MicroRNA-146a Negatively Regulates the Immunoregulatory Activity of Bone Marrow Stem Cells by Targeting Prostaglandin E<sub>2</sub> Synthase-2

Mariola Matysiak, Maria Fortak-Michalska, Bozena Szymanska, Wojciech Orlowski, Anna Jurewicz and Krzysztof Selmaj

*J Immunol* published online 15 April 2013
http://www.jimmunol.org/content/early/2013/04/14/jimmunol.1202397
MicroRNA-146a Negatively Regulates the Immunoregulatory Activity of Bone Marrow Stem Cells by Targeting Prostaglandin E2 Synthase-2

Mariola Matysiak, Maria Fortak-Michalska, Bożena Szymanska, Wojciech Orlowski, Anna Jurewicz, and Krzysztof Selmaj

The molecular mechanisms that regulate the immune function of bone marrow–derived mesenchymal stem cells (BMSCs) are not known. We have shown previously that freshly isolated BMSCs when induced to express neuronal stem cell markers lose immunoregulatory function when transferred into mice sensitized to develop experimental autoimmune encephalomyelitis. Recently, microRNAs (miRs) have been shown to be involved in the regulation of several immune responses in both innate and acquired immunity. We now show that among several differentially expressed miRs, miR-146a was strongly upregulated in neuronally differentiated when compared with miR-146a expression in freshly isolated BMSCs or control BMSCs cultured in parallel but in nondifferentiating medium. Inhibition of miR-146a with a selective antagonim restored the immunoregulatory activity of nBMSCs. We mapped miR-146a to its multiple predicted target mRNA transcripts and found that miR-146a was predicted to block PGE2 synthase (ptges-2). We then showed that Ptges-2 was directly targeted by miR-146a using a luciferase reporter assay. Furthermore, increased expression of miR-146a in BMSCs correlated with inhibition of PGE synthase-2 and inhibition of PGE2 release. Accordingly, inhibition of miR-146a restored synthesis of PGE2. These data support the conclusion that miR-146a plays a critical role in the control of the immunoregulatory potential of BMSCs. The Journal of Immunology, 2013, 190: 000–000.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating condition of the human CNS characterized by recurrent episodes of immune-mediated demyelination and axonal loss (1). Among recently proposed therapeutic strategies, transplantation of myelin-forming precursor cells or stem cells (SC) has generated both interest and reservations (2–4). Bone marrow–derived mesenchymal stem cells (BMSC) provide the structural and functional support for the generation of blood cell lineages from hematopoietic SCs (5). BMSCs expanded in vitro can generate progeny that differentiate into multiple cell lineages (6). Several recent reports have demonstrated that under specific experimental conditions, BMSCs can differentiate into bone cells (7), fat cells (8), hepatocytes (9), glia, and neurons (10, 11). The neuron-differentiation potential of BMSCs has attracted intense interest in the possible application of BMSCs in cell and gene therapy strategies for neurologic diseases (12). BMSCs have also been shown to modulate immune responses by influencing some T cell, B lymphocyte, NK, and dendritic cell functions (13–15). The mechanisms underlying these effects are largely unknown but are likely to be mediated either by direct cell–cell contact and/or soluble factors such as TGF-β1 (16), hepatocyte growth factor (17), NO (18), and PGE2 (19–21).

MicroRNAs (miRs) have become recognized as important gene regulators in eukaryotic organisms, including SCs (22). miRs are a class of nonprotein-coding small RNAs that have a specific secondary stem-loop hairpin structure within their primary transcripts with a higher minimal folding free energy index (23). miRs negatively regulate gene expression at the posttranscriptional level through direct mRNA cleavage (24), translational repression (25), or mRNA decay mediated by miR deadenylation (26). Although many endogenous miRs have been identified in mammals, specific functions have remained largely undefined. However, recent studies indicate that the expression profiles of miRs in SCs are different from other tissues (27). This suggests that miRs may play an essential role in SC self-renewal, differentiation, and other functions (28). With respect to immunoregulation, miRs have been shown to be involved in the regulation of several immune responses in both innate and acquired immunity.

In this study, we analyzed the expression profile of miRs in BMSCs as a correlate of their immunoregulatory potential. We found that several miRs were significantly upregulated, including miR-146a, in neuronally differentiated BMSCs that had lost immunoregulatory activity in mice with experimental autoimmune encephalomyelitis (EAE), an animal model for MS. We found that miR-146a targeted PGE2-synthase 2, which resulted in decreased secretion of PGE2. Because PGE2 was shown to contribute to the immunosuppressive activity of BMSCs (19–21), we analyzed whether the functional relation between miR-146a and BMSC-induced immunosuppression depended on PGE2.
miR-146a REGULATES THE IMMUNOREGULATORY ACTIVITY OF BMSC

Materials and Methods

Isolation of BMSCs

Normal SJL mice, 6–8 wk old, were used for the isolation of BMSC. Bone marrow cells were obtained from the femurs and tibias of euthanized mice by flushing with PBS. Cells were washed twice in sorting medium that consisted of PBS supplemented with 0.5% BSA (Sigma-Aldrich, St. Louis, MO), and subjected to negative magnetic sorting using the Lineage Cell Depletion Kit (Milteny Biotech, Bergisch Gladbach, Germany). Depletion of cells expressing lineage Ags by mAbs and magnetic beads resulted in a pure fraction of Lin− cells. Purity of the Lin− fraction was invariably >98%, as assessed by flow cytometry using Lineage Cocktail (anti-CD3e, anti-CD11b, anti-CD45RB/B220, anti-Ly-6G and Ly-6C, and anti-TER-119; BD Biosciences, San Jose, CA) (29). Cells retained differentiation capacity and were characterized by lack of CD34, CD11b, and CD45. Isolation of murine bone marrow Sca-1+ cells was performed using MACS Sca-1 MultiSort Kit (Milteny Biotec., Bergisch Gladbach, Germany). After enrichment, Sca-1 expression was up to 20%, compared with <1% in Lin− BMSCs, and in this population, c-kit was <2%. Sca-1 defines pluripotent capacity of SC population (30, 31). In addition, examining these cells gene expression profile with Affymetrix GeneChip, we found numerous mesenchymal SC (MSC)–specific markers, such as cd44, cxcl4, cxcr4, bmi-1, c-kit, cxcl12, cxcl13, ccl2, il2, itgav, il10, il17, il1cam, and il7. Moreover, several other genes associated with MSCs were also identified: aoxa5, bglap, cttnb1, gga3, hgf, icam1, il1b, nud6, pgs, pprc, tf, vegfα, vim, and vwf. These data indicate that isolated lineage-negative murine bone marrow cell population enriched with Sca-1 possesses differentiation potential and meets criteria for MSCs (32).

Neural differentiation of SCs

BMSCs were cultured for 3 d in medium (DMEM: F12:1 [1:1]; -glutamine [1.9 mM], insulin [4.7 μg/ml], putrescine dihydrochloride [15.3 μg/ml], proges- terone [58 ng/ml], apo-transferrin [94 μg/ml], sodium selenite [38 ng/ml], -thyroxine [380 ng/ml], tri-iodo-thyronine [317 ng/ml], basic fibroblast growth factor [bFGF; 10 ng/ml], and gentamicin [47 μg/ml]). The clinical course of EAE was evaluated daily on a 0 to 5 scale (0 = healthy, 1 = limp tail, 2 = ataxia and/or paresis of hind limbs, 3 = paralysis of hind limbs and/or paresis of forelimbs, 4 = tetraparesis, 5 = moribund). Mice were observed for 90 d after immunization. Isolation of BMSCs

Female SJL mice, 6–8 wk old, were obtained from the Animal Care Department, Medical University of Lodz. All animals were housed in pathogen-free conditions and were treated according to the guidelines of the local Animal Ethics Committee. Mice were immunized s.c. according to a previously published protocol using 0.15 mg PLP139–151 in IFA (Difco Laboratories), supplemented with 4 mg/ml Mycobacterium tuberculosis. Immunization with PLP peptide was followed by i.v. administration of 400 ng pertussis toxin (Sigma-Aldrich), on days 0 and 2. Freshly isolated Lin−Sca-1− BMSCs (BMSCs), neural-differentiated Lin−Sca-1− BMSCs (nBMSCs), and cultured nondifferentiated Lin−Sca-1− BMSCs (cBMSCs), 2 × 106 in 200 μl PBS were transferred i.v. into EAE mice at the peak of disease. Control mice with EAE received a sham injection of the same volume (200 μl) of PBS. The clinical course of EAE was evaluated daily on a 0 to 5 scale (0 = healthy, 1 = limp tail, 2 = ataxia and/or paresis of hind limbs, 3 = paralysis of hind limbs and/or paresis of forelimbs, 4 = tetraparesis, 5 = moribund). Mice were observed for 90 d after immunization.

Proliferation assay

Spleen cells were obtained 2 wk after immunization for EAE and cultured (2 × 106 cells/well) in triplicate for 72 h in medium supplemented with 10 μg/ml PLP139–151. BMSCs irradiated in vitro (30 Gy) were cultured with spleno- cytes from EAE mice at a ratio 1:5. For the final 16 h, 1 μCi [3H]thymidine (Tdr; Amersham Biosciences, Buckinghamshire, U.K.) was added to each well. At the end of the culture period, incorporation of [3H]thymidine was determined in a Wallac Betaplate liquid scintillation counter (Perkin Elmer Life Sciences, Wellesley, MA). Results were expressed as cpm.

ELISA

Quantitative analysis of PGE2 was performed by ELISA, using commercially available kits (R&D Systems). Supernatants were derived from BMSC cultures during differentiation and in nondifferentiated medium (on 4 and 7 d) or in 24-h cultures of BMSCs. Supernatants were frozen and analyzed according to the manufacturer’s instructions. Control, standard, and test samples were added to each well and incubated for appropriate time with adequate primary Ab and conjugate. After washing, substrate solution was added to each well for 30 min at room temperature in the dark. Finally, Stop Solution was added and the OD of each well was determined within 30 min using a microplate reader (wavelength 450 nm).

Western blotting

Cell lysates were obtained from cultures of BMSCs (24 h), nBMSCs, and cBMSCs. Cells were pelleted and lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM PMSF, 1 μg/ml apolipoprotein and leupeptin). Lysates were centrifuged at 14,000 rpm for 5 min. An equal amount of protein from each cell lysate was separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (Immobilon, Millipore, MA). Membranes were blocked with 5% dried milk in TBS-TBS (Sigma Aldrich) overnight, followed by incubation for 1–2 h with PGE2, synthease-1, PGE2, synthease-2, and PGE2, synthease (cytosolic) (rabbit 1:500; Cayman Chemical, Ann Arbor, MI), and GAPDH (Abcam, Cambridge, U.K.). After washing with TBS, blots were incubated with peroxidase-coupled secondary anti-rabbit Ab (1:2,000; Sigma-Aldrich). Proteins were detected by ECL–ECL (Amersham Biosciences). Densitometry was performed for comparison of Western blot data (Alpha Innotec, San Leandro, CA).

miR analysis

Total RNA samples were isolated from BMSCs and nBMSCs by using the miRVana RNA Isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Reverse transcription was performed with miR-146a–specific primer, mumu miR-146a (TaqMan MicroRNA Assays; Applied Biosystem, Foster City, CA). Real-time PCR with TaqMan probes was normalized by small nuclear RNA levels. To make cDNA for each TaqMan miR assay, we incubated 10 ng total RNA with 0.15 μl dNTPs (100 mM), 1.5 μl 10× reaction buffer, 0.19 μl RNase inhibitor, 1 μl reverse transcriptase, and 3 μl gene-specific primer in a 15-μl reaction. Real-time PCR was performed using an Applied Biosystems 7500 Sequence Detection System. Quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. The ratio of miR-146a to 18S of each control sample was set at 1 and was used to calculate the fold change in target genes.

For miRCURY LNA Array miR, RNA samples were concentrated to at least 1 μg/ml. The Exiqon miRCURY LNA array version 10.0 (http://www.exiqon.com) (33) was used for data analysis in this study. All array data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE43621.

Anti–miR treatments

A 22-oligonucleotide, anti-miR-146a (antagomir; 5′-AACCAUGGAA-UUCAUGUUCUA-3′) was used at 10-nM concentration complexed with the transcription reagent Lipofectamine 2000 (2 μl/cell; Invitrogen, Carlsbad, CA). Anti-miR-146a (or Lipofectamine control) was used in nBMSCs cultured in 24-wells (0.5 × 106 cells/well). After 4 d of culture, cells were used for the transplantation procedure. After 48 and 96 h posttransfection, samples were analyzed by RT-PCR for miR-146a expression (as described earlier).

Reverse transcription with miR-146a–specific primer, mumu-miR-146a (TaqMan MicroRNA Assays; Applied Biosystem), was performed to assess miR expression after antagomir transfection. To make cDNA for each TaqMan miR assay, we incubated 10 ng total RNA with 0.15 μl dNTPs (100 mM), 1.5 μl 10× reaction buffer, 0.19 μl RNase inhibitor, 1 μl reverse transcriptase, and 3 μl gene-specific primer in a 15-μl reaction. TaqMan MicroRNA Assay is a target-specific stem-loop, reverse-transcriptase primer highly specific only for mature miR and resulted in longer reverse transcription amnon in range 50–150 bp according to manufactured specification. PCR fragments were resolved using MultiNA Microchip Electrophoresis System on gel for visualization of miR expression (Shimadzu Biotech, Milton Keynes, U.K.).

Luciferase reporter transfection and dual luciferase assay

In 3′-untranslated region (UTR) assay, 5 × 104 HEK293 cells were transfected with 500 ng of the UTR reporter (mIR-Report) with inserted PGES-2 (casp2 420 bp, 20 pmol pre–mIR-miR (miR-146a), and pre-miR (negative control; Ambion, Austin, TX), and 2 μl Lipofectamine 2000 (Invitrogen). Lysates were harvested 24 h after transfection, and reporter activity was measured with Dual Luciferase Reporter Assay (Promega).
Statistical analysis

All data are expressed as the mean ± SD, and differences between groups were determined using the Student t test and Mann–Whitney U test; p values <0.05 were considered significant.

Results

miR expression profile in BMSCs in relation to their immunoregulatory potential for EAE

In a previous study, we demonstrated that mice transplanted i.v. with BMSCs showed significantly lower clinical scores and faster recovery compared with control mice with EAE (34). However, transplantation of Lin− Sca-1+ BMSCs that had been induced to differentiate into cells expressing GFAP, NeuN, and O4 (nBMSCs) resulted in a diminished immunoregulatory effect on the course of EAE compared with the transplantation of fBMSCs. Therefore, in this study, we tested with unbiased approach both BMSC populations, fBMSCs and nBMSCs, for miR expression profiles to search for miR associated with their immunoregulatory potential. When clustering samples and genes, 30 of 757 miRs passed the filtering criteria on variation across samples (SD > 1.0) (35) (Table I). Hence, these 30 miRs were used in the two-way hierarchical clustering analysis of genes. Analysis of log2(Hy3/Hy5) ratios showed that the most upregulated miR during BMSCs differentiation of PLP-activated T cells (Fig. 2C). These results indicate that miR-146a represents a critical regulatory element in molecular mechanisms involved in the immunosuppressive activity of BMSCs in the EAE model.

miR-146a inhibition reversed the loss of immunoregulatory function of nBMSCs

To confirm further the role of miR-146a in loss of immunoregulatory function of nBMSCs in EAE, we applied a selective miR-146a antagonist (antagomir). Transfection of nBMSCs with miR-146a antagomir resulted in stable decreased expression of up to 90% of miR-146a (Fig. 2A, 2B). Transplantation of these nBMSCs that had been transfected with the miR-146a antagomir resulted in amelioration of EAE comparable with that obtained with fBMSCs (Fig. 2C). These results indicate that miR-146a represents a critical regulatory element in molecular mechanisms involved in the immunosuppressive activity of BMSCs in the EAE model.

miR-146a inhibition restored the regulatory activity of differentiated nBMSCs on PLP-induced proliferation

In Fig. 3, we show that the immunoregulatory activity of fBMSCs in EAE correlated with an inhibitory effect on PLP-induced T cell proliferation (p < 0.013), whereas nBMSCs, which were not efficient in EAE immunoregulation, did not show an effect on PLP-induced T cell proliferation. However, nBMSCs transfected with the miR-146a antagomir showed an inhibitory effect on proliferation of PLP-activated T cells (p < 0.0274) that was comparable with fBMSCs (Fig. 3).

Table I. miR expression using MiRCURY array

<table>
<thead>
<tr>
<th>miR</th>
<th>Log Median Ratios</th>
<th>nBMSC4d</th>
<th>nBMSC7d</th>
<th>fBMSC</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-451</td>
<td>−5.56</td>
<td>−4.81</td>
<td>1.44</td>
<td>3.84</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-146a</td>
<td>0.45</td>
<td>0.69</td>
<td>−4.98</td>
<td>3.21</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-99b</td>
<td>0.89</td>
<td>0.51</td>
<td>−4.65</td>
<td>3.09</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-22</td>
<td>0.39</td>
<td>0.51</td>
<td>−4.77</td>
<td>3.02</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-144</td>
<td>−3.62</td>
<td>−4.00</td>
<td>1.14</td>
<td>2.86</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-125a-5p</td>
<td>0.54</td>
<td>0.27</td>
<td>−3.87</td>
<td>2.47</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-155</td>
<td>0.56</td>
<td>1.21</td>
<td>−3.30</td>
<td>2.44</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-21</td>
<td>0.74</td>
<td>1.04</td>
<td>−3.23</td>
<td>2.38</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-146b</td>
<td>0.35</td>
<td>0.68</td>
<td>−3.42</td>
<td>2.28</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-342-3p</td>
<td>1.34</td>
<td>0.34</td>
<td>−2.43</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-29c</td>
<td>0.97</td>
<td>1.16</td>
<td>−2.00</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-222</td>
<td>0.63</td>
<td>0.61</td>
<td>−2.12</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-20a</td>
<td>−1.81</td>
<td>−1.19</td>
<td>1.13</td>
<td>1.55</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-221</td>
<td>0.59</td>
<td>0.53</td>
<td>−2.08</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-652</td>
<td>0.96</td>
<td>0.71</td>
<td>−1.55</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-24</td>
<td>0.72</td>
<td>0.42</td>
<td>−1.80</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-19a</td>
<td>−1.54</td>
<td>−0.63</td>
<td>1.15</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-27b</td>
<td>0.29</td>
<td>0.55</td>
<td>−1.93</td>
<td>1.36</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-23b</td>
<td>0.54</td>
<td>0.44</td>
<td>−1.83</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-23a</td>
<td>0.59</td>
<td>0.43</td>
<td>−1.75</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-20b</td>
<td>−1.81</td>
<td>−1.08</td>
<td>0.71</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-17</td>
<td>−1.61</td>
<td>−1.03</td>
<td>0.81</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-20b</td>
<td>−1.77</td>
<td>−1.14</td>
<td>0.65</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-763</td>
<td>−1.58</td>
<td>−1.44</td>
<td>0.59</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-19b</td>
<td>−1.29</td>
<td>−0.56</td>
<td>1.07</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-29b</td>
<td>0.52</td>
<td>0.72</td>
<td>−1.37</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-18a</td>
<td>−1.37</td>
<td>−0.61</td>
<td>0.87</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-27a</td>
<td>0.14</td>
<td>0.47</td>
<td>−1.59</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-100a</td>
<td>−1.08</td>
<td>−0.45</td>
<td>0.89</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>mmu-miR-29a</td>
<td>0.24</td>
<td>0.52</td>
<td>−1.33</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>
Mir-146a is predicted to regulate the ptges2 gene

We next mapped mir-146a to its multiple predicted target mRNA transcripts in relation to their role in BMSC immunoregulatory function. We used several mirTarget software (www.mirbase.org [36], www.targetscan.org, and www.microRNA.org) and consistently found that mir-146a was predicted to block PGE2 synthase-2. It was of a particular interest that the ptges2 gene for PGE2 synthase-2 was selected for further testing in relation to miR-146a control of immunoregulatory function of BMSCs. To investigate whether ptges2 can be directly targeted by mir-146a, we engineered a luciferase reporter construct that contained a 420-bp fragment of the ptges2 gene. In HEK293 cells, the luciferase reporter was cotransfected with the mir-146a precursor (pre-miR-146a) or a nonspecific control pre-miR. The results showed that the pre-miR-146a significantly reduced luciferase activity, whereas the control pre-miR did not (Fig. 4B).

Increased expression of miR-146a in nBMSCs correlates with inhibition of PGE synthase-2 and inhibition of PGE2 release

fBMSCs, which are capable of inhibiting EAE, showed high levels of PGE2 production, whereas nBMSCs that lack EAE immunoregulatory function showed significantly lower secretion of PGE2 (Fig. 5A). PGE2 is synthesized by three different PGE synthases: microsomal PGE synthase (mPGES)-1 and -2, and cytosolic PGE synthase (cPGES). Using specific Abs for all PGE2 synthases, we detected expression of all three synthases in fBMSCs, but in nBMSCs, expression of mPGES-2 coded by ptges2 was significantly decreased compared with fBMSCs (Fig. 5B). We did not observe differences in expression levels of known miR-146a targets, IL-1R–associated kinase 1 and TNFR-associated factor 6, between BMSCs and nBMSCs (Fig. 5C).

Antagonism of mir-146a restores PGE synthase-2 expression and PGE2 production in nBMSCs

To establish the role of miR-146a in regulation of PGE2 production in BMSCs, we assessed the effect of the miR-146a antagomir on PGE2 release. The results showed that nBMSCs transfected with miR-146a antagomir had decreased expression of miR-146a (~90%) compared with control BMSCs cultured under the same conditions but not transfected with antimir-146a (Fig. 6A). Most importantly, in nBMSCs transfected with miR-146a antagomir, we observed restoration of PGE2 production (Fig. 6B). Furthermore, PGE2 release in nBMSCs transfected with miR-146a antagomir correlated with increased expression of mPGES-2 (Fig. 6C). fBMSC coculture with PLP-reactive spleen cells induced strong inhibition of PLEP-induced proliferation. nBMSCs showed a significantly milder inhibitory effect, but transfection with antimir-146a enhanced inhibition of PLP-reactive T cells (p < 0.003; Fig. 6D).

To correlate further mir-146a expression levels with PGE2 synthesis, we performed the reverse experiment and attempted to not find prediction of miR-146a for other genes related to PGE2 synthesis; ptges3, ptges1, or ptgs2. Thus, PGE2 synthase-2 was selected for further testing in relation to miR-146a control of immunoregulatory function of BMSCs. To investigate whether ptges2 can be directly targeted by mir-146a, we engineered a luciferase reporter construct that contained a 420-bp fragment of the ptges2 gene. In HEK293 cells, the luciferase reporter was cotransfected with the mir-146a precursor (pre-miR-146a) or a nonspecific control pre-miR. The results showed that the pre-miR-146a significantly reduced luciferase activity, whereas the control pre-miR did not (Fig. 4B).

Increased expression of miR-146a in nBMSCs correlates with inhibition of PGE synthase-2 and inhibition of PGE2 release

fBMSCs, which are capable of inhibiting EAE, showed high levels of PGE2 production, whereas nBMSCs that lack EAE immunoregulatory function showed significantly lower secretion of PGE2 (Fig. 5A). PGE2 is synthesized by three different PGE synthases: microsomal PGE synthase (mPGES)-1 and -2, and cytosolic PGE synthase (cPGES). Using specific Abs for all PGE2 synthases, we detected expression of all three synthases in fBMSCs, but in nBMSCs, expression of mPGES-2 coded by ptges2 was significantly decreased compared with fBMSCs (Fig. 5B). We did not observe differences in expression levels of known miR-146a targets, IL-1R–associated kinase 1 and TNFR-associated factor 6, between BMSCs and nBMSCs (Fig. 5C).

Antagonism of mir-146a restores PGE synthase-2 expression and PGE2 production in nBMSCs

To establish the role of miR-146a in regulation of PGE2 production in BMSCs, we assessed the effect of the miR-146a antagomir on PGE2 release. The results showed that nBMSCs transfected with miR-146a antagomir had decreased expression of miR-146a (~90%) compared with control BMSCs cultured under the same conditions but not transfected with antimir-146a (Fig. 6A). Most importantly, in nBMSCs transfected with miR-146a antagomir, we observed restoration of PGE2 production (Fig. 6B). Furthermore, PGE2 release in nBMSCs transfected with miR-146a antagomir correlated with increased expression of mPGES-2 (Fig. 6C). fBMSC coculture with PLP-reactive spleen cells induced strong inhibition of PLEP-induced proliferation. nBMSCs showed a significantly milder inhibitory effect, but transfection with antimir-146a enhanced inhibition of PLP-reactive T cells (p < 0.003; Fig. 6D).

To correlate further mir-146a expression levels with PGE2 synthesis, we performed the reverse experiment and attempted to
induce its expression in cBMSCs cultured in parallel with nBMSCs but without the neuronal differentiation conditions. cBMSCs produced ample amounts of PGE2 and maintained immunoregulatory function similar to fBMSCs. cBMSCs transfected with pre–Mir-146a construct resulted in an increase in miR-146a expression by 200-fold (Fig. 7A). Furthermore, cBMSCs transfected with pre–Mir-miR-146a showed a decrease in PGE2 production (Fig. 7B), which correlated with mPGES-2 synthesis inhibition (Fig. 7C). Proliferation assay revealed loss of immunosuppressive potential of cBMSCs transfected with pre–miR-146a (Fig. 7D).

Discussion

In a previous publication, we showed that freshly isolated Lin− BMSCs enriched for Sca1 cells transplanted i.v. into mice sensitized for EAE ameliorated the course of disease (40). Freshly isolated Lin− Sca1+ cells expressed a wide panel of molecules characteristic for MSCs. The immunoregulatory activity of Lin−Sca1+ BMSC was dependent on a nondifferentiated state and required secretion of PGE2 (34). In this study, we searched for molecular mechanisms that contributed to the immunoregulatory properties of freshly isolated nondifferentiated BMSCs. Based on recent progress in research identifying the role of miRs in the dynamic processes involved in cell differentiation and proliferation, we focused our studies on miRs (22). miR profiling of fBMSC population, with high immunoregulatory activity, analyzed against BMSCs differentiated toward a neuronal phenotype (nBMSC), without immunoregulatory activity, showed a number of miRs that were differentially expressed. Nineteen miRs were upregulated and 11 downregulated as a result of the neuronal differentiation process. The most highly differentially expressed miR was miR-146a, which in BMSCs cultured under neuronal differentiation conditions for 4–7 d was increased 500-fold. Because nBMSCs...
expressing high miR-146a lost immunoregulatory function, we hypothesized that miR-146a might negatively regulate this property of BMSCs. We verified this assumption by experiments in which we selectively inhibited miR-146a in nBMSCs by transfection with a specific anti–miR-146a antagomir. nBMSCs transfected with the miR-146a antagomir showed a reduction in miR-146a expression and restoration of immunoregulatory activity in EAE.

The miR-146 family is composed of two members, miR-146a and miR-146b. To date, a role for miR-146 has been demonstrated in the development of multiple cancers (41) and in the negative regulation of inflammation induced via the innate immune response (42). miR-146a expression has been shown to be induced in response to LPS, TNF-α, or IL-1β activation of TLR-2, -4, or -5 (43, 44). Searching for miR-146a targets that might be of significance in the negative regulation of the immunoregulatory activity of BMSCs, we mapped miR-146a to its multiple predicted target mRNA transcripts using three independent miRs databases. Interestingly, miR-146a was found to interfere with the Ptges2 gene that codes for PGE2 synthase-2, in all three databases. PGE2 in earlier studies was found to maintain the immunosuppressive activity of BMSCs toward allogeneic reactions. The immunosuppressive function of MSCs was noted in the absence of direct cell contact, indicating a role of soluble mediators like PGE2 in dendritic cell maturation and inhibition of activated T lymphocyte proliferation (21); PGE2 also contributed to the modulation of cytokine production by T cells (20). MSC-mediated immunoregulation by PGE2 was implicated in inhibition of TNF-α and IFN-γ production by activating dendritic cells and T cells (19). Of particular interest was that, in our previous study, we found that immunoregulation of EAE by BMSCs was dependent on PGE2.
secretion (34). Freshly isolated BMSCs produced high amounts of PGE2 and suppressed EAE development, whereas neuronal differentiated BMSCs lost PGE2 secretion and were not able to immunoregulate EAE. Thus, PGE2 synthases represent relevant targets for a role for miR-146a in the downregulation of the immunoregulatory activity of BMSCs. The functional dependence on the downregulation of ptgse2 by miR-146a was directly shown in experiments using a luciferase reporter assay.

Biosynthesis of PGE2 is mediated by three isoforms of PGE synthases (PGESs): two pPGESs (pPGES-1 and pPGES-2) (44) and one cPGES (45). pPGES-2 is an enzyme capable of coupling with both COX isoenzymes and is constitutively expressed in tissues (46). The pPGES-2 transcript is abundantly distributed in cells in the brain, heart, skeletal muscle, kidney, and liver (44), whereas pPGES-1 transcript is primarily detected in organs related to the immune response (47), suggesting that these two enzymes exhibit tissue specific functions. In experiments with nBMSCs transfected with an miR-146a antagonist, we detected increased expression of pPGES-2, but not pPGES-1 or cPGES. Thus, we hypothesized that miR-146a negatively controls BMSC immunoregulatory function by targeting ptgse2 leading to diminished secretion of PGE2. To verify this hypothesis, we performed a series of loss- and gain-of-function experiments. For the former, we transfected nBMSCs with a selective miR-146a antagomir and demonstrated that an increase in PGE2 secretion occurred in parallel to diminished miR-146a expression. In the gain-of-function experiments, we induced miR-146a expression in BMSCs for engineering SCs with a high immunoregulatory profile that can potentially be used for the treatment of human autoimmune diseases.

In summary, we have shown for the first time, to our knowledge, that the molecular mechanism controlling the immunoregulatory activity of BMSCs depends on miR-146a. Increased expression of miR-146a after BMSC differentiation negatively controls the immunoregulatory properties of BMSCs by targeting the pPGES-2 gene. These results contribute to our understanding of the immunoregulatory properties of BMSCs and provide a target for engineering SCs with a high immunoregulatory profile that can potentially be used for the treatment of human autoimmune diseases.

Disclosures

The authors have no financial conflicts of interest.

References


