RESEARCH COMMUNICATION

miR-29b is activated during neuronal maturation and targets BH3-only genes to restrict apoptosis

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The execution of apoptosis is critical for proper development of the nervous system. However, it is equally important that neurons strictly inhibit apoptosis after development to ensure their survival throughout the lifetime of the organism. Here we show that a microRNA, miR-29b, is markedly induced with neuronal maturation and functions as a novel inhibitor of neuronal apoptosis. The prosurvival function of miR-29b is mediated by targeting genes in the proapoptotic BH3-only family. Our results identify a unique strategy evolved by maturing neurons that uses a single microRNA to inhibit the multiple, redundant BH3-only proteins that are key initiators of apoptosis.

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During normal development of the nervous system, a period of massive neuronal apoptosis occurs to precisely match neurons to their respective target cells (Oppenheim 1991). However, once appropriate neuronal connections are in place, it is imperative that neurons strictly inhibit their apoptotic program, since these cells do not divide, have limited capability for regeneration, and must survive for the lifetime of the organism (Benn and Woolf 2004). In mammalian cells, apoptosis is triggered when cells encounter cytotoxic stresses, such as nutrient withdrawal, DNA damage, or endoplasmic reticulum (ER) stress. These insults initiate signaling cascades that activate proapoptotic BH3-only members of the Bcl-2 family of proteins and cause the release of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm (Wang 2001). The release of cytochrome *c* from mitochondria is a key event that triggers the rapid activation of caspases, the key cellular proteases that ultimately execute cell death (Hengartner 2000). Although some changes in apoptotic machinery have been identified during neuronal maturation (Tsui-Pierchala and Ginty 1999; Putcha et al.

2000; Walsh et al. 2004; Wright et al. 2007), it is unclear whether other mechanisms exist in mature neurons to strictly disable apoptosis.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression (Bartel 2009). While misexpression of some miRNAs has been linked with apoptosis and cancer (Kent and Mendell 2006), very little is known about how miRNAs regulate cell death during normal development. Here, we examined whether miRNAs function as critical regulators of survival in mature neurons. We show that miR-29b becomes induced with neuronal maturation and potentially inhibits apoptosis in normal, healthy neurons. Importantly, we report that miR-29b mediates its function by targeting multiple members of the BH3-only family of proapoptotic genes. Together, these data identify miR-29b as the first mammalian miRNA capable of inhibiting neuronal apoptosis.

Results and Discussion

In contrast to developing P5 (postnatal day 5) sympathetic neurons, mature P28 neurons are strikingly resistant to nerve growth factor (NGF) deprivation, DNA damage, and ER stress (Fig. 1A; Easton et al. 1997; Wright et al. 2007). To determine whether miRNAs may have a role in restricting apoptosis, we profiled their expression during neuronal maturation and reasoned that miRNAs that are most highly expressed in mature neurons would likely function to prevent cell death. Sympathetic neurons were obtained from the superior cervical ganglia (SCG) of mice at four developmental stages: embryonic day 18 (E18), P5, P13, and P28. Each of these stages occurs after postmitotic differentiation, thereby focusing our study on a time period when increasing restriction of apoptosis is a major known event occurring in these neurons (Glebova and Ginty 2005). Using significance analysis of microarrays (SAM) (Tusher et al. 2001) to compare young neurons (E18 and P5) with mature neurons (P13 and P28), we found that the expression of only one miRNA family, miRNA-29 (miR-29), was significantly increased in mature neurons (Fig. 1B; Supplemental Table S1).

The miR-29 family consists of three members (miR-29a, miR-29b, and miR-29c) that map to two distinct genomic loci in clusters. Since these miRNAs have extensive sequence homology, especially at the 5' seed region important for mRNA target recognition (Lewis et al. 2003), we focused on miR-29b, as it was the most highly expressed and is found at both genomic loci (Supplemental Fig. S1A). To confirm our microarray data, we performed quantitative RT-PCR (qRT-PCR) on isolated ganglia from P5 and P28 mice and found the levels of miR-29b to be increased dramatically in P28 ganglia (Supplemental Fig. S1B). In addition, using pure neuronal cultures, we found that miR-29 levels increased in P0 neurons cultured for 28 d *in vitro* versus neurons cultured for 5 d (Fig. 1C), indicating that the increase in miR-29 occurs specifically in neurons. The increase in miR-29b with neuronal maturation was not specific to sympathetic neurons, as a similar increase in expression was also observed during cerebellar and cortical maturation (Supplemental Fig. S1C,D). Together, these data indicate that miR-29b levels are induced at a time when neurons become increasingly resistant to apoptosis.

[**Keywords:** apoptosis; neurons; miRNAs; BH3-only; miR-29; Bcl-2 family]

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Figure 1. Neuronal maturation is associated with a marked increase in miR-29 and restriction of apoptosis. (A) P0 sympathetic neurons were cultured for 5 d (P5) or 28 d (P28) in vitro and either maintained in NGF-containing media (+NGF), deprived of NGF (–NGF), treated with 20 μ M etoposide to induce DNA damage (Etop), or treated with 2.5 μ M tunicamycin to induce ER stress (Tuni) for 72 h. Cell survival was quantified by cell morphology and was expressed as a percentage of viable cells prior to cell treatment. (B) miRNA microarray expression data for sympathetic ganglia isolated from E18, P5, P13, and P28 mice. Yellow denotes high expression and blue denotes low expression. Complete expression data is included in Supplemental Table S1. (C) qRT-PCR for miR-29a, miR-29b, and miR-29c using RNA collected from P0 sympathetic neurons maintained in culture for 5 d (P5) or 28 d (P28). Expression of each miRNA is plotted relative to levels in P5 neurons. Data in A and C are mean $\pm$ SD of three independent experiments.

Since the marked increase in miR-29 expression during maturation correlates with a time when strict restrictions on neuronal apoptosis are engaged, we hypothesized that introducing miR-29b in young P5 neurons may provide enhanced resistance to apoptotic stimuli. miR-29b or a control miRNA (nonconserved *Caenorhabditis elegans* miRNA cel-miR-67) was microinjected into P3 neurons and, after 48 h, neurons were subjected to NGF deprivation. Remarkably, microinjection of miR-29b was sufficient to protect neurons from apoptosis, while cells injected with the control miRNA died at the expected rate (Fig. 2A,B). Treatment of P5 neurons with NGF deprivation alone did not have a significant effect on the endogenous expression of miR-29b (Supplemental Fig. S2). The ability of miR-29b to inhibit neuronal apoptosis was not specific to NGF deprivation, as miR-29b expression also effectively inhibited apoptosis in response to DNA damage (Fig. 2C,D) and ER stress (Fig. 2E,F). These data indicate that miR-29b is a potent inhibitor of neuronal apoptosis induced by multiple stimuli.

To determine precisely how miR-29b functions to inhibit apoptosis, we analyzed the effect of miR-29b expression on key steps in the pathway activated by NGF deprivation. Upon NGF withdrawal, neurons activate the transcription factor c-Jun by phosphorylation at Ser 63, causing the induction of proapoptotic BH3-only proteins in the Bcl-2 family (Eilers et al. 1998; Whitfield et al. 2001), which results in cytochrome *c* release, caspase activation, and cell death (Fig. 3A). We examined the phosphorylation status of c-Jun in neurons injected with miR-29b after NGF withdrawal. NGF deprivation induced robust nuclear staining for phospho-Ser 63-c-Jun in both control and miR-29b-expressing neurons, indicating that miR-29b expression did not affect c-Jun phosphorylation (Supplemental Fig. S3). Next, we tested the effect of miR-29b on the release of cytochrome *c* from mitochondria. Following NGF deprivation, while uninjected or control-injected neurons showed very faint cytochrome *c* staining, consistent with its release to the cytoplasm (Deshmukh and Johnson 1998), neurons injected with miR-29b maintained cytochrome *c* at the mitochondria (Fig. 3B,C; Supplemental Fig. S4). Thus, miR-29b expression potentially inhibited apoptosis in neurons down-

stream from c-Jun phosphorylation but upstream of cytochrome *c* release. The identification that miR-29b acts at this step in the apoptotic pathway is consistent with the

Figure 2. miR-29b expression potentially inhibits neuronal apoptosis induced by multiple stimuli. P3 sympathetic neurons were microinjected with miR-29b or cel-miR-67 (ctrl miRNA, each 30 μ M) together with rhodamine to mark injected cells. (A,C,E) Forty-eight hours following injection, neurons were subjected to NGF deprivation (A), 20 μ M etoposide (C), or 2.5 μ M tunicamycin (E), and survival of injected cells was assessed at various time points following cell treatment. Survival was expressed as a percentage of viable cells prior to treatment. (B,D,F) Representative phase-contrast images of the exact field of sympathetic neurons before (untreated) or after 3 d of NGF deprivation (B), etoposide (D), or tunicamycin (F). Rhodamine marks cells injected with 30 μ M miR-29b (arrows). Data in A, C, and E are mean $\pm$ SD of at least three independent experiments. Bar, 20 μ m.

Figure 3. Inhibition of apoptosis by miR-29b occurs upstream of cytochrome *c* release. (A) Schematic representation of the apoptosis pathway activated after NGF deprivation in P5 neurons. (B,C) P3 sympathetic neurons were either uninjected (uninj) or microinjected with a GFP-expressing plasmid and either miR-29b or cel-miR-67 (ctrl miRNA, each 30 μ M). After 48 h, cells were left untreated (+NGF) or deprived of NGF (–NGF). Cells were fixed and immunostained 48 h after cell treatment. (B) Fluorescence intensity of cells after cytochrome *c* staining. (C) Representative photographs of cytochrome *c* staining in neurons; GFP expression indicates injected cells (arrows). Data in B are mean $\pm$ SEM of at least three independent experiments. Bar, 10 μ m.

fact that mature neurons, which we found to express high levels of miR-29, also phosphorylate c-Jun but do not release cytochrome *c* after NGF deprivation (Easton et al. 1997; Putcha et al. 2000).

The BH3-only proteins are a family of proapoptotic regulators that are critical for inducing cytochrome *c* release from mitochondria. This gene family comprises at least eight members, many of which act redundantly (Giam et al. 2008). For example, although *Bim* and *Hrk* (also known as *DP5*) are transcriptionally induced and are important for NGF deprivation-induced apoptosis, the knockout of either gene has only a modest effect on survival (Putcha et al. 2001; Imaizumi et al. 2004). Thus, an effective block of apoptosis at this point would require inhibition of multiple BH3-only proteins. To assess whether miR-29b was capable of functioning in this manner, we used miRNA target prediction programs (TargetScan, MicroCosm Targets, and PicTar) to determine whether any BH3-only mRNAs were putative targets of miR-29b. To our surprise, we found that multiple BH3-only mRNAs had predicted miR-29b-binding sites in their 3' untranslated regions (UTRs) (Fig. 4A,B).

To directly test whether miR-29b could target BH3-only mRNAs, we used a luciferase reporter assay in which the 3'UTRs of BH3-only genes, with or without the putative miR-29b-binding sites, were each fused downstream from the firefly luciferase gene. Each luciferase construct was cotransfected with either miR-29b or a control miRNA into HEK293T cells and luciferase activity was measured. Indeed, miR-29b was able to effectively reduce luciferase activity in cells transfected with constructs containing 3'UTRs of wild-type, but not

mutant, *Bim*, *Bmf*, *Hrk*, and *Puma* (Fig. 4C). Furthermore, miR-29b was also able to target the 3'UTR of *N-Bak*, a BH3-only splice variant of Bak that is expressed exclusively in neurons (Fig. 4C; Ham et al. 2005). Together, these data identify miR-29b as a single molecule capable of targeting multiple BH3-only mRNAs.

The observation that miR-29b is able to target the 3'UTRs of the BH3-only family of genes suggested to us that apoptosis is blocked in mature neurons due to the repression of BH3-only protein induction. Thus, we examined the status of several BH3-only proteins in P5 and mature P28 neurons after NGF deprivation or etoposide and tunicamycin treatment. Indeed, while *Bim* and *Puma* are induced after NGF deprivation in P5 neurons (Putcha et al. 2001), these proteins fail to be induced in P28 neurons (Fig. 5A). Equally important, while etoposide and tunicamycin treatment each robustly induced *Bim* and *Puma* in P5 neurons, an induction of these proteins was not seen in P28 neurons (Fig. 5B,C). In addition, we found that *Bmf*, a BH3-only protein whose function has not been well characterized in neurons, is also induced after all three treatments in P5 neurons, but not significantly in

P28 neurons (Fig. 5A–C). miRNAs are known to suppress gene expression through a combination of mRNA cleavage and translational repression (Bartel 2009). Interestingly, although BH3-only mRNAs became induced in P28 neurons after each treatment, the amount of BH3-only mRNA that was detected in treated P28 neurons was as low as that seen in healthy P5 neurons (Supplemental Fig. S5). Taken together, our data show that, in mature neurons (which have high endogenous levels of miR-29), the induction of BH3-only proteins is effectively blocked after multiple apoptotic insults.

A prediction of our model (Fig. 5F) is that expression of miR-29b should directly block the expression of BH3-only proteins in a situation where endogenous BH3-only proteins are induced. We tested this hypothesis in young P5 neurons and focused on *Bim*, since this is the best-characterized BH3-only protein shown to be induced after NGF deprivation. As expected, injection of the control miRNA had no effect on the induction of *Bim* following NGF deprivation in P5 neurons. Strikingly, however, P5 neurons expressing miR-29b showed a marked reduction in *Bim* staining after NGF deprivation (Fig. 5D,E). Together, these results show that miR-29b can block apoptosis in neurons by directly inhibiting the critical step of BH3-only protein induction.

While it is important for developing neurons to be sensitive to apoptotic stimuli for proper formation of the nervous system, the apoptotic pathway must be strictly inhibited after development to ensure that mature neurons can survive long-term. The observation that Bax remains inactive in the cytoplasm after NGF deprivation in mature neurons has been described, although the

Figure 4. miR-29b targets multiple members of the BH3-only family. (A) Schematic representation of predicted miR-29b-binding sites in the 3'UTRs of multiple mouse BH3-only mRNAs. Nucleotides of the 3'UTR containing miR-29b-binding sites are listed in parentheses. (B) Sequence and alignment of the miR-29b-binding sites in the 3'UTRs of multiple BH3-only mRNAs. The predicted base-pairing of miR-29b with target recognition seed sequence is shown in bold. (C) Luciferase activity was measured 48 h after transfection of HEK293T cells with reporter plasmids in which regions of either wild-type (wt) or mutant (mut) 3'UTRs of genes listed in A were each fused downstream from the firefly luciferase gene. Reporter plasmids were transfected either alone (vector), together with 20 nM miR-29b (miR-29b), or together with 20 nM cel-miR-67 (ctrl). Expression was normalized by taking the ratio of firefly to renilla luciferase, and is plotted relative to vector alone. Data are mean $\pm$ SEM of at least three independent experiments.

molecules responsible for this phenomenon were unknown (Putcha et al. 2000). Here, we identified miR-29b as a key molecule that is induced during neuronal maturation and functions to repress translation of the BH3-only family of proteins, thus preventing death in response to apoptotic stimuli. These results are the first to identify a mammalian miRNA that strictly inhibits apoptosis in normal, healthy neurons.

A recent study found that expression of the miR-29 family is reduced in sporadic Alzheimer's disease (AD) patients' brains (Hebert et al. 2008). This study identified β site APP-cleaving enzyme 1 (*BACE1*), a critical molecule in the release of β -amyloid peptides from APP, as a target of miR-29. Thus, loss of miR-29 expression in sporadic AD could lead to an increase in *BACE1* expression and, ultimately, β -amyloid plaques, which are the characteristic protein aggregates of AD. Our results identifying miR-29b as an important inhibitor of apoptosis in neurons provide additional insight as to why loss of miR-29 expression may leave neurons more vulnerable to neurodegeneration, and emphasize the importance of miR-29 for long-term neuronal survival.

After cytotoxic stress, proapoptotic BH3-only proteins are crucial for triggering apoptosis by either inhibiting the anti-apoptotic proteins Bcl-2, Mcl-1, and Bcl-xL, or directly activating proapoptotic Bax and Bak (Willis and Adams 2005; Chipuk and Green 2008). In *C. elegans*, only a single BH3-only protein, EGL-1, is necessary for activating apoptosis during development (Conradt and

Horvitz 1998). In contrast, mammals contain at least eight BH3-only proteins, distinct subsets of which are activated after different apoptotic stimuli (Giam et al. 2008). While this large repertoire of BH3-only proteins allows for increased regulation of apoptosis, it also leads to a redundancy in their function. In fact, loss of either *Bim* or *Hrk* alone in sympathetic neurons provides only a modest survival advantage over wild-type neurons (Putcha et al. 2001; Imaizumi et al. 2004). Thus, in order to efficiently inhibit apoptosis at the level of BH3-only activity, it is necessary to block multiple members of this pathway simultaneously. Indeed, we found that miR-29b is able to target at least five unique members of the BH3-only family. Interestingly, while we were unable to find predicted miR-29-binding sites in the 3'UTRs of *Bid*, *Bad*, or *Noxa*, evidence also shows that these BH3-only family members do not play a major role in sympathetic neurons (Putcha et al. 2002; Wytenbach and Tolkovsky 2006).

Why would miR-29b evolve to inhibit apoptosis in neurons by repressing several BH3-only genes when, for example, targeting Bax alone would lead to similar, if not greater, resistance to apoptosis by intrinsic stimuli (Deckwerth et al. 1996)? One possibility is that targeting Bax may have undesirable consequences because of its nonapoptotic role in regulating mitochondrial fusion (Karbowski et al. 2006). Also, by targeting the BH3-only members of the Bcl-2 family, miR-29b may have evolved to fine-tune apoptosis regulation, as opposed to completely disengaging apoptotic signaling.

Figure 5. miR-29b expression in neurons blocks induction of endogenous BH3-only proteins. (A–C) The protein levels of Bim_{EL}, Bmf, and Puma were determined by Western blot in P0 neurons cultured for 5 d (P5) or 28 d (P28) in vitro. Neurons were either left untreated (+NGF) or deprived of NGF (–NGF) (A), untreated (–Etop) or treated with 20 μ M etoposide (+Etop) (B), or untreated (–Tuni) or treated with 2.5 μ M tunicamycin (+Tuni) (C). All treatments were performed for 48 h in the presence of the caspase inhibitor Q-VD-OPH (25 μ M) before lysates were collected. Representative Western blots are shown. Bmf isoforms were detected at ~25 kDa and ~30 kDa. (D,E) P3 sympathetic neurons were microinjected with a GFP-expressing plasmid and either miR-29b or cel-miR-67 (ctrl miRNA, each 30 μ M). After 48 h, cells were left untreated (+NGF) or deprived of NGF (–NGF). Cells were fixed and stained 48 h after cell treatment. (D) Representative photographs of Bim staining in neurons; GFP expression indicates injected cells (arrows). Bar, 10 μ m. (E) Fluorescence intensity of cells after Bim staining. Data are mean $\pm$ SEM of three independent experiments. (F) Proposed model showing that high miR-29b levels in mature neurons prevent induction of BH3-only proteins after apoptotic stimuli. Apoptotic stimuli cause cytochrome *c* release and death in young neurons, while mature neurons remain resistant.

Intriguingly, miR-29 function in cancer cells appears to be complex. While miR-29 expression is elevated in some cancers where it appears to function as an oncogene (Gebeshuber et al. 2009; Han et al. 2010), others have found miR-29 to have tumor suppressor functions (Pekarsky et al. 2006; Wang et al. 2008), notably by indirectly activating p53 or targeting the anti-apoptotic protein Mcl-1 (Mott et al. 2007; Park et al. 2009). We examined levels of Mcl-1 in P5 versus P28 neurons and found that, paradoxically, Mcl-1 levels were down-regulated upon neuronal maturation, despite these neurons being strikingly resistant to apoptosis (Supplemental Fig. S6). Thus, although the consequence of miR-29 expression in various cancer cells may depend on cellular context, its ability to inhibit the BH3-only family proteins has a clear anti-apoptotic function in primary neurons.

miRNAs have been described to modulate a variety of cellular processes—including differentiation, proliferation,

and apoptosis (Kent and Mendell 2006; Stefani and Slack 2008)—and may regulate nearly two-thirds of the entire mammalian genome (Friedman et al. 2009). Our results here identify miR-29b as being induced during the physiologically normal process of neuronal maturation, and demonstrate the ability of a single miRNA to inhibit apoptosis by targeting multiple members of a key proapoptotic gene family.

Materials and methods

Cell culture conditions, primers, plasmids, antibodies, and other standard methods are described in detail in the Supplemental Material.

RNA extraction and microarray analysis

For miRNA microarray and qRT-PCR analysis, total RNA was extracted using Trizol Reagent (Invitrogen) or the miRNeasy kit (Qiagen). miRNA microarray was performed essentially as described previously (Thomson et al. 2004). Normalized log₂ data were clustered hierarchically by sample and gene and represented as a heat map using Cluster 3.0 and TreeView software programs, respectively (Michael Eisen, Stanford University).

Microinjection and quantification of cell survival

Cells were injected with 30 μ M miR-29b or a control *C. elegans* miRNA (cel-miR-67) that is not conserved in mammalian cells (miRIDIAN mimics; Dharmacon) along with rhodamine dextran (8 μ g/ μ L) and EGFP-expressing plasmid (50 ng/ μ L) in microinjection buffer containing 100 mM KCl and 10 mM KPi (pH 7.4) as described previously (Potts et al. 2003). This concentration of miR-29b was estimated to elevate miR-29b to approximately the levels seen in P28 neurons (see the Supplemental Material for more information). The number of viable rhodamine-positive cells with intact phase-bright cell bodies was counted prior to treatment with NGF deprivation, etoposide, or tunicamycin and then counted at indicated times after cell treatment. Cell survival was expressed as a percentage of the number of cells prior to treatment. This method of assessing survival correlates well with other cell survival assays such as trypan blue exclusion and calcein AM staining, and follows recent guidelines for assessment of death in neuronal cells (Potts et al. 2003; Galluzzi et al. 2009).

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