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Applying Lessons from Development to Neuronal Stem Cell Biology

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This report describes recent findings in developmental neurobiology focusing on recent progress in understanding how cells acquire their specific identities and how neurons become wired up correctly. Over the last few years tremendous progress has been made in identifying extrinsic factors that mediate cell-to-cell communication during development and in defining the intrinsic cellular pathways that mediate neuronal/glial development and connectivity. The fast pace of this research is exhilarating and the details can become daunting to new investigators in the field, but it is important not to loose track of the profound opportunities of developmental neurobiology research such as understanding how circuits for specific behaviors are assembled, defining the basis for developmental defects of the nervous system, and understanding the rules that will govern repair and regeneration of the nervous system following injury or disease.

Neurogenesis: Neural induction to Neuronal Specification

Throughout the animal kingdom, a common feature in neurogenesis is the sequential generation of different types of neurons by the same precursor cell through a series of asymmetric cell divisions. Recent studies have begun to define the network of transcription factors that control the orderly production of specific neuronal types from neuroblasts. A Cartesian-like patterning mechanism specifies the expression of combinations of transcription

factors that serve as intrinsic regulators of cell fate. Thus, the concept of master regulators has given way to the notion of combinatorial codes for fate specification. This new understanding has important consequences in regard to neuronal regeneration, since the generation of particular neuronal subtypes from stem cells will require the activation of an appropriate set of intrinsic determinants. Numerous studies have established the antagonistic role of bone-morphogenic proteins (BMPs) on neural differentiation in embryos, and interestingly BMPs appear to also act as inhibitors of neurogenesis in the adult CNS. Consequently inhibitors of BMPs such as noggin unmask the neurogenic potential of stem cells in adults. Thus, BMPs might act as stem cell maintenance factors by promoting the proliferative capacity of stem cells and antagonizing differentiation. During development, this region-specific maintenance of proliferation and inhibition of neurogenesis could contribute to the selective growth of brain regions such as the midbrain and the telencephalon.

Patterning and Neurogenesis

The spinal cord is an area of the nervous system where considerable progress has been made over the last decade in defining the pathways that control neuronal subtype specification. These studies have identified opposing gradients of tumor growth factor β family members dorsally and sonic hedgehog ventrally as important signaling molecules for patterning the neural tube. Combinatorial transcription codes are prevalent in the developing central nervous system and are used to produce a large number of distinct cell types from a limited repertoire of factors. The LIM homeodomain (LIM-HD) code is a prototypical example, in which overlapping combinations of LIM factors dictate numerous aspects of neuronal development. Simultaneously, proneural basic helix-loop-helix (bHLH) transcription factors are thought to regulate general

aspects of neural development such as expression of generic neuronal traits and exit from the cell cycle (neurogenesis). Nevertheless, the molecular basis for linking the transcription factors that control cell identity with those that regulate general properties of neuronal differentiation are not well understood. And in fact, it remains controversial whether the bHLH factors contribute directly to the regulation of cell identity or act only to control neurogenesis.

To address these questions we have examined the molecular basis for the regulatory coordination between LIM-HD and bHLH factors. We have focused on a class of spinal cord neurons involved in locomotion, termed motor neurons, because the development and function of these cells is well established. Inductive signaling leads to the co-activation of regulatory pathways for specifying general neuronal traits in parallel with instructions for neuronal subtype specification. Nevertheless, the mechanisms that ensure these pathways are synchronized have not been defined. To address this we examined how bHLH proteins Ngn2 and NeuroM controlling neurogenesis functionally converge with LIM-homeodomain (LIM-HD) factors Isl1 and Lhx3 involved in motor neuron subtype specification. We found that Ngn2 and NeuroM transcriptionally synergize with Isl1 and Lhx3 to specify motor neurons in the embryonic spinal cord and in P19 stem cells. The mechanism underlying this cooperativity is based on interactions that directly couple the activity of the bHLH and LIM-HD proteins, mediated by the adaptor protein NLI. This functional link acts to synchronize neuronal subtype specification with neurogenesis. By examining the transcriptional regulatory mechanisms in these cells, we have found that the LIM-HD factors and bHLH factors interact synergistically to control gene expression, thus revealing how cell fate and neurogenesis are coupled.

Adult Neurogenesis and Regeneration

While it is common to view neural development as something that ceases at a particular stage, numerous studies have begun to highlight the ongoing generation of neurons in adult animals. There are nevertheless important differences in the ability to generate neurons in adults. Neurogenesis is restricted to niches within the mammalian telencephalon where neuronal generation can occur. What is special about these sites? It appears that endogenous neural precursors can migrate from the telencephalon to replace lesioned neurons in the basal ganglia. In addition, quiescent precursors can be re-activated to generate an enormous number of new projection neurons in the hippocampus after growth factor infusion into an ischemic hippocampus.

The hope for successful connection of newly generated neurons hinges on the finding that CNS axons are not intrinsically incapable of extending in the adult CNS, but are subject to specific inhibitory molecules. Three molecules that are potent inhibitors of axon outgrowth, Nogo, MAG and OMgp. Surprisingly, they all appear to act via the Nogo receptor (NgR) and the low affinity neurotrophin receptor p75 serving as a coreceptor. These findings provide hope that the targeted inhibition of this pathway will unmask the axon growth potential of new neurons in the adult CNS, thereby helping to facilitate regenerative strategies involving stem cells.

A cascade of signaling events triggers the differentiation of specific neuronal and glial cell populations that comprise the central nervous system (CNS). Epidermal ectoderm deprived of bone morphogenic protein (BMP) signaling differentiates into "neural" ectoderm [1], the precursor of the CNS. These neural cells are multipotential and respond to signals in their environment in order to generate the appropriate types of neurons and glia at the correct

positions. In this section we focus on the spinal cord, the most caudal region of the CNS, since it has served as a useful model in which to investigate signaling events that give rise to neuronal and glial populations within the developing neural tube.

Emerging from a combination of modern molecular studies and classical cellular studies, a central theme in spinal cord development is one in which inductive factors signal along the dorsoventral and rostrocaudal axes of the developing spinal cord to specify cell fate in a Cartesian coordinate-like manner [2]. This signaling leads to the generation of dorsal spinal cord interneurons that process sensory information and relay it to the brain, while the ventral spinal cord forms interneurons and motor neurons involved in locomotor control (Figure 1A). Along the rostrocaudal axis, discontinuous subclasses of motor neurons are generated in register with the peripheral targets that they innervate. In addition, numerous glial cell types are formed including the roof plate and floor plate, which act as organizing centers within the spinal cord, and astrocytes and oligodendroctyes, which support neuronal function and myelinate neurons, respectively.

Patterning Along the Dorsoventral Axis

Two classes of factors play prominent roles in specifying distinct cell types along the dorsoventral axis of the spinal cord: members of the transforming growth factor β (TGFβ) superfamily acting dorsally, and Sonic hedgehog (Shh) ventrally (Figure 1A) [3, 4]. TGFβ signaling from the epidermal ectoderm flanking the dorsal neural tube leads to the differentiation of the roof plate [5], and Shh expression from the notochord below the neural tube triggers the formation of the floor plate [6]. These two glial structures in the spinal cord then express TGFβ

dorsally and Shh ventrally. In this way, signals from the periphery are propagated into the spinal cord to control cell differentiation locally.

The dividing progenitor cells within the ventricular (medial) region of the spinal cord monitor the types and concentrations of TGFβ and Shh in order to determine their position, and consequently their fate, as they become postmitotic and migrate laterally into the mantle region (Figure 1A). The signaling pathways triggered by these inductive factors lead to the activation of transcriptional networks that first define distinct domains along the dorsoventral axis of the ventricular zone and ultimately lead to the expression of genes involved in controlling cell function (Table 1) [7-10].

Dorsal Spinal Cord Development

In the embryonic dorsal spinal cord, four classes of interneurons (INs) termed D1-D4 arise in an orderly fashion from specific regions of the progenitor zone (Figure 1A). These INs , while not absolutely defined, consist of association and commissural cells that process and relay sensory information and depend upon the formation of the roof plate in order to develop [5, 11]. The roof plate and adjacent neural epithelial cells express overlapping and nested combinations of several TGFβ members including BMP4/5/7, Gdf6/7, and Dsl1 [5, 9]. How might the TGFβs produce different cell types in the spinal cord? Several strategies are likely to be involved including quantitative, qualitative, and timing differences in TGFβ activity. The expression pattern of the TGFβs suggests that a high-dorsal to low-ventral gradient of these proteins is present within the neural tube (Figure 1A). Although more studies are needed to determine whether graded levels of TGFβs contribute to spinal cord patterning, *in vitro* experiments with

neural explants have detected concentration-dependent activities for Activin A in the induction of D1 and D2 IN classes [5].

The clearest example of how TGFβs trigger the differentiation of specific IN types is based on the finding that individual members of this family have different qualitative activities [5]. The most convincing evidence for such a mechanism is found in the GDF7 mutant mice where a specific subpopulation of D1 INs fails to be generated [12]. These results suggest that the specificity of dorsal IN patterning is mediated, at least in part, by various TGFβ signaling molecules, some of which act directly to render class specificity (Table 1). An additional mechanism for generating cellular diversity in the dorsal spinal cord involves a temporal switch in the way neuroepithelial cells respond to TGFβ signals. Early in development, progenitor cells produce neural crest cells when exposed to BMP4 or Activin A, but later, they give rise to dorsal INs in response to the same signals [5]. The basis for qualitative differences in TGFβ signaling and the mechanisms underlying the developmental switch in TGFβ responsiveness by neural epithelial cells remain important questions.

Shh signaling for ventral cell differentiation is attenuated by TGFβ signaling [13]. What limits the range of TGFβ activity to the appropriate areas of the developing spinal cord? Several TGFβ antagonists have been identified including noggin, chordin, and follistatin which bind directly to and sequester specific TGFβs [14]. These antagonists are expressed by the somites and notochord near the ventral surface of the neural tube, and therefore are expected to limit the exposure of ventral cells to certain TGFβs. In *noggin* mutant mice, TGFβ signaling in the ventral

neural tube is unmasked (Figure 1A), which leads to a progressive loss of ventral cell differentiation [15].

The receptors of the TGFβs are serine/threonine kinases comprised of type I and type II dimers. These receptor complexes have not been well characterized in the spinal cord, but may select for different ligands and serve as the basis for the qualitative differences in cell differentiation induced by different TGFβ family members. The best known transducers of TGFβ signaling are the SMAD transcription factors [16], though the role of SMADs in spinal cord development also requires further characterization. Recently, a better understanding of the downstream targets of TFGβs has begun to emerge (Table 1). For instance, it is now known that D1 INs, characterized postmitotically by the markers Lhx2/9, arise from progenitor cells that express the bHLH transcription factor mATH1 involved in establishing the fate of these cells [9, 10]. Likewise, D3 IN progenitor cells marked by Lhx1/5 arise from progenitors that express the bHLH protein Ngn1. Thus, the identification of target genes activated by TGFβ signaling should help to work backwards to characterize the signal transduction pathways.

Ventral Spinal Cord Development

Genetic studies as well as *in vitro* explant experiments have implicated Shh in the differentiation of ventral spinal cord cell types involved in locomotor control (V0-V3 INs and MNs), as well as oligodendroctyes (Figure 1A) [3, 8]. Unlike the nested combinations of TGFβ molecules in the dorsal spinal cord, however, only one hedgehog member appears to be involved in ventral spinal cord patterning in higher vertebrates. This raises the question of how different ventral cell types are induced by a single factor. Extensive studies with *in vitro* explants have

shown that Shh concentration differences of ~2-3 fold dramatically influence the types of cells that are triggered to differentiate. Decreasing concentrations of Shh progressively induce cell types found further from the ventral midline, recapitulating the normal organization of cells in the ventral spinal cord [17]. As with TGFβ signaling, there are also important temporal mechanisms that modify progenitor cell responses to Shh signaling during development. At early stages Shh acts on progenitor cells to trigger MN differentiation, but, later in development oligodendrocytes are produced instead of MNs. The basis for this switch is not well understood but seems to involve the regulation of the transcription factor Nkx2.2 [18, 19].

The active Shh signaling molecule is autoprocessed and cholesterol modified, and binds to the patched/smoothened receptor complex [20]. In the absence of Shh, patched is thought to inhibit smoothened from signaling, and this inhibition is relieved when Shh binds patched. Many additional components of the Shh signaling pathway have been identified through genetic studies in *Drosophila*, including the downstream Gli family of zinc finger transcription factors [20]. Genetic studies of *Gli3* and *Gli3/Shh* compound mutants indicate that this transcription factor is likely an intermediary in the Shh pathway, although it seems to function indirectly as a transcriptional repressor [21, 22].

How might small gradations in the level of Shh signaling produce sharp progenitor cell domains that serve as the precursors for different ventral cell types? Studies of the factors regulated by Shh in the ventricular zone have uncovered a network of homeodomain proteins that mark distinct progenitor domains (Table 1) [7]. The expression of these factors is controlled at two levels. First, Shh either represses (Class I) or activates (Class II) the expression of the homeodomain factors. If this were the only mechanism operating to control these factors, it

might be expected that the interpretation of the fine Shh gradient would lead to imprecise boundaries of gene expression. However the domains appear to be further refined by cross-repressive transcriptional interactions between factors from different domains. In this two step manner, graded Shh leads to the activation of unique combinations of homeodomain transcription factors in precise progenitor cell domains [3, 8]. The combinatorial activities of these homeodomain factors lead to the activation of downstream transcriptional regulators involved in cell specification and function (Table 1) [23].

The opposing nature of the ventral Shh gradient meeting the dorsal TGFβ factors suggests that inhibitors of Shh activity might constrain its activity, much like the inhibitors of TGFβs. Hedgehog interacting protein (Hip) is a surface membrane protein that binds Shh and attenuates its activity [24]. In addition, characterization of the mouse *open brain* (*opb*) mutant, in which ventral cell types form inappropriately in the dorsal region of the spinal cord, has led to the identification of a member of the Rab family of vesicular transporters, Rab23, important in limiting the activity of Shh dorsally (Figure 1A) [25]. Interestingly, mice deficient in both Shh and Rab23 regain many of the ventral cell types lost in Shh mutants. This, together with the observation that *Gli3/Shh* double mutants also regain many ventral cell types [22], suggests additional Shh-independent pathways might contribute to ventral spinal cord development. Studies to understand the basis for Shh-independent signaling have uncovered a parallel pathway involving retinoic acid (RA) expressed by paraxial mesoderm beside the neural tube [26]. It will be interesting to examine in more detail the interplay between Shh and RA signaling pathways in order to fully understand the molecular basis for neuronal specification along the dorsoventral axis of the neural tube.

Rostrocaudal Specification

The spinal cord can be subdivided into four broad, functional regions along the rostrocaudal axis: cervical, brachial, thoracic, and lumbar/sacral. The IN classes of the spinal cord extend continuously throughout these regions, while specific MN subclasses are found at each level [27]. Individual MN subclasses form discontinuous columns in register with their targets, such that MNs of the cervical region innervate axial muscles, brachial region MNs innervate the forelimb, MNs of the thoracic region innervate body wall muscle, and lumbar MNs innervate hindlimbs. Much like the initiation of dorsoventral patterning in the spinal cord, embryonic manipulations and *in vitro* explant studies suggest that members of several families of signaling molecules originating initially from sources outside the spinal cord contribute to the diversification process that leads to the generation of specific classes of MNs along the rostrocaudal axis [28-31].

Studies of the signals that control segmental identity along the rostrocaudal axis have used *Hox* gene expression patterns as downstream molecular correlates of the regional specification of cell identity (Table 1). Furthermore, there is increasing functional data to suggest that *Hox* genes contribute to the proper development of MN subclasses [27, 32, 33]. As neuroepithelial cell identity is first established, it is thought to have a rostral identity which is then modified by "caudalizing" signals [4]. Hindbrain studies have found that increasing levels of RA activate more caudal-type *Hox* genes [34-36]. Likewise, the pattern of *Hox* gene expression in the cervical spinal cord is regulated by RA synthesized by the cervical paraxial mesoderm flanking the neural tube (Figure 1B) [31].

However, at more caudal regions of the spinal cord, RA is insufficient to confer positional identity. A major source of additional regionalizing signals is detected in Hensen's node (HN), a precursor of the axial mesoderm that moves in a caudal direction below the nascent neural tube as development progresses. Interestingly, HN tissue taken from different stages (ie. different rostrocaudal levels) is able to specify different regional values in neural explants [31]. Studies utilizing FGF receptor antagonist SU5402 and expression of constitutively active FGF receptors are found to alter the Hox coding in neural cells, implicating fibroblast growth factor (FGF) signaling as a mediator of HN activity. FGF8 is expressed by the HN and *in vitro* studies have found that this factor can act in a concentration-dependent manner to induce progressively more caudal positional values in neural explants. The Cdx family of transcription factors represent possible downstream mediators of both RA and FGF8 signaling in the regulation of Hox expression [36].

Taken together, these findings suggest that the signaling activity of FGF8 increases as the HN moves caudally (Figure 1B) [31]. An additional mechanism that appears to contribute to the increased activity of FGF8 at more caudal positions is the involvement of accessory factors that enhance FGF signaling. One such example is the TGFβ superfamily member Gdf11. This factor is expressed in HN as it progress through lumbar/sacral levels, where FGF signaling is expected to be highest (Figure 1B). Unlike other TGFβ members, Gdf11 does not influence the dorsoventral pattern of the spinal cord, but rather acts to enhance FGF8 signaling activity. In this way, progressively more caudal regions of the spinal cord are defined by the composite functions of FGF8 and Gdf11 through the regulation of Hox codes involved in establishing regional levels of the spinal cord that will generate different MN subclasses (Table 1).

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Figure Legends

Figure 1: Patterning signals along the dorsoventral and rostrocaudal axes of the developing spinal cord. **(A)** Transverse view of the spinal cord in which the gray area represents the ventricular zone containing progenitor cells and the white area the mantle layer with mature cell types. FP, floor plate: RP, roof plate. **(B)** Rostrocaudal view of the spinal cord and the graded pattern of signals expressed by paraxial and axial mesoderm adjacent to the neural tube, based on work from Liu et al. [31].

Table 1: Signaling Events in the Developing Spinal Cord.