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MicroRNA-9 Regulates the Differentiation and Function of Myeloid-Derived Suppressor Cells via Targeting Runx1

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Myeloid-derived suppressor cells (MDSCs) play a critical role in tumor-associated immunosuppression, thus affecting effective immunotherapies for cancers. However, the molecular mechanisms involved in regulating the differentiation and function of MDSCs remain largely unclear. In this study, we found that inhibition of microRNA (miR)-9 promoted the differentiation of MDSCs with significantly reduced immunosuppressive function whereas overexpression of miR-9 markedly enhanced the function of MDSCs. Notably, knockdown of miR-9 significantly impaired the activity of MDSCs and inhibited the tumor growth of Lewis lung carcinoma in mice. Moreover, miR-9 regulated MDSCs differentiation by targeting the runt-related transcription factor 1, an essential transcription factor in regulating MDSC differentiation and function. Furthermore, the CREB was found to regulate miR-9 expression in MDSCs. Taken together, our findings have identified a critical role of miR-9 in regulating the differentiation and function of MDSCs. The Journal of Immunology, 2015, 195: 1301–1311.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells, including myeloid progenitors, precursors of macrophages, dendritic cells and granulocytes, which are characterized by their strong ability to suppress T cell functions (1, 2). In mice, MDSCs are broadly defined as cells coexpressing two phenotypic markers CD11b and Gr-1 (1). Murine MDSCs can be further subdivided into two different subsets based on their expression of Ly-6C and Ly-6G (3, 4). CD11b+Ly-6G+Ly-6C− cells showing granulocyte-like morphology are termed granulocytic MDSCs (G-MDSCs), whereas CD11b+Ly-6C−Ly-6G+ cells with monocytic-like morphology are termed monocytic MDSCs (M-MDSCs). Unlike murine MDSCs, the human MDSCs are less well defined owing to the lack of specific markers. MDSCs are commonly defined as CD11b+CD33+HLA-DRlow/− cells. MDSCs in humans are also subdivided into two main subsets: CD15+CD14+CD11b+CD33+HLA-DRlow/− G-MDSCs and CD15−CD14+CD11b+CD33+HLA-DRlow/− M-MDSCs (5–7). Different subsets of MDSCs use differential pathways to suppress T cell function. G-MDSCs suppress T cell proliferation mainly by expressing high levels of arginase 1 (arginase 1 (ARG1)) and reactive oxygen species (ROS) whereas M-MDSCs express inducible NO synthase (iNOS) in addition to high levels of ARG1 (1, 4, 8, 9). In healthy individuals, MDSCs are generated in bone marrow and quickly differentiate into mature macrophages, dendritic cells, or granulocytes. However, a partial block in the differentiation of MDSCs into mature myeloid cells often occurs under pathological conditions, resulting in the expansion of this population (1). Because MDSCs are critically involved in tumor-associated immune suppression, many studies have focused on exploring therapeutic strategies to eliminate these cells or to modulate their function. As the immunosuppressive activity of MDSCs is mostly associated with their immature state, further studies on the modulation of MDSCs differentiation will be of great interest in reversing their immunosuppressive functions within the tumor microenvironment.

β-Glucans have been recognized as biological response modifiers, which can enhance both innate and adaptive immune responses (10, 11). Dectin-1, a non–Toll-like pattern recognition receptor for β-glucan, is predominantly expressed on myeloid cells, including dendritic cells, monocytes/macrophages, neutrophils, and a subset of T cells (12, 13). Our previous studies have shown that both M-MDSCs and G-MDSCs express dectin-1 (14). Moreover, whole β-glucan particles (WGPs), the particulate β-glucan derived from yeast Saccharomyces cerevisiae, could promote M-MDSC differentiation into more mature myeloid cells via the dectin-1 pathway, thus effectively abrogating M-MDSC–mediated immune suppression and enhancing antitumor responses (14). However, the molecular mechanisms underlying the functional modulation of MDSCs remain largely unclear.

The microRNAs (miRNAs) are short and single-stranded noncoding RNAs (20–23 nucleotides) that play crucial roles in regulating gene expression at the posttranscriptional level. It becomes clear that miRNAs bind to the specific cognate sequences in the 3′ untranslated region (UTR) of target mRNAs to mediate posttranscriptional gene repression by degrading target transcripts or inhibiting protein translation. The CREB was found to regulate miR-9 expression in MDSCs. Taken together, our findings have identified a critical role of miR-9 in regulating the differentiation and function of MDSCs. The Journal of Immunology, 2015, 195: 1301–1311.
miRNAs have been shown to regulate the differentiation, maturation, and function of immune cells. Moreover, miRNAs play an essential role in the maintenance of the cellular homeostasis and the development of various hematopoietic systems. Many miRNAs identified from mouse bone marrow cells are differentially regulated in various hematopoietic lineages. However, it is still not clear whether and how miRNAs are involved in regulating MDSC differentiation and function.

In this study, we found that microRNA (miR)-9 played a critical role in regulating the differentiation and function of MDSCs. Treatment with WGP markedly downregulated miR-9 expression in mouse MDSCs, an effect specifically abolished by supplementing miR-9. Moreover, downregulation of miR-9 can promote the differentiation of MDSCs and decrease their suppressive capacity. Furthermore, we identified runt-related transcription factor 1 (Runx1), a regulator in differentiation and function of MDSCs, as the direct target of miR-9. Thus, these findings provide new insights into understanding the molecular mechanisms that regulate the differentiation and function of MDSCs.

Materials and Methods

Cell line, mice, and tumor models

The Lewis lung carcinoma (LLC) cells, human embryonic kidney cell line 293T cells, and mouse monocyte/macrophage cell line RAW264.7 were purchased from the American Type Culture Collection. Male C57BL/6 mice were housed in a specific pathogen-free animal facility and used at 6–8 wk of age. For tumor models, mice were implanted s.c. with LLC (3 × 10^6/mouse) (14).

Isolation of MDSCs

Murine MDSCs were isolated from the spleens of LLC tumor-bearing mice using a mouse MDSC isolation kit (Miltenyi Biotec) following the manufacturer’s protocol. The purity of CD11b^+Ly-6G^−Ly-6Ch^low G-MDSCs was >98% and the purity of CD11b^+Ly-6G^−Ly-6Ch^high M-MDSCs was >90%. Total MDSCs were isolated using anti-CD11b conjugated to biotin followed by anti-biotin microbeads (Miltenyi Biotec), and the purity of CD11b^+Gr-1^+ cells was >98%. For MDSC stimulation, β-glucan WGP (Biothera) was provided by Dr. Jun Yan from the University of Louisville School of Medicine (22).

Flow cytometry

For surface markers, single-cell suspensions were stained with relevant fluorochrome-conjugated CD11c, F4/80, CD40, CD80, CD86, and MHC class II mAbs (eBioscience). Cells were stained with above Abs in PBS for 30 min, and then analyzed by flow cytometry.
RNA isolation and quantitative real-time PCR

Total RNA of cells was extracted in TRIZol (Invitrogen). Then, cDNA was synthesized with ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer’s instructions. The RT-PCR and quantitative real-time PCR (qRT-PCR) were performed as previously described (23). β-Actin was used as an internal control. The primers are listed in Supplemental Table 1. For qRT-PCR analyses of miRNA using SYBR RT-PCR kit (Takara Bio), sequences of RT primers with stem-loop structure are: U6, 5'-CCCTTCGACGTAATCAGGAC-3'; mmu-miR-9, 5'-GCTGATCACGATGCTGTCGGTGGAGTCGGCAATTGCACTGGATACGAC-3'; CACCCAC-3'; GAGTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGA-3'. qRT-PCR primers are listed in Supplemental Table 1. The relative expression of miRNAs was normalized to that of internal control U6. Relative quantification of mRNA expression was calculated by the comparative threshold cycle method.

Microarray analysis

The miRNA microarray assay was conducted at KangChen Bio-tech. Total RNA was extracted using TRIZol and an miRNeasy mini kit (Qiagen) according to the manufacturer’s instructions. After passing RNA quantity measurement by the NanoDrop 1000, samples were labeled using the miRCURY LNA array (v.16.0). Following the washing steps, the slides were scanned with the Axon GenePix 4000B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs that had intensities >50 in all samples were chosen for calculating normalization factor. Expressed data were normalized using median normalization. After normalization, differentially expressed miRNAs were identified through fold change filtering. The threshold used to screen up- or down-regulated miRNAs was fold change ≥ 2.0. The microarray data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67578) under accession number GSE67578.

Western blot analysis

Proteins extracted from cells were prepared as previously described (23). Protein samples were separated by SDS-PAGE, then transferred onto Immobilon polyvinylidene difluoride membranes (Bio-Rad) and probed with rabbit Runx1 Ab (Cell Signaling Technology) and mouse β-actin Ab (Abcam) followed by chemiluminescent detection (Champion Chemical).

Detection of arginase activity and ROS levels

Arginase activity, determining the conversion of arginine to ornithine and urea, was detected by a quantitative colorimetric assay employing a QuantiChrom arginase assay kit (BioAssay Systems). The arginase activity was calculated according to the manufacturer’s instructions.

The oxidation-sensitive dye 2',7'-dichlorofluorescin diacetate (Invitrogen) was used to detect ROS production by MDSCs. Cells were simultaneously cultured with 2.5 μM 2',7'-dichlorofluorescin diacetate with 30 ng/ml PMA in PBS for 30 min before flow cytometric analysis.

MDSC suppression assay

For evaluation of MDSC suppressive function, MDSCs isolated from spleens of tumor-bearing mice were transfected with miR-9 mimics, miR-9 inhibitors, and Runx1 small interfering RNA (siRNA) or a negative control for miRNA mimics, inhibitor, and siRNA (Ribobio). For responder cells, splenic CD4⁺ T cells and CD8⁺ T cells were sorted from wild-type (WT) C57BL/6 mice using CD4 microbeads (Miltenyi Biotec), FITC-conjugated anti-CD8 mAb (BD Pharmingen), and anti-FITC microbeads (Miltenyi Biotec). The purities of CD4⁺ T cells and CD8⁺ T cells were >95%. Responder cells were cocultured with different ratios of transfected MDSCs (without gamma irradiation) in U-bottom 96-well plates (Costar) in the presence of anti-CD3 (BioLegend, 10 μg/ml) and anti-CD28 (Bio-Legend, 5 μg/ml) mAbs for 72 h and pulsed with [³H]thymidine (Pharmacia Biotech, 1 μCi/well) for the last 16 h of culture. The capacity of MDSCs to suppress T cells was calculated by cpm MDSCs plus T cells – cpm MDSCs, and the average values of MDSC cpm under different treatment were <120.

Transfection

miR-9 mimics, miR-9 inhibitors, miR-9 agomir, miR-9 antagonist, Runx1 siRNA, and negative controls were synthesized by Ribobio. Oligonucleotide transfection was performed with Entranster-R (Engreen Biosystem) according to the manufacturer’s instructions.

3'UTR luciferase reporter assays

The WT Runx1 3'UTR luciferase reporter vectors were constructed by amplifying the mouse Runx1 3'UTR and cloned into the XhoI site of the psiCHECK-2 vector (Promega). The mutation type of Runx1 3'UTR was obtained from the WT construct by overlap-extension PCR. 293T cells were cotransfected with 100 ng luciferase reporter plasmid and 100 nM miR-9 mimics using Lipofectamine 2000 reagents (Invitrogen) according
to the manufacturer’s instructions. After 24 h, lysates of 293T cells were harvested using lysis buffer (Promega), and luciferase activities were measured using Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

Promoter analysis

A 2-kb fragment located upstream of the miR-9 transcription start site was PCR amplified and inserted into pGL3-Basic vector (Promega). The mutation type lacking CREB binding sites was obtained from the WT construct by overlap-extension PCR. The WT plasmids (pGL3-miR-9 promoter) or mutation-type plasmids (Mut pGL3-miR-9 promoter), control plasmid (pRL-TK) together with CREB overexpression plasmids, or control plasmids (pcDNA3.1) were cotransfected into 293T cells. After 24 h, cells were assayed with the Dual-Luciferase assay (Promega).

In vivo MDSC suppressive experiments

MDSCs transfected with miR-9 agomir, miR-9 antagonir, or negative controls were injected into the tumors of mice on days 1 and 7 (2 \( \times 10^6/\)mouse) after palpable tumors were formed. The live MDSCs transfected with BLOCK-iT fluorescent oligonucleotides (Invitrogen) can be measured in vivo even after injection for 5 d (Supplemental Fig. 1A–H).

Patients and tissue samples

Human primary tumor tissues and distal noncancerous tissues were collected from patients clinically diagnosed with lung cancer at the Affiliated People’s Hospital of Jiangsu University (Zhenjiang, China). Tissue samples were immediately snap frozen in liquid nitrogen. Both tumors and distal noncancerous tissues were histologically examined.

**FIGURE 3.** miR-9 suppresses Runx1 expression by directly targeting its 3’UTR. (A) Schematic representation of the predicted Runx1 3’UTR indicating the binding sites for miR-9, and designed mutated version of the Runx1 3’UTR. (B) Constructed luciferase reporter vectors. Luciferase reporter vectors were constructed in psiCHECK-Runx1 3’UTR with the following: 1) seed sequence 1 (185–190, Runx1 3’UTR1) and seed sequence 2 (653–658, Runx1 3’UTR2); or Runx1 3’UTR(1+2); 2) mutated seed sequence 1 and seed sequence 2, or Runx1 3’UTR1 Mut+2; 3) seed sequence 1 and mutated seed sequence 2, or Runx1 3’UTR2 Mut+1; and 4) mutated seed sequence 1 and mutated seed sequence 2, or Runx1 3’UTR(1+2) Mut. The Runx1 3’UTR was cloned and mutated by PCR-based mutagenesis according to the protocol in Materials and Methods. (C) 293T cells (1 \( \times 10^6 \)) were cotransfected with 100 nM miR-9 mimics (miR-9) or miR control for 24 h, and relative expression of miR-9 (D) and Runx1 (E) was analyzed by qRT-PCR. (F) RAW264.7 cells were transfected with different amounts of miR-9 mimics (0, 10, 50, 100 nM) or miR control for 24 h, and relative expression of miR-9 and Runx1 were determined. Data are shown as mean \( \pm \) SD of three independent experiments. **p < 0.01, ***p < 0.001. Ctrl, control.
**Immunofluorescence microscopy**

Paraffin sections were incubated with FITC-conjugated anti-CD11b mAb (eBioscience) and allophycocyanin-conjugated anti-CD33 mAb (eBioscience). Matched isotype controls (eBioscience) were used for control staining. After three washes in PBS, the sections were incubated with Hoechst 33342 (Beyotime) for labeling nuclei.

**RNA extraction from serum and qRT-PCR analysis for miRNA**

Blood samples of lung cancer patients and healthy subjects were collected and then centrifuged to obtain serum. A NucleoSpin miRNA plasma kit (Macherey-Nagel) was used to extract miRNA in plasma. cDNA of the miRNA was synthesized with miRNA-specific RT primers in the transcription RT mixture (Takara Bio) (RT buffer, dNTP mixture, Moloney murine leukemia virus, RNase inhibitor), with the procedure including 42°C for 60 min and 70°C for 10 min. qRT-PCR analyses of miRNA used the SYBR RT-PCR kit (Takara Bio) and followed the recommended cycling conditions (denaturation at 95°C for 30 s followed by 35 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 15 s). The relative expression of miRNAs was normalized to that of internal control U6. Relative quantification of mRNA expression was calculated by the comparative threshold cycle method.

**Computational prediction**

Two target prediction databases, TargetScan 6.1 (http://www.targetscan.org) and miRanda (http://www.microrna.org/), were used to analyze the interactions between miR-9 and Runx1 3’UTR. Putative CREB binding site within the 2-kb 5’ flanking sequence of pri-miR-9 was predicted with TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html).

**Statistical analysis**

The statistical significance of differences between groups was determined by the Student t test and two-way ANOVA. For survival analysis, the Kaplan–Meier method and log-rank test were performed. Correlations between variables were determined by a Spearman correlation coefficient. All analyses were performed using SPSS 16.0 software. Differences were considered significant at a p value of <0.05.

**Study approval**

All animal protocols were approved by the Institutional Committee on the Use of Animals for Research and Teaching. All human samples were obtained with informed consent in accordance with the regulations approved by the Affiliated People’s Hospital of Jiangsu University.

**Results**

**Runx1 is involved in the differentiation and function of MDSCs**

Runx1 is an essential transcription factor that controls the development of multiple hematopoietic lineage expression and modulates several genes underlying myeloid differentiation (24). In our experiments, G-MDSCs and M-MDSCs were isolated from spleens of mice bearing LLC for flow cytometric analysis, and the ratio of G-MDSCs to M-MDSCs was ∼3:1 (Supplemental Fig. 2A, 2B). We previously showed that β-glucans could promote the differentiation of M-MDSCs into more maturate myeloid cells with reduced immunosuppressive functions. In the present study, we further found that WGPS could also downregulate the sup-

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**FIGURE 4.** Downregulation of miR-9 in MDSCs promotes the differentiation of the cells and impairs their suppressive capacity. Isolated M-MDSCs and G-MDSCs from tumor-bearing mice spleens were transfected with 100 nM miR-9 mimics (miR-9), 200 nM miR-9 inhibitors (miR-9i), or miRNA control; after 24 h, (A) relative expression of miR-9 was detected by qRT-PCR. (B) M-MDSCs transfected with miR-9 inhibitors or negative control were stained with specific Abs against CD11c, F4/80, CD40, CD80, CD86, and MHC class II (isotype: solid gray). (C and D) Transfected M-MDSCs (C) and G-MDSCs (D) were harvested, and arginase activity and ROS levels were measured as described in Materials and Methods. RNA from each group was extracted and reverse transcribed for qRT-PCR for iNOS. (E and F) M-MDSCs (E) and G-MDSCs (F) were transfected with miR-9 mimics, miR-9 inhibitors, or miRNA control; after 24 h, cells were harvested and then cocultured with responder cells, CD4+ T cells (left), or CD8+ T cells (right) (MDSC/T cell ratio of 2:1, 1:1, 0.5:1) in the presence of anti-CD3 mAb and anti-CD28 mAb for 72 h. Suppressive function of MDSCs on T cell proliferation was measured by incorporation of [3H]thymidine. Statistical significance is evaluated between miR-9/miR-9i group and control group. Data are shown as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Ctrl, control.
pressive effect of G-MDSCs (Supplemental Fig. 2C–F). Notably, Runx1 expression in M-MDSCs and G-MDSCs was upregulated after β-glucan stimulation (Fig. 1A). Knockdown of Runx1 in MDSCs by siRNA significantly blocked the β-glucan-mediated effect. As shown in Fig. 1C, WGP-induced M-MDSC differentiation was partially blocked upon the inhibition of Runx1 expression. Levels of CD11c, F4/80, CD40, CD80, CD86, and MHC class II expression were decreased upon downregulation of Runx1 expression. Furthermore, levels of arginase, iNOS, and ROS secreted by M-MDSCs and G-MDSCs were markedly upregulated after Runx1 was knocked down (Fig. 1D, 1E). Additionally, knocking down Runx1 expression in both M-MDSCs and G-MDSCs significantly enhanced the capacity of MDSCs to suppress CD4+ and CD8+ T cell proliferation (Fig. 1F, 1G). Thus, these data suggest that Runx1 is involved in regulating the differentiation and function of MDSCs.

Expression of miR-9 is decreased in MDSCs after WGP treatment

To characterize the differential expression of miRNAs in MDSCs upon WGP stimulation, we first performed miRNA microarray analysis on RNA samples isolated from MDSCs treated with or without WGPs. It was found that 61 miRNAs were downregulated whereas 40 miRNAs were upregulated in MDSCs stimulated by WGPs. To identify the possible miRNAs involved in the differentiation and function of MDSCs, we used TargetScan 6.1 and miRanda software to predict miRNAs that could bind to the 3' UTR of Runx1. According to the data from miRNA microarrays and predictive software analysis, three miRNAs that were downregulated in MDSCs upon WGP stimulation and could also bind to Runx1 3'UTR were selected: miR-9, miR-181d, and miR-181b. We confirmed the expression of these miRNAs by qRT-PCR analysis, among which the expression of miR-9 and miR-181d was consistent with the data from microarray (Fig. 2A). To further verify the expression of miR-9 and miR-181d in M-MDSCs and G-MDSCs after WGP stimulation, we found that miR-181d expression was decreased in G-MDSCs but upregulated in M-MDSCs (Fig. 2B). However, only miR-9 was decreased in both M-MDSCs and G-MDSCs stimulated by WGPs (Fig. 2C). To verify whether the reduction of miR-9 was mediated via the dectin-1 pathway, we found that miR-9 expression was reversed to high levels after blocking dectin-1 with anti-dectin-1 Ab (Fig. 2D). Taken together, these data suggest that miR-9 may be involved in the regulation of MDSC differentiation and function.

miR-9 directly targets Runx1 in MDSCs

Next, we investigated whether Runx1 was a direct target of miR-9. Using TargetScan 6.1 combined with miRanda analyses, we found that the 3' UTR of the Runx1 gene had two highly conserved seed sequences (position 185–190 and position 653–658 in mice) for miR-9 (Fig. 3A). Because there existed two seed sequences in the Runx1 3' UTR, we first tested the contribution of each seed sequence for regulating the expression of Runx1. Mouse Runx1 3'UTR (1+2) containing position 185–190 (Runx1 3'UTR1) and...
position 653–658 (Runx1 3′UTR) or mutated versions (Runx1 3′UTR1 mutant with WT seed sequence 653–658 [Runx1 3′UTR1 Mut+2] and Runx1 3′UTR2 mutant with WT seed sequence 185–190 [Runx1 3′UTR2 Mut+1]) (Fig. 3B) were cloned into a psiCHECK luciferase reporter vector downstream from Renilla luciferase and then used to assess whether miR-9 could repress luciferase gene expression in 293T cells. As shown in Fig. 3C, luciferase expression in cells transfected with Runx1 3′UTR (1+2) was repressed by miR-9. Moreover, mutation of either position 185–190 or position 653–658 in the Runx1 3′UTR did not significantly affect the luciferase expression in the presence of miR-9, further indicating that both seed sequences regulated the expression of Runx1. The mutation of both position 185–190 and position 653–658 of the 3′UTR seed match sequences relieved the inhibition by miR-9. These data suggest that both seed sequences of Runx1 3′UTR play an important role in regulating the expression of Runx1. To determine whether binding of miR-9 to Runx1 3′UTR could regulate Runx1 expression, a monocytic/macrophage cell line RAW264.7 was transfected with miR-9 mimics at different doses. Transfection was confirmed using qRT-PCR (Fig. 3D), and Runx1 expression was measured at both mRNA and protein levels. As shown in Fig. 3E and 3F, the protein levels of Runx1 in RAW264.7 cells transfected with miR-9 mimics were lower than in negative control transfected cells, whereas the transcription level of Runx1 was not affected. Notably, Runx1 siRNA not only downregulated the transcription but also protein levels of Runx1. Taken together, these results demonstrate that miR-9 directly targets Runx1 to downregulate Runx1 expression.

**Downregulation of miR-9 promotes MDSCs differentiation and impairs their suppressive capacity**

To define the function of miR-9 in MDSC differentiation and function, M-MDSCs and G-MDSCs were transfected with miR-9 mimics or inhibitors. As shown in Fig. 4, downregulation of miR-9 in M-MDSCs could enhance the expression of CD11c, F4/80, CD40, CD80, CD86, and MHC class II, indicating a mature phenotype of MDSCs upon miR-9 inhibition. Furthermore, the suppressive factors secreted by M-MDSCs and G-MDSCs, including arginase, iNOS, and ROS, were decreased after miR-9 was knocked down. In contrast, overexpression of miR-9 significantly enhanced the production of these suppressive factors by MDSCs (Fig. 4C, 4D). To determine whether the suppressive capacity of MDSCs was regulated by miR-9, isolated splenic M-MDSCs and G-MDSCs from mice bearing Lewis lung carcinoma were transfected with miR-9 mimics or inhibitors. As shown in Fig. 4E and 4F, miR-9 mimics remarkably enhanced the ability of MDSCs to suppress CD4+ and CD8+ T cells whereas inhibition of miR-9 reduced their suppressive function. Thus, these results demonstrate that downregulation of miR-9 promotes MDSCs differentiation and impairs their suppressive capacity.

**Exogenous miR-9 reverses the regulatory effect of WGPs on MDSC differentiation and function**

To further confirm that miR-9 regulates the differentiation and function of MDSCs, exogenous miR-9 was supplemented to cultured MDSCs in the presence of WGPs. It was found that the WGP-induced effect on MDSCs was reversed by upregulation of miR-9. As shown in Fig. 5A, levels of CD11c, F4/80, CD40, CD80, CD86, and MHC class II molecule on MDSCs were reduced after miR-9 was enhanced. Moreover, both arginase activity and levels of iNOS and ROS were also enhanced together with the increased miR-9 in MDSCs (Fig. 5B, 5C). Therefore, these data suggest that miR-9 inhibits the regulatory effect of WGPs on MDSC differentiation and function.

**Downregulation of miR-9 in MDSCs suppresses tumor growth in vivo**

To examine the function of miR-9 in MDSCs in vivo, we established a mouse model with LLC and intratumorously injected miR-9 mimic– or inhibitor–modified MDSCs to observe tumor progression. As shown in Fig. 6A, tumors in mice treated with miR-9...
inhibitor (antagonir)–modified MDSCs grew slower than did mice treated with control transfected MDSCs. Conversely, tumors in mice treated with miR-9 mimic (agomir)–transfected MDSCs were found to grow faster than those in mice treated with control transfected MDSCs. Moreover, tumor-bearing mice treated with miR-9 mimic–modified MDSCs showed significantly reduced survival when compared with the control group. However, there was no obvious prolonged survival in miR-9 inhibitor–modified MDSCs (Fig. 6B). To further determine whether miR-9 exerts any effect on tumor growth, miR-9 mimics or inhibitors were directly injected into tumors. We first confirmed that miR-9 mimics or inhibitors could cross the membrane of MDSCs (Supplemental Fig. 1I). As shown in Fig. 6C and 6D, upregulation of miR-9 significantly promoted tumor growth. However, downregulation of miR-9 did not show any significant effects on survival rate. Interestingly, miR-9 mimics significantly increased the level of arginase in tumor tissues whereas miR-9 inhibitors reduced arginase mRNA expression (Fig. 6E). Similar effects were also observed in the CT26 colon carcinoma model (Supplemental Fig. 3).

**CREB controls the expression of miR-9**

CREB is a transcription factor that regulates various cellular responses, including proliferation, differentiation, and survival (25–27). CREB is induced by many growth factors and inflammatory signals and subsequently mediates the transcription of genes containing a cAMP-responsive element (28). In our study, we found that CREB expression was significantly decreased in both M-MDSCs and G-MDSCs upon WGP stimulation (Fig. 7A). Using anti–dectin-1 Ab to block the dectin-1 pathway further confirmed that WGP-induced CREB decrease was mediated by the dectin-1 pathway. As shown in Fig. 7B, CREB expression was recovered to a high level after inhibition of the dectin-1 pathway. Interestingly, after CREB in MDSCs was knocked down (Fig. 7C), miR-9 levels were also significantly decreased (Fig. 7D). To further delineate the relationship between CREB and miR-9, we established the WT and Mut of luciferase reporter vectors containing the pri-miR-9 promoter region using TFSEARCH for prediction. Thus, we obtained the WT and Mut of luciferase reporter vectors. The predictive binding site for CREB was 5'-TGACGTCA-3’, and the mutated binding site was changed to 5'-AATTCCGG-3’ (Fig. 7E). We next cotransfected CREB overexpression plasmids or control plasmid pcDNA3.1, WT-miR-9 promoter luciferase reporter vectors, or mutant miR-9 promoter vector together with thymidine kinase promoter Renilla luciferase reporter (pTK-RL) plasmids into 293T cells. As shown in Fig. 7F, ectopic expression of CREB

**FIGURE 7.** miR-9 is under the control of CREB. (A) Sorted M-MDSCs and G-MDSCs were cultured in the presence or absence of WGs at 100 µg/ml for 24 h. qRT-PCR was used to detect CREB mRNA expression. (B) The purified M-MDSCs and G-MDSCs were pretreated with anti–dectin-1 mAb or rat IgG (5 µg/ml) for 1 h at 37°C and then treated with 100 µg/ml WGs. After 24 h stimulation, cells were subjected to analyze CREB mRNA levels. (C and D) Isolated M-MDSCs and G-MDSCs were transfected with CREB siRNA (siCREB) or negative control for 24 h, and relative expression of CREB (C) and miR-9 (D) was analyzed by qRT-PCR. (E) Construction of luciferase reporter vectors containing pri-miR-9 promoter region. The binding site for CREB (5'-TGACGTCA-3’) was predicted according to TFSEARCH browser, and the mutation type was mutated by PCR-based mutagenesis (5'-AATTCCGG-3’). (F) 293T cells (1 × 10⁵) were cotransfected with WT or mutant pGL3-miR-9 promoter luciferase reporter vectors, thymidine kinase promoter Renilla luciferase reporter (pTK-RL) plasmids, together with CREB overexpression plasmids or control plasmids (pcDNA3.1). After 24 h, firefly luciferase activity was measured and normalized by Renilla luciferase activity. Data are shown as mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01. Ctrl, control.
enhanced the transcriptional activity of the 5’ flanking sequence of miR-9-1, and mutation of the binding site for CREB abolished the enhancing effect of CREB. Thus, these data suggest that miR-9 expression is directly regulated by CREB.

**High levels of miR-9 inversely correlate with Runx1 expression in lung cancer patients**

We further evaluated miR-9 expression and its correlation with Runx1 in human lung cancers. First, we found markedly increased numbers of MDSCs in tumor tissues (lung cancer) when compared to those in distal nontumorous tissues (distal lung) (Fig. 8A). Furthermore, miR-9 expression in tumor tissues was significantly higher than controls. However, there was no obvious elevation of miR-9 in plasma of lung cancer patients when compared with healthy individuals (Fig. 8B). As shown in Fig. 8C, expression of Runx1 was extremely low in tumor tissues, and there was a modest correlation between Runx1 and miR-9 in tumor tissues (Fig. 8E). Additionally, tumor tissues expressed high levels of arginase (Fig. 8D), and arginase had negative correlation with Runx1 but positive correlation with miR-9 (Fig. 8E). All of these data indicate that miR-9 is highly expressed in lung cancers and is associated with MDSC-derived suppressive factors.

**Discussion**

In this study, we show that miR-9 regulates MDSC differentiation and function by targeting Runx1 expression. Downregulation of miR-9 could significantly promote the differentiation of MDSCs and impair their suppressive capacity. Moreover, miR-9 level in MDSCs was controlled by CREB transcription factor. Taken together, our results have demonstrated a key role of miR-9 in regulating the differentiation and function of MDSCs.

Recent studies have shown that miRNAs control myeloid cell proliferation, differentiation, maturation, and activation (29). It has been reported that miRNAs, including miR-17-5p, miR-20a (30), miR-223 (31), and miR-494 (32), are involved in regulating the differentiation and function of tumor-expanded MDSCs. miR-17-5p and miR-20a alleviate the immunosuppressive potential of MDSCs by modulating STAT3 expression (30). Additionally, miR-223 suppresses differentiation of tumor-induced MDSCs from bone marrow cells via targeting MEF2C. Moreover, miR-494 promotes the accumulation and functions of tumor-expanded MDSCs via regulating PTEN expression. However, the molecular events controlling the differentiation and maturation of tumor-expanded MDSCs are largely unknown.

miR-9 has been characterized as an essential element in regulating immune responses (33), neuronal differentiation (34), posttraumatic stress (35), and various cancers (36, 37). Our previous studies have demonstrated that the immunomodulator β-glucan could promote MDSC differentiation into more mature myeloid cells and inhibit the regulatory function of MDSCs. In the present study, we found that miR-9 expression was significantly downregulated in MDSCs stimulated by WGP. Moreover, inhibition of miR-9 in M-MDSCs upregulated the expression of CD11c, F4/80, CD40, CD80, CD86, and MHC class II molecule, indicating that downregulation of miR-9 level in M-MDSCs could promote differentiation of M-MDSCs into a more mature state. Additionally, after inhibition of miR-9 in both M-MDSCs and G-MDSCs, levels of suppressive factors, including arginase, iNOS, and ROS, were significantly decreased. Notably, the capacity of MDSCs to suppress CD4+ or CD8+ T cell proliferation was reduced when these cells were transfected with miR-9 inhibitors. On the contrary, upregulation of miR-9 in MDSCs significantly enhanced

**FIGURE 8.** miR-9 is increased in human lung cancer. (A) MDSCs (arrow, yellow spots) accumulation in tumor tissues or distal noncancerous tissues of lung cancers were analyzed by immunofluorescent staining with FITC-labeled anti-CD11b mAb and allophycocyanin-labeled anti-CD33 mAb (original magnification ×200). The number of MDSCs was quantified. (B) miR-9 relative expression was detected in tumor tissues and distal noncancerous tissues (n = 18). Plasma from 15 healthy persons and lung cancer patients was collected to detect miR-9 levels. (C and D) Runx1 (C) and arginase (D) mRNA expression was analyzed in tumor tissues or distal noncancerous tissues. (E) Correlation of miR-9, Runx1, and arginase between each other was analyzed. Results are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
miR-9 plays a critical role in modulating MDSC differentiation and function. To further define a role of miR-9 in the differentiation and function of MDSCs, we found that the WGP-induced effect on MDSCs was reversed when miR-9 was exogenously supplemented in culture. Consistently, our in vivo experiments further showed that miR-9 inhibitor–modified MDSCs exhibited poor suppressive capacity, resulting in suppressed tumor growth whereas MDSCs with high miR-9 expression promoted tumor development in vivo. Thus, these data demonstrate that miR-9 is a critical regulator in MDSC differentiation and function.

In this study, we identified Runx1 as a functional target of miR-9 in the regulation of MDSC differentiation and function. Runx1 is a well-known transcription factor during the formation of hematopoietic stem cells from the hemogenic endothelium (38–40), which is considered as an important differentiation inducer. Runx1 is required for hematopoietic stem cell differentiation into endothelial cells and promotes the differentiation of myeloid, lymphoid, and megakaryocytic lineages (41) by controlling the expression of several genes regulating myeloid differentiation, including GM-CSF (42), myeloperoxidase (43), IL-3 (44), and M-CSFR (45). In this study, we found that Runx1 was markedly upregulated in MDSCs stimulated by WGPs. However, after knocking down the expression of Runx1 by siRNA, the WGP-induced effect was partially blocked. The expression levels of costimulatory molecules (CD40, CD80, CD86) and MHC class II molecule on M-MDSCs were reduced after knocking down Runx1, which indicates that Runx1 is involved in the differentiation of M-MDSCs. Additionally, suppressive factors secreted by MDSCs, including arginase, iNOS, and ROS, were restored to high levels in WGP-treated MDSCs with knocking down Runx1, and the capacity of MDSCs to suppress CD4+ and CD8+ T cells was enhanced after knocking down Runx1, which suggests that Runx1 also plays an essential role in regulating the function of MDSCs. Furthermore, our results revealed that Runx1 3′UTR had two “seed regions” for miR-9 and that miR-9 can bind to these two regions to regulate the target gene expression. Another interesting finding in our study is that miR-9 expression is controlled by another transcription factor, that is, CREB. CREB is a member of the activating transcription factor/CREB family of transcription factors, which can regulate diverse cellular responses, including proliferation, survival, and differentiation (25–27). For instance, CREB has been reported to be a proto-oncogene, leading to increased proliferation of myeloid cells and myeloproliferative diseases (46, 47). In the present study, we excitedly found that CREB level was downregulated in MDSCs after WGP treatment, which was a recent discovery and raises the question whether miR-9 expression was regulated by CREB. Interestingly, the predictive software showed that CREB’s binding site was in miR-9 promoter region. As indicated in Fig. 7F, the luciferase reporter assay further confirmed the prediction and demonstrated that CREB could bind to the promoter of miR-9 and regulate its expression. Effective cancer immunotherapy requires the elicitation of potent antitumor T cell responses and elimination of immunosuppressive cell populations such as MDSCs and regulatory T cells. As the suppressive function of MDSCs is closely associated with their immature state, the induction of MDSC differentiation could be an effective approach in reversing their suppressive capacity to normal immune responses. Thus, promoting the differentiation of MDSCs and inhibiting their suppressive function appear to be effective strategies to target MDSCs in cancer immunotherapy. In this study, we first demonstrated that miR-9 could regulate the differentiation and function of MDSCs by targeting Runx1. Downregulation of miR-9 could promote the differentiation of MDSCs and impair their suppressive capacity. Importantly, downregulating miR-9 level in MDSCs could significantly delay the tumor development in the mouse LLC model. Moreover, the clinical data also showed that high levels of miR-9 were observed in tumor tissues. Furthermore, miR-9 positively correlated with arginase whereas Runx1 negatively correlated with arginase. Although tumor-bearing mice treated with miR-9 inhibitors (antagomir)–modified MDSCs showed reduced tumor growth, the direct injection of miR-9 inhibitors into tumors did not show any significant effect on suppressing tumor growth. Further studies are warranted to establish an optimized protocol for prolonging the survival of tumor-bearing mice treated with miR-9 antagonist–modified MDSCs and to determine whether the direct injection of miR-9 inhibitors may represent an effective strategy for antitumor therapy.

In summary, our results indicate that targeting of miR-9 can reduce MDSC-mediated suppression and then enhance antitumor immunity, which might be further validated as a potential therapeutic target.

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Disclosures
The authors have no financial conflicts of interest.

References

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**microRNA-9 regulates the differentiation and function of myeloid-derived suppressor cells via targeting Runx1**

**Supplementary Table 1. Sequences of qRT-PCR primers**

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<tr>
<th>Name</th>
<th>Forward primer</th>
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<td>GGTAGGGCTTGCTGAGTG</td>
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<tr>
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<td>GCACCAGACTTGCCCTCCAA</td>
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<tr>
<td>m β-actin</td>
<td>TGGATCTCTGCTGGCAGTGAGA</td>
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<tr>
<td>m ARG1</td>
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<tr>
<td>m CREB</td>
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Supplementary Fig. 1. Viability of transfected MDSCs in vivo and miR-9 expression in MDSCs upon miR-9 agomir/antagomir treatment. Murine Lewis lung carcinoma cells (3×10^6) were injected into C57BL/6 mice (n=3/group). Isolated MDSCs were transfected with BLOCK-iT fluorescent oligos or control unlabeled oligos and then injected into the tumors of mice (2×10^6/mouse). In vivo imaging of tumor-bearing mice was performed under light microscope (A, D) and fluorescence
microscope (B, C, E, F) on day 3 (A, B, C) and day 5 (D, E, F) after injection. Fluorescent signals of oligos were detected after eliminating the autofluorescence interference in mice (C, F). Further analysis confirmed that the emission wavelength of the fluorescent was 518nm, which was exactly the same as fluorescent labeled oligos (G). (H) Three days after injection, the mice were sacrificed for the preparation of tumor cell suspensions. Flow cytometry was performed to detect CD11b^Gr-1^FITC^+^-AAD^- cells in tumors, which represented the grafted viable MDSCs containing the RNA oilogs. (I) 1 µg miR-9 mimics (miR-9 agomir), 2 µg inhibitors (miR-9 antagomir) or negative control (Ctrl) were injected into tumors of mice after palpable tumors were formed. 3 days later, MDSCs infiltrated in tumors were isolated and qRT-PCR was performed to analyze the miR-9 levels in MDSCs. Results are expressed as mean ± SD. **p<0.01, *p<0.05.
Supplementary Figure 2

Supplementary Fig. 2. WGP down-regulates the suppressive capacity of G-MDSCs. 3×10^6 LLC cells were injected s.c. into C57BL/6 mice. After 4 weeks, splenocytes were stained with anti-CD11b, anti-Ly6G and anti-Ly6C Abs to analyze the proportions of CD11b^+Ly6G^+Ly6C^{low} G-MDSCs and CD11b^+Ly6G^+Ly6C^{high} M-MDSCs. (A) Representative flow cytometric profile showing the frequencies of G-MDSCs and M-MDSCs in spleens from mouse Lewis lung carcinoma model. (B) Statistical analysis on the frequencies of G-MDSCs and M-MDSCs in spleens from mice bearing Lewis lung carcinoma. Data are shown as mean± SD pooled from three
independent experiments. **p<0.01. (C-F) Splenocytes were collected to isolate G-MDSCs. Sorted G-MDSCs were cultured in the presence or absence of WGP (100 µg/ml) for 48 h. (C, D) Arginase activity and ROS levels were measured as described in Materials and Methods. (E, F) WGP s were removed and the cells were harvested, and then co-cultured with responder cells, (E) CD4+ T cells or (F) CD8+ T cells (MDSC: T cell ratio of 2:1, 1:1, 0.5:1) in the presence of anti-CD3 mAb and anti-CD28 mAb for 72 h. Suppression of T-cell proliferation was measured by 3H-thymidine incorporation. All data are shown as mean± SD pooled from three independent experiments. Student’s t-test was used for the statistical analysis. **p<0.01, *p<0.05, WGP versus the corresponding medium. ###p < 0.001.
Supplementary Fig. 3. Down-regulation of miR-9 in MDSCs delays tumor development. Murine CT26 colon carcinoma cells (2×10^5) were injected into C57BL/6 mice (n=18). (A, B) Then, sorted MDSCs from tumor-bearing mice spleens were transfected with 100 nM miR-9 mimics (miR-9 agomir-MDSC), 200 nM inhibitors (miR-9 antagonim-MDSC) or negative control (Ctrl-MDSC), and these modified MDSCs (2×10^6 per mouse) were injected into the tumors of mice after palpable tumors were formed. 5 days later, the modified MDSCs were administered for the second time. (A) Tumors were measured with a caliper at indicated time (n=8) and (B) mice in each group were monitored for survival (n=10). (C-E) 1 µg miR-9 mimics (miR-9 agomir), 2 µg inhibitors (miR-9 antagonim) or negative control (Ctrl) were injected into tumors of mice after palpable tumors were formed. 5 days later, each group was administered for the second time. (C) Tumors were measured with a caliper at indicated time (n=8) and (D) mice in each group were monitored for survival (n=10). (E) Arginase activity of cells from tumor tissues was measured.
Results are expressed as mean ± SD. **p<0.01, *p <0.05.