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MicroRNA Expression Patterns in Human Astrocytes in Relation to Anatomical Location and Age

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Abstract

Anatomic distribution and age are variables linked to functions of astrocytes under physiologic and pathologic conditions. We measured the relative expression of a panel of microRNAs (miRNAs) in astrocytes captured by laser micro-dissection from normal human adult white and grey matter, human fetal white matter and germinal matrix samples. Although expression of most miRNAs was comparable between adult and fetal samples, regional differences were observed. In the adult cerebral cortex, expression of miRNAs in morphologically distinct inter-laminar astrocytes underlying the glial limitans differed from those in deeper cortical layers, suggesting functional specialization possibly related to structural stability and defense from potentially harmful factors in the cerebrospinal fluid. Differences between adult white and grey matter miRNA expression included higher expression of pro-inflammatory miRNAs in the former, potentially contributing to differences in inflammation between grey and white matter plaques in multiple sclerosis. Lower expression of miRNAs in fetal versus adult white matter astrocytes likely reflects the immaturity of these migrating cells. Highly expressed miRNAs in the fetal germinal matrix are probably relevant in development and also recapitulate some responses to injury. Future studies can address regional alterations of miRNA expression in pathological conditions.

Key Words: Adult, Fetal, Brain, Human astrocytes, Laser micro-dissection, MicroRNAs

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INTRODUCTION

The wide array of functions performed by astrocytes under physiological and pathological conditions is influenced by anatomic distribution and age (1–3). The physiological roles of astrocytes during development include participation in neurogenesis, guidance of neural cell migration, blood-brain barrier and glial limitans formation, and regulation of myelination. In the mature CNS, astrocytes contribute to structural stability, maintenance of blood-brain barrier function and neuronal synapses, tissue homeostasis and myelin maintenance. Under pathological conditions, astrocytes and their secreted products participate in tissue reactions to injury, neuroprotective responses, and repair processes by acting on specific neural cell targets and regulating immune cell reactivity.

Since the time of Cajal, astrocytes have been categorized into 2 broad morphologies: protoplasmic with short and highly branched processes, and fibrous astrocytes with long unbranched processes. Astrocytes in the white matter are usually described as fibrous, whereas grey matter astrocytes are protoplasmic. White matter fibrous astrocytes in the mature brain are largely derived from the fetal radial glial cells as well as other germinal matrix precursors; grey matter astrocytes have migrated from the germinal matrix in the second half of gestation. It is not clear whether these morphologic differences reflect intrinsic cell properties or result from the distinct anatomical structural features of the cellular grey matter as opposed to the isomorphic nature of the axonal tracts in the white matter. White matter astrocytes are less vulnerable to injury compared to grey matter astrocytes in MCAO (middle cerebral artery occlusion) mouse models of ischemia (4). This may reflect an overall lower metabolic demand on cells in the white matter compared to those in the cortex. *In situ* studies have demonstrated that fetal astrocytes produce less scarring than adult astrocytes in response to injury (5). Human fetal-derived astrocytes *in vitro* have a significantly higher rate of proliferation and an increased expression of tumor necrosis factor and interleukin-6 (IL-6) transcripts compared to cells derived from the adult CNS (6). This is seen both under basal conditions and in response to interferon-γ and lipopolysaccharide.

The aim of this study was to compare microRNA (miRNA) expression profiles in astrocytes captured directly *in situ* from white and grey matter samples of normal human adult and fetal brains. miRNAs are small non-coding RNA

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molecules that post-transcriptionally regulate gene expression. We measured levels of a panel of 20 miRNAs that were selected based on the following criteria: a) miRNAs whose function in astrocytes is already known; b) miRNAs that are highly expressed in astrocytes; c) miRNAs that are upregulated in ischemic tissue injury; d) miRNAs with differential regional distribution in brain; and e) inflammation-related miRNA in cells other than astrocytes (Table). We used laser capture micro-dissection (LCM) to obtain glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes from adult white and cortical grey matter, and from cerebral white matter and germinal matrix of the fetal brain samples.

The known relative absence of GFAP-positive identifiable astrocytes in the grey matter of second trimester fetal brains precluded their inclusion in our analyses. Developing astrocytes in these regions express the fetal intermediate filament protein vimentin, which is only later co-expressed with and eventually totally replaced by GFAP (7). In adult samples, given the distinct morphology of astrocytes, including their relationship to the glial limitans, we compared miRNA expression in astrocytes dissected from the superficial cortical layers (inter-laminar, layers 1-2) (8), from deeper cortical layers, and from white matter. In fetal samples, we also included astrocytes found within the germinal matrix because these cells play important roles in the proliferation, differentiation and migration of neural progenitor cells (9).

MATERIALS AND METHODS

Immunohistochemistry and LCM

Adult human brain specimens ages 24 to 76 years without recognizable pathology were obtained from the archives of the pathology laboratory of the Montreal Neurological Institute; second trimester fetal specimens (ages 17-20 weeks) were obtained from the Department of Pathology, Alberta Children's Hospital, University of Calgary (Supplementary Table). The fetal ages were selected based both on their correspondence with existent *in vitro* studies (i.e. fetal astrocytes cultures are mostly derived from second trimester brains) and as a time of vulnerability to pre-natal insults. The use of the tissue samples in this study received the approval of McGill Research Ethics Board (Montréal Neurological Institute).

Formalin-fixed paraffin-embedded (FFPE) tissue sections were immunostained with the Ventana Benchmark XT stainer using an antibody against GFAP (Ventana cat # 760-4345, clone #EPG724, Cell Marque, CA). Cells were visualized using secondary antibody conjugated to 3,3'-diaminobenzidine (DAB). DAB immunostaining has been previously used for LCM-based transcriptome analysis (10). Captures were performed on 2 slides from each of 3 adult and fetal cases. GFAP-positive cells (~25 cells) were captured separately from different brain regions using the PALM-LCM system (Carl Zeiss, North York, Ontario, Canada). Cells were captured from grey and white matter of normal adult brain sections. In the grey matter, astrocytes were captured separately from cortical layers 1-2 (inter-laminar astrocytes), and from the deeper layers (layers 2-6) (Figs. 1, 7). White matter and germinal matrix astrocytes were captured from fetal brain sections (Figs. 1, 7). We used hematoxylin and eosin-stained sections as guides from each case to identify different regions for capture. Captures across samples were normalized for laser power as well as for area of capture. Collected samples were immediately lysed in Isol-RNA lysis reagent (5 Prime, Hamburg, Germany) and stored at −80°C until further processing.

RNA Isolation, Preamplification Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction

Total RNA was extracted from astrocytes using standard Trizol® protocols followed by DNase treatment according to manufacturer's instructions (Qiagen, Valencia, CA). Multiplexed reverse transcription (RT) reactions were performed with a pool of miRNA-specific RT primers (a pool that included RT primers for all the miRNAs in the study) using a TaqMan® miRNA RT kit. cDNAs from laser-captured samples were subjected to a pre-amplification reaction using a pooled set of TaqMan® miRNA-specific probes to increase the number of target copies, as previously described for laser-captured FFPE samples (11, 12). Individual miRNA expression assays were performed using specific miRNA TaqMan probes to assess expression relative to RNU48, a stable human small nuclear RNA, which is a widely used endogenous reference (12–15). We found stable expression of this small nuclear RNA in all the samples analyzed (Supplementary Graph 1). miRNA expression analyses were performed using negative Δ cycle time, –(Δ Ct) values. Although the Δ Ct values have been previously used for analyzing miRNA expression data (14, 16), we used –(Δ Ct) values, which allow easier visualization of expression data on the graphs (i.e. a higher –(Δ Ct) value represents higher expression and a lower value depicts lower expression of individual miRNA). The panel of 20 miRNAs analyzed is listed in the Table.

miRNA Data and Statistical Analyses

The color gradient map depicting –(Δ Ct) values was prepared using the SAS software (JMP® 11). The principal component analysis (PCA) biplot and the hierarchical cluster data were generated using NIA (National Institute of Aging) array analysis tool available online (http://lgsun.grc.nia.nih.gov/ANOVA/index.html). The –(Δ Ct) values are presented as mean ± SEM in all graphs. For comparisons spanning the different regions of the adult brain, an one-way ANOVA was performed. For all other comparisons, an unpaired Student t test was used for the evaluation of all comparisons. All tests resulting with a p value <0.05 were considered significant. These tests were done using GraphPad Prism software (version 5.0).

RESULTS

Morphologic Comparisons of GFAP-Positive Cells in Adult and Fetal Brains

In adults, the protoplasmic astrocytes in the deeper layers of grey matter (layers II/III-VI) showed an abundant radial network of GFAP-positive processes (Fig. 1A, B); the processes of fibrous astrocytes in white matter (Fig. 1C) were elongated and

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TABLE. miRNA Panel

miRNA	Function	PMID
Category #1: miRNAs Functionally Characterized in Astrocytes		
21	Overexpression of miR-21 in astrocytes attenuates the hypertrophic response to spinal cord injury (SCI), and may reduce chronic scarring	23238710
29a	Negatively regulates proapoptotic protein, BCL2 *in vitro*. MiR-29a mimic protects and miR-29a inhibitor aggravates cell injury and mitochondrial function after ischemia-like stresses *in vitro*	24038396
100	Negatively regulates the expression of aquaporin 4 (AQP4). Down-regulation of AQP4 in astrocytes is neuroprotective in cerebral ischemia/reperfusion injury	18258830
124a	Positively regulates the expression of glutamate transporter, GLT1 protein *in vitro* potentially enhancing protection	23364798
125b	Positively regulates astrocytic reaction to pro-inflammatory insults (interleukin-6) correlating with enhanced GFAP expression	20347935
129	Negatively regulates the expression of expression of Caspase-6 *in vitro* inhibiting apoptosis and may be neuroprotective (Inhibits cell proliferation in GI cancer)	25187728
145	Negative regulator of astrogliosis after SCI (increased levels of this miRNA leads to decreased reaction, proliferation and migration and process formation)	25139829
146a	Negatively regulates the expression of Interleukin-1 receptor-associated kinase 1 (IRAK-1), IRAK-2 and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) *in vitro* (inflammation resolving function)	23028621
155	Negatively regulates the expression of SOCS1 gene expression *in vitro* (proinflammatory function)	22170100
181a	Represses expression of the methyl CpG binding protein 2 (MeCP2), implicating an anti-inflammatory role	23650073
320	Negatively regulates the expression of aquaporin 1 and 4 *in vitro* and *in vivo* supporting a neuroprotective role	20628061
Category #2: miRNAs Highly Expressed in Astrocytes		
99a	Higher expression in astrocytes, microglia and neurons compared to oligodendrocytes	24316888
	Known as a tumor suppressor in glioblastoma	23409016
143	Higher expression in astrocytes compared to microglia, neurons and oligodendrocytes	23516279
	Known as a tumor suppressor in glioma	24980823
449	Higher expression in astrocytes compared to microglia, neurons and oligodendrocytes	23516279
	A miRNA found essential for normal mouse brain development. A lack leads to dysregulated microtubule dynamics with effects on migration	24982181
Category #3: miRNAs Upregulated in Ischemic Tissue Injury		
34a	Upregulated in middle cerebral artery occlusion (MCAo) model of ischemia	18258830
	Has a role in cell cycle, differentiation, and apoptosis.	22162084
	Under SIRT1 activation by pharmacologic treatment with resveratrol, miR-34a promoted astrocytic differentiation	21857907
210	Upregulated in MCAo model of ischemia	18258830
	Highly expressed in astrocytes; overexpression of this miRNA induced neurogenesis in subventricular zone of adult mouse brain	24152581
214	Upregulated in MCAo model of ischemia	18258830
	Role in proliferation, migration and angiogenesis in glioma. However potentially neuroprotective in non-neoplastic conditions	24277415
Category #4: miRNAs With Differential Regional Distribution in Brain		
338	Enriched in human white matter compared to grey matter	20936480
	Upregulated in multiple sclerosis and expeirmental autoimmune encephalomyelitis (not specifically in astrocytes). Pro-inflammatory through neuro-steroid inhibition	21908875
365	Enriched in human white matter compared to grey matter	20936480
	In microglia, acts as a pro-inflammatory miRNA that interferes with the interleukin-6 and STAT3 pathway determining increased tumor necrosis factor (TNF) transcription	24336079
Category #5: Inflammation-related miRNA in Cells Other Than Astrocytes		
146b	Acts as a pro-inflammatory molecule in microglia in amyotorophic lateral sclerosis, operating through P2X7 receptor activation. Acts in synergy with other pro-inflammatory miRNAs such as miR-155 and miR-125b.	24336079

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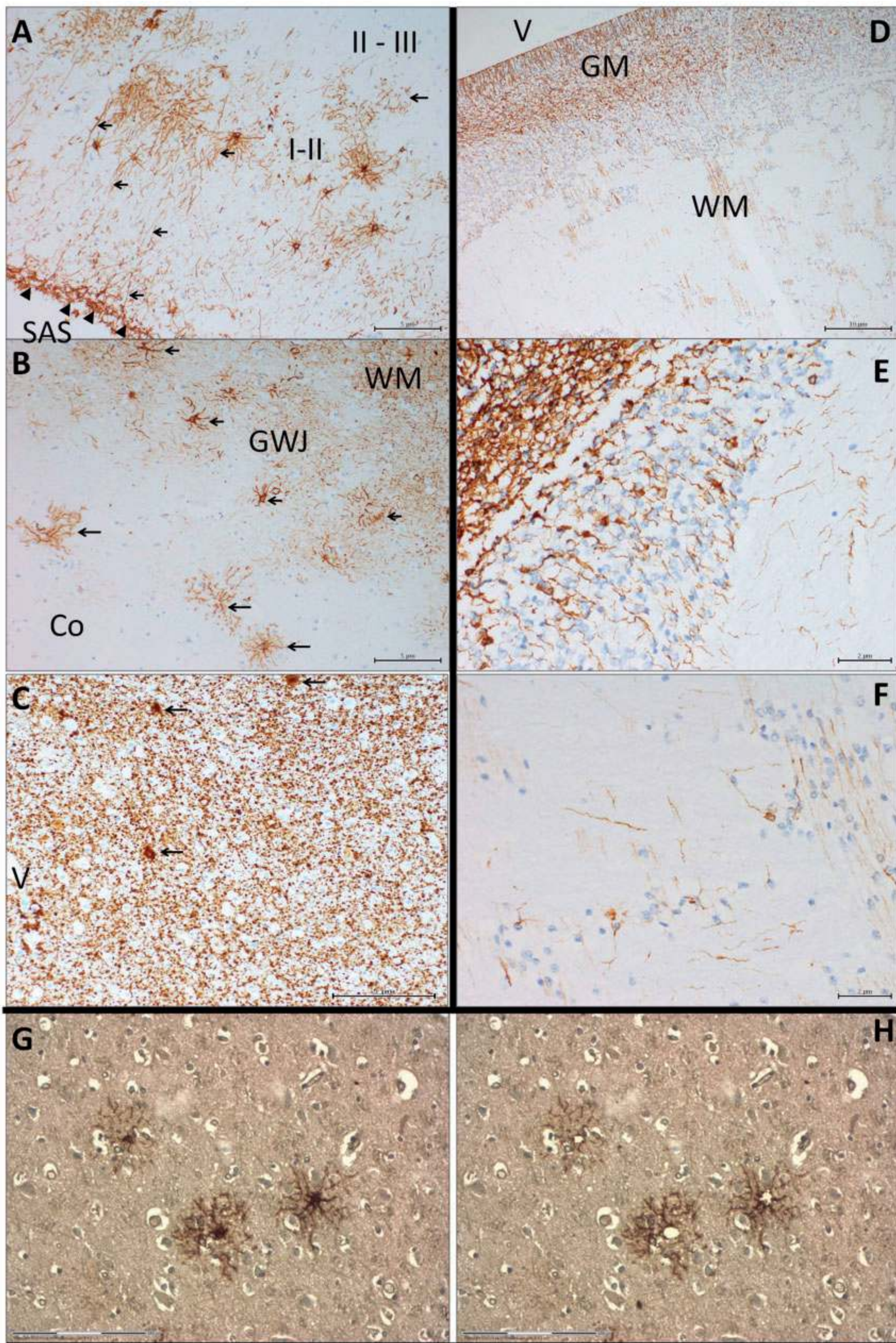

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FIGURE 1. Morphology of glial fibrillary acidic protein (GFAP)-immunopositive astrocytes in different regions of human adult and fetal brain. **(A)** The superficial layers of the adult cortex. The glia limitans (arrowheads) is composed of astrocyte foot-processes and underlies the sub-arachnoid space (SAS). It is formed by numerous prominent radial glial fibers with thick cytoplasmic processes

oriented along the axonal fibers. The inter-laminar (layers I-II) astrocytes were distinguished by their number, distinctive linear arrangement, prominence of GFAP staining, and prominent processes often in a radial distribution extending to the glial limitans (Fig. 1A). The cell bodies of adult astrocytes in white and grey matter were larger and more readily recognizable than those in fetal brains (Fig. 1D-F). The germinal matrix had many cells with filamentous radial processes thicker than those of the fetal white matter. GFAP-positive cells were rarely observed in fetal grey matter.

Comparison of miRNA Expression in Astrocytes From Different Regions of Adult and Fetal Brains

Comparisons were made between miRNA expression profiles in cells captured from the superficial (inter-laminar or layers I/II), the deeper cortical grey matter (layers II/III-VI), and the white matter of adult brain, as well as the white matter and germinal matrix (subventricular zone) of the fetal brain by LCM (Figs. 2-7). Figure 2A provides a color gradient map representing –(Δ Ct) values of all the miRNAs studied in GFAP-positive cells from the selected regions in adult and fetal tissue. The PCA was performed to visualize the variances in the pattern of overall miRNA expression across all regions and samples (Fig. 2B, C). Lower variances were observed between cells from adult deep grey and white matter. The miRNA expression of cells from the fetal germinal matrix region more closely resembled those from adult deep grey matter and adult white matter. Although differing greatly from each other, adult inter-laminar astrocytes and fetal white matter astrocytes were both outliers compared to the other 3 regions (Figs. 2B, C). Hierarchical clustering of the miRNA expression data reflects these same similarities and variances (Fig. 2C).

The following significant individual differences were observed between various regions and developmental time points. The functional implications of these expression differences are summarized in the Table.

miRNAs Differentially Expressed in Inter-laminar Astrocytes

miRNAs -129, -181a, -210, -214 and -365 were not detected in inter-laminar astrocytes captured from layers 1-2, a contrast with either deeper grey or white matter (Fig. 3A). In addition, inter-laminar astrocytes showed lower expression of miRNAs -34a, -124a, -338, -320 and -449 compared to the other 2 regions. Of these 5 latter miRNAs, the difference between inter-laminar astrocytes and astrocytes from the other 2 regions was more pronounced for miRNAs -34a, -124a and -338 (Fig. 3B).

In contrast, miRNAs -125b, -143 and -146a were more highly expressed in inter-laminar astrocytes than in astrocytes from deep grey matter. Astrocytes from deeper grey matter did not express miR-125b; however, miR-146a was the only miRNA in our panel that was found to be more highly expressed in inter-laminar astrocytes compared to those from both the deeper cortical layers and the white matter (Fig. 3C).

Comparison of miRNA Expression Between Astrocytes From Adult Deep Grey and White Matter

Significant differences were only observed for 3 miRNAs. MiR-181a was more highly expressed in deeper grey matter astrocytes. MiR-125b was not detected in deeper grey matter astrocytes (Fig. 3C), whereas miR-449 was more highly expressed in white matter astrocytes (Fig. 3A, C).

Comparison of Astrocyte miRNA Expression Between Adult and Fetal White Matter

Adult white matter captured cells showed higher expression of miRNAs -34a, -124a, -143, -145, -146a, -155, -320, -338 -365 and -449 compared to those from the fetal white matter (Fig. 4). Of these miRNAs, the difference in expression of miR-449 was the most significant. Of the miRNAs examined, none were more highly expressed in fetal white matter compared to adult white matter.

Comparison of Astrocyte miRNA Expression Between Fetal White Matter and Germinal Matrix

miRNAs -21, -124a, -145, -365 and -449 were less highly expressed in astrocytes from the white matter compared to cells from the germinal matrix (Fig. 5). Again, of all

(small arrows) emanating from inter-laminar astrocytes between layers I and II. In the top right is part of the deeper cortex (layers II/III-VI), which contains fewer stained astrocytes with more delicate network of cell processes. **(B)** In the adult, the grey-white junction (GWJ) lying between the cortex (Co) and the white matter (WM) incorporates parts of layer VI and the superficial white matter. It has numerous prominent astrocytes, often with thickened processes (short arrows). In contrast, the cortical astrocytes (long arrows) are sparser and have a delicate radial network of processes. **(C)** In adult white matter there is a linear arrangement of astrocytic fibers that run longitudinally along the tracts. The astrocytes are smaller and their processes lack the radial arrangement present in cortical astrocytes but they extend their processes around the parallel axons. **(D)** Overview micrograph depicting both the white matter (WM) and the germinal matrix (GM) of a fetal human brain. The germinal matrix underlies the ventricle (V). **(E)** Fetal germinal matrix. Cells in the deep germinal matrix (top left), including radial glia, astrocytes, subependymal astrocytes, and ependymal cells are immunostained for GFAP; there is a heavily stained network of processes emanating from these cells. In the more superficial germinal matrix, parallel radial glial fibers and astrocyte cell bodies are seen among the unstained germinal matrix cells. **(F)** Fetal white matter astrocytes. The stained cells are smaller than in the adult, and have delicate processes. Radial GFAP-positive glial fibers are also seen traversing the white matter toward the cortex. Non-stained migrating cells are associated with the radial fibers. **(G, H)** Micrographs showing adult grey matter astrocytes before and after laser-capture, respectively. Scale bars: **A–C**, 5 μm; **D**, 10 μm; **E, F**, 2 μm; **G, H**, 150 μm.

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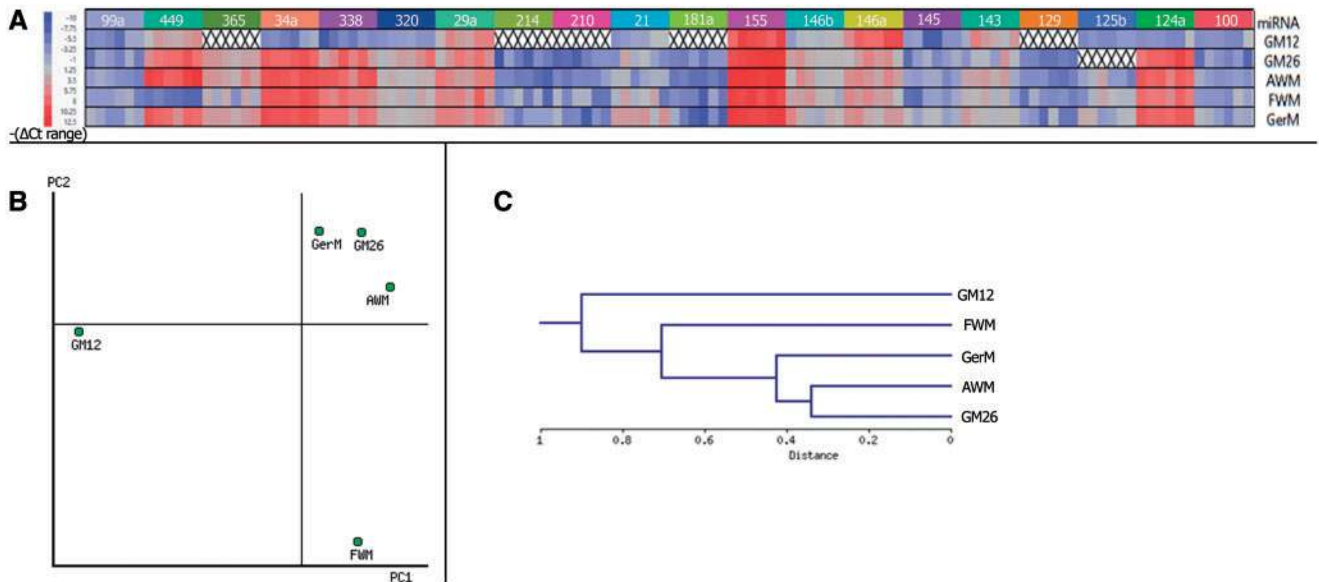

FIGURE 2. Color gradient map and principal component analysis (PCA) of microRNA (miRNA) expression (P.S- GM1/2 = adult inter-laminar, GM2/3-6 = adult deep grey matter (layers 2/3-6), AWM = adult white matter, FWM = fetal white matter and GerM = fetal germinal matrix). **(A)** Color gradient map representing the average negative Δ cycle time (-[Δ Ct]) values of miRNA panel in astrocytes from fetal and adult human brains. The scale bar indicates the range of color variation in relation to variation in -(Δ Ct) values. Lower detection of miRNAs is depicted in blue and more highly detected miRNAs are shown in red. Non-detection is marked 'X'. **(B)** A bi-plot or a 2-dimensional depiction of the PCA of miRNA expression data sets. **(C)** Hierarchical clustering of samples based on PCA of miRNA expression data sets.

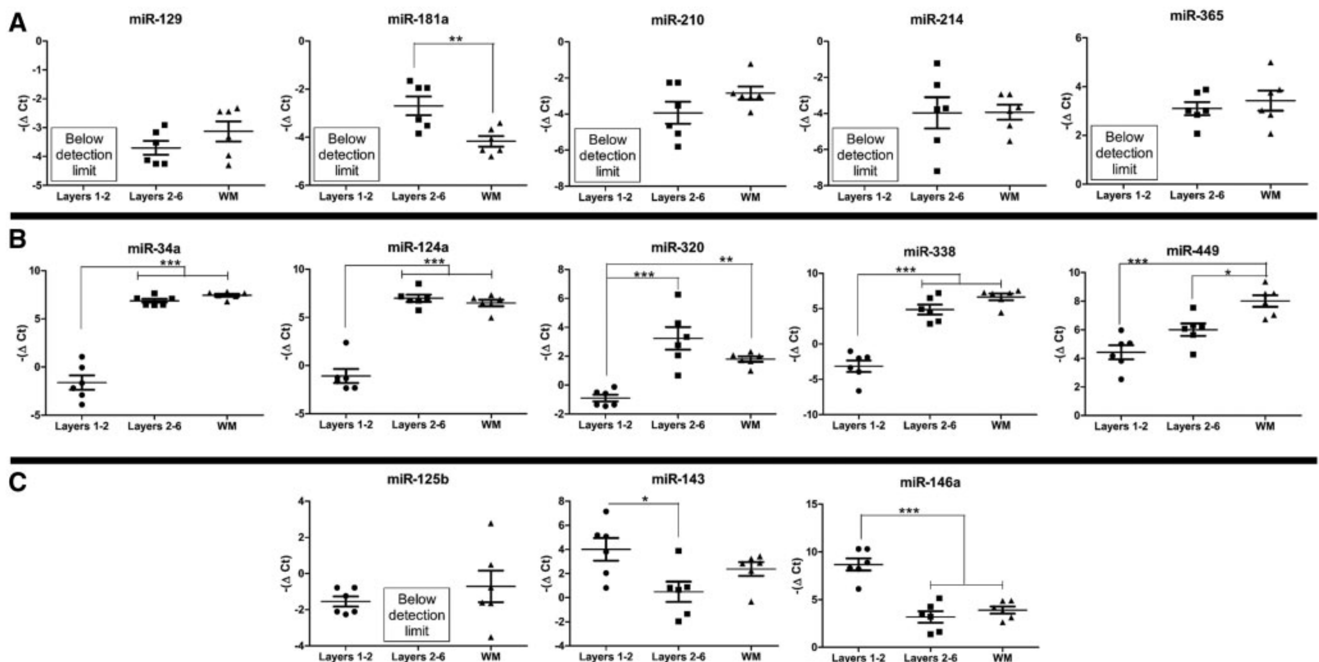

FIGURE 3. MicroRNA (miRNA) expression profiles in astrocytes captured from 3 different regions of the adult brain: Layers 1-2 (inter-laminar/molecular layer), layers 2/3-6 and white matter. **(A)** miRNAs not detected in astrocytes captured from layers 1-2. **(B)** miRNAs with lower expression in astrocytes captured from layers 1-2 relative to those from layers 2/3-6 and from white matter. **(C)** miR-125b is not detectable in deeper grey matter astrocytes (layers 2/3-6); miRNAs -143 and -146a are more highly expressed in grey matter astrocytes from layers 1-2 compared to those from layers 2/3-6. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

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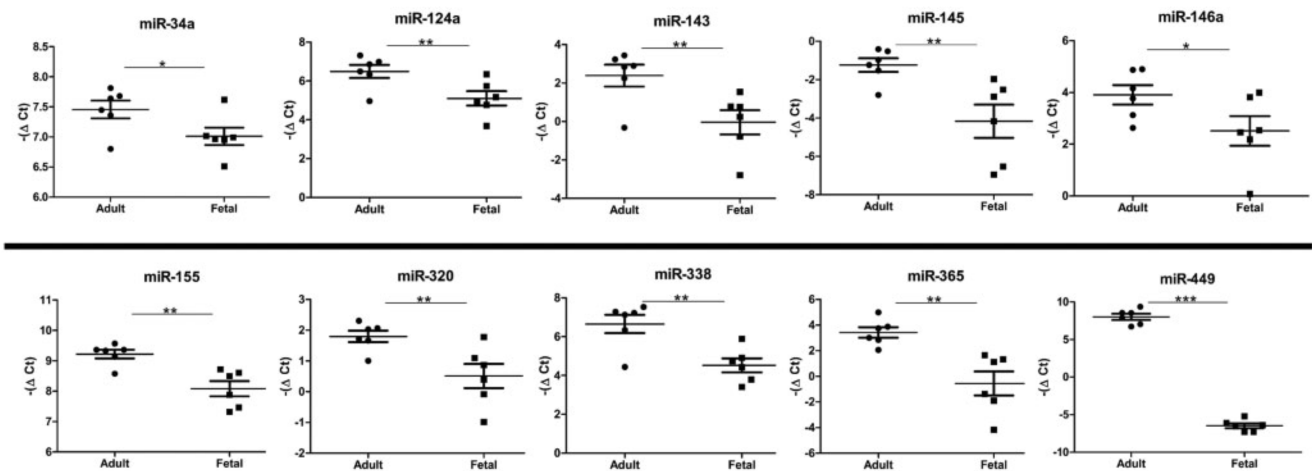

FIGURE 4. MicroRNAs more highly expressed in astrocytes captured from white matter of adult brains compared to those captured from developing fetal white matter. The most significant difference was found for miR-449. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

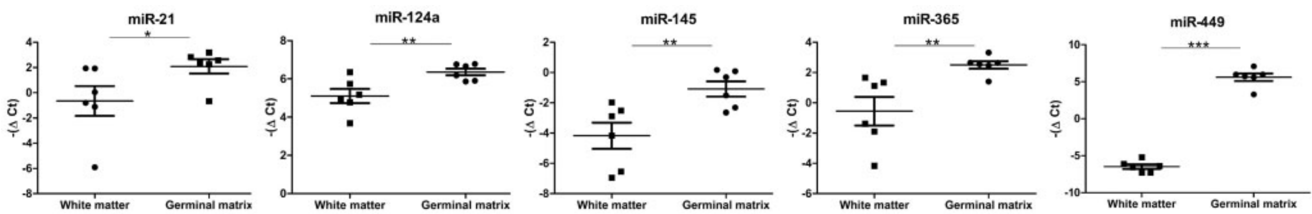

FIGURE 5. MicroRNAs with lower expression in astrocytes captured from fetal white matter compared to those from germinal matrix of fetal brains. The most significant difference was found for miR-449. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

of these miRNAs, the difference in expression of miR-449 was most significant.

Comparison of miRNA Expression Between Astrocytes in Adult Inter-Laminar Region and Those in Fetal Germinal Matrix

miRNAs -129, -181a, -210, -214 and -365 were detected in germinal matrix astrocytes but not in inter-laminar astrocytes (Fig. 6A). In addition, the astrocytes in the germinal matrix showed a higher expression of miRNAs -34a, -124a, 320 and -338 compared to adult inter-laminar astrocytes (Fig. 6B). In contrast, adult inter-laminar astrocytes had a higher expression of miRNAs -29a, -143, -146a and -155 compared to germinal matrix astrocytes (Fig. 6C).

DISCUSSION

Our study is the first to demonstrate differences in miRNA expression profiles of astrocytes specifically and individually isolated by LCM from different regions of normal adult and fetal human brains. This approach circumvents limitations of studies analyzing miRNA expression from whole tissue samples and *in vitro* studies. In whole tissue samples, the specific cell type represents only part of the overall tissue composition. Two previous reports have focused on the miRNA profiles of adult human astrocytes. The first report detected miR-155 in astrocytes captured from multiple sclerosis (MS) lesions (16), and the second was an *in vitro* study on miR-125b using commercially available frozen human astrocytes (17). In the present study, we used GFAP immunoreactivity to define astrocytes *in situ* using FFPE tissue sections from cases without specific recognizable pathologies to extract positively identified cells. The stability of miRNAs permits efficient expression profiling even from FFPE tissue samples (18, 19). A possible limitation of this strategy could be that some astrocytes, particularly those under non-activated conditions, may not have expressed detectable levels of GFAP. Similar limitations also apply to other markers such as aldehyde dehydrogenase 1 and glutamine synthetase.

In all of our mid-second trimester fetal samples, we detected and isolated GFAP-positive cells in developing white matter (Fig. 1D, F) and germinal matrix (Fig. 1D, E); however, as expected, few GFAP-positive cells were identified in developing grey matter (20, 21). In the human fetal brain, it has been previously noted that GFAP-positive cells start appearing in the internal capsule and thalamus by 14 weeks, in the ependyma of the frontal lobe by 14 to19 weeks, and in the germinal matrix starting at 17 weeks (7, 22–24). Compared to astrocytes in the adult samples, the cell bodies in the fetal samples were significantly smaller in all regions, likely reflecting the requirements for increased mobility of astrocytes during development.

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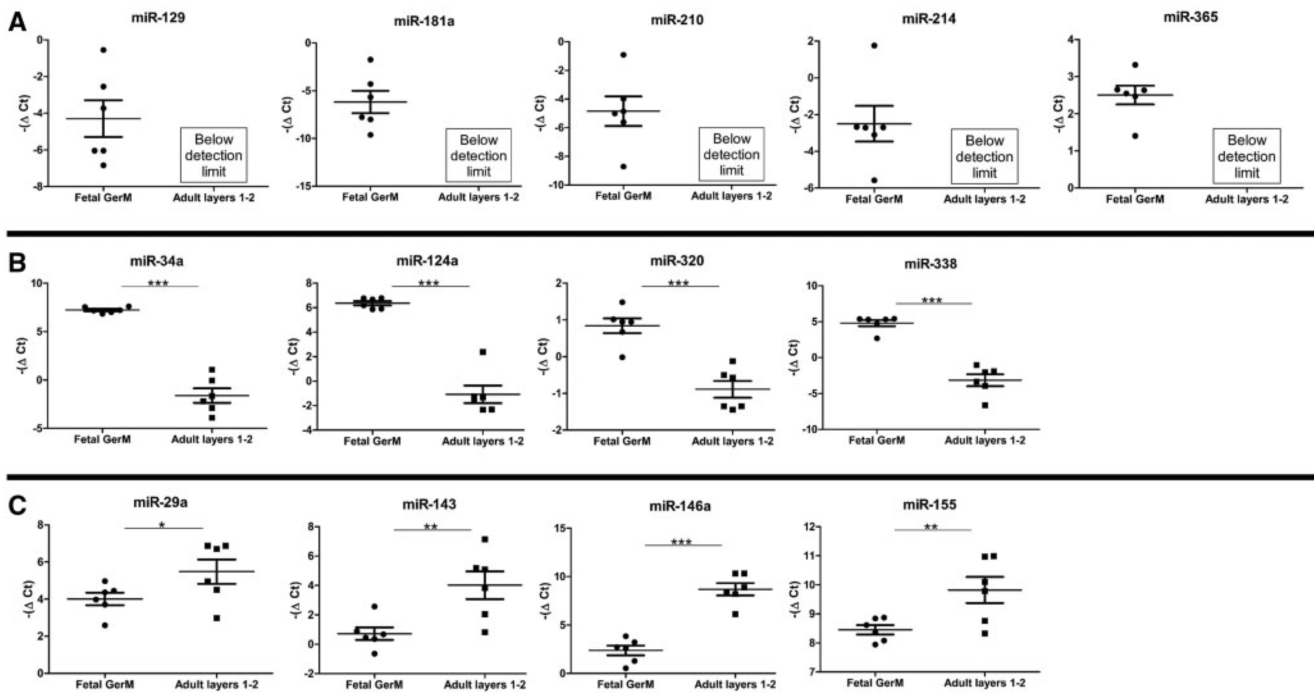

FIGURE 6. MicroRNA (miRNA) expression profiles in astrocytes captured from germinal matrix of fetal brains compared to inter-laminar (layers 1-2) astrocytes of adult brains. **(A)** miRNAs not detected in astrocytes captured from layers 1-2. **(B)** miRNAs more highly expressed in fetal germinal matrix astrocytes. **(C)** miRNAs more highly expressed in astrocytes captured from layers 1-2. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

We based our selection of the 20 miRNAs used in this study on their known functional role in astrocytes because they were known to be enriched in astrocytes, or for their predicted or proven roles in regulating inflammatory or neuroregenerative responses, which may play major roles in normal surveillance and in disease (Table). Many of the miRNAs showed little variation of expression from one area to another (Fig. 2A), suggesting (as would be expected) that there are functions common to all astrocytes. However, for those that showed significant regional variation (Fig. 7), we can infer some individual functional characteristics specific to the various regions.

Our studies have shown that the inter-laminar astrocytes (Fig. 1A) appear to have a unique overall miRNA profile, in addition to their specialized morphologic features. In contrast to astrocytes in the deeper cortical layers and in the white matter of the adult, miRNAs -129, -181a, -210, -214 and -365 were undetectable in the inter-laminar astrocytes. In addition, miRNAs -34a, -124a, -338, -320, and -449 were expressed significantly lower in the inter-laminar astrocytes than in the other 2 adult regions. As indicated in the Table, these miRNAs have important roles in cell proliferation, development, migration, and angiogenesis, as well as in both pro- and anti-inflammatory processes. Overexpression of miR-34a under pharmacological activation of SIRT1 (Sirtuin 1) in mouse neural stem cells promoted astrogliogenesis (25). The lower expression of miR-34a in the inter-laminar layer could contribute to the stability of these cells, which have lower proliferative properties than the other regions. The inter-laminar cells have a lower expression of miR-124a, which regulates EAAT2 (excitatory amino acid transporter 2, rodent analog GLT1) protein (26). Glutamate transporters are critical for homeostasis of neurons and axons; and the low levels of miR-124a probably reflect the relative paucity of neurons in this anatomic location. In agreement with our results, miR-338 is reported to be less highly expressed in grey matter than white matter; its function is thought to enhance pro-inflammatory responses (Table) (27). The lower expression of miR-320, a negative regulator of aquaporin-4 (28), may account for the very prominent staining of this protein in the glial limitans (29). This would contribute to the observed increased aquaporin expression in the cell processes of the glial limitans. MiR-449 is essential for proper brain development (30), probably involving multiple cell processes such as proliferation and migration by ensuring efficient microtubular function; reduced expression of miR-449, a modulator of microtubule dynamics, may contribute to the architecture of this region.

In contrast, miRNAs -125b, -143, and -146a were enriched in inter-laminar astrocytes compared to those in the deeper layers and white matter. MiR-125b has been previously described as a brain enriched miRNA (31), which is implicated in astrogliosis based on *in vitro* studies (17). Inter-laminar astrocytes have enhanced expression of miR-125b, which is a modulator of GFAP expression. The increased expression of miR-146a, an anti-inflammatory miRNA (32), coincides with reduced expression of the pro-inflammatory miRNAs -338 and -365. The potential importance of this finding in explaining the lower level of inflammation in the grey matter of MS patients is noted below. MiR-143 acts as a tumor suppressor in glioma cells (33). Our data overall suggest that the inter-laminar cells

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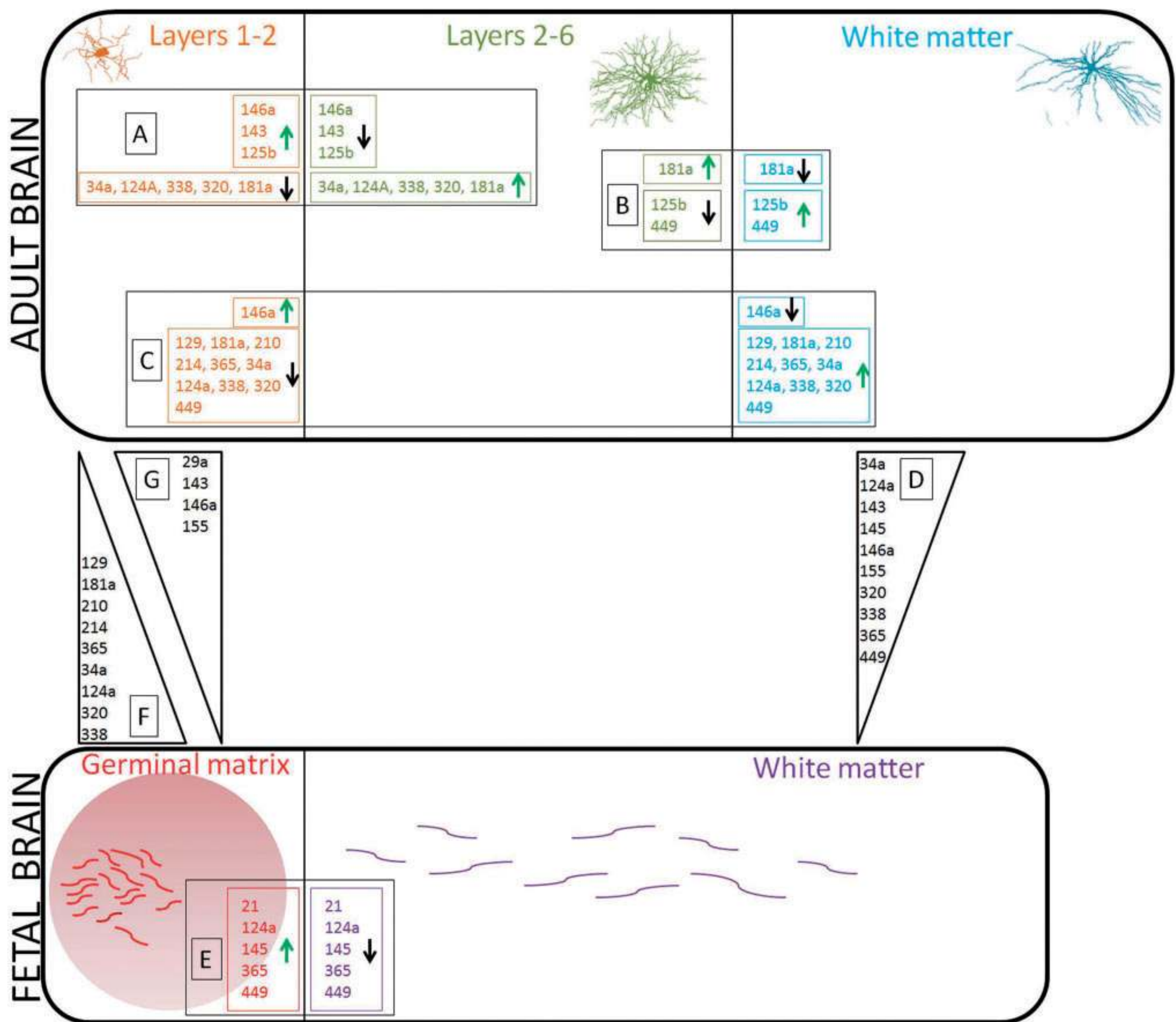

FIGURE 7. A cartoon summarizing the overall picture of all the significant microRNA (miRNA) expression differences in astrocytes captured from different regions of adult and fetal brains. Different regions from which the astrocytes were separately captured for comparison are depicted in different colors. Layers 1-2, layers 2-6 and white matter astrocytes are represented in amber, green and blue colors respectively in the adult brain. Germinal matrix and white matter astrocytes are represented in red and purple colors respectively in the fetal brain. **(A)** miRNAs differentially expressed between astrocytes captured from layers 1-2 and layers 2-6 of the adult brain. **(B)** miRNAs differentially expressed between astrocytes captured from layers 2-6 and the white matter of the adult brain. **(C)** miRNAs differentially expressed between astrocytes captured from layers 1-2 and the white matter of the adult brain. **(D)** miRNAs more highly expressed in white matter astrocytes captured from adult brain compared to those from fetal brain. **(E)** miRNAs differentially expressed between astrocytes captured from the germinal matrix region and the white matter of the fetal brain. **(F)** miRNAs more highly expressed in germinal matrix astrocytes compared to those captured from layers 1-2 of the adult brain. **(G)** miRNAs more highly expressed in astrocytes from layers 1-2 of the adult brain compared to those captured from germinal matrix.

represent a more stable population of astrocytes, with less capacity for cell proliferation, apoptosis and angiogenesis, and very little role in neuron-axon metabolic homeostasis In addition to other functions that these cells may have, miRNA expression results suggest that they act as a first-line of defense against substances or agents entering the CNS from the cerebrospinal fluid.

Astrocytes in the deep cortical layers and the white matter showed similar expression of numerous miRNAs, suggesting that their functions, in spite of the anatomic differences, were also similar (Fig. 3). However, there are some differences between the 2 regions. miR-181a was more highly expressed in deeper grey matter astrocytes compared to those from white

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matter. This may be of significance in helping to understand the relatively lesser extent of inflammatory activity of grey matter lesions in MS compared with those of the white matter (34). In contrast to our findings using laser-captured astrocytes, miR-181a was upregulated in the white matter in a study that used whole tissue samples (27). Such results may be attributed to the expression of this miRNA in cells other than astrocytes, including oligodendrocytes or neurons at the grey and white matter border. MiR-125b remained undetected in deeper grey matter astrocytes, whereas white matter astrocytes expressed this miRNA. Similarly, deeper grey matter astrocytes had lower expression of miR-449 compared to white matter astrocytes. As discussed above, both miRNAs -125b (17) and -449 (30) are relevant in the context of structural functions in astrocytes. Higher expression of these miRNAs in white matter astrocytes may perhaps be an indication of more pronounced structural requirements/demands of this region.

We found that miRNAs -146a, -155, -365, -338, -145, -124a, -320, -143, -34a and -449 were more highly expressed in adult white matter astrocytes compared to fetal astrocytes (Fig. 4). The functional roles of miR-146a (anti-inflammatory) and miR-155 (pro-inflammatory) have been previously characterized in astrocytes *in vitro*. MiR-146a is an inflammation-resolving miRNA and targets IRAK-1 (interleukin-1 receptor-associated kinase-1) mRNA (32); miR-155 is a pro-inflammatory molecule that targets SOCS-1 (suppressor of cytokine signaling-1) mRNA (35). MiR-365 is a key inflammation mediator that directly targets mouse IL-6 mRNA and represses the expression of IL-6 protein in microglia (36). MiR-338 is expressed in astrocytes, is upregulated in MS patient tissue and CNS tissue of animals with experimental autoimmune encephalomyelitis, and has been postulated to be pro-inflammatory (37). The low expression of pro- and anti-inflammatory miRNAs in the fetal white matter may explain the poor response of the white matter to injury seen in fetal brains (5). In rodent spinal cord astrocyte cultures, overexpression of miR-145 has been shown to be a negative regulator of astrocytic size, morphology, and migratory behaviors (38). Lower expression of miR-145 in fetal white matter astrocytes may contribute to increased migratory abilities of these cells during development. MiR-124, the regulator of GLT1 protein (26), plays an important role in tissue homeostasis, as described above (Table). The low levels of expression of miR-124 in micro-dissected fetal astrocytes is consistent with reports that GLT1 is not detectable in human fetal astrocyte cultures (39) but is detected in human adult brain-derived astrocytes in culture (40). The functional relevance of miRNAs -320, -143, -34a and -449 has been described above and in the Table. No miRNAs were found to be more highly expressed in the fetal white matter than in the adult white matter. This finding is perhaps not unexpected in that the astrocytes in the fetal white matter are migrating and have not yet established fixed anatomical and functional relationships with the axons, neurons, blood vessels or the sub-pial brain tissue.

Compared to astrocytes in the fetal white matter, a number of miRNAs were upregulated in the fetal germinal matrix, including miRNAs -21, -145, -124a, -365 and -449 (Fig. 5). In contrast to the fetal white matter, which contains large numbers of migrating astrocytes, the germinal matrix is a highly active tissue with proliferating and differentiating cells, leading to gliogenesis and neurogenesis (41). We speculate that these miRNAs may also participate in the reaction that occurs in this region in response to systemic infection in premature infants (42). MiR-21 has been associated with properties related to astrocytic scarring *in vitro* (43); this is a structural function. The early development of GFAP-positive radial glial astrocytes provides scaffolding for migration of other cell types (5). There is, however, a cell culture-based study using rodent astrocytes that showed that miR-21 negatively regulates GFAP expression and cell size (44). The functional findings of increased miR-145 are contradictory. Although migration in the white matter could be explained by lower levels, the decrease in proliferation in the germinal matrix is puzzling (Table). At present, we have no explanation for this but future studies may provide clarification. The functional relevance of miRNAs -124a, -365 and -449 have been described above and are also noted in the Table. All of these miRNAs would be required for the successful proliferative and protective properties of a highly active developing area. The cellular modalities underlying inflammation and development are often common to both.

We also compared the miRNA profiles of the adult inter-laminar astrocytes with those of fetal germinal matrix layer astrocytes since both these cells form radial glial fibers and provide structural support. The inter-laminar cells provide processes subtending the glial limitans and confer some rigidity, whereas the radial processes of the germinal matrix astrocyte provide structural support for cell migration. miRNAs 129, -181a, -210, -214 and -365 were only detected in the germinal matrix astrocytes. The expression of miRNAs -129, -210 and -214 in the germinal matrix is not unexpected because they have anti-apoptotic and neurogenic/proliferative functions (Table). miRNAs -181a and -365 are anti- and pro-inflammatory, respectively, and have been discussed above. miRNAs -34a, -124a, -320 and -338 were detected with lower expression in cells from the inter-laminar region compared to the fetal germinal matrix. Higher expression of these miRNAs in the germinal matrix confirms the dynamic nature of this region (Table). miRNAs -29a, -143, -146a and -155 all had higher expression in inter-laminar astrocytes than the germinal matrix. miR-29a has a cell protective function demonstrated *in vitro* (45). There is no obvious reason why this miRNA should be more highly expressed in the inter-laminar cells than those in the germinal matrix. The anti-proliferative function of miR-143 can be related to the stable nature of inter-laminar astrocytes; miRNAs -146a and -155 are inflammation-related (Table).

The results of PCA and hierarchical clustering analyses show an overall pattern of similarity between the multiple dynamic functional activities of the astrocytes in the deeper cortex, the white matter, and the fetal germinal matrix. In contrast, the astrocytes from the inter-laminar layer and the fetal white matter, although differing greatly from each other, appear to have more restricted functional roles (Fig. 2B, D). The PCA was performed using 2 different statistical analysis tools: the NIA array tool (Fig. 2B, D) and with JMP® software (data not shown); we obtained the same results from these analyses using 2 different tools.

Overall, the present study suggests a wide variation of miRNAs governing the functional properties of astrocytes, which

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are dependent to a large degree on age and anatomical location (Fig. 7). Increasing insights into the astrocytic functions of distinct miRNAs will provide a greater opportunity to determine their roles in disease, as well as to devise therapeutic agents to target and alter pathological processes.

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