microRNA regulation of neural precursor self-renewal and differentiation

Laura I Hudish and Bruce Appel*

Department of Pediatrics; University of Colorado School of Medicine; Aurora, CO USA

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Abbreviations: Par, partitioning defective; Prkci, protein kinase c, iota; MO, morpholino oligonucleotide; UTR, untranslated region

© Laura I Hudish and Bruce Appel

*Correspondence to: Bruce Appel; Email: Bruce.Appel@ucdenver.edu

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During early stages of development of the vertebrate central nervous system, neural precursors divide symmetrically to produce new precursors, thereby expanding the precursor population. During middle stages of neural development, precursors switch to an asymmetric division pattern whereby each mitosis produces one new precursor and one cell that differentiates as a neuron or glial cell. At late stages of development, most precursors stop dividing and terminally differentiate. Par complex proteins are associated with the apical membrane of neural precursors and promote precursor self-renewal. How Par proteins are down regulated to bring precursor self-renewal to an end has not been known. Our investigations of zebrafish neural development revealed that the microRNA *miR-219* negatively regulates apical Par proteins, thereby promoting cessation of neural precursor division and driving terminal differentiation.

One of the most fundamental questions in developmental neurobiology is this: What determines whether dividing neural precursors produce more precursors or cells that differentiate as neurons or glia? This seemingly simple “decision” has profound consequences for neuronal and glial cell production and, consequently, brain development and disease. For example, changing the balance between new precursor and differentiated cell production would have a direct influence on brain size and, therefore, likely represent a key constraint on brain evolution.^{1–3} On the other hand, failure to maintain an appropriate balance between new

precursor and differentiated cell production contributes significantly to developmental disorders of the brain.⁴

Neural precursors undergo only 3 fundamental types of division. First, a precursor can divide to generate 2 new precursors. This symmetric proliferative pattern predominates during early neural development to expand the precursor population. Second, a precursor can divide to produce one cell that remains as a precursor and one that differentiates, either directly or after one to several divisions as a transit amplifying cell.¹ This asymmetric self-renewing division is generally thought to predominate during the mid point of neural development to permit formation of neurons while maintaining precursors for production of later born neurons and glia. Third, both progeny of a precursor can differentiate as neurons or glia. This symmetric differentiative pattern likely predominates near the end of neural development to bring neurogenesis and gliogenesis to a halt. Therefore, mechanisms that determine which of these division patterns a precursor undergoes are key regulators of brain development.

In recent years considerable attention has been given to working out the mechanisms that regulate symmetric proliferative and asymmetric self-renewing divisions. Much of this work has been inspired by a beautiful model whereby the plane of precursor division determines whether a division is symmetric or asymmetric.⁵ In this model planar divisions, in which the mitotic spindle is oriented in the plane of the neuroepithelium, are symmetric whereas vertical divisions, in which the spindle is perpendicular to the neuroepithelium, are asymmetric. How might the division plane determine the fate of the

progeny cells? One possibility is that if the cell is polarized and different factors localize to different sides of the cell, the division plane will determine which of these factors are inherited by each of the daughters. If factors important for keeping a precursor cell in a self-renewing state are associated with apical membrane, then a planar division could distribute the factors equally to both progeny cells, resulting in a symmetric, proliferative division. By contrast, a vertical division would distribute apical membrane-associated self-renewal factors to only one of the 2 progeny cells resulting in an asymmetric division in which one cell remains as a precursor and the other differentiates. However, reality has turned out to be a bit more complicated than this simple model. For instance, many planar divisions are asymmetric with respect to fate and many divisions are oblique to the plane of the neuroepithelium with no clear correlation of inheritance of apical membrane and cell fate.⁶⁻⁸ Consequently, the relationship between division plane, distribution of apical factors and cell fate remain unclear.

Nevertheless, it seems that apical-membrane associated factors can influence neural precursor self-renewal. One important group of such factors is the "Partitioning defective" (Par) proteins first discovered in nematodes.⁹ Three of these proteins, Pard3, Pard6 and Prkci (also known as atypical protein kinase C) form a complex that can interact with a variety of other proteins at the apical membrane. In the nervous systems of both flies and vertebrates, apical Par proteins are associated with precursor cell self-renewal. Thus, in flies, progeny cells that acquire apical Par proteins from a dividing precursor maintain a precursor, or neuroblast, fate, whereas progeny cells that do not receive apical Par proteins differentiate.¹⁰ In vertebrates this relationship also holds in that high levels of apical Par proteins have been associated with precursor self-renewal and low levels with differentiation.^{7,8,11}

If apical Par proteins promote neural precursor self-renewal, what brings self-renewal to an end, resulting in terminal differentiation? An obvious answer might be down regulation or inactivation of apical Par proteins. Indeed, Par proteins

disappear from apical membranes of neural precursors at the end of embryogenesis in mice and zebrafish, correlating with their exit from the cell cycle and differentiation.^{11,12} However, the mechanisms by which apical Par proteins are downregulated, resulting in the end of neurogenesis and gliogenesis, have not been known.

One of the ways that protein production can be regulated is through control of mRNA translation or degradation by microRNAs.¹³ Could microRNAs contribute to Par protein down regulation to promote terminal differentiation of neural precursors? Our path toward answering this question began with a collaborative project with Richard Lu's lab to investigate microRNA regulation of myelination. Richard's group had identified 2 microRNAs, *miR-219* and *miR-338*, expressed by oligodendrocytes, the myelinating cell type of the vertebrate central nervous system. To help test a hypothesis that these microRNAs promote oligodendrocyte differentiation and myelination, Richard asked us to interfere with their functions in zebrafish by injecting embryos with antisense morpholino oligonucleotides (MOs). *miR-338* MO had no obvious effect, but *miR-219* MO produced an interesting phenotype. First, *miR-219* deficient larvae expressed mRNA encoding Myelin basic protein at very low level, supporting the idea that *miR-219* promotes myelination.¹⁴ Second, we noticed that *miR-219* deficient larvae also had fewer oligodendrocytes. This raised the prospect in our minds that, in addition to its role in oligodendrocyte differentiation, *miR-219* also regulates an earlier step in oligodendrocyte development.

To investigate this possibility, we carried out a more extensive analysis of spinal cord development. This revealed that, in the absence of *miR-219* function, spinal cord precursors remained in a proliferative state and produced fewer late-born neurons and oligodendrocytes than control animals.¹² This indicated that *miR-219* promotes cell cycle exit and differentiation. How might it do this? To identify possible mechanisms, we used prediction programs to search for mRNAs that might be targeted by *miR-219* to control transition of neural precursors from self-renewing to terminally differentiated status. Of course,

these prediction programs produce long lists of possible targets, but we were struck by the fact that *pard3*, *prkci*, *pard6b* and *pard6ga* all turned up as candidates. With this information in hand, we hypothesized that *miR-219* promotes cell cycle exit and terminal differentiation by reducing production of apical Par polarity proteins.

To test our hypothesis we focused our attention on Pard3 and Prkci. Reporter assays in cultured mammalian cells and zebrafish embryos gave evidence that *miR-219* negatively regulates Pard3 and Prkci expression by targeting single sites within their 3' UTRs. Immunohistochemical staining revealed that, rather than be depleted by the end of embryogenesis, proteins associated with apical membrane, including Prkci, were maintained in *miR-219* deficient embryos, consistent with the abnormally long persistence of neural precursors. But, of all the possible *miR-219* targets, could these effects on neural precursors be attributed simply to misregulation of Par proteins? To answer this question we performed 3 experiments. First, we predicted that if neural precursors failed to differentiate because they maintained high levels of Par proteins in the absence of *miR-219* function, then simultaneous reduction of Par proteins should rescue the *miR-219* loss of function phenotype. Indeed, injections of a *pard3* antisense MO partially rescued oligodendrocyte formation. Second, we predicted that blocking the *miR-219* target sites on the *pard3* and *prkci* 3' UTRs with antisense, or target protector, MOs, should phenocopy loss of *miR-219* function. Embryos injected with either *pard3* or *prkci* target protector MOs maintained excess neural precursors through the end of embryogenesis, similar to the effect of blocking *miR-219* function. Third, we predicted that overexpression of Pard3 would also phenocopy the effect of blocking *miR-219* function. Conditional expression of Pard3 encoded by a heat-responsive transgene also promoted the maintenance of precursor characteristics. Altogether, our data support the idea that *pard3* and *prkci* mRNAs are functionally relevant targets of *miR-219* and that *miR-219* brings self-renewing divisions of neural precursors to an end by reducing Pard3 and Prkci protein levels.

One important problem requiring resolution is the exact role of apical Par proteins in neural development. Although we interpreted our data in a way that is consistent with the well known function of apical Par proteins in *Drosophila* neuroblast self-renewal, it is important to note that there are exceptions to this relationship. In particular, studies of zebrafish embryonic hindbrain and forebrain revealed that in many vertical divisions the basal sibling cell remains as a precursor whereas the apical cell differentiates and that *Pard3* can inhibit self-renewal and promote neuronal differentiation.^{15,16} Similarly, apical Par proteins promote differentiation in asymmetric divisions occurring in the *Drosophila* intestine.¹⁷ Therefore, whether apical Par proteins promote self-renewal or differentiation appears to be context-dependent, likely reflecting context-specific interaction with other self-renewal and differentiation factors. For example, in some contexts *Pard3* might prevent Numb from inhibiting Notch signaling activity, thereby promoting a Notch-dependent self-renewal precursor state.⁷ In other contexts segregation of Mind bomb to the apical sibling cell by *Pard3* might promote high Notch signaling in the basal sibling, thereby specifying basal, and not apical, sibling cells as self-renewing precursors.¹⁶

What might be the context within which apical Par proteins promote self-renewal in the zebrafish spinal cord, as our work indicates? Recent work has made interesting connections between the mother centriole, ciliary membrane, ciliogenesis and self-renewal in asymmetric divisions.^{18,19} Apical Par proteins promote ciliogenesis in polarized epithelial cells^{20,21} and primary cilia have important roles in transduction of developmental signals such as Sonic Hedgehog.²² Perhaps, then, apical Par proteins promote rapid reassembly of cilia following division of spinal cord precursors, permitting transduction of signals that favor self-renewal over differentiation. Indeed, our current, unpublished work indicates that Sonic Hedgehog signaling is elevated in *miR-219*-deficient embryos, in which apical Par proteins are maintained at high levels.

Notably, our manipulations of *miR-219* function had the greatest affect on

dorsal spinal cord, with little impact on more ventral cells. At early stages of neural development, a primitive lumen extends throughout the dorsoventral extent of the spinal cord. The lumen is lined by the apical membranes of dividing neuroepithelial cells, at which Par proteins are localized. By late development the lumen transforms into a small central canal, which occupies ventral spinal cord. This transformation coincides with loss of apical Par protein localization and neuronal differentiation dorsal to the central canal. *miR-219* drives this transformation, because blocking its function preserves the primitive lumen and apical Par protein localization and interferes with differentiation. However, cells lining the central canal retain apical Par proteins. Our studies did not reveal any obvious differences of *miR-219* expression in dorsal and ventral spinal cord and we do not know why ventral spinal cord cells seem refractory to *miR-219* function. In earlier studies we found that central canal-associated radial glial cells that express the transcription factor *Olig2* persist into adulthood, during which they divide and potentially give rise to new oligodendrocytes.²³ Following injury to the adult spinal cord, these cells produce motor neurons.²⁴ Therefore, mechanisms that prevent *miR-219* downregulation of apical Par proteins might help preserve specific subpopulations of embryonic neural precursors for adult stem cell fates.

Previous studies revealed that microRNAs *miR-9*, *let7b* and *miR-124* can promote neurogenesis by regulating expression of various transcription factors, the nuclear receptor TLX and the RNA-binding protein PTBP.²⁵⁻³¹ By identifying the apical Par proteins as targets of *miR-219*, our work has added a new layer to our understanding of the ways that microRNAs can influence neural precursor maintenance and specification. Learning how these various microRNA functions are integrated and coordinated will be an important goal of future work. Additionally, learning whether *miR-219* regulates neural precursors in the brain similarly to the spinal cord and determining how apical Par proteins might act as both self-renewal and differentiation factors should reveal interesting new features of nervous system development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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