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MicroRNA-301a inhibition enhances the immunomodulatory functions of adipose-derived mesenchymal stem cells by induction of macrophage M2 polarization

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Abstract

MicroRNAs (miRNAs) are a class of short non-coding RNAs that play a significant role in biological processes in various cell types, including mesenchymal stem cells (MSCs). However, how miRNAs regulate the immunomodulatory functions of adipose-derived MSCs (AD-MSCs) remains unknown. Here, we showed that modulation of miR-301a in AD-MSCs altered macrophage polarization. Bone marrow (BM)-derived macrophages were stimulated with LPS (1 μg/ml) and co-cultured with miRNA transfected AD-MSCs for 24 h. The expression of M1 and M2 markers in macrophages was analyzed. Inhibition of miR-301a induced M2 macrophage with arginase-1, CD163, CD206, and IL-10 upregulation. Additionally, toll-like receptor (TLR)-4 mRNA expression in macrophages was downregulated in co-cultures with AD-MSCs transfected with a miR-301a inhibitor. Nitric oxide (NO) in the supernatant of AD-MSC/macrophage co-culture was also suppressed by inhibition of miR-301a in AD-MSCs. We further found that suppression of miR-301a in AD-MSCs increased prostaglandin E_2 (PGE2) concentration in the conditioned medium of the co-culture. Taken together, the results of our study indicate that miR-301a can modulate the immunoregulatory functions of AD-MSCs that favor the applicability as a potential immunotherapeutic agent.

Keywords

immunomodulatory, macrophage polarization, mesenchymal stem cells, miR-301a

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Introduction

Liver transplantation is a therapeutic option for end-stage liver disease. Even with modern immunosuppressive treatment protocols, allograft rejection is still of concern.[1] Mesenchymal stem cells (MSCs) have been demonstrated unique immunomodulatory effects on innate and adaptive immune cells.[2–5] Recently, MSCs have a wide rage application in regenerative medicine, even in an allogeneic context, because of their immunomodulatory properties. A pro-inflammatory environment (presence of *interferon* (IFN)-γ, *tumor necrosis*

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factor (TNF)-α, *interleukin* (IL-1)-β) rather enhances the potency of MSCs to suppress T-cell proliferation and activates regulatory T cells.[6–8] This phenomenon is suggested to be mediated by direct cell-cell contact and/or through paracrine factors such as transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), and prostaglandin E_2 (PGE2).[9,10] Of these, indoleamine-2, 3-dioxygenase (IDO) and TNF-stimulated gene 6 (TSG-6) have been shown to be critical for MSCs' immunomodulatory function.[9] These regulatory properties have attracted the attention for potential clinical utility of MSCs to treat immune diseases, as well as to prevent allograft rejection in organ transplantation.[11] Moreover, the therapeutic advantage of MSCs has rapidly been recognized because of the relatively convenient isolation and expansion, and the low toxicity. As MSCs exist only in a few tissue populations, further MSC use for cell-based therapies and tissue regeneration need prior expansion. However, evidence has been accumulating during the past decade indicating that the expansion of MSCs is highly inconsistent in culture conditions in comparison with freshly isolated MSCs.[10,12] It has also been shown that external factors can influence the behavior and function of MSCs.[10]

In recent years, microRNAs (miRNAs) have been shown to regulate multiple differentiation and immunomodulatory functions of MSCs.[13–20] For instance, miR-196a and miR-204 have been demonstrated to regulate osteogenic differentiation through target of homeobox (HOX)-C8 and runt-related transcription factor 2 (Runx2) pathways, respectively.[15,17] Also, miR-21 enhances adipogenic differentiation of human adipose-derived (AD)-MSCs through modulation of *signal transducer and activator of transcription 3* (*STAT3*) signaling.[14] However, the role of miRNAs on MSCs' immunomodulatory activity remains poorly explored. In our experimental orthotopic liver transplantation (OLT) model, DA-LEW OLT (DA rats: liver donor; Lewis rats: recipients) resulted in acute rejection, whereas PVG recipients with DA livers survive without immunosuppression (tolerogenic). Comparison of the miRNA profile in AD-MSCs between the two OLT models using a miRNA microarray revealed a number of potential immunomodulatory miRNA candidates. From those, we observed that miR-301a was not only highly expressed in AD-MSCs but also upregulated in the liver of rejection OLT model.[21] MicroRNA-301a (miR-301a), together with miR-130a, miR-130b, and miR-301b, belongs to the miR-130/301 family in the mouse genome.[22] miR-301a has been reported to be overexpressed in several tumor types and acts as an oncogenic miRNA facilitating tumorigenesis.[23–25] In addition, miR-301a has also been reported to play a regulatory role in the immune system. Increased levels of miR-301a were observed following T cell activation.[22] However, the immunoregulatory function of miR-301a in AD-MSCs was unclear. As an essential component of the innate immunity, macrophages make crucial contributions to the *adaptive immune* response and play immunoregulatory roles in immune homeostasis and pathogenic infection.[26] Recent studies have suggested that MSCs are capable of educating normal macrophages into an M2 phenotype with increased IL-10/TGF-β1 and decreased TNF-α/*inducible nitric oxide synthase* (*iNOS*) expression.[27,28] Therefore, in the current study we addressed whether miR-301a is involved in the immunoregulatory function of AD-MSCs on macrophage polarization.

Materials and methods

Preparation of rat adipose tissue derived stem cell (AD-MSC) suspensions

The abdominal adipose tissue was obtained from 8-week-old LEW rats. AD-MSCs were isolated as previously described.[29] All animal experimentation was reviewed and approved by our institutional experimental animal committee.

Transfection of miR-301a mimic and inhibitor

Transfection of miRNA into the AD-MSCs was conducted when they reached 70% confluence. AD-MSCs were transfected with a miR-301a mimic (20 nM) or inhibitor (40 nM) (Ambion®, Thermo Fisher Scientific Inc.) using GenMute™ Transfection Reagent (SignaGen Laboratories, MD, USA) following the manufacturer's protocol.

Bone marrow (BM)-derived macrophage cultures

Thighbones obtained from LEW rats were cleaned of muscle tissue and flushed with PBS. BM-derived macrophages were placed in fresh complete

medium (DMEM with 10% FBS, 1% PSA, and 2 mM L-glutamine) containing 10 ng/ml recombinant macrophage colony-stimulating factor ((r) M-CSF) (PeproTech, Rehovot, Israel). On day 7, cells were treated with LPS (1 μg/ml), and analyzed for the relative gene expression and the release of nitric oxide using real-time PCR and NOA 280i, respectively.

Co-culture of macrophages with AD-MSCs

For Trans-well culture, Millicell cell culture inserts with a pore size of 1.0 μm (Millipore Corporation, MA, USA) were placed into 6-well plates with macrophages seeded at the bottom of the wells and treated with LPS (1 μg/ml), while 2×10^5 miRNA-transfected AD-MSCs were seeded onto the inserts and continued to culture for 24 h.

Reverse transcription-polymerase chain reaction (RT-PCR)

TaqMan® MicroRNA Assays (Applied Biosystems, CA, USA) were used to quantitatively detect the levels of miR-301a using target-specific probes (000528, Applied Biosystems) according to the manufacturer's instructions. RNU6B (001973, Applied Biosystems) was used to normalize the data. The rat specific primers were summarized in Supplemental Table 1. The quantitative RT-PCR reaction was performed on an ABI 7500 Fast Real-Time PCR System.

Nitric oxide (NO) measurement in supernatant of macrophages co-cultured with miRNA-modified AD-MSCs

NO-derived products such as nitrite, nitrate, and nitrosothiols were determined in culture supernatant by reacting with $VaCl_3$ using an NOA 280i NO analyzer (GE Analytical Instruments Inc.).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for PGE2 were purchased from R&D Systems. The measurement was conducted according to the manufacturer's protocol. ELISA was run in duplicate and plates were read at 450 nm on a Microplate ELISA reader (PerkinElmer VICTOR X4).

Immunoblotting

The proteins were separated using 10% SDS-PAGE and immunoblotted according to standard protocols. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with primary antibodies prepared in TBST at 4°C overnight. Primary antibodies used in this study include: STAT3, p-STAT3 (Abcam, MA, USA), and β-actin (Millipore Corporation, Bedford, MA, USA). Secondary antibody was peroxidase-conjugated goat anti-mouse or rabbit IgG (Jackson ImmunoResearch; PA, USA) prepared in TBST. Detection was performed using an enhanced chemiluminescence (ECL) detection kit (Millipore). Images were captured using a G:BOX Image Station iChemi XL device (SYNGENE, Cambridge, UK), and the relevant bands were quantified by densitometry using GeneTools (SYNGENE).

Statistical analysis

Descriptive statistics, means, standard deviations, and ranges were used where appropriate. The results are shown as the mean value ± the standard deviation (SD) of the mean. Statistical comparisons between different groups were calculated by SPSS version 20 software (IBM Corporation, Armonk, New York). A value of $P < 0.05$ was considered significant.

Results

Inhibition of miR-301a expression in AD-MSCs induced M2 macrophage polarization

Transfection of the miR-301a mimic strikingly up-regulated miR-301a expression, whereas transfection of the miR-301a inhibitor down-regulated miR-301a in AD-MSCs (Figure 1). To assess the effect of miR-301a-transfected AD-MSCs on macrophage polarization, the BM-derived macrophages were stimulated with LPS (1 μg/ml) and co-cultured with AD-MSCs. Both the expression of M1 and M2 markers in macrophages were analyzed. M1 markers such as IL-12β, CXCL-10, MHCII and MCP-1 were significantly decreased by co-culturing with AD-MSCs transfected with the miR-301a inhibitor (Figure 2(a)). M2 markers including Arginase-1, CD163, CD206 and IL-10

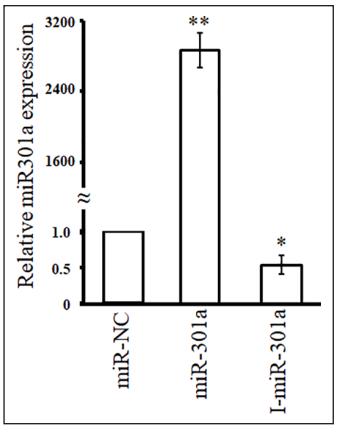

Figure 1. Transfection of the miR-301a mimic and inhibitor in AD-MSCs. AD-MSCs were transfected with the miR-301a mimic (20 nM) and inhibitor (40 nM) for 24 h. The expression of miR-301a was quantified by real time-PCR. *,**Denotes significantly different when compared with the control group ($P < 0.05$ and 0.01, respectively).

were strongly induced by co-culturing with AD-MSCs transfected with the miR-301a inhibitor (Figure 2(b)). Moreover, the percentage of M2 surface marker CD206 was significantly increased in macrophages co-cultured with AD-MSCs transfected with the miR-301a inhibitor (Figure 2(c) and (d)). Consistent with this observation, the macrophages showed a rounded and/or spindle shaped morphology in the miR-301a inhibitor group (Figure 2 (e)).

Inhibition of miR-301a expression in AD-MSCs suppressed toll-like receptor (TLR)-4 expression in macrophages

As TLR-4 activation promotes M1 polarization,[30] we next explored whether miRNA-modulated AD-MSCs affects TLR-4 activation in macrophages. We found that expression of TLR-4 in macrophages was significantly reduced in co-cultures with AD-MSCs transfected with the miR-301a inhibitor (Figure 3). These data indicate that inhibition of miR-301a in AD-MSCs have the immunomodulatory ability to alter the macrophages towards an M2 phenotype.

Inhibition of miR-301a expression in AD-MSCs suppressed NO production in macrophages

Next, we assayed the nitric oxide production in macrophages co-cultured with AD-MSCs using a NOA 280i NO analyzer. The NO production was significantly decreased in macrophages co-cultured with AD-MSCs transfected with the miR-301a inhibitor compared to the control group (Figure 4). Additionally, the NO production was suppressed in all co-culture groups compared to the group in which macrophages were treated with LPS only.

Increased PGE2 levels in macrophages co-cultured with AD-MSCs transfected with the miR-301a inhibitor

M2 macrophages can be induced by MSCs through soluble factors, such as TGF-$\beta 1$ and PGE2.[9] Hence, we measured the PGE2 level in the supernatant of macrophages co-cultured with AD-MSCs. Secretion of PGE2 was increased when macrophages were co-cultured with AD-MSCs transfected with the miR-301a inhibitor compared to the control group (Figure 5(a)). Next, STAT3 phosphorylation was determined using Western blotting. As shown in Figure 5(b), the levels of p-STAT3 were not significantly different in AD-MSCs transfected with either miR-301a miRNA or the inhibitor.

Discussion

There is growing evidence that miRNAs mediate a multitude of biological processes in MSCs.[15,17,19] In this study, we revealed that miR-301a regulated the immunomodulatory functions of AD-MSCs on macrophage polarization. Macrophages are ubiquitously present innate immune cells, they have a central role in inflammation and host defense in almost all tissues.[26,31] Macrophage polarization and the ratio of M1 (inflammatory):M2 (immunomodulatory) macrophages are likely to be regulated by paracrine signals from the environment.[32] Our results showed that inhibition of miR-301a in AD-MSCs modulated macrophage polarization, with significantly decreased M1 phenotype and enhanced M2 phenotype including upregulation of arginase-1, CD163, CD206, and IL-10. However, over-expression of miR-301a in AD-MSCs had no significant effect on the expression of M1 phenotype such as IL-12β, CXCL-10, and MCP-1 compared to control group. Additionally, activation of the TLRs, especially TLR4, predominantly drives the macrophages towards an M1 phenotype.[30] Our data also showed that TLR-4 mRNA expression in

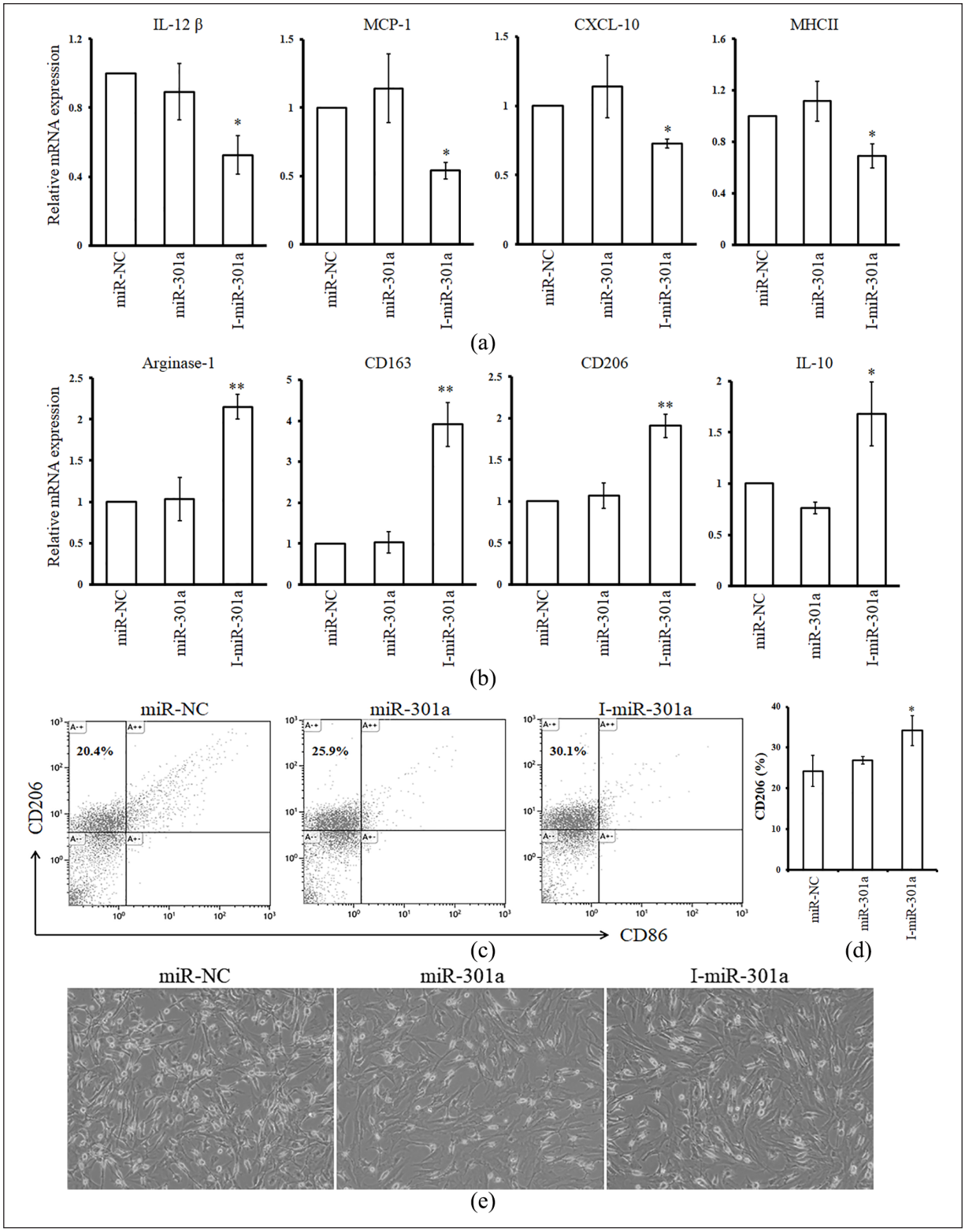

Figure 2. The regulatory effect of miRNA-modulated AD-MSCs on polarization of BM-derived macrophages. Macrophages were stimulated with LPS (1 μg/ml) for 24 h in the presence of miRNA-transfected AD-MSCs in the Trans-well system. The mRNA expression of macrophages was analyzed by real-time PCR. The gene expression of (a) IL-12β, CXCL-10, MHCII and MCP-1; (b) arginase-1, CD163 and CD206 in LPS-stimulated macrophages co-cultured with miRNA transfected-AD-MSCs. (c and d) Comparison of CD206 expression on macrophages co-cultured with miRNA-transfected AD-MSCs. (e) The morphology of macrophages co-cultured with miRNA-transfected AD-MSCs. *,**Denotes significantly different when compared with the control group ($P < 0.05$ and 0.01, respectively).

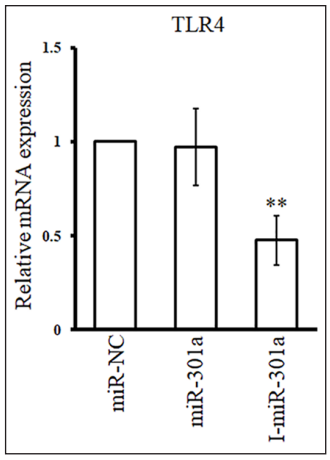

Figure 3. The expression of TLR4 in BM-derived macrophages co-cultured with miRNA-transfected AD-MSCs. Macrophages were stimulated with LPS (1 μg/ml) for 24 h in the presence of miRNA-transfected AD-MSCs in the Trans-well system. The expression of TLR-4 was analyzed using real-time PCR. $^{**}P < 0.01$ compared with the control group.

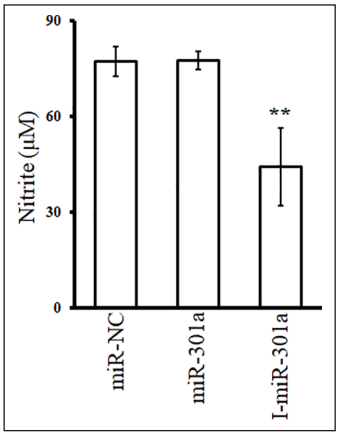

Figure 4. NO production in BM-derived macrophages co-cultured with AD-MSCs transfected with the miR-301a mimic and inhibitor. After BM-derived macrophages were differentiated for 8 days, cells were co-cultured with miRNA-modulated AD-MSCs for 24 h in the presence of LPS (1 μg/ml). The supernatant was collected for NO analysis. $^{**}P < 0.01$ compared with the control group.

macrophages was downregulated in co-cultures with AD-MSCs transfected with a miR-301a inhibitor. Additionally, NO in the supernatant of AD-MSC/macrophage co-culture was suppressed by inhibition of miR-301a in AD-MSCs. M1 macrophages express high levels of iNOS, which converts L-arginine to NO and competes with arginase-1, the enzyme that further converts, NO to urea and ornithine. By inhibiting iNOS, arginase-1 can promote the M2 phenotype and lead to M1 suppression.[30]

Recent data also suggest that MSCs can mediate suppressive effects in numerous ways.[5,33] The mechanisms proposed to mediate the immunosuppressive effect of MSCs, including increased activities of IDO, iNOS, and cyclooxygenase 2 (COX2), as well as the secretion of PGE2, have been reported to play a role in this process.[34,35] Moreover, MSCs could cause alternative activation of macrophages, and induce macrophages to adapt to an enhanced regulatory phenotype via secretion of paracrine factors.[9] Nemeth et al.[36] have shown that the immunosuppressive mechanisms of MSCs identified PGE2 as the main factor in the differentiation of the monocyte fraction of PBMC into M2 immunosuppressive macrophages. Our data showed that suppression of miR-301a could affect PGE2 concentration in conditioned medium of AD-MSCs co-cultured with macrophages. Our present study showed that miR-301a could affect macrophage polarization via paracrine factors such as PGE2 in AD-MSCs.

MiRNAs function as endogenous regulators of gene expression by targeting the 3′-untranslated region (3′-UTR) of target genes. Recently, several studies have demonstrated immunomodulatory functions of MSCs regulated by miRNA. MSCs from miR-21 knockout mice show enhanced immunomodulatory capacity by induction of regulatory T cells and secretion of TGF-β1 compared with wild-type MSCs.[19] Over-expression of miR-30a in MSCs stimulated with IL-1β inhibited the activation of nuclear factor κB (NF-κB) and JNK signaling pathways and subsequently decreased the expression of IL-6, COX2, and IL-8. Additionally, the immunomodulatory effects of MSCs on macrophages were also impaired by miR-30a.[18] Matysiak et al.[20] reported that miR-146a plays a critical role in the control of the immunomodulatory potential of MSCs through regulating PGE synthase-2 expression and PGE2 release. The role of miR-301a in MSCs was unknown. It was previously reported that inhibition of miR-301a suppressed IL-17 secretion in T helper 17 cells.[37] Mycko et al.[38] demonstrated that miR-301a influenced the development of Th17 cells via inhibition of the IL-6–STAT3 pathway and found that PIAS3, a STAT3 inhibitor, was a target of miR-301a in $CD4^+$ T cells. Our previous results have demonstrated induction of IL-6 production through overexpression hepatic miR-301a in rat primary hepatocytes. Moreover, splenocytes co-cultured with hepatocytes overexpressing

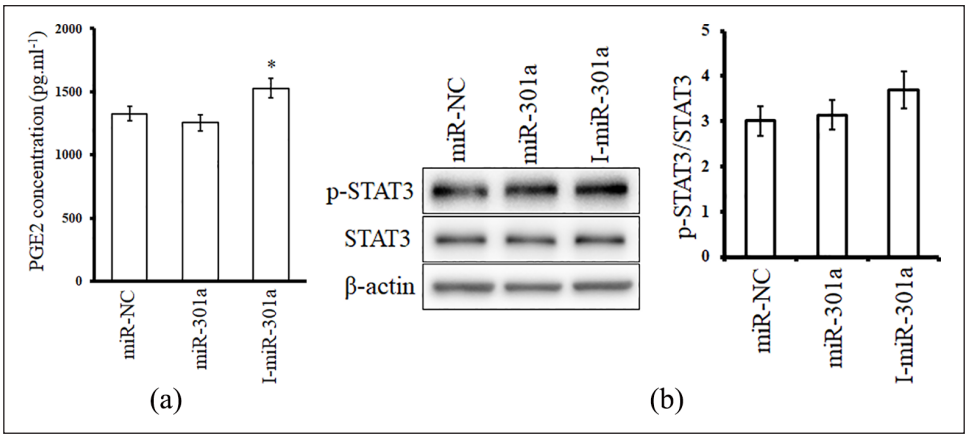

Figure 5. The PGE2 level in supernatant of BM-derived macrophages co-cultured with miRNA-modulated AD-MSCs. Differentiated BM-derived macrophages were co-cultured with miRNA-modulated AD-MSCs for 24 h in the presence of LPS (1 μg/ml). (a) The supernatant was collected and PGE2 concentration was measured by ELISA. (b) p-STAT3 protein levels were determined using immunoblotting. *$P < 0.05$ compared with the control group.

miR-301a showed induction of T helper 17 cell differentiation via downregulation of PIAS3.[21] However, the activation of STAT3 was not significantly different in AD-MSCs transfected with either miR-301a miRNA or the inhibitor. The targets of miR-301a in AD-MSCs will be investigated in further studies.

Conclusion

In summary, our work has demonstrated that miR-301a regulates immunomodulatory functions of AD-MSCs. Inhibition of miR-301a in AD-MSCs induces M2 macrophage polarization. We further found that immunomodulatory effects of miR-301a inhibition in AD-MSC, were through modulating PGE2 secretion, modulation of TLR4 expression and NO production in macrophages. Our results have provided an interest in understanding the role of miR-301a in controlling the therapeutic activity of AD-MSCs.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

Ethics approval for this study was obtained from Institutional Animal Care and Use Committee (approval No. 2014062501, 2019102202) in Kaohsiung Chang Gung Memorial Hospital.

Animal welfare

The present study followed the Animal Protection Law by the Council of Agriculture, Executive Yuan, R.O.C., and the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, USA for humane animal treatment and complied with relevant legislation.

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Supplemental material

Supplemental material for this article is available online.

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