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# MICRORNA-29A/PTEN PATHWAY MODULATES NEURITE OUTGROWTH IN PC12 CELLS

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**Abstract—PTEN serves as an intrinsic brake on neurite outgrowth, but the regulatory mechanism that governs its action is not clear. In the present study, miR-29a was found to increase neurite outgrowth by decreasing PTEN expression. Results showed that miR-92a-1, miR-29a, miR-92b, and miR-29c expression levels increased during nerve growth factor (NGF)-induced differentiation of PC12 cells. Based on in silico analysis of possible miR-29a targets, PTEN mRNA may be a binding site for miR-29a. A protein expression assay and luciferase reporter assay showed that miR-29a could directly target the 3′-UTRs (untranslated regions) of PTEN mRNA and down-regulate the expression of PTEN. PC12 cells infected with lentiviral pLKO-miR-29a showed far higher levels of miR-29a and Akt phosphorylation level than those infected with control. This promoted neurite outgrowth of PC12 cells. Collectively, these results indicate that miR-29a is an important regulator of neurite outgrowth via targeting PTEN and that it may be a promising therapeutic target for neural disease.** 

**Key words: miRNA, neurite outgrowth, miR-29a, PTEN, Akt.**

## INTRODUCTION

Deletion of the phosphatase and tensin homologs on chromosome ten (PTEN) anti-oncogene is among the most frequently mutations in high-grade brain tumors such as gliomas, glioblastoma, and medulloblastoma (Rasheed et al., 1997; Holand et al., 2011). Although the importance of PTEN in cancer etiology is clear, its role in the nervous system remains unclear (Stolarov et al., 2001; Weng et al., 2002; Musatov et al., 2004). Recent studies have suggested that PTEN plays an important role in neural development and differentiation. PTEN is expressed in the brains of mice, starting at approximately postnatal day 0, preferentially in neurons, especially Purkinje neurons, olfactory mitral neurons, and large pyramidal neurons in the brains of adult mice (Lachyankar et al., 2000a). Loss of PTEN expression is associated with cerebellar dysplasia in human patients (Liaw et al., 1997). However, PTEN deletion does not seem to facilitate intrinsic regenerative outgrowth of adult peripheral axons (Christie et al., 2010). Rather, it enhances regeneration of axons after CNS injury (Park et al., 2008) and in adult cortico-spinal neurons after spinal cord injury (Liu et al., 2010). These studies highlight the importance of the role of PTEN in neuronal development and possibly axon outgrowth.

A considerable amount of evidence supports the hypothesis that many cellular processes, including cell proliferation, apoptosis, and cytokine release, are regulated by miRNAs (Qureshi et al., 2012). It has been estimated that miRNAs, which constitute only 1% of the genes in the human genome, can regulate at least 20–30% of all human genes (Gimm et al., 2000). Emerging evidence suggests that miRNAs not only increase proliferation and inhibit cell apoptosis in tumor cells in vitro but also promote chemotherapeutic drug resistance and tumor metastasis by down-regulating the expression of PTEN. Previous studies have confirmed that miRNAs can regulate the expression of PTEN in neural cells and promote axonal outgrowth (Li et al., 2013). Results showed that nerve growth factor (NGF) can normally induce differentiation of PC12 cells into a neuron-like phenotype and induce up-regulation of miR-92a-1, miR-29a, miR-29c, and miR-92b expression. MiR-29a is involved in the regulation of migration of hepatoma cells mediated by hepatitis B virus X protein (HBx) through modulation of Akt phosphorylation (Kong et al., 2011b). It is here proposed that the miR-29a/PTEN pathway exists and may be a regulatory factor on neurite outgrowth via regulating Akt pathway. However, the details underlying this mechanism are not known.

In this study, results showed that miR-29a expression levels were higher during NGF-induced differentiation of PC12 cells than at other times. For this reason, PC12 cells with stable miR-29a expression were generated. Results demonstrated that alteration of miR-29a expression regulates neurite outgrowth by modulating PTEN expression and increasing Akt phosphorylation.

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*Abbreviations:* HBx, hepatitis B virus X; MREs, miRNA response elements; NGF, nerve growth factor; UTR, untranslated region.

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## EXPERIMENTAL PROCEDURES

### Cell culture and treatments

To cause the PC12 cells to differentiate, cells were plated onto 6-well tissue culture plates at a relatively low density ($2 \times 10^3$ cells $cm^{-2}$) in DMEM/F12 (Hyclone, Logan, UT, USA) medium with 5% FBS. After plating cells for 24 h, a concentrated stock of NGF (human recombinant NGF, Sigma, MO, USA) was added to the above culture medium to a final concentration of 50 ng/ml. Cells exposed to vehicle alone (5% FBS culture medium) were used as controls. To inhibit Akt signaling, the cells were pretreated with the PI3K inhibitor LY294002 at 20 μM for 2 h after being starved of FBS for 12 h.

### Target prediction

Several freeware programs are available on the internet. These are commonly used to search for potential miRNA–mRNA binding sites. Three such software products were used here, TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de), and microT (http://www.microrna.gr/microT). All three identified PTEN had relatively likely putative binding sites for miR-29a.

### Real-time polymerase chain reaction [RT-PCR] (miRNA)

Total RNA was extracted from $5 \times 10^6$ cells using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's manual. We used the poly(A) tailing kit (New England Biolabs, MA, USA) for miRNAs detection. The ingredients were as follows: 1-μg total RNA was mixed with 2 μl poly(A) buffer (10×), 2 μl ATP (10 nM), 0.5 μl *E coli* poly(A) Polymerase I (E-PAP). The total volume was adjusted to 20 μl with RNase-free $ddH_2O$. Single-stranded cDNA was obtained from RNA by reverse transcriptase (TAKARA, China). The ingredients were as follows: 0.5-μg total poly(A)-tailing RNA was mixed with 2 μl M-MLV buffer (5×), 0.5 μl dNTP mixture (10 mM), 0.25 μl RNase inhibitor (40 U/μl), 0.25 μl RTase M-MLV (RNase H-) (200 U/μl), and 1 μl adaptor(dT)15 (50 μM). The total volume was adjusted to 20 μl with RNase-free $ddH_2O$. Reverse transcription process was performed at 42 °C for 60 min, followed by an inactivation reaction at 70 °C for 15 min. The PCR mixture contains 10 μl qPCR Master Mix (2×) (Bio-Rad, USA) and 1 μl cDNA. The total volume was increased to 20 μl with $ddH_2O$. The primer powder was fixed to the bottom of the 96-well plate. To each well 20 μl of PCR mixture was added. RT-PCR was performed using a CFX96™ Real-Time Instrument (Bio-Rad). Real-Time PCR (RT-PCR) was conducted using the iQ™ SYBR Green q-PCR Super-mix (Bio-Rad) with the following primers (Table 1). Thermal cycle parameters were as follows: 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s, and 65–95 °C drawing dissociation curve. The expression of each gene was defined from the threshold cycle (Ct), and the melting temperatures (Tm) were recorded. Using the ΔΔCt method analyzes relative changes in gene expression. Here, $\Delta\Delta Ct = (Ct_{target\text{-}gene/miRNA} - Ct_{ref\text{-}genes/miRNA})$ PC12-NGF- $(Ct_{target\text{-}gene/miRNA} - Ct_{ref\text{-}genes/miRNA})$ PC12. Fold change $2^{-\Delta\Delta Ct}$ = target gene/

**Table 1.** Primers for Q-PCR

| Primer | Sequence(5′–3′) | Base (bp) |
|---|---|---|
| rno-miR-29b-1 Forward | GCCGCCTTTCATATGGTGGTTTAGATTT | 28 |
| rno-miR-29b-1 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-29b-2 Forward | CTGGTTTCACATGGTGGCTTAG | 22 |
| rno-miR-29b-2 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-29a Forward | GCGCACTGATTTCTTTTGGTGTTCAG | 26 |
| rno-miR-29a Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-29c Forward | TGACCGATTTCTCCTGGTGTTC | 22 |
| rno-miR-29c Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-32 Forward | GCGCTATTGCACATTACTAAGTTGCA | 26 |
| rno-miR-32 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-363 Forward | CGGGTGGATCACGATGCAATTT | 22 |
| rno-miR-363 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-25 Forward | AGGCGGAGACACGGGCAATTGC | 22 |
| rno-miR-25 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-92b Forward | AGGGACGGGACGCGGTGCAGTGTT | 24 |
| rno-miR-92b Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-92a-1 Forward | AGGTTGGGATTTGTCGCAATGCT | 23 |
| rno-miR-92a-1 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| rno-miR-92a-2 Forward | AGGTGGGGATTAGTGCCATTAC | 22 |
| rno-miR-92a-2 Reverse | GCGAGCACAGAATTAATACGAC | 22 |
| PTEN Forward primer | AAGGACGGACTGGTGTAA | 18 |
| PTEN Reverse primer | CCTGAGTTGGAGGAGTAGAT | 20 |
| GAPDH Forward primer | AACGGATTTGGTCGTATTG | 19 |
| GAPDH Reverse primer | GGAAGATGGTGATGGGATT | 19 |
| u6 Forward | CTCGCTTCGGCAGCACA | 17 |
| u6 Reverse | GCGAGCACAGAATTAATACGAC | 22 |



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