survival pathways that are compromised in disease.

Conclusion

Understanding how TFs and TF-complexes regulate MN identity is integral to decrypt the pathways that govern the precise differentiation, maturation, and diversification of MNs. Combining insights from increasingly reductionist model systems (moving from vertebrates to invertebrates to in-vitro ESC/iPSC derived MNs), we now have a better understanding of how TFs dictate specific MN identities and their regulatory mechanisms. Based on the broad expression of these TF classes, it is likely that principles gleaned from MN development will apply to the development of other neuronal subtypes in the nervous system.

Acknowledgments

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Highlights

- Hox transcription factors are upstream of motor neuron axon guidance cues necessary for proper peripheral innervation.
- New findings in *C. elegans* reveal an elegant system of gene repressors and activators controlling motor neuron diversification.
- Single-cell RNA sequencing reveals how different motor neuron induction protocols can utilize distinct gene transcription pathways to converge on a shared motor neuron identity.

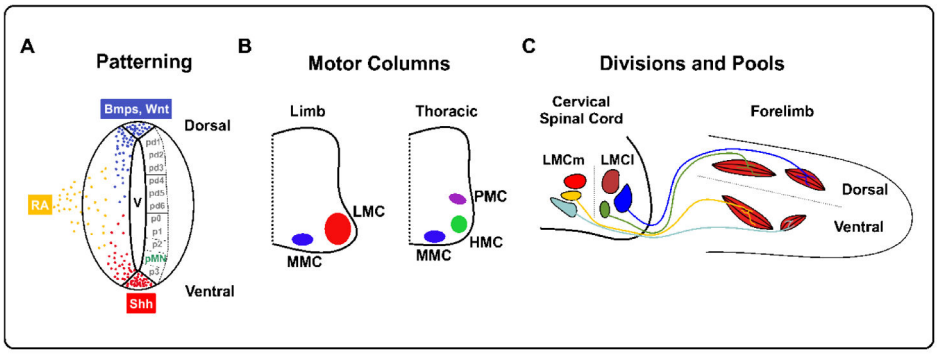

Figure 1. Motor Neuron Organization in the CNS

(A) Embryonic progenitors in the ventricular zone are patterned into discrete dorsal-ventral domains by opposing morphogen gradients. Motor neurons are generated from the ventral pMN domain. V = ventricle, Shh = Sonic Hedgehog, RA = Retinoic Acid. (B) Post-mitotic MNs are organized into motor columns that project to muscles in the limbs (LMC), trunk (MMC), intercostal muscles (HMC), or sympathetic ganglia (PMC). (C) Medial and lateral divisions of the LMC project to ventral and dorsal limb muscles, respectively. Within these divisions, motor pools innervate specific muscle groups.

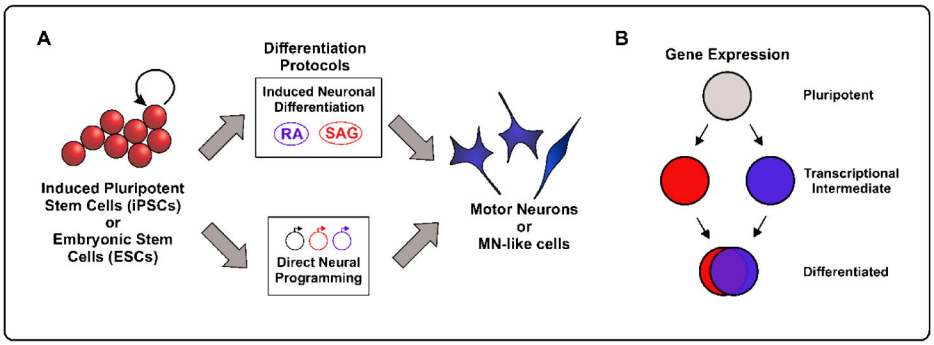

Figure 2. In-vitro Motor Neuron Generation

(A) Pluripotent ESCs or iPSCs can be differentiated into MNs in-vitro through multiple protocols. Induced neuronal differentiation relies on the delivery of exogenous signaling molecules such as retinoic acid (RA) and Shh pathway agonists (SAG), mirroring embryonic development. MNs can also be differentiated by the forced expression of terminal MN TFs for direct neural programming. (B) RNA sequencing experiments have recently revealed that differentiation via direct neural programming (red circles) versus induced neuronal differentiation (blue circles) can take distinct transcription paths; however, their terminal gene expression patterns are largely convergent.