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Transcription Factor Hand-offs "Enhance" Motor Neuron Differentiation

Clinton Cave[1] and Shanthini Sockanathan[1,*]
[1]The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, 725 N. Wolfe Street, PCTB 1004, Baltimore, MD 21205, USA
*Correspondence: ssockan1@jhmi.edu
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Rhee et al. (2016) in this issue of *Neuron* and Velasco et al. (2016) in *Cell Stem Cell* find that the activity of transcription factors binding sequentially to a series of transient early and late enhancers directs gene expression that is essential for motor neuron differentiation and function.

Neuronal differentiation, the transition from a dividing progenitor into a postmitotic neuron, is orchestrated by a series of transcription factors (TFs) whose sequential and coordinate activation is regulated by a complex interplay between diffusible morphogens, such as Sonic Hedgehog (Shh) and retinoic acid (RA), and intercellular signaling pathways, such as Notch/Delta (Dasen and Jessell, 2009; Alaynick et al., 2011; Pierfelice et al., 2011). These so-called programming TFs regulate the expression of terminal effector genes that produce all the requisite machinery for neuronal function, for example, neurotransmitters and ion channels, as well as additional TFs that fine-tune gene expression to assemble the diverse neuronal subtypes of the mature nervous system. These TFs must navigate the extraordinary complexity of the chromatin landscape in order to bind their appropriate enhancers to promote or repress gene expression. In many instances, programming TF expression is downregulated during neuronal differentiation and maturation, but terminal effector gene expression is stably maintained. How can the temporary expression of key TFs lead to sustained gene transcription? Perhaps alternate TFs are recruited to stabilize the initial enhancers or certain TFs could act in a relay—transferring to new enhancers within the same gene to continue gene expression. Unfortunately, our inability to precisely quantify TF-genomic interaction on a large scale has clouded the clarification of these possibilities.

In this issue of *Neuron*, Rhee and colleagues (Rhee et al., 2016) have surmounted this barrier by analyzing chromatin accessibility and utilizing high-resolution mapping of TF binding sites during stepwise differentiation of embryonic stem cells (ESCs) into hypaxial motor neurons. This is a well-defined, in vitro system that reproduces the dynamic expression of TFs seen in development, wherein Isl1 and Lhx3 are required for the transcription of mature neuronal effector genes, but Lhx3 is downregulated without changes in terminal gene expression. ESCs supplied with patterning factors will differentiate into nascent motor neurons expressing Isl1 and Lhx3 before maturing into hypaxial motor neurons that retain Isl1 expression but downregulate Lhx3. Rhee et al. (2016) employ a combination of transposase-accessible chromatin with sequencing (ATAC-seq), a novel technique that probes the accessibility of genomic regions via transposon insertion (Buenrostro et al., 2013), and chromatin immunoprecipitation sequencing (ChIP-seq) of acetylated Histone 3-Lysine 27 (H3K27) to measure chromatin accessibility over the course of ESC differentiation. They observe that there is a remarkably dynamic organization of active enhancers between nascent and mature hypaxial motor neurons, although the net changes in gene expression are relatively low. What could be the purpose of these enhancers that only have a small window of accessibility? Rhee et al. (2016) discovered that Isl1 and Lhx3 use a surprising strategy to ensure stable gene expression during motor neuron maturation. They demonstrate that, in nascent motor neurons, Isl1 forms temporary heterodimers, first with Lhx3, that bind to transient enhancers in close proximity to terminal neuronal effector genes. Subsequently, as Lhx3 is downregulated during neuronal maturation, Isl1 is freed to associate with enhancers occupied by Onecut TFs at new loci among the same neuronal effector genes (Figure 1A). This relay of Isl1 binding to nascent and mature enhancers works together to ensure the continuous expression of effector gene products essential for mature neuronal function. In contrast, Ngn2, which is expressed transiently at the start of differentiation and is necessary for generic neuronal identify, shares very few binding sites with Isl1 heterodimers, suggesting that it works independently to activate a largely non-overlapping set of neuronal-required genes.

In a series of elegant experiments to investigate whether the temporal specificity of nascent and mature enhancers exists in vivo, Rhee et al. (2016) deploy a set of artificial enhancers expressed in differentiating motor neurons in the embryonic chick spinal cord. Controlling the expression of destabilized GFP, these constructs reveal how nascent enhancers produce GFP within 24 hr post-electroporation but are unable to maintain GFP expression by 48 hr, while mature enhancers do not produce GFP until 48 hr following electroporation. Importantly, in a configuration analogous to endogenous neuronal genes, a reporter with combined nascent and mature enhancers generates continual GFP expression in differentiated motor neurons. This principle of stage-specific enhancer use was further validated in motor neuron differentiation of ESCs using CRISPR/Cas9-guided removal of nascent and mature enhancers associated with Isl1 and the motor neuron gene Hb9. How does the stepwise transfer of Isl1 from nascent to mature enhancers occur

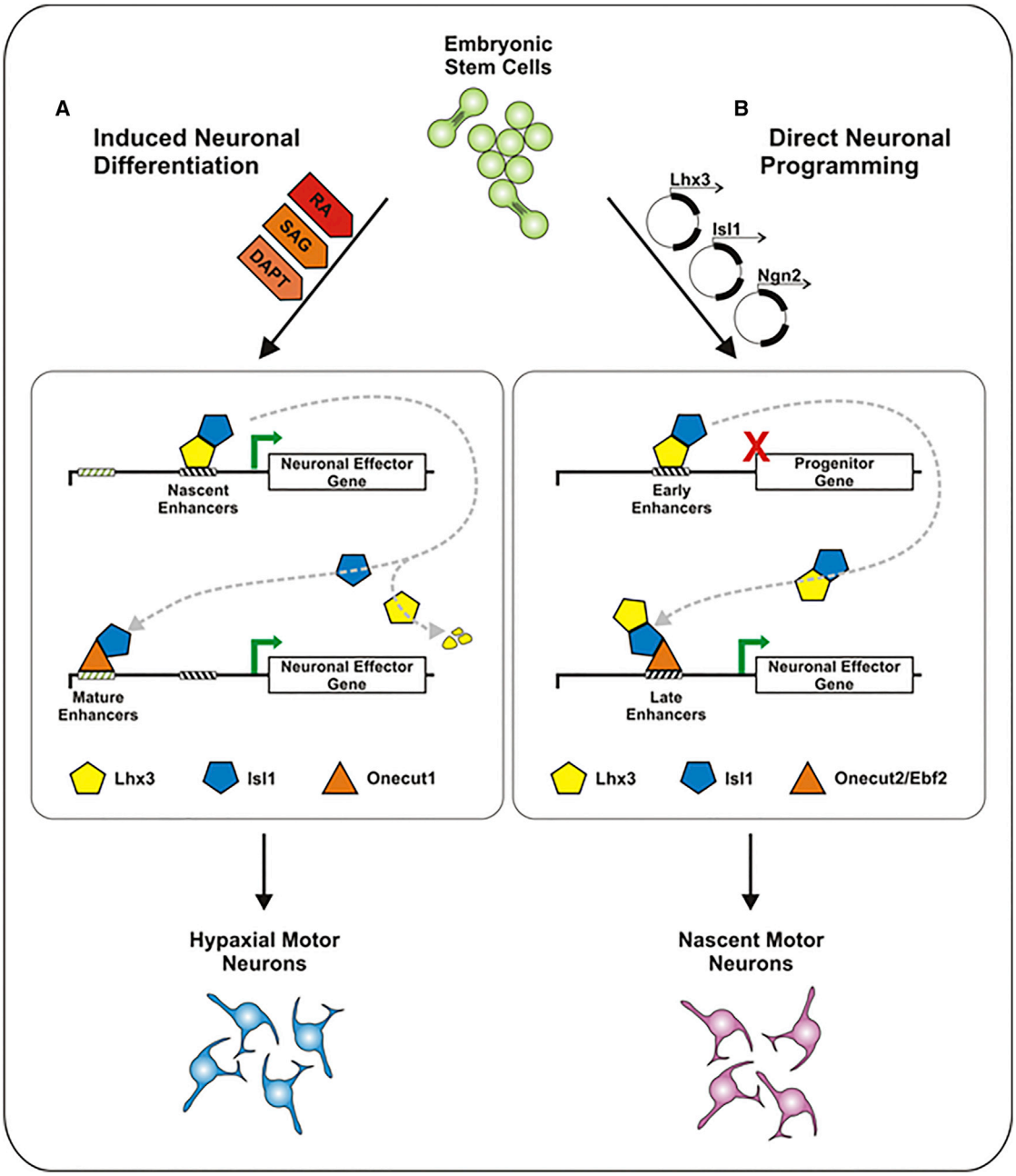

Figure 1. Sequential Occupancy of Transient Enhancers Regulate Gene Expression during ESC Differentiation

(A) During induced neuronal differentiation, exogenous application of retinoic acid (RA), smoothened agonist (SAG), and γ-secretase inhibitor (DAPT) promote embryonic stem cells (ESCs) to differentiate into hypaxial motor neurons over the course of 6 days. During differentiation, the expression of neuronal effector genes is maintained by Isl1/Lhx3 heterodimers acting on nascent transient enhancers, followed by downregulation of Lhx3 and transfer of Isl1 to Onecut1-bound mature enhancers.

(B) Direct neuronal programming differentiates ESCs into nascent motor neurons within 48 hr via the forced expression of programming transcription factors—Lhx3, Isl1, and Ngn2. Lhx3/Isl1 binding to early enhancers inhibits expression of proliferative genes, and subsequent transfer to late-stage enhancers with Onecut and Ebf transcription factors promotes neuronal effector gene expression.

mechanistically? Rhee et al. (2016) demonstrate that Isl1 transfer occurs through a competitive binding model, wherein Lhx3 competes with Onecut1 for Isl1 binding, with transfer to Onecut1 likely aided by the downregulation of Lhx3 upon maturation. Interestingly, Isl1 interacts mostly with Lhx3 or Onecut1 that is already bound to their enhancers and the recruitment of Isl1 correlates with gene activation. Together, this work raises the compelling idea that a gene can be primed for expression by factors like Onecut1 and Lhx3 and subsequently activated and stabilized over time by the competitive

transfer of Isl1 between the relay enhancers. This model adds another dimension to gene transcriptional states, wherein Isl1 surveys for enhancers with sufficient levels of a cognate "anchor" (e.g., Lhx3 or Onecut1) to induce binding and gene transcription.

Nascent motor neurons can be generated from ESCs by a completely different strategy involving the forced expression of Isl1 and Lhx3 together with Ngn2. This direct neuronal programming leads to the expression of neuronal effector genes within 48 hr (Mazzoni et al., 2013). How can the same class of TFs induce effector gene transcription in a vastly different landscape of chromatin accessibility and available co-factors compared to the progressive differentiation of ESCs over the course of several days? In *Cell Stem Cell*, Velasco and colleagues (Velasco et al., 2016) analyze the dynamics of Ngn2, Lhx3, and Isl1 binding and their effects on the transcriptome and chromatin behavior during the first 48 hr of direct motor neuron programming. Remarkably, the overall logic of TFs employing a temporal system of enhancer occupancy also applies to direct motor neuron programming; however, some interesting differences emerge. Using ChIP-seq, Velasco et al. (2016) also find an abundance of early transient enhancers bound by Lhx3/Isl1 heterodimers, but these enhancers are associated with *progenitor* genes rather than neuronal effector genes. Additionally, these early interactions lead to the *decommissioning* of the proliferative genes and, accordingly, a more restricted chromatin state. Subsequently, Lhx3/Isl1 associate with late enhancers on *terminal effector* genes that are associated with neuronal function, and these late-stage interactions *induce* gene expression. This system allows the programming factors, in a two-step process, to dampen progenitor character and promote neuronal differentiation. Further, Velasco et al. (2016) demonstrate that, under conditions of directed differentiation, Isl1 interacts with both Lhx3 and Onecut at late enhancers (Figure 1B). These contrasting observations diverge from the competitive binding model put forth by Rhee et al. (2016) but likely arise from the abrupt and sustained expression of Isl1/Lhx3 during direct differentiation, which lacks the broader differentiation program induced by exogenous RA-Shh and Notch inhibition. In this context, Isl1/Lhx3 have access to binding sites (i.e., a chromatin configuration) that are never available in ESC-induced differentiation. Moreover, the competitive binding of Isl1 to either Onecut1 or Lhx3 may be inconsequential if there is an overabundance of Isl1 and maintenance of Lhx3, allowing for simultaneous occupancy of Isl1 and Lhx3 with late-stage, Onecut-bound enhancers. The controlled expression of TFs in the direct differentiation paradigm does afford the investigators an opportunity to understand the effects of each TF independently. By removing Lhx3 and Isl1 from the programming milieu, Velasco et al. (2016) reveal that Ngn2 binding promotes the subsequent expression of Onecut and Ebf TFs. Furthermore, if Lhx3/Isl1 are expressed without Ngn2, they show severely reduced ability to transfer to late enhancers. This mechanism elaborates how the initial binding of Ngn2 and Isl1/Lhx3 to mutually exclusive enhancers observed by both groups can ultimately converge on late stage enhancers leading to a feed-forward system of gene regulation. Together, both studies elucidate the importance of transient enhancers as cells acquire their post-mitotic neuronal identities.

Is there biological value for utilizing sequential relays and/or feed-forward TF systems to regulate gene expression? In development, motor neuron subtype specification requires the maintenance of general neuronal genes and the refinement of subtype specific features (e.g., ion channel expression to control spiking frequency). Transient occupancy of early enhancers may trigger a cascade of TFs, each binding to transient enhancers to generate the next set of TFs that continue to refine gene expression. As this feed-forward process proceeds, relay enhancers could stabilize gene expression among neuronal subtypes. As proposed by Rhee et al. (2016), one could easily imagine chromatin accessibility guiding anchor TF binding on a subtype-by-subtype basis, therefore allowing the differential recruitment of "activator" TFs and stable gene expression. In addition, whether the use of transient enhancers persists in mature neurons will greatly inform efforts for cell reprogramming and cell replacement therapies. For example, medial motor column (MMC) neurons maintain Lhx3, while lateral divisions of limb innervating lateral motor column (LMC) neurons lose expression of Isl1 but retain Isl2 (Shirasaki and Pfaff, 2002). Does the forced maintenance of Lhx3 in programmed neurons create a more MMC state? Could the paralogous relationship described between Isl2 and Onecut2 use relay enhancers to ensure gene expression in the absence of Isl1? Understanding how in vivo configurations relate to the terminal transcriptional states following stepwise and direct differentiation of motor neurons will be of critical importance when considering the utility of these neurons in clinical and research settings alike. Collectively, these studies provide a welcome insight into the mechanisms necessary to ensure the expression of post-mitotic genes and the reorganization of the chromatin landscape during cellular differentiation.

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