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Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

MicroRNA signature of central nervous system-infiltrating dendritic cells in an animal model of multiple sclerosis

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doi:10.1111/imm.12934
Received 5 October 2017; revised 28 February 2018; accepted 23 March 2018.
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Summary

Innate immune cells are integral to the pathogenesis of several diseases of the central nervous system (CNS), including multiple sclerosis (MS). Dendritic cells (DCs) are potent CD11c$^+$ antigen-presenting cells that are critical regulators of adaptive immune responses, particularly in autoimmune diseases such as MS. The regulation of DC function in both the periphery and CNS compartment has not been fully elucidated. One limitation to studying the role of CD11c$^+$ DCs in the CNS is that microglia can upregulate CD11c during inflammation, making it challenging to distinguish bone marrow-derived DCs (BMDCs) from microglia. Selective expression of microRNAs (miRNAs) has been shown to distinguish populations of innate cells and regulate their function within the CNS during neuroinflammation. Using the experimental autoimmune encephalomyelitis (EAE) murine model of MS, we characterized the expression of miRNAs in CD11c$^+$ cells using a non-biased murine array. Several miRNAs, including miR-31, were enriched in CD11c$^+$ cells within the CNS during EAE, but not LysM$^+$ microglia. Moreover, to distinguish CD11c$^+$ DCs from microglia that upregulate CD11c, we generated bone marrow chimeras and found that miR-31 expression was specific to BMDCs. Interestingly, miR-31-binding sites were enriched in mRNAs downregulated in BMDCs that migrated into the CNS, and a subset was confirmed to be regulated by miR-31. Finally, miR-31 was elevated in DCs migrating through an *in vitro* blood–brain barrier. Our findings suggest miRNAs, including miR-31, may regulate entry of DCs into the CNS during EAE, and could potentially represent therapeutic targets for CNS autoimmune diseases such as MS.

Keywords: dendritic cells; microRNAs; multiple sclerosis; neuroinflammation.

Introduction

Multiple sclerosis (MS) – the prototypical inflammatory disease of the central nervous system (CNS) – is characterized by widely distributed inflammatory demyelinating plaques within the brain and spinal cord that can be associated with severe neurological disability.[1] A hallmark feature of MS plaques is engagement of leucocytes with the blood–brain barrier (BBB) coupled with an influx of immune cells into the CNS from the periphery.[2,3] Using the common animal model for MS, experimental autoimmune encephalomyelitis (EAE), a variety of innate immune cells, including dendritic cells (DCs) and microglia, have been implicated in the promotion of

Abbreviations: AD, Atopic dermatitis; Ago2, argonaute 2; APCs, antigen-presenting cells; BBB, blood–brain barrier; BMDCs, bone marrow-derived dendritic cells; CNS, central nervous system; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; LPS, lipopolysaccharide; miRAP, miRNA affinity and tagging purification; miRNAs, microRNAs; MS, multiple sclerosis; Tregs, T regulatory cells

neuro-inflammation.[4,5] The mechanisms by which innate cells regulate adaptive immune responses in the CNS, coordinate damage to myelin and neurons, and respond to injury remain unclear.

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate the stability and translation of mRNAs.[6] While numerous physiological processes are regulated by the ever-expanding number of recognized miRNAs, the cell-specific expression of miRNAs is elemental to their ultimate function.[7] Furthermore, several miRNAs expressed by innate immune cells have been implicated in dysregulation of immune function that is associated with, and may be causal to, autoimmunity.[8,9] Thus, identification of miRNA signatures for various immune cells is likely to provide insight into the mechanisms by which innate immunity is involved in the pathogenesis of neuro-inflammatory diseases such as MS. For example, a small number of miRNAs has been described as a signature for microglia in comparison to other innate immune cells.[10] What remains to be determined is the role for miRNA in the specialization of different innate immune cell subsets, particularly during inflammatory diseases of the CNS.

We have previously found that DCs are critical regulators of adaptive immune responses in the CNS, and that the bone marrow-derived fraction of DCs is necessary for T-cell-dependent neuro-inflammation during EAE.[11] Several miRNAs, including Let-7c,[12] miR-142-3p[13] and miR-34a,[14] have been shown to regulate DC function. In what manner miRNA expression directs DC autoimmune behaviour during EAE has yet to be established. Therefore, we aimed to determine the miRNA signature of DCs that accumulate within the CNS during EAE.

To characterize the molecular regulation of DCs in EAE, we utilized an *in vivo* Cre-LoxP murine system in which a tagged miRNA-processing protein, argonaute 2 (Ago2), is expressed only in CD11c-expressing cells. Immunoprecipitation of tagged Ago2 (tAgo2) from these cells facilitates isolation of CD11c-specific miRNAs.[15,16] Using this system in combination with miRNA arrays, we found that miR-31 and miR-301 are significantly enriched in CD11c-expressing mononuclear cells isolated from the CNS during EAE. Furthermore, the enrichment of miR-31 is driven by entry of bone marrow-derived DCs (BMDCs) and miR-31 regulates their mRNA expression profiles. Moreover, miR-31 serves as a signature of BMDCs migrating through endothelial cells in an *in vitro* BBB system.

Materials and methods

Mice

Animal protocols were in accordance with the ARRIVE guidelines, and were approved by the Institutional Animal Care and Use Committee of Washington University at St Louis. Wild-type (WT) C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MiRNA tagging and affinity purification (miRAP) mice[16] expressing a loxP-flanked myc-tagged Ago2 construct under the control of CMV beta-actin enhancer promoter inserted in the *ROSA* locus (JAX ID: 017626) were crossed to $CD11c^{Cre}$ mice ($Itgax^{Cre}$)[17] obtained from The Jackson Laboratory (JAX ID: 008068) to generate $miRAP^{CD11c}$ mice. Likewise, $miRAP^{LysM}$ mice were generated by breeding miRAP mice to $LysM^{Cre}$ mice[18] obtained from The Jackson Laboratory (JAX ID: 004781). Radiation bone marrow chimeras were generated by intravenous injection of 1×10^6 donor bone marrow cells into hosts irradiated with 1000 Gy.[11] Bone marrow from mice with ubiquitous GFP expression (JAX ID: 003291)[19] or eYFP driven by CD11c (JAX ID: 008829)[20] was transplanted into irradiated mice. Mice were provided 0·1% Enrofloxacin (Baytril®; Bayer Healthcare, Shawnee Mission, KS) antibiotic PO *ad libitum* for 7 days post-irradiation. Irradiated mice were used for experimentation after full immune reconstitution at 8 weeks following bone marrow transplantation.

EAE

We induced active EAE in standard fashion.[11] Briefly, mice were immunized with residues 35–55 of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) emulsified in complete Freund's adjuvant. Two-hundred nanograms of pertussis toxin was administered at the time of immunization and 2 days later. Mice were graded on a scale of 0 (unaffected) to 5 (moribund or dead), with 1 representing a limp tail, 2 indicating hind limb paresis, 3 reflecting more severe hind limb paresis along with righting difficulty, and 4 indicative of hind limb paralysis.[11]

Atopic dermatitis (AD)

Six mice were treated with 50 μM MC903 (calcipotriol) or vehicle (ethanol) on each ear for 8 days to stimulate an inflammatory response in the skin.[21] Lymph nodes and ears were harvested on the 8th day. Ears were separated with forceps and digested in 0·25 mg/ml Liberase TL (Roche/Sigma, St. Louis, MO) in Dulbecco's modified Eagle's minimum essential medium (DMEM) for 90 min at 37°. Single cell suspensions from lymph nodes and digested ear skin were filtered through 100-μm filters and washed in media before staining for FACS as described above. Cells from individual mice or pooled mice were then used to isolate RNA.

miRAP and miRNA arrays

At day 21 post-immunization, mice with a clinical score of 2 or greater were anaesthetized with an overdose of

inhaled isoflurane and subsequently perfused with ice-cold 1 × phosphate-buffered saline (PBS). Spleen (unperfused) and spinal cord tissue were flash-frozen in liquid nitrogen and frozen at −80°. MiRAP immunoprecipitation using an anti-myc antibody (9E10 sc-40; Santa Cruz, Dallas, TX) was performed as previously described.[15,16] A BCA assay was performed on all tissue homogenates to normalize protein input; 700 µl of Qiazol (Qiagen, Germantown, MD) was added directly to the beads and stored at −20° before proceeding with RNA isolation (miRNeasy 217004; Qiagen). Taqman low-density rodent miRNA microarrays (version 3.0; Life Technologies, Waltham, MA) were performed with pre-amplification on spinal cord and spleen tissue from three male mice. Microarrays were run on a 7900HT quantitative polymerase chain reaction (qPCR) machine (Thermo Fisher; Waltham, MA) for 40 cycles.

mRNA microarrays

Radiation bone marrow chimeras with BMDCs expressing eYFP were immunized with $MOG_{35–55}$ as described above to induce active EAE. $CD11c^+eYFP^+$ cells were sorted by FACS at day 18 post-immunization from the spleen and perfused CNS. RNA was isolated and amplified with the Nugen Ovation WTA kit (Nugen, San Carlos, CA) according to the NuGEN Ovation Pico WTA system v2 user guide and the Affymetrix GeneChip Expression Analysis Technical Manual, then hybridized to Affymetrix Mouse Gene 1·0ST arrays (Affymetrix, Santa Clara, CA). A GeneChip 3000 7G scanner was used to collect fluorescence signal. In order to determine the fold-change of the genes of interest, Affymetrix normalized, background-subtracted.cel files were imported into Partek Genomic Suite (v 5). In order to determine genes whose expression is altered between radiation-resistant and radiation-sensitive DCs, as well as radiation-sensitive DCs within the CNS versus spleen, two-way ANOVA analysis was employed on log2-transformed data. *P*-values were corrected using step-up multiple testing correction.[22]

miRNA expression validation

MiRNA array expression data were normalized to a geometric mean of all miRNAs expressed at CT < 30. DC miRNAs enriched in the CNS (spinal cord) as compared with periphery (spleen) were confirmed at a higher power ($n = 6$) with individual reverse transcription (RT)-qPCR assays on a 7500 fast Real-Time PCR system.

Flow cytometry and FACS sorts

Mice were perfused with 25 ml of ice-cold PBS. Subsequently, brains and spinal cords were collected from perfused mice and homogenized to obtain single cell suspensions. CNS cells were purified by centrifugation for 30 min in a 95% Percoll (GE Healthcare, Chicago, IL) solution with EBSS (Sigma, St Louis, MO).[11] Cells were incubated with the anti-Fc receptor antibody 2·4G2 prior to the addition of antibodies. The following antibodies were purchased from BD Biosciences (Franklin Lakes, NJ): CD45-FITC, CD8a-APC-H7, CD19-APC-H7, CD19-BV510, B220–PE-CF594, CD11b-AlexaFluor-700, MHCII-v450. The following antibodies were purchased from eBioscience (San Diego, CA): MHCII-Pacific Blue, CD11c-PECy7. The following antibodies were purchased from BioLegend (San Diego, CA): MHCII (I-A/I-E)-Pacific Blue, Thy1·1-PerCP, CD4-APC. Cells were acquired on a Gallios flow cytometer (Beckman Coulter, Brea, CA) and analysed with FLOWJO software (TreeStar, Ashland, OR) with doublets being excluded. Cells were sorted on a SY3200 cell sorter (Sony Biotechnology, San Jose, CA), and 700 µl of Qiazol was added directly to the cells before proceeding with RNA isolation (miRNeasy 217004; Qiagen).

To identify BMDCs that entered into the CNS during EAE, the bone marrow of mice expressing enhanced GFP under the actin promoter were used to generate chimeras. During EAE, we isolated cells using FACS by first gating on singlets from the lymphocyte population of cells isolated, as shown in Fig. S2. To isolate microglia, we excluded GFP^+ cells and sorted the $CD45^{int}CD11b^{int}$ population of cells. To isolate BMDCs, we gated the GFP^+ cells and sorted the $CD11c^+$ population of cells. CD11c−/CD11b− cells from the GFP^+ population were also gated to sort $CD3^+$ T-cells. $CD45^+$ GFP^+ $CD11b^+$ $CD11c^+$ DCs and $CD45^+$ GFP^+ $CD3^+$ T-cells were sorted similarly from the single cell suspensions of the spleens from the same mice with EAE.

To identify $CD11c^+$ resident and migratory DCs in the draining lymph from mice with AD-induced skin inflammation, we isolated cells using FACS by first gating on singlets and then excluding $CD3^+$, $CD9^+$ and $NK1{\cdot}1^+$ cells from the $CD45^+$ population. We then sorted $CD11c^+$ $MHCII^{low}$ (resident DCs) and $CD11c^+$ $MHCII^{high}$ (migratory DCs) populations.[23] To isolate $CD11c^+$ cells that infiltrated the ear, we again excluded the $CD3^+$ $CD9^+$ $NK1{\cdot}1^+$ cells from the $CD45^+$ population, and isolated $CD11c^{hi}$, $CD207^{low}$ cells.

Migration assays

Bone marrow-derived dendritic cells were generated *in vitro* as previously reported.[24] Briefly, bone marrow from tibia and fibulae were cultured in RPMI medium containing 10% fetal bovine serum and supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) for 7–10 days. BBB cultures were utilized as described previously;[25] $0{\cdot}5–1{\cdot}0 \times 10^6$ BMDCs were added to the top of the BBB

cultures, and 16 hr later media was removed from the top and bottom chambers of the transwell apparatus. Cells were spun down and 700 µl of Qiazol was added directly to the cells before proceeding with RNA isolation (miRNeasy 217004; Qiagen).

Luciferase assays

The 3′-untranslated regions (UTRs) of putative miR-31 targets were cloned into the Dual Glo luciferase vector (Promega, Madison, WI) using the primers listed in Table S1. Mouse miR-31 was cloned into the pSilencer vector (Thermo Fisher) using the primers: GGACACTCT AAGGACCACTGC (forward) and CGAGACAGACCAAG TCACAGG (reverse). Site-directed mutagenesis was used to mutate the first four nucleotides of the miR-31-binding site in the IL-34 3′-UTR; 30 000 HEK 293 cells were plated in a 96-well dish (Thermo Fisher), and transfected with 100 ng of dual glo vector and 100 ng of miR-31 using FuGENE 6 (Promega). Firefly luciferase (Fluc) luminescence was monitored 24 hr post-transfection using the Dual Glo assay kit (Promega, E2920), and was normalized to a control renilla luciferase (Rluc). Three–six technical replicates for each condition were performed for each biological replicate ($n = 3$).

Results

Migratory CD11c$^+$ cells have a miRNA expression profile distinct from microglia in EAE spinal cord tissue

Several distinct antigen-presenting cells (APCs) are present within the CNS during inflammatory demyelination.[5,26] DCs, generally identified by expression of the integrin CD11c, are potent APCs involved in the initiation and propagation of neuro-inflammation in MS and EAE.[11,27,28] We sought to examine miRNA expression by DCs to define the potential regulatory mechanism of this important APC subset during EAE. We utilized the murine miRAP system[15,16] to selectively isolate miRNA from CD11c$^+$ DCs during EAE. MiRNA from the spleens and spinal cords of miRAP^{CD11c} mice with EAE was isolated at day 21 post-immunization (Fig. 1a). MiRNA microarray analysis of miRAP^{CD11c} spinal cord tissue identified several miRNAs with elevated expression compared with the spleen (Data S1; Fig. S1). As confirmed by individual RT-qPCR assays, miR-31, miR-301a, miR-301b and miR-339-5p expressions were elevated in DCs within the CNS of miRAP^{CD11c} mice during EAE (Fig. 1b). To explore the selective nature of miRNA expression by CD11c$^+$ cells, we compared the expression of these miRNAs with innate cells expressing the myeloid marker LysM, which, in a perfused mouse, labels resident microglia.[29,30] In comparison to miRAP^{LysM} mice at the peak of EAE, significant elevations of miR-31, miR-301a and miR-339-5p were observed in CNS of miRAP^{CD11c} mice, suggesting a selective enrichment in CD11c$^+$ DCs compared with microglial cells expressing LysM[31] (Fig. 1c). Consistent with isolation of LysM$^+$ microglia, the expression of miR-155, a miRNA important in myeloid cells during EAE,[32–34] was enriched in miRAP^{LysM} as compared with miRAP^{CD11c} EAE spinal cord tissue (Fig. 1d). These data suggest that migratory CD11c$^+$ cells in EAE spinal cord tissue have a miRNA expression profile distinct from LysM$^+$ microglia.

MiR-31 expression in migratory CD11c$^+$ cells in EAE spinal cord is driven selectively by BMDCs and not resident microglia

Expression of CD11c by both infiltrating DCs and CNS resident innate immune cells can confound the identification of DCs within the CNS during EAE.[35] As BMDCs are considered essential and sufficient for antigen presentation during EAE,[11,27,36] we generated radiation bone marrow chimeric mice in which miRAP^{CD11c} bone marrow was transplanted into lethally irradiated B6 or miRAP^{CD11c} host mice. We performed miRAP and isolated RNA at the peak of disease from the spinal cords and spleens of chimeric mice, and ran miRNA arrays (Data S1). In agreement with miRAP^{CD11c} mice with EAE, elevated expression of miR-31 was observed in the spinal cords of miRAP^{CD11c} → miRAP^{CD11c} chimeric mice (Fig. 2a). While this did not reach statistical significance ($P = 0{\cdot}1$), the magnitude of the elevation was nearly identical to the upregulation that was found in Fig. 1a. Furthermore, DC expression of miR-31 in miRAP^{CD11c} → WT mice was significantly elevated within the spinal cord in comparison to the spleen, suggesting the expression of miR-31 by CD11c-expressing cells in the spinal cord during EAE is driven by BMDCs, not CNS resident innate immune cells (Fig. 2a). To confirm this finding, we isolated bone marrow-derived CD11b^{hi}CD11c$^{int/hi}$ DCs and CD45^{int}CD11b^{int} microglia from the spinal cord of EAE mice by FACS. In these sorted cells, we found that miR-31 was highly and selectively enriched in BMDCs as compared with resident microglia (Fig. 2d). These data, however, do not distinguish BMDCs from CNS-infiltrating monocytes that differentiate into DCs.

Concomitant with the selective expression of miR-31 in BMDCs, we also found that miR-34b-3p was selectively depleted in CD11c$^+$ cells arising from the bone marrow (Fig. 2e). Thus, miR-34b-3p appears to be restricted to resident CNS immune cells that upregulate CD11c during EAE. Consistent with this hypothesis, we found that miR-34b-3p expression was higher in microglia than in bone marrow-derived CD11b^{hi}CD11c$^{int/hi}$ cells isolated by FACS from the CNS during EAE (Fig. 2f).

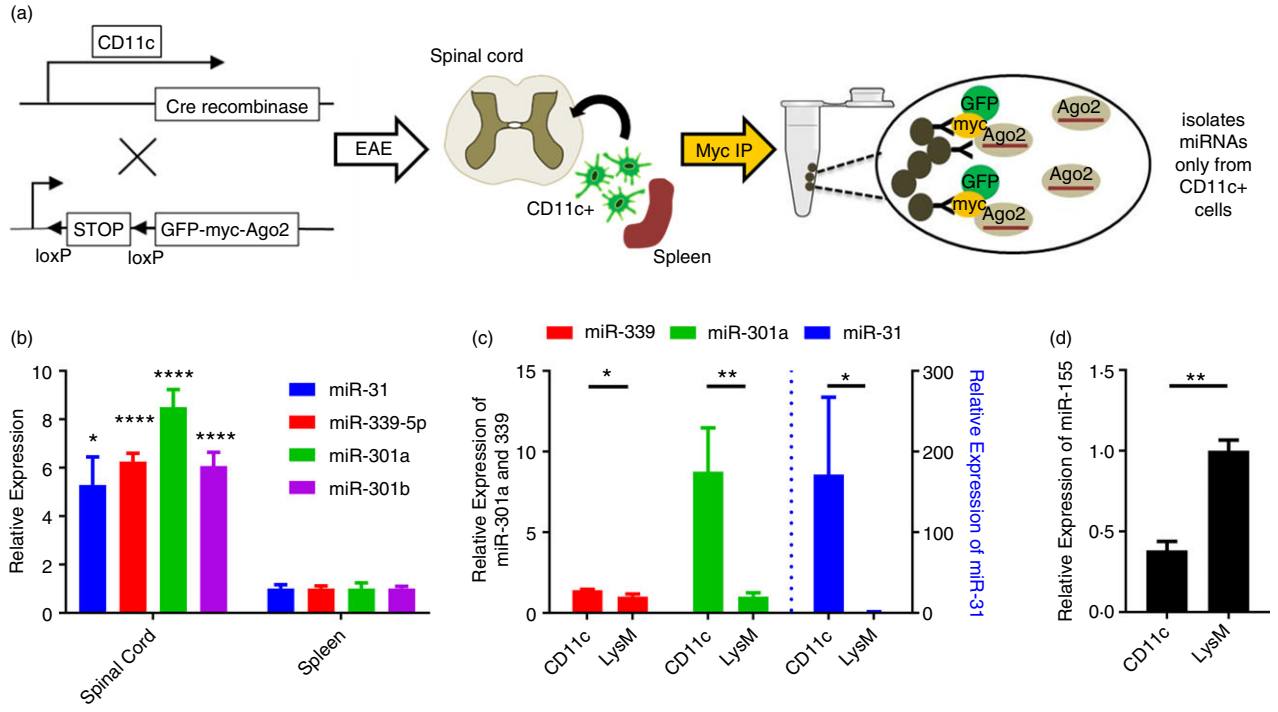

Figure 1. The miRNA signature of migratory CD11c$^+$ cells in experimental autoimmune encephalomyelitis (EAE) spinal cord tissue is distinct from LysM$^+$ microglia. (a) Schematic of miRNA affinity and tagging purification (miRAP) approach where CD11c-Cre mice are crossed with mice expressing LSL-tAgo2. miRAP from spleen and spinal cord of CD11c Cre, LSL-tAgo2 mice isolates miRNAs only from CD11c$^+$ DCs. (b) miR-31, 339-5p, 301a and 301b levels are enriched in CD11c$^+$ cells of EAE spinal cord tissue as compared to spleen; $n = 6$/tissue. (c, d) miRAP of CD11c$^+$ cells as compared with LysM$^+$ microglia in the spinal cord indicates elevated miR-339-5p, miR-301a and miR-31 is specific to CD11c$^+$ cells, whereas miR-155 is more enriched in LysM$^+$ microglia; $n = 6$/line. Values represented as mean ± SEM. Relative expression normalized to a geometric mean of miR-24 and miR-191. Student's two-tailed, unpaired *t*-tests with Bonferroni correction for multiple (4) comparisons (b, c–d). Adjusted *P*-values: $^*P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$, $^{****}P \leq 0{\cdot}0001$.

Because miR-31 has been reported to be elevated in T-cells,[37] we compared miR-31 expression in various mononuclear cells sorted by FACS from the spleens and CNS of mice with EAE. Intriguingly, compared with miR-31 expression by CD11b^{hi}CD11c$^{int/hi}$ DCs, CD3$^+$ T-cells within the periphery of mice with EAE expressed a low level of miR-31 (Fig. S2f). However, upon entry into the CNS, expression of miR-31 was higher in CD3$^+$ T-cells than CD11b^{hi}CD11c$^{int/hi}$ DCs (Fig. S2g).

miR-31-binding sites are enriched in mRNAs downregulated in BMDCs that migrate into the CNS during EAE

To determine the functional relevance of elevated miR-31 in BMDCs that migrate into the CNS during EAE, we generated chimeric mice expressing eYFP exclusively by bone marrow-derived CD11c$^+$ cells. At the peak of EAE induced by $MOG_{35–55}$ immunization, microarrays were performed on mRNA isolated from BMDCs in the spleen and CNS. Because miRNAs typically downregulate their target mRNAs, we queried mRNAs that were downregulated by ≥ 1·5-fold in brain BMDCs versus spleen BMDCs, and found a significant number were putative miR-31 targets (Fig. 3a; $P = 0{\cdot}013$, χ^2 test with Yates' correction). These data suggest that miR-31 elevation in BMDCs in CNS tissue during EAE functionally modulates BMDCs cellular expression profiles. To confirm miR-31 regulation of mRNAs downregulated in BMDCs that migrate into the CNS, we performed luciferase assays on a subset of targets. Of the four candidates tested, three were confirmed to be regulated by miR-31 (Fig. 3b–e). The full list of mRNA changes is included in Data S1.

Elevated miR-31 expression in Cd11c$^+$ DCs may be broadly representative of DCs that migrate into inflamed tissue

To test whether elevated miR-31 expression is specific to DCs that migrate into the CNS during EAE or whether elevated miR-31 is generally representative of BMDCs infiltrating inflamed tissues, we used a murine model of skin inflammation resembling AD. In this paradigm, leucocytes, including DCs, migrate to the site of irritation in the ears induced by the vitamin D3 analogue, MC903. We isolated migratory DCs from the ear and lymph nodes, as well as

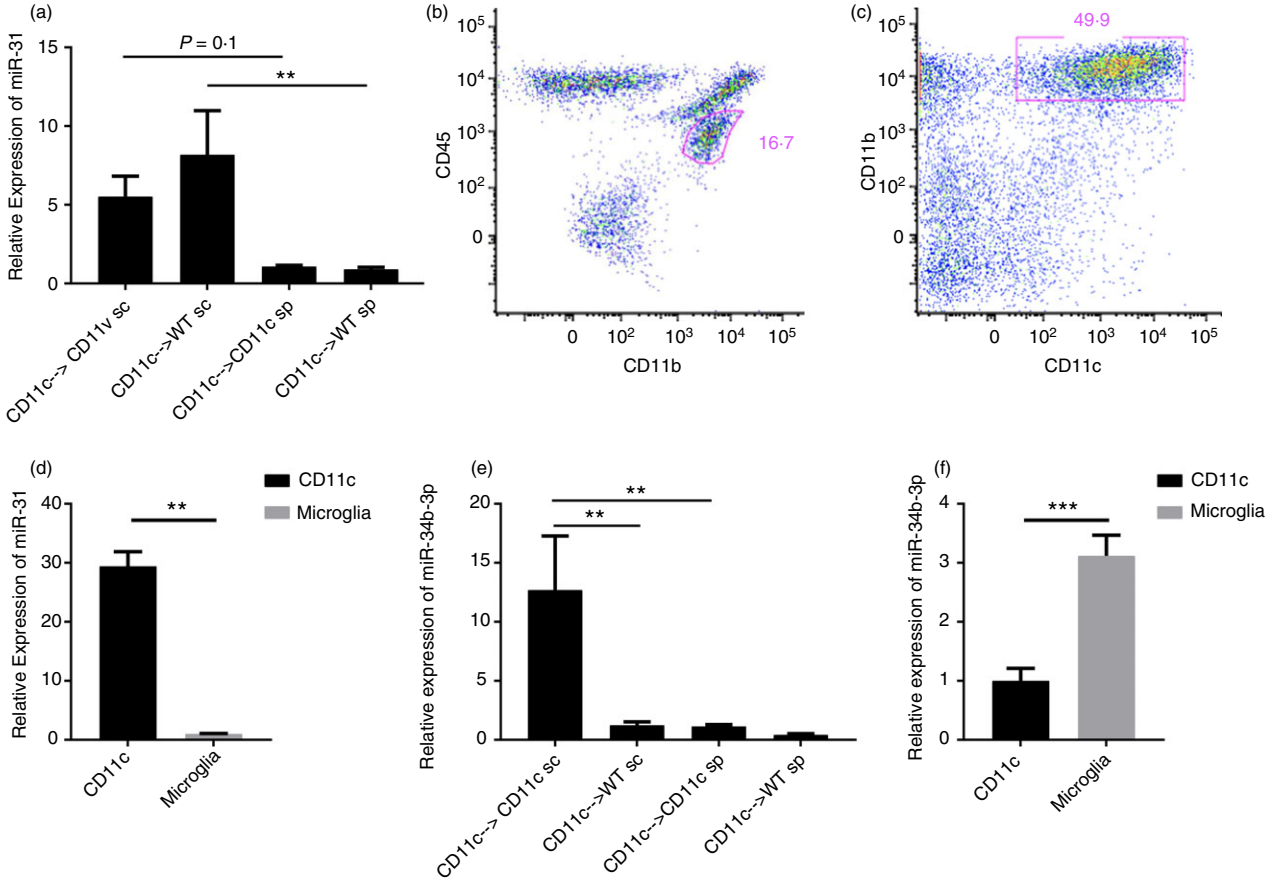

Figure 2. miR-31 enrichment in central nervous system (CNS) localized CD11c$^+$ cells is driven by bone marrow-derived dendritic cells (BMDCs), not resident CD11c$^+$ cells during experimental autoimmune encephalomyelitis (EAE). (a) miRNA affinity and tagging purification (miRAP) of all CD11c$^+$ cells or specifically CD11c$^+$ BMDCs from EAE tissue confirms enrichment of miR-31 in the spinal cord as compared with spleen, particularly in CD11c$^+$ BMDCs; $n = 6$/condition/tissue. CD45^{int}CD11b^{int} microglia (b) and CD11b^{hi}CD11c$^{int/hi}$ BMDCs (c) were isolated by FACS from the spinal cord ($n = 4$–8) of mice with EAE. (d) Expression of miR-31 by CD11b^{hi}CD11c$^{int/hi}$ BMDCs and CD45^{int}CD11b^{int} microglia isolated by FACS from the spinal cord of mice with EAE; $n = 4$–8. (e) miR-34b-3p is selectively depleted from CNS-infiltrating BMDCs as compared with resident CNS CD11c$^+$ cells. (f) Expression of miR-34b-3p by CD11c$^{int/hi}$ BMDCs and CD45^{int}CD11b^{int} microglia isolated by FACS from the spinal cord of mice with EAE; $n = 4$–8. Values represented as mean ± SEM. Relative expression normalized to a geometric mean of miR-24 and miR-191 (a, e) or U6 snRNA (d, f). One-way ANOVA with multiple comparisons (Dunnett's) (a, e) or Student's two-tailed, unpaired *t*-tests (d, f). *P*-values: $^*P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$.

resident DCs in the lymph nodes, by FACS (Fig. S3a–d). Interestingly, we found that migratory DCs in the lymph nodes did not have elevated miR-31 expression, but DCs infiltrating into the skin did have elevated miR-31 expression as compared with resident DCs in the lymph nodes (Fig. S3e). These data suggest that elevated miR-31 may be representative of DCs that localize to inflamed tissue rather than those migrating in general.

miR-31 is enriched in BMDCs that migrate through an *in vitro* BBB irrespective of lipopolysaccharide (LPS)-mediated DC activation

Because the expression of miR-31 by DCs is derived from DCs originating outside of the CNS compartment, we hypothesized that engagement of DCs with tissue resident cells may prompt an increase in miR-31 expression. To test this hypothesis, we used cultured BMDCs in an *in vitro* BBB model.[25] DCs were incubated in the top chamber of the BBB system, and were harvested from both top and bottom compartments after 16 hr. Expression of the innate cell-associated miRNA, miR-155, was similar in DCs from both the top and bottom wells (Fig. 4a). In contrast, DCs traversing the BBB into the bottom chamber had a nearly two-fold increase in miR-31 (Fig. 4b). To determine whether DC activation further influenced DC migration, we activated DCs with LPS prior to incubation with BBB cultures. To confirm that DCs were responding to LPS stimulation, we measured the levels of miR-155 and miR-31 in LPS-activated and -non-activated DCs. As expected, LPS-activated DCs had a robust increase in miR-155 expression, whereas miR-31

(a)

	Ovelapping targets	Remaining mRNAs downregulated ≥ 1·5-fold in CNS BMDCs vs. spleen BMDCs	Total
Overlapping targets	101	4379	4480
Remaining miR-31 targets (Targetscan)	313	18207	18520
Total	414	22586	23000
χ^2 *P*-value = 0·0129 (with yates' correction)			

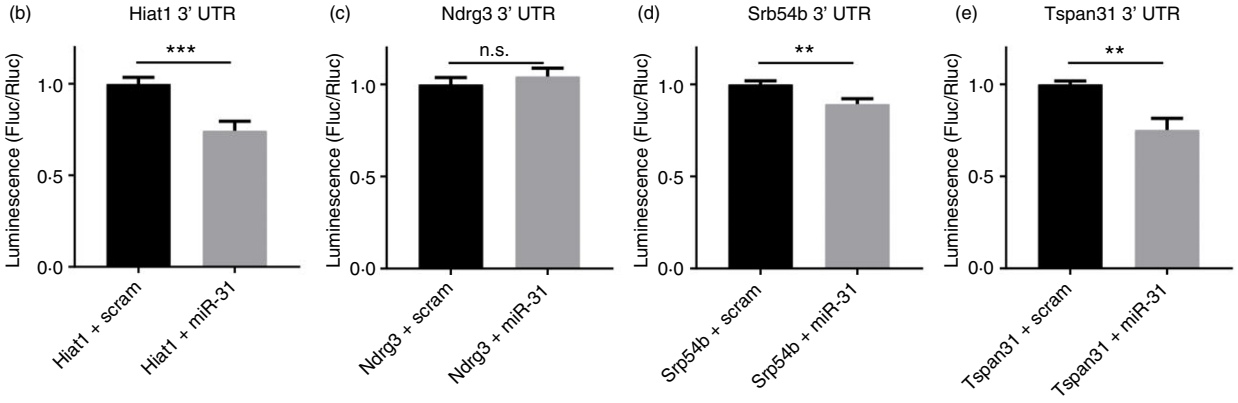

Figure 3. miR-31 sites are enriched in mRNAs downregulated in central nervous system (CNS) bone marrow-derived dendritic cells (BMDCs) compared with spleen BMDCs. (a) We found that of the 4480 mRNAs downregulated by ≥ 1·5-fold in CNS BMDCs compared with spleen BMDCs, 101 transcripts were putative miR-31 targets (Targetscan.org). This is a significant enrichment when compared with the mouse genome of ~23 000 genes. (b–e) Putative miR-31 targets, Hiat1 (b), Srp54b (d) and Tspan31 (e) were confirmed using luciferase assays; the luminescence activity of the reporter bearing the corresponding 3′-untranslated region (UTR) was downregulated in the presence of miR-31 as compared with scrambled miRNA control. Putative miR-31 target, Ndrg3, (c) was not regulated by miR-31. $n = 10$/condition (three biological replicates with three–four technical replicates each). Values represented as mean ± SEM. Student's two-tailed, unpaired *t*-tests (b–e). *P*-values: $^*P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}001$.

was not changed (Fig. 4c). This suggests miR-31 expression is not upregulated in response to DC activation by LPS. The application of these activated DCs resulted in a similar enriched expression of miR-31 in DCs that migrate into the lower chamber, while the miR-155 levels were still not influenced by passing through the *in vitro* BBB (Fig. 4d,e). These data suggest that miR-31 expression by DCs is not substantially driven by LPS-mediated activation, and its elevation in DCs that migrate into the CNS is irrespective of LPS-mediated activation status. However, other stimulants may differentially affect miR-31 expression in DCs or their migratory propensity.

Discussion

Dysregulation of DC function is considered an underpinning of autoimmunity in the brain and spinal cord during MS. An important consideration regarding the role of DCs in MS autoimmunity is the specialized compartmental nature of the CNS. At steady-state, DCs are found scattered sparsely within the CNS, primarily affiliated with the vasculature and meninges.[27,38] In EAE, entry and accumulation of DCs within the CNS is driven in large part by recruitment from bone marrow progenitors.[11,39] Because BMDCs are critical and sufficient for antigen presentation during EAE,[11,27,36] understanding their entry into the CNS is of great therapeutic interest. However, one limitation to studying DC function in EAE is the possible contamination of microglia that upregulate the DC marker CD11c during neuroinflammation.

MicroRNAs regulate hundreds of targets and, accordingly, shape the expression profile of a cell. Thus, miRNAs often exhibit cell type-enriched expression and have been shown to distinguish various innate immune cell

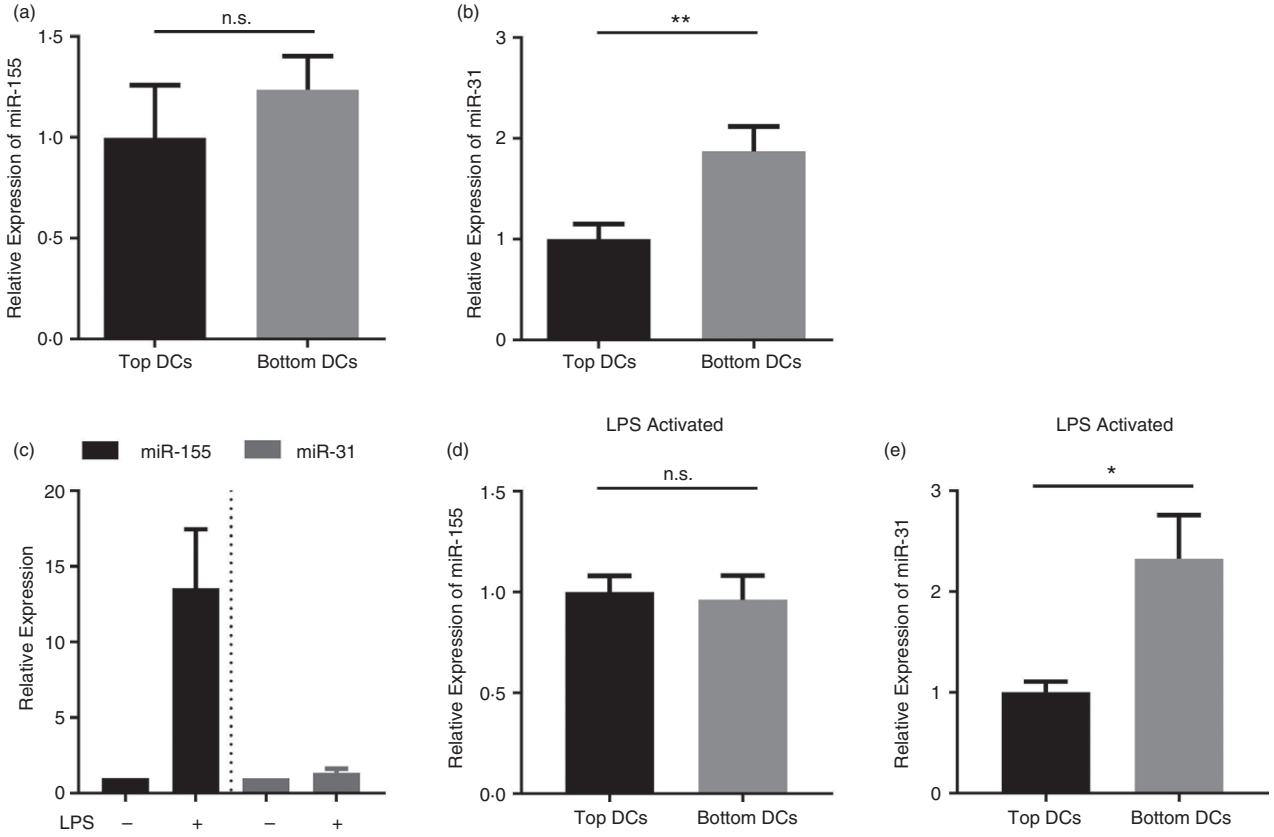

Figure 4. MiR-31 is increased in dendritic cells (DCs) that migrate through an *in vitro* blood–brain barrier (BBB). (a, b) miR-31, but not miR-155, is elevated in cultured bone marrow-derived dendritic cells (BMDCs) that migrate through (bottom DCs) an *in vitro* BBB assay, $n = 6$–9/condition. (c) Lipopolysaccharide (LPS) stimulation of BMDCs results in increased expression of miR-155, but not miR-31; $n = 2$/condition. (d, e) LPS-activated DCs also have elevated miR-31, but not miR-155, upon migrating through (bottom DCs) an *in vitro* BBB, $n = 6$/condition. Values represented as mean ± SEM. Relative expression normalized to U6 snRNA. Student's unpaired, two-tailed *t*-test (a, b, d, e). *P* -values: $^{*}P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$.

populations.[10] Furthermore, miRNAs can also influence cellular migration, most notably in cancer metastasis.[40,41] To this end, we sought to characterize the miRNA expression profile of migratory BMDCs, distinct from microglia, in the CNS compartment during EAE.

Our finding that miR-31 expression is enriched in BMDCs that pass through the BBB both *in vivo* and *in vitro* suggests that miR-31 may participate in CNS, or general inflamed tissue, localization of peripheral DCs. A precedent for this concept was reported in vascular development, whereby miR-126-3p mediates perivascular and endothelial interactions.[42] More abundant evidence for miRNA regulation of cellular migration stems from the field of tumour biology. For example, miR-223 facilitates gastric cancer cell migration and invasion,[40] while miR-515-5p expression is associated with reduced migration and metastasis of multiple cancer lines.[41] Thus, miR-31 may act to regulate the trafficking of DCs through the BBB during inflammation. Consistent with a functional role for elevated miR-31 in modulating a DC phenotype associated with infiltration of inflamed tissue, we found that miR-31-binding sites were enriched in mRNAs downregulated in DCs that migrated into the CNS during EAE (Fig. 3a). The confirmed miR-31-downregulated mRNAs (Hiat1, Srp54b and Tspan31) will be interesting candidates for further studies into the phenotypic changes that contribute to DC migration into inflamed tissue.

Alternatively, the milieu of the inflamed CNS microenvironment may drive DC expression of miR-31 upon invasion of the CNS and other inflamed tissue. Again, similar observations have been made in tumour studies, including metastatic infiltration into the brain.[43,44] The potential for specific miRNA changes in DCs based on interactions beyond the BBB during EAE and MS raises the intriguing possibility that miR-31 acts to perpetuate inflammation within the local tissue environment. Indeed, miR-31 targets IL-34, which is a critical component for microglial survival as well as maintaining BBB integrity.[45,46] Using a luciferase reporter assay, we found that miR-31 robustly represses IL-34 (Fig. S4), which predicts a loss of IL-34 by infiltrating DCs within the CNS during EAE. While IL-34 is expressed most abundantly in

neurons within the naive CNS, it is possible that miR-31-mediated suppression of IL-34 by DCs assists ongoing BBB damage during EAE. Further exploration of the effects from miR-31-dependent IL-34 expression changes *in vivo* during EAE is warranted.

Intriguingly, activation of DCs using LPS did not induce miR-31 expression, nor influence the migration of DCs through an *in vitro* BBB (Fig. 4c,e). While several miRNAs have been implicated in DC activation, including miR-34a, let-7c and miR-155,[12,47,48] miRNA regulation of DC migration through the BBB has not been characterized extensively. Expression of both chemokine receptors[49] and adhesion molecules[50] is considered critical for DC migration into the CNS, and both have been shown to be targets of miRNA regulation.[51] The maturation state of DCs influences the combination of chemokine and cytokine expression,[52] thus adding to the complexity of DC trafficking into the CNS. In dissecting the steps involved in DC infiltration through the BBB, it is feasible that distinct molecular pathways for activation and trafficking are utilized, thus enabling additional levels of regulation, some of which may be miRNA-mediated. Indeed, the separation of differentiation and activation of DCs from their migration was recently described by Rhee and colleagues, who found that PTPN12 is essential for DC migration from the periphery to secondary lymphoid organs without influencing differentiation, maturation or cytokine production.[53] Our data suggest that miR-31 induction in DCs may be regulated by factors other than those involved in DC maturation, thus identifying a distinct miRNA-dependent regulatory mechanism for DC migration during neuro-inflammation.

Recently, a report demonstrated the importance of miR-31 in EAE based on its role in T-cell lineage commitment.[37] Using a murine conditional miR-31 knockout, Zhang and colleagues demonstrated that miR-31 serves to inhibit the generation of peripheral T regulatory cells (Tregs).[37] The function of miR-31 during EAE may thus serve dual effects on driving the pathogenesis of MS by coordinating a layered inflammatory response in both the innate as well as adaptive immune responses. Our data found that miR-31 expression is higher in $CD11c^+$ DCs in the peripherary as compared with $CD3^+$ T-cells (Fig. S2f), but that upon entry into the CNS, $CD3^+$ T-cells have increased expression of miR-31 (Fig. S2g). Because DCs have higher miR-31 expression than T-cells in spleen and DCs are among the first cells to enter the CNS during EAE,[11,54] it is possible that DC entry, and expression of miR-31, may be requisite for T-cell engagement and subsequent induction of miR-31 expression. Moreover, if DC expression of miR-31 influences BBB permeability, then migration of miR-31-expressing DCs could enable entry of T-cells. To elucidate the complex interplay between miR-31 expression in DCs and T-cells, the conditional knockout mice generated by Zhang and colleagues[37] should be used to study the role of miR-31 in $CD11c^+$ DCs during EAE.

More fundamental is the question of what defines DCs versus microglia within the inflamed CNS. Compared with peripheral myeloid cells, microglia have a specific miRNA expression pattern.[10] Importantly, use of radiation bone marrow chimeras segregates microglia that express CD11c from BMDCs.[11,35] Our data demonstrate a signature of infiltrating BMDCs within the CNS during EAE distinct from microglia (Fig. 2a). In contrast, miR-34b-3p was found to be significantly elevated in the spinal cord of $miRAP^{CD11c} \rightarrow miRAP^{CD11c}$ chimeric mice, but not in the spinal cord of $miRAP^{CD11c} \rightarrow WT$ mice (Fig. 2e), suggesting miR-34b-3p may signify a subset of microglia adopting a DC phenotype. Differentiating unique miRNA expressed by DCs from microglia represents one essential step toward targeting individual contributions by each innate cell during EAE and MS. This work could be further expanded upon by differentiating BMDCs from DCs derived from CNS-infiltrating monocytes.

Evidence continues to build for a critical role of miRNA in DC function in MS. Our results indicate miR-31 may function in directing DC migration into the CNS or may be induced in DCs by the inflamed tissue environment, which could negatively influence DC mRNA expression profiles and/or BBB permeability. Interestingly, the induction of miR-31 does not seem to be influenced by LPS-mediated DC activation, indicating potentially separate levels of regulation for DC maturation and migration. Furthermore, while resident microglia can upregulate CD11c in EAE, miR-31 is sufficient to distinguish BMDCs from these resident immune cells. Finally, our work clarifies the role of miR-31 in EAE, demonstrating a functional role of miR-31 in DCs, in addition to the previously described role of miR-31 in Tregs. This study further highlights the importance of miR-31 in EAE, and suggests miR-31 inhibition may serve as a therapeutic for reducing inflammatory responses within the CNS by targeting DC expression.

Acknowledgements

MLH, ASA, TMG, LKO and MDC performed experiments. MLH and GFW designed the study. RSK, SDC, BSK and TMM provided new techniques and reagents. MLH, ASA, TMG, MDC, LKO, SDC, BSK, RSK, TMM and GFW edited the manuscript. MLH and GFW wrote the manuscript. The authors thank The Molecular Profiling Facility at The University of Pennsylvania for quantitative mRNA profiling. The authors also thank Cassie A.-L. Parks for assistance with these studies. The authors are grateful to Dr Anne Cross for critical review of the manuscript. Funding was provided by the Hope Center for Neurologic Diseases, and the National Institute of

Neurological Disorders and Stroke [R01NS083678 (GFW), R01NS078398 (TMM), R25NS09098502 (TMG), and F31NS092340 (MLH)].

Disclosure

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Raw microRNA array data and mRNA array data.

Figure S1. Volcano plot of miRNA microarray data generated from spleen and spinal cords of CD11c-Cre, LSL-tAgo2 mice following miRAP. Several miRNAs were significantly enriched ($P < 0.1$, log2FC > 1.5) in $CD11c^+$ DCs in the spinal cord versus spleen. miR31, 339-5p and miR-301a/b were selected for validation because they were abundant in both tissues (CT < 30).

Figure S2. MiR-31 expression in $CD3^+$ T-cells and $CD11c^+$ cells in EAE spleen and spinal cord.

Figure S3. miR-31 is elevated in DCs that migrate into inflamed tissue.

Figure S4. miR-31 regulates IL-34 expression through 3′-UTR binding.

Table S1. Primers used to generate putative miR-31 target 3′-UTR constructs.

