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Micro RNA-125b (miRNA-125b) function in astrogliosis and glial cell proliferation

A.I. Pogue[a], J.G. Cui[a], Y.Y. Li[a], Y. Zhao[b], F. Culicchia[c], W.J. Lukiw[a,*]

[a] LSU Neuroscience Center, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA
[b] Department of Structural Biology, University of Pittsburgh, Pittsburgh, PA 15260, USA
[c] LSU Department of Neurosurgery, LSU Health Sciences Center, New Orleans, LA 70112, USA

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ABSTRACT

Micro RNAs (miRNAs) are post-transcriptional modulators of gene expression that regulate the stability and translation of their target messenger RNAs (mRNAs). Here we report that the levels of a human brain-enriched miRNA-125b are up-regulated in interleukin-6 (IL-6)-stressed normal human astrocytes (NHA), a treatment known to induce astrogliosis. *In vitro*, anti-miRNA-125b added exogenously to IL-6-stressed NHA cultures attenuated both glial cell proliferation and increased the expression of the cyclin-dependent kinase inhibitor 2A (CDKN2A), a miRNA-125b target and negative regulator of cell growth. A strong positive correlation between miRNA-125b abundance and the glial cell markers glial fibrillary acidic protein (GFAP) and vimentin, and CDKN2A down-regulation was noted in advanced Alzheimer's disease (AD) and in Down's syndrome (DS) brain, chronic neurological disorders associated with astrogliosis. The results suggest that miRNA-125b up-regulation contributes to astrogliosis and to defects in the cell cycle that are characteristic of degenerating brain tissues.

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Astrocytes, constituting the major glial cell population in the CNS, play important physiological, signaling and immunological roles in central nervous system (CNS) structure and function [18,1,6]. Astrocytic proliferation or astrogliosis occurs in acute or chronically damaged areas of the CNS and is associated with regional neuronal loss; astrocytes tend to accumulate where neurons have been damaged. The reactivity and rapid proliferation of astrocytes is seen in pro-inflammatory cytokine-stressed astroglial cells, after acute, focal mechanical trauma and viral infection, and is associated with chronic neurodegenerative diseases including Alzheimer's disease (AD) and Down's syndrome (DS) [18,1,6,19]. Typically, astroglial proliferation is accompanied by significant increases in the expression of the cytoarchitectural proteins glial fibrillary acidic protein (GFAP) and vimentin, and these are considered as specific markers for astrogliosis and the degree of astroglial cell expansion [1,6,19,2].

Molecular triggers for astrogliosis in neurodegenerative disease are not well understood. In this study we examined β-actin, GFAP, vimentin and miRNA profiles in control normal human astrocytic (NHA) cells in primary culture and in cytokine IL-6-stressed NHA cells, and found a significant up-regulation in the brain abundant miRNA-125b strongly associated with glial cell proliferation. miRNA-125b has multiple mRNA targets in the brain, and has also been found to be up-regulated in AD and DS brain tissues, neurodegenerative disorders associated with astrogliosis [11,12,9,20]. The up-regulation of miRNA-125b correlated with (a) significant increases in GFAP and vimentin gene expression, (b) a striking decrease in the expression of a miRNA-125b target, a cyclin-dependent kinase CDK2NA, a negative regulator of cellular proliferation, and (c) an increase in NHA cell proliferation. Further, anti-miRNA-125b was found to attenuate NHA cellular proliferation associated with IL-6-induced stress and was associated with increases in CDK2NA. The results suggest a miRNA-125b-directed, CDK2NA-mediated role in astrocyte proliferation that may contribute to astrogliosis in neurodegenerative brain disease.

Reagents were purchased from commercial suppliers and were used without further purification. RNA isolation reagents (isopropanol, nucleic acid grade ethanol, diethyl pyrocarbonate water) and RNAse-free vials were purchased from Invitrogen (Carlsbad, CA). miRNA-125b and anti-miRNA-125b were obtained through Ambion (Austin, TX). Human recombinant IL-6 (10 μM) was instilled into 37 °C astrocyte basal medium (ABM) according to the manufacturer's protocols (I1395; Sigma–Aldrich, St. Louis, MO). Westerns were performed using primary antibodies directed against the control β-actin (3598-100; Sigma), CDKN2A (p16; c-20; SC-468); GFAP (C-19; sc-6170); and vimentin (C-20; sc-7557; Santa Cruz Biotechnologies, Santa Cruz, CA) using techniques previously described [20,7,14,17].

Cryopreserved NHA cells (CC-2565; Lonza Corp., Walkersville, MD) were grown in ABM containing epidermal growth factor (EGF), insulin, ascorbic acid, gentiamycin-amphoteracin-1000 (GA-1000),

* Corresponding author. Tel.: +1 504 599 0842; fax: +1 504 599 0891.
E-mail address: wlukiw@lsuhsc.edu (W.J. Lukiw).

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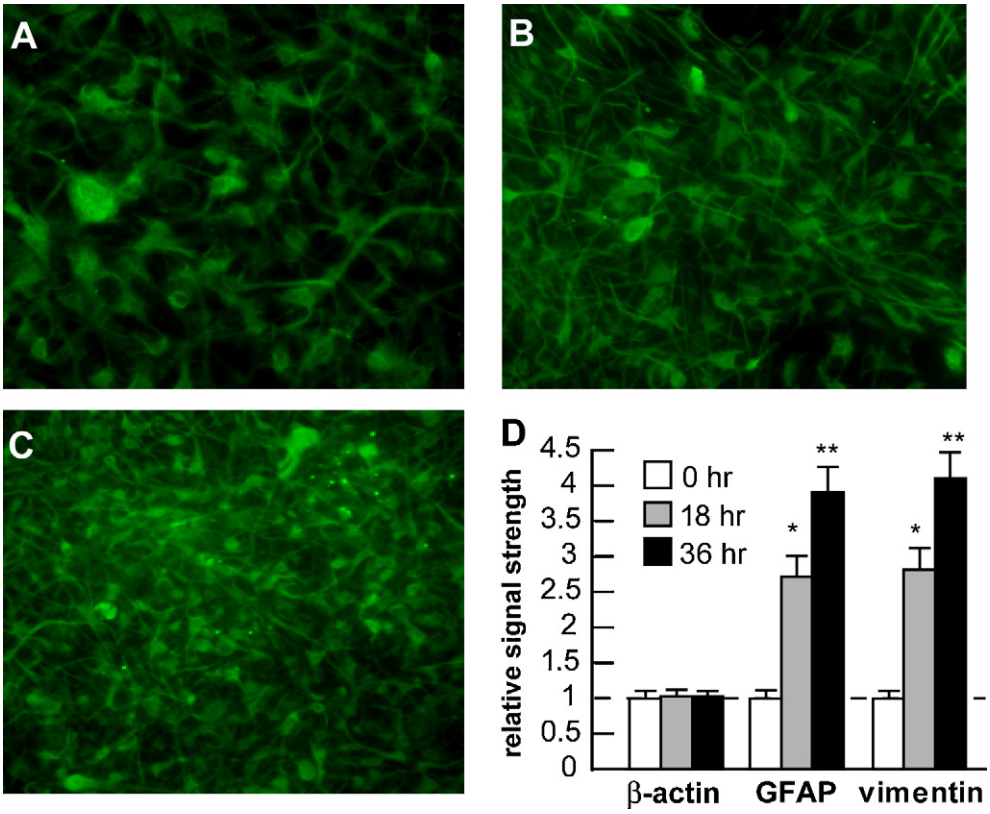

Fig. 1. Proliferation of primary NHA cells after treatment with IL-6 (10 μM). Morphology of NHA cells stained with a antibody to GFAP at time "0" control (CON) (A), 18 h (B) and 36 h; magnification 20× (C). Levels of β-actin, GFAP and vimentin for each time period were assayed using Western analysis (D). NHA cells are slightly larger in early growth stages and multiply in number but decrease in average cell size after IL-6-induced proliferation. A horizontal dashed line at 1.0 indicates mean β-actin signal strength for ease of comparison. Error bars = 1 SD; $N = 4$; $^{*}p < 0.05$; $^{**}p < 0.01$ (ANOVA).

L-glutamine, fetal bovine serum (FBS) with and without IL-6, as described by the manufacturer (Lonza) [7,14,22] (Fig. 1). NHA cells were seeded at 5000 cells/cm^2 and grown to 45% confluency; ABM was changed every 2 days; total RNA and proteins were extracted at 0, 18, and 36 h after IL-6 treatment.

Brain tissues were used in accordance with the institutional review board guidelines at LSUHSC and donor institutions [11,12,14]. Stringent parameters were used in the selection of tissues employed as post mortem interval (PMI) is a factor affecting RNA quality [17,22,5,13]. All RNA was derived from superior temporal lobe (Brodmann A22) tissues having a PMI of ≤1.5 h. A pool of human control brain tissues ($N = 6$) had a mean age of 69.0 ± 1.8 yrs, an age range of 66–71 yrs, a PMI range of 0.8–1.2 h (death to brain freezing interval at −81 °C), an RNA $A_{260/280}$ ranging from 2.08 to 2.10, an RNA 28S/18S of 1.4–1.5 and an RNA yield of 1.2 total μg RNA/mg wet weight of brain tissue. Brain tissues from mild (clinical dementia rating, CDR 1.5) AD patients ($N = 4$) had a mean age of 72.0 ± 1.6 yrs, an age range of 65–75 yrs, a PMI range of 0.9–1.5 h, an RNA $A_{260/280}$ ranging from 2.1 to 2.15, an RNA 28S/18S of 1.4–1.6 and an RNA yield of 1.3 total μg RNA/mg wet weight. Brain tissues from an advanced (CDR 5) group of AD patients ($N = 6$) had a mean age of 71.2 ± 1.4 yrs, an age range of 67–73 yrs, a PMI of 0.8 to 1.3 hrs, an RNA $A_{260/280}$ ranging from 2.09 to 2.11, an RNA 28S/18S of 1.4–1.55 and an RNA yield of 1.25 total μg RNA/mg wet weight of tissue. Human DS tissues ($N = 4$) had a mean age of 66.8 ± 1.7 yrs, an age range of 63–73 yrs, and a PMI range of 1.1–1.5 h. Total RNA extracted from DS brain had RNA $A_{260/280}$ ranging from 2.05 to 2.15, RNA 28S/18S ranging from 1.3 to 1.6 and an RNA yield ranging from 1.2 total μg RNA/mg wet weight of brain tissue.

The effects of exogenously added anti-miRNA-125b (AM-125b) levels on β-actin, miRNA-125b, CDKN2A, GFAP, and cell proliferation were studied in NHA cells after transfection with AM-125b oligo (25–75 nmol/L; Ambion) and Lipofectamine 2000 (Invitrogen), and were compared to transfection with miRNA and anti-miRNA controls as previously described (Fig. 5) [23]. Proliferation of NHA cell lines was determined using an EdU (5-ethynyl-2′-deoxyuridine)-Alexa Fluor® alkyne-azide cell proliferation assay (Invitrogen) (Fig. 4).

After 0, 18, or 36 h of IL-6 treatment, total RNA was extracted from NHA cells using TRIzol (Invitrogen) and was quality controlled using an Agilent Bioanalyzer 2100 (Lucent Technologies/Caliper Technologies, Palo Alto, CA) [11,12,20,7,14]. An electropherogram was generated for each total RNA sample; if the ratio for 28S/18S was larger than 1.4 (indicating high RNA spectral quality) samples were further processed. As a preliminary screen, miRNA panels (711 human miRNA targets and 52 controls; MRA-1001; LC Sciences, Houston TX) were probed with total miRNA extracted from IL-6-stressed or control NHA cells [20,7,14,17]. Specific miRNAs showing strong hybridization signals were further subjected to Northern dot blot analysis [20,14,5]. Total miRNA extracts (25 μg) were spotted onto GeneScreen membranes, transferred, cross-linked, baked, hybridized, and probed with specific DNA oligomers corresponding to specific small RNAs and miRNAs [14,17,22,5]. Samples were analyzed individually or as pooled samples to examine trends. There were no significant differences in the total RNA yield or RNA quality between control and IL-6-stressed NHA cells at any time-point examined.

Total proteins were simultaneously isolated using TRIzol and concentrations determined using dotMETRIC microassay (sensitivity 0.3 ng protein/ml; Chemicon Temecula, CA) [7,14,17]. To ascertain whether IL-6 was associated with an increase in the expression of inflammatory or pathogenic proteins, West-