Perspective

Stem Cell INVESTIGATION

Identification of neural stem cell differentiation repressor complex *Pnky*-PTBP1

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Abstract: Splicing increases immensely the complexity of gene products expressed in the cell. The precise regulation of splicing is critical for the development, homeostasis, and function of all tissues in the body, including those comprising the neural system. Ramos *et al*. recently identified *Pnky* as a long noncoding RNA expressed selectively in neural tissues that was implicated in the transition of neural stem cells (NSCs) to mature neurons. *Pnky* actions appeared to be mediated by its interaction with the splicing factor and RNA-binding protein (RBP) polypyrimidine tract-binding protein (PTBP1), as silencing either *Pnky* or PTBP1 modulated in similar ways the patterns of spliced and expressed mRNAs in the cell. Strikingly, lowering the expression levels of *Pnky* or PTBP1 in NSCs actually enhanced neurogenesis, suggesting that the *Pnky*-PTBP1 complex elicited a splicing program of suppression of neurogenesis. With rapid progress in the design and delivery of RNA-based therapies, interventions to reduce *Pnky* levels may prove beneficial towards enhancing neurogenesis in disease states characterized by aberrant neuronal loss.

Keywords: Neural stem cells (NSCs); *Pnky*; PTBP1; ribonucleoprotein complex; long non-coding RNAs (lncRNAs); splicing

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Neural differentiation is a complex process involving numerous cell types and transition programs. Both the embryonic cortical ventricular zone (VZ) and the adult ventricular-subventricular zone (V-SVZ) of the brain contain neural stem cells (NSCs). NSCs are multipotent cells of glial origin capable of giving rise to intermediate progenitor cells that divide before producing migratory young neurons (1). The importance of this process is underscored by the fact that targeting developing neurons locally in specialized brain regions would permit treatment of neurodevelopmental or neurodegenerative diseases in which specific mature neurons are lost or absent (2).

Many molecular factors and pathways govern differentiation programs in all human tissues, organs, and systems. Among these, long non-coding RNAs (lncRNAs) are emerging as critical regulatory molecules in differentiation processes that affect broadly human physiology and pathology (3). LncRNAs are RNA molecules longer than 200 nucleotides lacking apparent protein-coding ability. Thousands of lncRNAs are expressed in the mammalian genome, and they are capable of controlling gene expression programs on many different levels: they can modulate chromatin organization and function, influence gene transcription, alter pre-mRNA metabolism, and affect mRNA turnover and translation (4,5). LncRNAs have been implicated specifically in neural development and differentiation (6). Early examples of this influence include lncRNAs *Cyrano* and *Megamind*, which were shown to be necessary for neuronal development, as knockdowns of these lncRNAs decreased the numbers of neurons in zebrafish and mouse embryos, respectively (7). However, lncRNAs playing a role in NSCs and their transition to neurogenic progenitors are only now beginning to emerge (6).

Recently, Ramos and colleagues (8) reported *Pnky*, a predominantly nuclear and evolutionarily conserved lncRNA expressed only in neural tissues. Expression

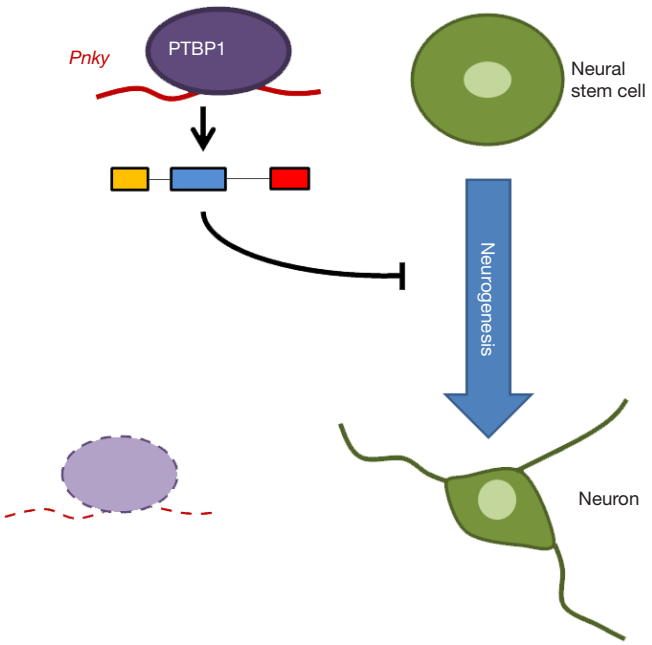

Figure 1 Proposed impact of *Pnky* and PTBP1 on neural differentiation. In neural stem cells (NSCs), the splicing factor PTBP1 can bind the neural-specific lncRNA *Pnky* and control alternative splicing and gene expression programs that suppress differentiation. Reductions in the levels of PTBP1 and *Pnky* in NSCs, which occur during neural differentiation, enhance neurogenesis.

of *Pnky* decreased during V-SVZ NSC differentiation into neuronal cells, suggesting that *Pnky* might be the first known neuronal lncRNA that inhibits neuronal development. This finding contrasts with earlier evidence of other neuronal lncRNAs such as *Cyrano* and *Megamind* that instead promoted neuronal development, as revealed when their reduced expression prevented neuronal differentiation [reviewed in (6)]. In contrast, *Pnky* loss-of-function interventions (e.g., using lentiviral particles with shRNA targeting *Pnky*) were found to trigger the development of higher numbers of neurons from cultured post-natal V-SVZ NSCs (8). Time-lapse microscopy unexpectedly revealed that suppressing *Pnky* expression did not increase NSC proliferation, but instead produced more transit-amplifying cells, the intermediate cell type that eventually produces neuroblasts (8). Thus, *Pnky* knockdown promoted neural lineage largely by decreasing cell death. The authors observed similar results in another model of *in vivo* neurogenesis. Injection of *Pnky* shRNA in the embryonic brain of mice led to a relative increase in neurons and decrease in NSCs compared to control shRNA injections, supporting the hypothesis that *Pnky* represses the production of young neurons in the developing brain (8).

In search for a mechanism that might explain how *Pnky* elicited this effect, the authors performed affinity pull-down assays using tagged *Pnky* as RNA bait followed by proteomic analysis to isolate bound proteins. This was a likely approach, as lncRNAs often carry out their function by interacting with RNA-binding proteins (RBPs) (4,5,9,10). Through their association with coding and noncoding RNAs, RBPs have been implicated in all aspects of gene regulation, including nuclear functions like splicing and maturation, and cytoplasmic functions like transport, stability, storage and translation (10,11). RBPs are ubiquitous regulators of cell functions such as division, apoptosis, differentiation, and senescence; however, they are also specialized in the functions of certain tissues, like insulin production, myogenesis, immune cell activation, and adipogenesis. A subset of RBPs is specifically implicated in neural development and in particular in the alternative splicing that leads to isoform diversity (9,12). Numerous studies have uncovered major roles of splicing regulators in the brain such as NOVA and heterogeneous ribonuclear proteins (hnRNPs), including the polypyrimidine tract-binding protein 1 (PTBP1, also known as hnRNP I). PTBP1 was shown to play a role in fibroblast reprogramming to neurons and PTBP1 knockdown led to precocious cortical development (13,14).

Ramos and coworkers identified PTBP1 as a specific binding partner of *Pnky* in nuclear extracts from V-SVZ NSCs (8). Since nuclear PTBP1 plays a role in controlling splicing, the authors proposed that the complex *Pnky*-PTBP1 may affect alternative splicing in NSCs leading to neuronal differentiation (*Figure 1*). In support of this idea were several of the authors' findings: (I) PTBP1 knockdown in NSCs increased the number of neurons, just as *Pnky* knockdown increased the number of neurons; (II) many of the same mRNAs were differentially expressed and differentially spliced following PTBP1 knockdown as were following *Pnky* knockdown; (III) gene ontology analysis revealed that the shared regulated mRNAs encoded proteins with roles in biological processes relevant to neuronal differentiation, such as cell-cell adhesion, synaptogenesis, and neurogenesis.

The finding that the *Pnky*-PTBP1 lncRNP (long noncoding ribonucleoprotein) complex controls splicing during differentiation of NSCs to neurons raises several questions for immediate consideration. It will be important to disrupt PTBP1 or *Pnky* abundance by alternative

methods (e.g., by genomic editing using CRISPR/Cas9 or by alternative silencing RNAs) in order to solidify their observations. It will also be interesting to understand the mechanism whereby this lncRNA-RBP complex modulates splicing, as it could potentially shed light broadly on the field of splicing. For example, does the lncRNA *Pnky* guide the complex to specific sites of the pre-mRNA, possibly via complementarity with the pre-mRNA, in order for PTBP1 to initiate splicing? Does binding of *Pnky* to PTBP1 change the affinity of PTBP1 for pre-mRNA sequences?

Among the list of NSC pre-mRNAs selectively spliced by the *Pnky*-PTBP1 complex, it will be important to identify the actual effectors of *Pnky*-PTBP1-regulated neurogenesis. In this regard, splicing events that modulate the production of apoptosis regulators might be particularly relevant, given the authors' finding that silencing *Pnky* enhanced lineage commitment of NSCs, elevated the number of neurogenic progenitors, and suppressed cell death. Validation that the protein (PTBP1) and the lncRNA (*Pnky*) components of the complex function jointly to splice specific targets using splicing assays of endogenous and ectopic pre-mRNAs will further strengthen the authors' model.

The full significance of the interaction of *Pnky* and PTBP1 also deserves further scrutiny. For example, the *Pnky*-PTBP1 complex may affect other nuclear functions, such as target mRNA mobilization, maturation, or degradation. Additionally, *Pnky* likely interacts with other RBPs besides PTBP1, perhaps to regulate subsets of mRNAs outside of those identified in the Ramos report (8) as being coordinately regulated when either PTBP1 or *Pnky* were silenced. Similarly, PTBP1 associates with other lncRNAs [(15); http://starbase.sysu.edu.cn/)] and these interactions could further influence splicing or other dimensions of gene regulation in NSCs affecting neuronal development.

The significance of the Ramos study (8) is threefold. First, it identifies *Pnky* as the first lncRNA implicated in controlling neuronal differentiation specifically by modulating the transition of NSCs to intermediate progenitor cells. These results were confirmed in two models of neurogenesis, post-natal and embryonic NSCs. In this process, a critical role is played by the RBP PTBP1. *Pnky* may also bind to other proteins in order to inhibit neurogenesis.

Second, *Pnky* is an example of an lncRNA that interacts with a splicing factor to modulate splicing. This model of action contrasts with the well-known example of the splicing-regulatory lncRNA *MALAT1*, which was shown to function by 'capturing' the serine/arginine (SR) splicing factor. By complexing with SR, *MALAT1* rendered SR unavailable for endogenous target pre-mRNAs (16,17). By contrast, the *Pnky*-PTBP1 appears to actively modulate splicing patterns, since losing the lncRNA or the RBP component had similar consequences on gene expression patterns.

Finally, the Ramos report (8) paves the way for the design of possible pharmacological interventions. When targeting specific RNAs or proteins for therapy, it is far easier to silence or inactivate a given molecule that it is to overexpress it in the appropriate tissue, time, and concentration. Therefore, in neurological disease states in which it is desirable to increase the number of differentiated neurons, it may be advantageous to inhibit the *Pnky*-PTBP1 complex. Although the delivery methods need to improve greatly, one can envision inhibitory therapy directed at shutting off *Pnky*-PTBP1 activity in order to trigger the progression of NSCs into neurons. Further, since PTBP1 is expressed ubiquitously and in all developmental stages, targeting PTBP1 selectively in neuronal stem cells may be challenging. A more attractive strategy appears to be to target *Pnky* instead, as its expression is restricted to a small subset of cell types, primarily neuronal stem cells and cells in the subventricular zone and the developing brain. Indeed, in order to design such interventions effectively, it will be important to understand in detail the spatiotemporal pattern of *Pnky* expression in neural tissue. Such in-depth knowledge could potentially enable exceptionally precise *Pnky*-directed therapies.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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