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MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47

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We established microRNA profiles from active and inactive multiple sclerosis lesions. Using laser capture microdissection from multiple sclerosis lesions to pool single cells and *in vitro* cultures, we assigned differentially expressed microRNA to specific cell types. Astrocytes contained all 10 microRNA that were most strongly upregulated in active multiple sclerosis lesions, including microRNA-155, which is known to modulate immune responses in different ways but so far had not been assigned to central nervous system resident cells. MicroRNA-155 was expressed in human astrocytes *in situ*, and further induced with cytokines in human astrocytes *in vitro*. This was confirmed with astrocyte cultures from microRNA-155-l-*lacZ* mice. We matched microRNA upregulated in phagocytically active multiple sclerosis lesions with downregulated protein coding transcripts. This converged on CD47, which functions as a 'don't eat me' signal inhibiting macrophage activity. Three microRNA upregulated in active multiple sclerosis lesions (microRNA-34a, microRNA-155 and microRNA-326) targeted the 3′-untranslated region of CD47 in reporter assays, with microRNA-155 even at two distinct sites. Our findings suggest that microRNA dysregulated in multiple sclerosis lesions reduce CD47 in brain resident cells, releasing macrophages from inhibitory control, thereby promoting phagocytosis of myelin. This mechanism may have broad implications for microRNA-regulated macrophage activation in inflammatory diseases.

Keywords: multiple sclerosis; microRNAs; CD47; autoimmunity; inflammation

Abbreviations: CT = cycle threshold; GFAP = glial fibrillary acidic protein; LCM = laser capture microdissection; miRNA = micro-ribonucleic acid; qPCR = quantitative polymerase chain reaction; SIRP-α = signal regulatory protein; UTR = untranslated region

Introduction

A few hundred microRNAs (miRNAs) post-transcriptionally regulate the expression of about one-third of all protein-coding genes (Lewis *et al.*, 2005). miRNAs recognize partially complementary target sequences in cognate mRNAs and either destabilize their mRNA targets or inhibit protein translation. A single miRNA can regulate the expression of hundreds of target genes (Baek *et al.*, 2008; Selbach *et al.*, 2008). miRNA-mediated gene regulation is critical during development and adulthood, e.g. by regulating and

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maintaining functions of the immune (Taganov *et al.*, 2007; Lodish *et al.*, 2008) and nervous (Kosik, 2006) systems. Although the quantitative impact of miRNA 'fine tuning' on gene expression levels is relatively small, miRNAs are nevertheless implicated in the pathogenesis of different diseases, including cancer, cardiac failure and neurodegenerative diseases (Papagiannakopoulos and Kosik, 2008; Thum *et al.*, 2008; Hebert and De, 2009; Visone and Croce, 2009). Moreover, miRNAs represent promising novel targets for therapy (Krutzfeldt *et al.*, 2005; Czech, 2006; Elmen *et al.*, 2008).

In this study, we set out to identify miRNA profiles of multiple sclerosis lesions. Multiple sclerosis is a chronic disease of the CNS, characterized by inflammation and demyelination. A complex interplay between brain-invading immune cells and CNS resident cells determines lesion development (Fugger *et al.*, 2009; Goverman, 2009; Steinman, 2009). Activation of macrophages/microglia plays a central role in the effector phase of myelin breakdown (Sospedra and Martin, 2005; Trapp and Nave, 2008; Steinman, 2009). In previous investigations, unbiased approaches starting from the transcriptome (Lock *et al.*, 2002) or proteome (Han *et al.*, 2008) of multiple sclerosis lesions provided important new insights into the pathogenesis and yielded potential targets for therapy. Here we asked whether analysis of miRNA profiles in active and chronic inactive multiple sclerosis lesions could (i) link miRNAs to specific genes that are dysregulated in multiple sclerosis lesions; (ii) identify novel miRNA targets; and (iii) provide new mechanisms regarding the contribution of tissue-resident cells in the disease process.

Materials and methods

Tissue specimens

Twenty-one tissue blocks from 20 different multiple sclerosis patients and nine tissue blocks from nine subjects without any known neurological disease were used for miRNA analyses (Supplementary Table S1). These samples included four frozen active, 12 formalin-fixed, paraffin embedded active, one formalin-fixed, paraffin embedded inactive and four frozen inactive multiple sclerosis lesions. From control subjects, four frozen white matter and five formalin-fixed, paraffin embedded white matter specimens were used (Supplementary Table S1). Additionally, RNA from four active lesions was used for profiling of protein-coding transcripts. The study was approved by the Institutional Review Board of the Ludwig-Maximilians University, Munich.

Histology

Sections were stained with haematoxylin and eosin, luxol fast blue for myelin and Oil Red O for neutral lipids (frozen sections only). Antibodies against CD4, CD8, CD20, CD47, CD68 and glial fibrillary acidic protein (GFAP) (Supplementary Table S2) were used for immune staining, along with standard peroxidase procedures (DAKO, Hamburg, Germany) using diaminobenzidine as substrate. Double staining for proteolipoprotein and CD47 or GFAP and CD47 were performed with polyclonal rabbit antibodies to proteolipoprotein or GFAP and the mouse monoclonal antibody to CD47 LS-B1959 (Supplementary Table S2). Confocal microscopy was done with a Leica SP-2 microscope.

Tissue blocks were classified according to defined criteria: active lesions contained abundant macrophages with early (luxol fast blue and Oil Red O positive) myelin degradation products, either throughout the whole lesion or in a broad rim at the lesion edge. Inactive demyelinated lesions were sharply demarcated from the periplaque white matter, lacked a rim of microglia activation and were devoid of luxol fast blue- or Oil Red O-reactive myelin degradation products in immune cells.

Dissection of multiple sclerosis lesions, laser capture microdissection, RNA extraction and quantitative PCR

Frozen and paraffin sections were mounted on membrane-covered polyethylene naphthalate slides (Zeiss, Jena, Germany). Parallel sections were stained with luxol fast blue to allow identification of the lesions. For the analysis of miRNA profiles, white matter multiple sclerosis lesions or control white matter specimens were dissected from the slides with a scalpel and 10–15 sections were pooled for RNA extraction. This miRNA analysis was restricted to white matter tissue samples to limit possible confounding effects of neuronal miRNAs. Formalin-fixed, paraffin embedded tissue was deparaffinized and digested with proteinase K (Sigma-Aldrich, Steinheim, Germany) before RNA extraction.

Laser capture microdissection (LCM) was used to assign miRNAs to certain cell types using quick immunoflourescence staining as described previously (Junker *et al.*, 2007; for antibodies see Supplementary Table S2). RNA from 100 pooled LCM cells per sample was prepared from eight different sections. The miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used for macrodissected lesions and the RNeasy Micro Kit (Qiagen) for LCM cells. miRNAs were transcribed using the TaqMan® miRNA Reverse Transcription Kit and miRNA-specific stem-looped primers (Applied Biosystems, Darmstadt, Germany). These stem-looped primers bind to the miRNAs and generate a longer cDNA transcript in the RT reaction, which then can easily be amplified in a normal quantitative (q) PCR reaction. For analysis of RNA representing protein coding transcripts, random hexamer primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) were used for cDNA synthesis.

The qPCR was performed on the ABI 7900 (Applied Biosystems) using the qPCR Core Kit and uracyl *N*-glycosylase (both from Eurogentec, Cologne, Germany). miRNAs were detected with single TaqMan® miRNA Assays (Applied Biosystems) and with Taqman Low Density miRNA Arrays (TaqMan® Human MiRNA Array v1.0; Early Access; Applied Biosystems), which contains 365 different human miRNA assays. The total amount of transcribed RNA equivalent used per PCR reaction was 0.2–1 ng for miRNAs. Different methods were used for normalization of the measurements. For analysis of Low Density miRNA Arrays (Applied Biosystems), the median of the most abundant 43 miRNAs (43 miRNAs could be detected with a raw cycle threshold (CT) <30 in all samples) was used as a surrogate housekeeping gene. The relative expression of miRNAs in multiple sclerosis lesions versus control tissue was calculated with the $\Delta\Delta$CT method [$\Delta\Delta$CT = (median of CT miRNA multiple sclerosis lesions minus median of most abundant 43 miRNAs in the group of multiple sclerosis lesions) minus (CT miRNA control minus median of most abundant 43 miRNAs of group of controls)]. For the analysis of LCM material and cultured cells, single miRNA Taqman Assays (Applied Biosystems) were used,

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and the small nuclear RNA RNU6B was used for normalization in the $\Delta\Delta$CT method (Ng *et al.,* 2009).

Both frozen and formalin-fixed, paraffin embedded tissue specimens were used to quantify miRNAs by qPCR. We noted that the miRNA transcripts could be amplified with a comparable level from frozen and formalin-fixed, paraffin embedded tissue: the median of the most abundant (CT $<$ 30) 43 miRNAs was 24.0 in the frozen and 25.4 in the formalin-fixed, paraffin embedded tissue specimens, and the calculated $\Delta\Delta$CT was very similar in frozen and formalin-fixed, paraffin embedded tissue, which is in agreement with Doleshal *et al*. (2008). Therefore miRNA levels obtained from formalin-fixed, paraffin embedded and frozen tissue samples of the same lesion type or control tissue were placed into one group for statistical analyses.

The expression levels of 528 genes (519 genes of interest and nine slots for housekeeping genes) were determined by qPCR (custom-made low density arrays; Applied Biosystems). The 519 selected genes were related to immune function, myelin biology, extracellular matrix and neurotrophic factors.

Cell culture

Monocytes and T cells were isolated from peripheral blood mononuclear cells by negative isolation (MACS, Miltenyi, Bergisch Gladbach, Germany). Human astrocytes of embryonic origin (Krumbholz *et al.,* 2005) were used after the third or fourth passage. Owing to their embryonic origin, these astrocyte cultures are devoid of microglial cells or macrophages (Aloisi *et al.,* 1992; Krumbholz *et al.,* 2005). All cells were cultured in Roswell Park Memorial Institute medium supplemented with 5–20% foetal calf serum (PAN Biotech, Aidenbach and Biochrom, Berlin, Germany). In some experiments, human cells were stimulated overnight with gamma-interferon (0.1 U/μl; Roche, Diagnostics GmbH, Mannheim, Germany), interleukin-1β (0.05 μg/ml; R&D, Minneapolis, MN, USA), transforming growth factor-β1 (1 ng/ml; R&D) or tumour necrosis factor-α (0.01 μg/ml; R&D).

Luciferase assays for analysing miRNA targeting

Oligonucleotides of 50 base pair length (Metabion, Martinsried, Germany) containing specific miRNA binding sites (sequences shown in Supplementary Table S3) were cloned into the 3′ untranslated region (UTR) of luciferase in a reporter plasmid (pMIR-REPORT™ miRNA Expression Reporter Vector System Ambion/Applied Biosystems).

Pre-miRs™ or anti-miRs™ (Ambion/Applied Biosystems) were transfected into HeLa cells with Lipofectamin® 2000 (Invitrogen, Karlsruhe, Germany) along with the luciferase plasmid containing the predicted binding site of the respective miRNA. A control plasmid coding for β-galactosidase without any known miRNA binding site in its 3′UTR was used for normalization of the luciferase signal; 200 ng of each plasmid and 25 nmol pre- or anti-miR were applied to transfect 8×10^4 HeLa cells. Pre-miR control-1 (Ambion/Applied Biosystems) with a scrambled nucleotide sequence was used as a control.

Luciferase and β-galactosidase were measured 24 h after triple transfection (see above) using the Dual Light Luciferase Assay from Applied Biosystems and the Victor Luminometer from Perkin Elmer.

miRNA-155-deficient astrocytes

B6.Cg-Mirn155tm1.1Rsky/J mice (Thai *et al.,* 2007) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). In these miR-155 knockout mice, the *lac*Z reporter allows detection of bic/miR-155 promoter transcriptional activity. Astrocyte cultures from these mice were established essentially as described (Sasaki *et al.,* 1989). These astrocyte cultures were stimulated with murine tumour necrosis factor-α (10 ng/ml, PeProtech, Hamburg, Germany) for 24 h and bic/miR-155 promotor activity was detected with a β-galactosidase staining kit (Active Motif, Rixensart, Belgium). GFAP staining of cultured astrocytes was performed with GFAP antibody Z0334 (DAKO), which had been previously labelled with the Cy3-mAb Labelling Kit (GE, Amersham).

All animal procedures used in this report were in accordance with guidelines of the committee on animals of the Max Planck Institute for Neurobiology and with the license of the Regierung von Oberbayern (Munich, Germany).

Statistical analyses

For statistical analysis, we used the Mann–Whitney U-test. The significance levels are indicated in the individual figures and tables.

Results

miRNA pattern in different multiple sclerosis lesions

To identify miRNA profiles of multiple sclerosis lesions we quantified the expression level of 365 different mature miRNAs by qPCR in 16 active and 5 inactive white matter multiple sclerosis brain lesions and in 9 control white matter specimens. One hundred and sixty-seven miRNAs were detectable in all groups of examined specimens with a raw CT-value $<$35.

We considered abundance (CT $<$ 35), amount of regulation (at least 2-fold) and significance ($P < 0.01$; U-test) to establish miRNA signatures of the different types of multiple sclerosis lesions. Using these criteria, in active lesions 20 miRNAs were at least twice more abundant and 8 miRNAs at least twice less abundant than in normal white matter (Table 1). In inactive lesions, 22 miRNAs were at least twice more abundant and 13 miRNAs at least twice less abundant than in normal white matter (Table 1). The complete data set of all miRNAs expressed in the examined brain specimens is given in Supplementary Table S4.

Eight of our active multiple sclerosis brain specimens derive from multiple sclerosis cases with a very fulminant disease course called Marburg's variant. We analysed the group of Marburg's and non-Marburg's variant separately (Supplementary Table S5). We noted that the 28 most prominently regulated miRNAs ($P < 0.01$ in the entire group) were regulated in the same direction in both groups of active lesions. Eight of the 10 miRNAs upregulated the most in the entire group of active lesions were significantly upregulated in both Marburg and non-Marburg cases. Some miRNAs were more prominently regulated in the Marburg variant than in the other active lesions. This probably reflects the more intense tissue destruction in the Marburg variant.

Among the significantly altered miRNAs, some showed a differential regulation in active versus inactive lesions, whereas others were modified in the same direction (details in Fig. 1A and Table 1). As one example, details of the expression of miRNA-155, which we further analysed in detail (see below), are shown in Fig. 1B.

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Table 1 miRNA profiles in active and inactive multiple sclerosis lesions

miRNAs upregulated in lesions[a]	Percent surrogate housekeeping gene[b] in lesions	Fold regulation in lesions compared to normal brain white matter[c]	miRNAs downregulated in lesions[a]	Percent surrogate housekeeping gene[b] in lesions	Fold regulation in lesions compared to normal brain white matter[c]
miRNA profiles in active multiple sclerosis lesions					
miR-650	5.7	15.1**	miR-656	0.2	0.15**
miR-155	37.1	11.9**	miR-184	0.9	0.21**
miR-326	1.5	8.9**	miR-139	1.1	0.36**
miR-142-3p	68.5	7.7**	miR-23b	16.9	0.37**
miR-146a	73.7	6.4**	miR-328	34.1	0.46**
miR-146b	50.4	5.1**	miR-487b	4.7	0.46**
miR-34a	9.3	4.9**	miR-181c	2.1	0.48**
miR-21	82.7	3.9**	miR-340	7.2	0.50**
miR-23a	1.4	3.9**			
miR-199a	1.2	3.3**			
miR-27a	88.3	3.1**			
miR-142-5p	8.3	3.0**			
miR-193a	10.5	2.9**			
miR-15a	12.3	2.8**			
miR-200c	2.3	2.8**			
miR-130a	9.8	2.6**			
miR-223	167.9	2.4**			
miR-22	25.1	2.4**			
miR-320	49.2	2.2**			
miR-214	2.5	2.1**			
miRNA profiles in inactive multiple sclerosis lesions					
miR-629	1.4	10.1**	miR-219	0.9	0.02**
miR-148a	16.1	9.8**	miR-338	1.1	0.04**
miR-23a	2.9	8.8**	miR-642	0.3	0.06**
miR-28	15.4	6.9**	miR-181b	55.0	0.13**
miR-195	214.9	5.0**	miR-18a	0.7	0.14**
miR-497	11.2	4.8**	miR-340	3.9	0.15**
miR-214	4.5	4.3**	miR-190	0.5	0.16**
miR-130a	16.0	4.2**	miR-213	1.4	0.22**
miR-135a	37.8	3.7**	miR-330	3.5	0.24**
miR-204	127.0	3.2**	miR-181d	22.0	0.32**
miR-200c	2.7	3.1**	miR-151	20.7	0.37**
miR-660	32.5	3.1**	miR-23b	15.4	0.37**
miR-152	54.4	3.1**	miR-140	52.6	0.50**
miR-30a-5p	424.8	3.0**			
miR-30a-3p	73.7	3.0**			
miR-365	37.0	2.9**			
miR-532	16.2	2.9**			
miR-126	288.0	2.4**			
let7c	136.2	2.4**			
miR-20b	7.0	2.4**			
miR-30d	67.0	2.3**			
miR-9	239.8	2.2**			

a Those miRNAs are listed, which were at least 2-fold and significantly (**$P < 0.01$; U-test) regulated in active or inactive lesions in comparison to normal brain.
b Surrogate housekeeping gene: the median of the 43 most abundant miRNAs (CT < 30).
c Sixteen active lesions, five inactive lesions and nine control white matter specimens were examined.

Assignment of regulated miRNAs to specific cell types

In principle, these altered miRNA profiles might reflect the presence of infiltrating immune cells, changes in brain resident cells, or both. To assign the differentially expressed miRNAs to specific cell types, we performed three types of experiments. First, we analysed cultured human astrocytes, T cells and monocytes *in vitro*. Second, we used LCM to isolate CD68+ macrophages/microglia, CD8+ T cells, CD20+ B cells and GFAP+ astrocytes from human brain tissue. And

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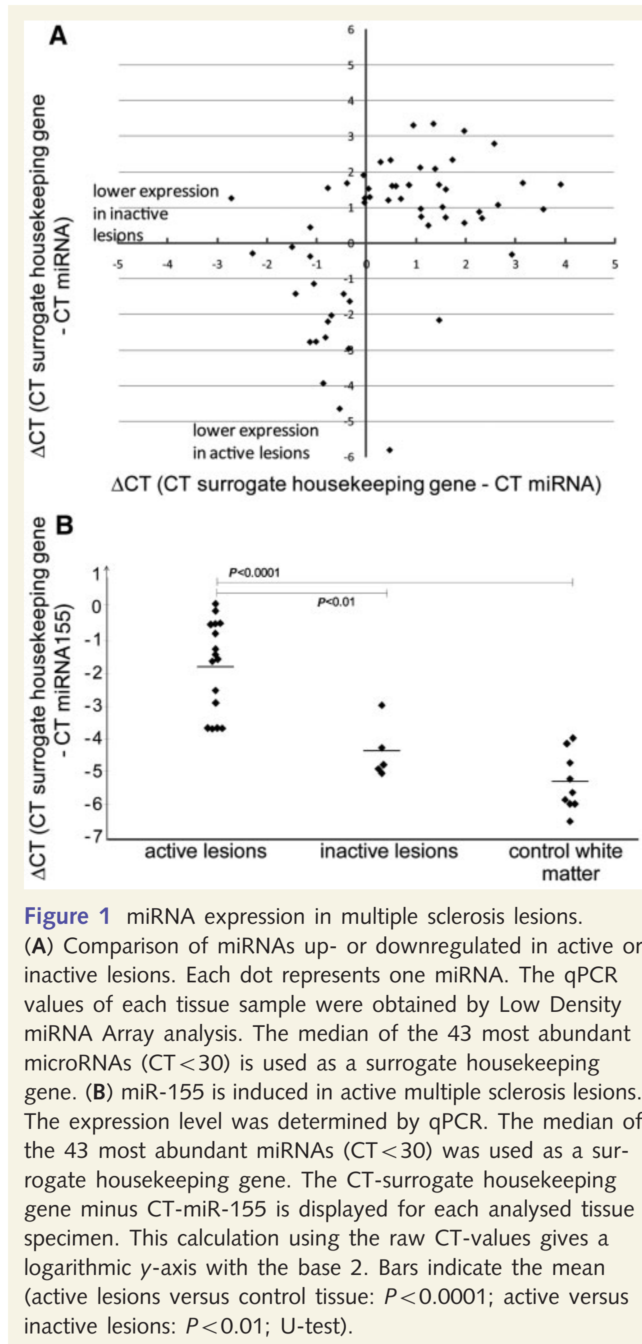

Figure 1 miRNA expression in multiple sclerosis lesions. (**A**) Comparison of miRNAs up- or downregulated in active or inactive lesions. Each dot represents one miRNA. The qPCR values of each tissue sample were obtained by Low Density miRNA Array analysis. The median of the 43 most abundant microRNAs ($CT < 30$) is used as a surrogate housekeeping gene. (**B**) miR-155 is induced in active multiple sclerosis lesions. The expression level was determined by qPCR. The median of the 43 most abundant miRNAs ($CT < 30$) was used as a surrogate housekeeping gene. The CT-surrogate housekeeping gene minus CT-miR-155 is displayed for each analysed tissue specimen. This calculation using the raw CT-values gives a logarithmic y-axis with the base 2. Bars indicate the mean (active lesions versus control tissue: $P < 0.0001$; active versus inactive lesions: $P < 0.01$; U-test).

third, we investigated the expression of miR-155, which we found to be particularly relevant for this study, in brain-derived cells from miR-155 deficient mice, which have the *lac*Z reporter instead of miR-155 (Thai *et al.*, 2007).

Based on the miRNA profiling of multiple sclerosis lesions (Table 1), we selected 21 miRNAs, including the 10 miRNAs most strongly upregulated in active lesions, for expression analysis in cultured cells. The majority of the 21 miRNAs analysed were expressed in T cells, monocytes and astrocytes (Supplementary Table S6). Some miRNAs, however, were differentially expressed in the studied cell types; miR-130a and miR-30a-3p were most abundant in astrocytes, miR-23a, miR-199a# and miR-152 in monocytes, and miR-146b in T cells and cytokine-stimulated astrocytes. Notably, 18 out of 21 miRNAs, selected because they were induced in multiple sclerosis lesions, were detectable in cultured astrocytes (Supplementary Table S6).

We analysed how miRNA expression levels were regulated in astrocytes by inflammatory cytokines. We stimulated human astrocytes with interleukin-1β, tumour necrosis factor-α, gamma-interferon and transforming growth factor-β, and investigated the expression of 19 miRNAs. These included the 10 miRNAs most upregulated in active multiple sclerosis lesions and the miRNAs predicted to target CD47 (which were also upregulated in active lesions). In particular, miR-23a, miR-146a and miR-155 were strongly induced by the applied cytokines (Fig. 2).

We applied LCM to assign selected miRNAs to specific cell types that are prominent in active and inactive multiple sclerosis lesions. We dissected $CD8^+$, $GFAP^+$, $CD20^+$ and $CD68^+$ cells from multiple sclerosis tissue (four active and four inactive lesions). Eight cell pools, each consisting of 100 individually microdissected cells, were compiled from astrocytes, monocytes, $CD8^+$ cells and four pools of $CD20^+$ B cells. Using this material, we found that the two miRNAs most prominently upregulated in active multiple sclerosis lesions, miR-155 and miR-650, were present in microdissected astrocytes (miR-155 in Fig. 3a; data for miR-650 not shown), and $CD8^+$ T cells, B cells and $CD68^+$ macrophages (raw $CT < 33$ in microdissected cells; data not shown); miR-34a and miR-326, other regulators of CD47 (see below), were detected both in astrocytes and infiltrating immune cells, with a CT-value between 33 and 37 (data not shown).

Because miR-155 has been described so far in different immune cells (Rodriguez *et al.*, 2007; Thai *et al.*, 2007), but not in brain resident cells, we elaborated this point using mice expressing *lac*Z instead of miR-155 (Thai *et al.*, 2007). We established astrocyte cultures from these mice and observed co-localization of *lac*Z encoded β-galactosidase and GFAP, in particular after cytokine stimulation of the astrocytes (Fig. 3B and C).

CD47 as a shared target of miRNAs upregulated in multiple sclerosis lesions

Having identified several miRNAs that are upregulated in multiple sclerosis lesions, we examined whether the *in silico* predicted target transcripts of these miRNAs are actually downregulated in the lesions. Using qPCR we determined the expression levels of 519 selected targets, including immune- and myelin-related genes from eight active multiple sclerosis lesions (four of which had also been used for miRNA analysis), four inactive lesions and control white matter. We detected around 40 genes that were downregulated in the active lesions; among them was CD47.

Transcripts of CD47 were downregulated by ~50% in active lesions compared to control white matter (data not shown), which is consistent with a previous report (Koning *et al.*, 2007). In contrast, in inactive lesions, the CD47 expression level was similar to normal white matter as seen by qPCR (data not shown). Immunohistochemistry with three different monoclonal antibodies specific for CD47 showed a reduced expression in active multiple sclerosis lesions (Supplementary Fig. S1; data not shown). Chronic inactive lesions showed CD47 expression in their

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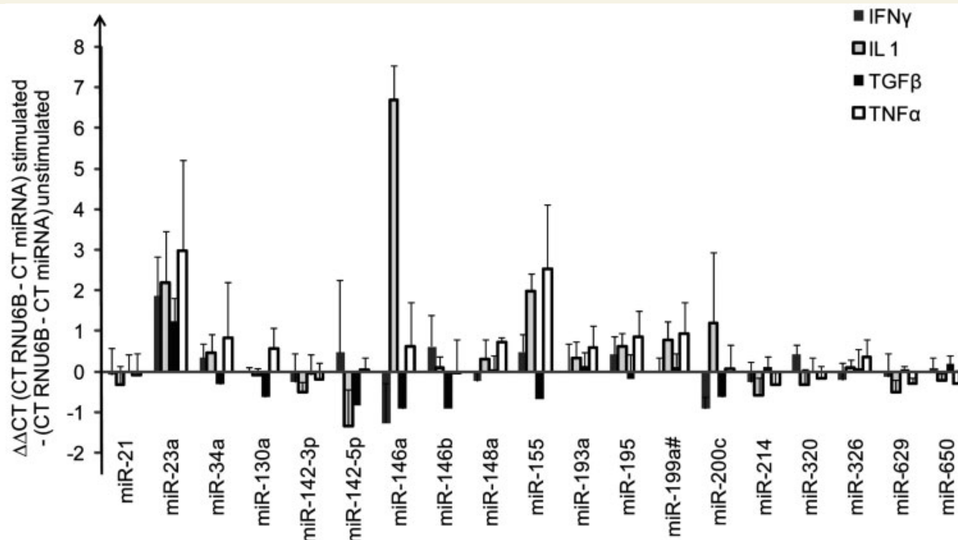

Figure 2 miRNA expression is regulated in astrocytes. Cultured human astrocytes were stimulated with interleukin (IL)-1, tumour necrosis factor (TNF)-α, gamma-interferon (IFN)-γ and transforming growth factor (TGF)-β for 24 h or left untreated. Subsequently the expression levels of the indicated microRNAs were determined by qPCR. The small nuclear RNA RNU6B was used as a housekeeping gene (Ng *et al.*, 2009) and the cytokine-induced regulation was calculated as ΔΔCT-value [ΔΔCT = (CT RNU6B minus CT miRNA of the stimulated samples) minus (CT RNU6B minus CT miRNA of the unstimulated samples)]. This calculation using the raw CT-values gives a logarithmic *y*-axis with the base 2. The mean ± SEM of 2–6 experiments is shown.

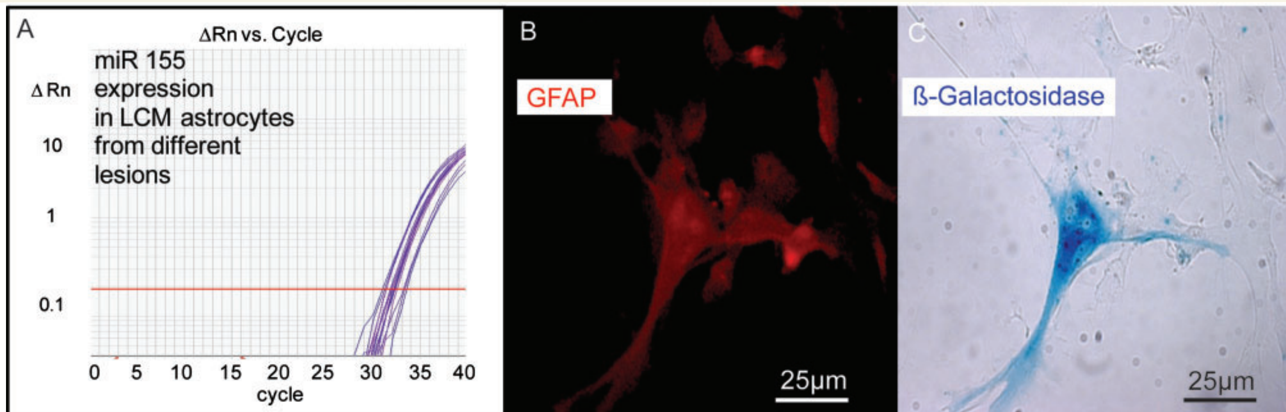

Figure 3 Astrocytes express miR-155. (**A**) GFAP+ astrocytes from multiple sclerosis lesions were obtained by LCM, 100 dissected cells from one lesion were pooled and analysed for miR-155 expression by qPCR. Depicted are the amplification curves in duplicates of dissected astrocyte samples from eight different multiple sclerosis lesions. (**B**, **C**) Cultured astrocytes from mice expressing the reporter *Lac*Z instead of miR-155 (Thai *et al.*, 2007) were stimulated with tumour necrosis factor-α for 24 h. The LacZ expression (**C**) co-localized with GFAP (**B**).

demyelinated lesion core (Supplementary Fig. S1). CD47 co-localized with both GFAP and proteolipoprotein (Supplementary Fig. S1), indicating that this broadly expressed protein is displayed by both astrocytes and oligodendrocytes.

We then searched the miRNAs induced in active lesions (Table 1) using Targetscan 5.0 (Lewis *et al.*, 2005; Grimson *et al.*, 2007) for highly conserved binding sites in the 3′UTR of these transcripts that were downregulated in active lesions. CD47 appeared to be most promising because Targetscan 5.0 detected two potential conserved and several unconserved miRNA binding sites in its 3′UTR assigned to those miRNAs that were most strongly upregulated in the active multiple sclerosis lesions (Supplementary Table S3).

Eight of the 20 miRNAs most upregulated in active multiple sclerosis lesions (Table 1) are predicted to target CD47 as assessed by Targetscan 5.0 (Lewis *et al.*, 2005; Grimson *et al.*, 2007), namely miR-155, miR-214, miR-34a, miR-21, miR-142-5p, miR-193a, miR-320 and miR-326. These eight miRNAs were tested in luciferase assays for targeting of the 3′UTR of CD47 (Fig. 4A and B). We found that miR-155 targeted both its highly conserved

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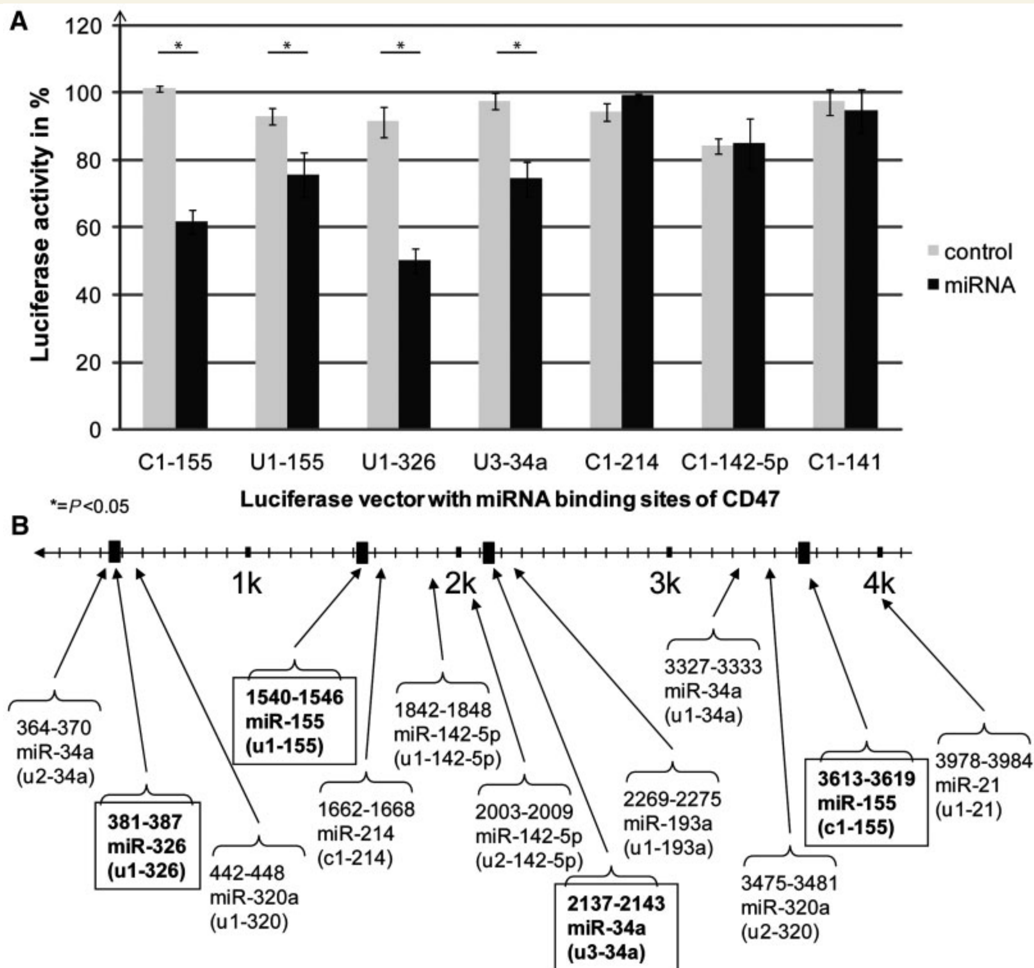

Figure 4 miRNAs target the 3′UTR of CD47. (**A**) The luciferase activity in HeLa cells transfected with the indicated luciferase vectors and the corresponding premiR or control miR (premiR with a scrambled RNA sequence) is shown. Details of the applied luciferase reporter vectors are in Supplementary Table S3. A control vector with β-galactosidase was used for normalization. Luciferase activity was divided by β-galactosidase activity and then normalized on a mock-transfected control. Luciferase measurements were done in quadruplicates in each individual experiment. The SEM of three to four experiments is shown. Significant differences between the scrambled control premiR and the premiRs predicted to target CD47 are indicated (U-test, $P < 0.05$). (**B**) 3′UTR of CD47 with predicted and proven binding sites of microRNAs upregulated in active multiple sclerosis lesions. MicroRNA target sites of human CD47 3′UTR NM_001025079 (length 4189) were predicted with Targetscan human (release 5.0: December 2008). Interaction of miR-34a, miR-155, miR-326 with their predicted target sites u1-326, c1-155, u1-155, u3-34a could be confirmed by luciferase assays (**A**) and are indicated with their position in CD47 3′UTR in the figure (**B**). Of each predicted binding site the first line indicates the position in 3′UTR of CD47. Thereby 'c' indicates a highly conserved binding site and 'u' indicates a less conserved binding site. The second line names the miRNA predicted to bind, and the third line is the designation of the luciferase vector that includes the binding site. Binding sites functional in the luciferase assay (**A**) are boxed.

and less conserved binding site in the 3′UTR of CD47 (Fig. 4A). Furthermore, miR-34a and miR-326 also targeted the 3′UTR of CD47. Nine additional potential miRNA binding sites were analysed in the same way, but the corresponding miRNAs did not significantly reduce the luciferase activity in our assay (Fig. 4A and B; data not shown).

Discussion

Establishing miRNA signatures of active and inactive multiple sclerosis lesions led us to identify the 'don't eat me' signal CD47 as a target of miRNA-mediated regulation. This extends the current concepts of multiple sclerosis lesion activity to the level of miRNA mediated gene regulation. Our observations may have broad implications for the regulation of macrophage activation in autoimmune and inflammatory diseases in general.

When we matched the miRNAs that are upregulated in active multiple sclerosis lesions with the pattern of transcripts that are downregulated in active multiple sclerosis lesions (own data), we noted that eight miRNAs found to be upregulated in active multiple sclerosis lesions are predicted to target CD47, which was one of the down-regulated transcripts in the active lesions in

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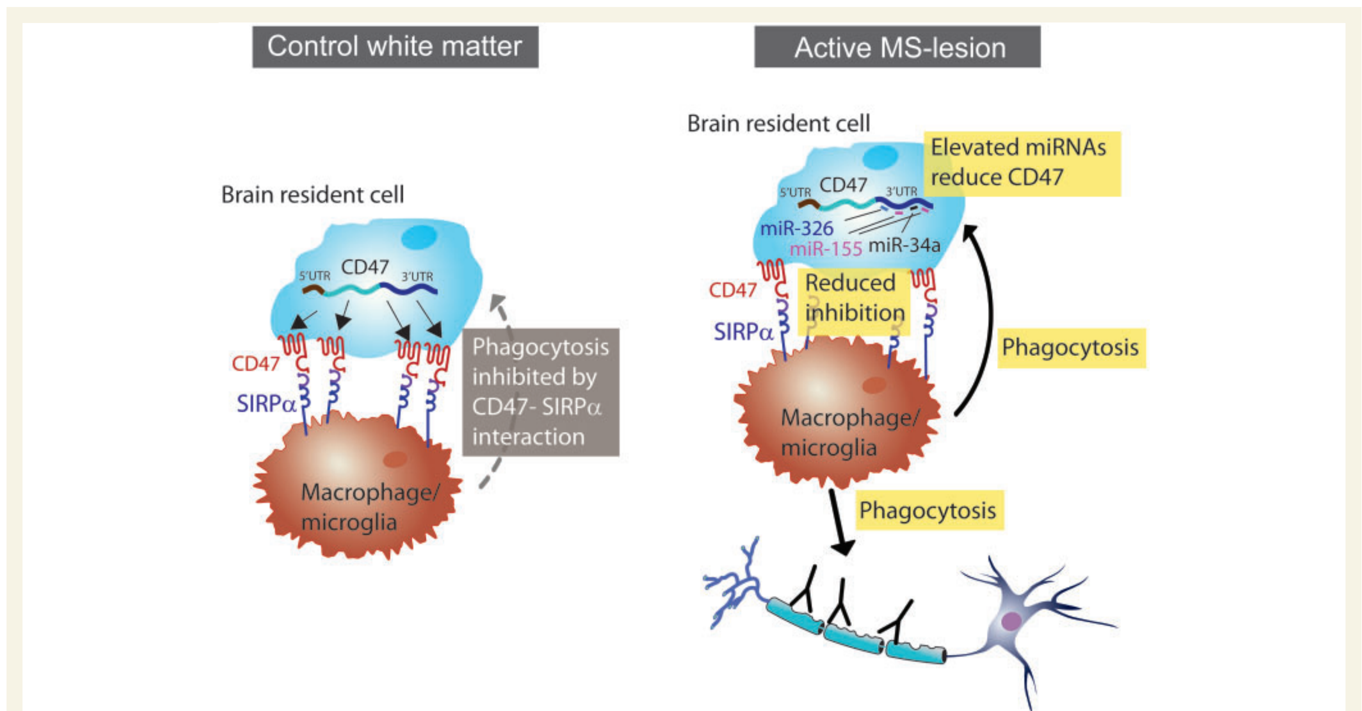

Figure 5 Hypothetical model of miRNA-regulated macrophage activity in multiple sclerosis lesions. In active multiple sclerosis (MS) lesions miRNA-155, miRNA-34a and miRNA-326 are upregulated in comparison with control white matter. These miRNAs target the 3′UTR of CD47 and might thereby reduce CD47 expression. These CD47-regulating miRNAs were found in astrocytes. Their expression in other brain resident cells needs to be explored. Reduced expression of CD47 might release macrophages/microglia from inhibitory control normally mediated by interaction of signal regulatory protein (SIRP)-α on macrophages/microglia with CD47 on potential targets. Reduced signalling via signal regulatory protein-α might then promote phagocytosis of CD47^{low} target cells, and possibly also of susceptible bystander cells, e.g. oligodendrocytes. 'Unleashed' phagocytosis will be directed particularly against opsonized (e.g. antibody-coated) targets, because reduced CD47 is known to promote phagocytosis of antibody-coated cells.

comparison to normal brain white matter. *In vitro* testing of these predicted binding sites revealed that miR-34a, miR-155 and miR-326 indeed target the 3′UTR of CD47, with miR-155 even at two sites. Notably, these three identified miRNA regulators of CD47 were among the 10 most strongly induced miRNAs in active multiple sclerosis lesions.

CD47 is ubiquitously expressed and can mediate multiple functions (Barclay, 2009; Matozaki *et al.*, 2009). It interacts in *cis* with integrins (hence its previous name, integrin activating protein, IAP) and in *trans* with the extracellular matrix molecule thrombospondin-1 and signal regulatory protein-α (CD172a), which is found on macrophages and dendritic cells. CD47 inhibits the phagocytic activity of macrophages (Oldenborg *et al.*, 2000; Yamao *et al.*, 2002; Ishikawa-Sekigami *et al.*, 2006) and cytokine production of dendritic cells via signal regulatory protein-α (Latour *et al.*, 2001). Owing to its inhibitory effect on macrophages, CD47 has been considered as a 'don't eat me signal' and as a 'marker of self' (Oldenborg *et al.*, 2000; Yamao *et al.*, 2002). In some apoptotic cells, CD47 is lost and the reduced level of this 'don't eat me' signal entitles macrophages to engulf these apoptotic cells (Gardai *et al.*, 2005). The importance of CD47 as a marker of self is also supported by the observation that interspecies incompatibilities of CD47 contribute to the rejection of xenogeneic grafts by macrophages (Ide *et al.*, 2007). The biological importance of macrophage inhibition by CD47 is further highlighted by the fact that most poxviruses encode a CD47 homologue. In the case of myxomavirus, it was shown that the viral CD47 homologue is a virulence factor acting by inhibition of macrophages (Cameron *et al.*, 2005).

The interaction of CD47 with signal regulatory protein-α on macrophages blocks Immunoglobulin G or complement-induced phagocytosis indicating a protective role of CD47 in immunopathology (Oldenborg *et al.*, 2001). Beyond this, expression of CD47 on haematopoietic stem cells, leukaemia cells or other tumour cells leads to prevention of phagocytosis (Blazar *et al.*, 2001; Chan *et al.*, 2009; Jaiswal *et al.*, 2009; Majeti *et al.*, 2009). CD47 expression is reduced in active multiple sclerosis lesions as shown in this study and by Koning *et al.* (2007). Active lesions are defined by the presence of myelin degradation products in macrophages, and phagocytosis of myelin by activated macrophages/microglia is a crucial step in tissue destruction in multiple sclerosis (Ozawa *et al.*, 1994).

We propose that the local up-regulation of the three miRNAs miR-34a, miR-155 and miR-326 in active multiple sclerosis lesions is linked to the local down-regulation of CD47 on brain resident cells, thereby unleashing macrophages for tissue destruction (Fig. 5). As shown here, CD47 is expressed by both astrocytes and myelin. The expression of CD47 on myelin indeed suggests

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that this directly regulates phagocytosis. Typically macrophages specifically select their target (frequently opsonized by complement or Ig). CD47 also regulates the phagocytosis of opsonized cells (Oldenborg *et al.*, 2002). Our work warrants further analysis of the local regulation of miRNAs in oligodendrocytes in active and newly forming multiple sclerosis lesions. The activation of phagocytosis by a disturbed CD47–signal regulatory protein-α interaction is not necessarily limited to a direct interaction of the phagocytes with their CD47 bearing target cells, but might also have an effect on bystander phagocytosis, since soluble CD47 reduces the phagocytosis of colloidal carriers (Hsu *et al.*, 2003). So it is also plausible that specific phagocytosis in multiple sclerosis lesions is enhanced by a reduced CD47 on bystander cells, e.g. astrocytes.

Owing to the multiple biological effects of CD47, including inhibition of macrophage activation (Oldenborg *et al.*, 2000, 2001; Yamao *et al.*, 2002; Gardai *et al.*, 2005; Ide *et al.*, 2007), extravasation (de Vries *et al.*, 2002) and T cell co-stimulation (Vallejo *et al.*, 2003; Piccio *et al.*, 2005), a simple CD47-deficient experimental autoimmune encephalomyelitis mouse model would not allow detailed conclusions. Cell-type-specific and/or conditional knockout mice could be used for experimental autoimmune encephalomyelitis models, but it is unclear whether this would reflect the human multiple sclerosis lesion environment. Additional mechanisms of CD47 regulation may add to the complexity. Surprisingly, despite the enormous biological importance of CD47 (Jaiswal *et al.*, 2009; Majeti *et al.*, 2009), details of its regulation are largely unknown. The three miRNAs we identify here provide some insight into the regulation of CD47 expression.

miRNAs typically repress target protein expression by less than 2-fold. It is therefore usually assumed that miRNA-mediated regulation is biologically relevant only if a 2-fold (down) regulation of the corresponding protein translates into biologically meaningful effects (Seitz, 2009). Indeed, CD47-dependent processes are sensitive to around 2-fold regulation, as seen by the dose dependency of macrophage uptake of immunoglobulin G-sensitized targets (Olsson *et al.*, 2007). $CD47^{+/-}$ platelets and erythrocytes were more prone to phagocytosis than their wild-type counterparts (Olsson *et al*., 2006, 2007). A recent paper (Jaiswal *et al.*, 2009) has shown that haematopoietic stem cells of such $CD47^{+/-}$ mice express just half the amount of CD47 and are more prone to phagocytosis. This lends support to our model.

Our present findings demonstrate that miRNA levels in astrocytes are highly regulated in response to inflammatory cytokines. We could assign miRNAs that are induced in multiple sclerosis lesions to astrocytes: the 10 most strongly induced miRNAs in active lesions were expressed by astrocytes and three miRNAs could be induced in astrocytes by *in vitro* stimulation with inflammatory cytokines. Our study provides further evidence for an involvement of astrocytes in shaping the environment in multiple sclerosis lesions.

Considering that post-transcriptional regulation by miRNAs affects approximately one-third of all genes, one has to assume that many of the already published downregulated proteins or transcripts observed in multiple sclerosis lesions are actually regulated, or at least ‘fine-tuned’, by miRNAs. We would therefore expect that beyond our observations on CD47, several additional differentially regulated miRNAs identified in this work may explain some other previous observations regarding transcript or protein regulation in multiple sclerosis lesions.

Therefore, we compared our miRNA profiles with previous microarray (Lock *et al.*, 2002) or proteome analyses (Han *et al.*, 2008) of multiple sclerosis tissue using Targetscan 5.0 for *in silico* searches (Lewis *et al.*, 2005; Grimson *et al.*, 2007). This revealed several interesting connections. For example, myocyte enhancer factor-2c, which is downregulated in multiple sclerosis lesions (Lock *et al.*, 2002), is a predicted target of three miRNAs (miR-23a, miR-223 and miR-27) that we found to be upregulated in active multiple sclerosis lesions. Myocyte enhancer factor-2c, a transcription factor that promotes (among other features) myeloid progenitor proliferation, is a proven target of miR-223 (Johnnidis *et al.*, 2008). Furthermore, microtubule-associated protein 1b is downregulated in active multiple sclerosis lesions (Han *et al.*, 2008) and is a predicted target of miR-130a and miR-27a, which are identified here in our miRNA signature. Microtubule-associated protein 1b is one of the major growth-associated and cytoskeletal proteins in neuronal and glial cells (Riederer, 2007).

While the implications of these proven or predicted interactions for multiple sclerosis lesion development need to be established, our miRNA profiles might explain another earlier observation related to macrophage activation: using *in situ* hybridization it has been noted that colony-stimulating factor receptor 1 was downregulated in macrophages/microglia in active multiple sclerosis lesions as compared to adjacent normal white matter (Werner *et al.*, 2002). Our finding that miR-155 is upregulated in active multiple sclerosis lesions, along with the previous finding that colony-stimulating factor receptor 1 is a proven target of miR-155 (O’Connell *et al.*, 2008), now suggests a mechanism that might at least contribute to the loss of colony-stimulating factor receptor 1 in active multiple sclerosis lesions.

In conclusion, we present miRNA signatures of active and inactive brain lesions of patients with multiple sclerosis. Our finding that three of the most upregulated miRNAs in multiple sclerosis lesions target CD47 extends the concept of tissue destruction in multiple sclerosis lesions to the level of miRNA-regulated gene expression. We postulate that downregulation of CD47 by miR-34a, miR-155 and miR-326 in the lesion environment releases macrophages/microglia from inhibitory control. This promotes myelin phagocytosis, the pathological hallmark of active multiple sclerosis lesions. Targeting these miRNAs might represent a promising therapeutic strategy to calm down lesion activity. Because of the ubiquitous expression of CD47 and its critical role in self-recognition and macrophage activation, our results may have implications for other immunopathological conditions.

Note added in proof

After this paper was accepted, miR-326 was reported to be higher in peripheral blood leukocytes of MS patients and to regulate TH-17 differentiation (Du *et al*. 2009). We found that this miRNA was one of the three most up-regulated miRNAs in active MS lesions (Table 1) lending further support to the relevance of this miRNA for MS pathogenesis.

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Supplementary material

Supplementary material is available at *Brain* online.

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