miR-181a Induces Macrophage Polarized to M2 Phenotype and Promotes M2 Macrophage-mediated Tumor Cell Metastasis by Targeting KLF6 and C/EBPα

Jia Bi¹, Xianxin Zeng¹, Lin Zhao¹, Qian Wei¹, Lifeng Yu¹, Xinnan Wang¹, Zhaojin Yu¹, Yaming Cao², Fengping Shan² and Minjie Wei¹

Macrophages can acquire a variety of polarization status and functions: classically activated macrophages (M1 macrophages); alternatively activated macrophages (M2 macrophages). However, the molecular basis of the process is still unclear. Here, this study addresses that microRNA-181a (miR-181a) is a key molecule controlling macrophage polarization. We found that miR-181a is overexpressed in M2 macrophages than in M1 macrophages. miR-181a expression was decreased when M2 phenotype converted to M1, whereas it increased when M1 phenotype converted to M2. Overexpression of miR-181a in M1 macrophages diminished M1 phenotype expression while promoting polarization to the M2 phenotype. In contrast, knockdown of miR-181a in M2 macrophages promoted M1 polarization and diminished M2 phenotype expression. Mechanistically, Bioinformatic analysis revealed that Kruppel-like factor 6 (KLF6) and CCAAT/enhancer binding protein-α (C/EBPα) is a potential target of miR-181a and luciferase assay confirmed that KLF6 and C/EBPα translation is suppressed by miR-181a through interaction with the 3′UTR of KLF6 and C/EBPα mRNA. Further analysis showed that induction of miR-181a suppressed KLF6 and C/EBPα protein expression. Importantly, miR-181a also diminishes M2 macrophages-mediated migration and invasion capacity of tumor cells. Collectively, our results suggest that miR-181a plays a significant role in regulating macrophage polarization through directly target KLF6 and C/EBPα.

Introduction

Macrophages are derived from tissue-residing precursors or circulating monocytes. Macrophages residing in distinct tissue microenvironments can display divergent phenotypes and functions.¹ In response to various signals macrophages undergo polarization into classically activated (M1 or proinflammatory) or alternatively activated (M2 or anti-inflammatory) phenotypes. M1 macrophages can be generated in the presence of Toll like receptor ligands, such as IFN-γ and/or Lipopolysaccharides (LPS) and they exhibit potent antimicrobial properties and promote inflammation responses. Th2 cytokines such as IL-4 and IL-13 activated M2 macrophages which can suppress immune response and induce angiogenesis.²,³ Transcriptional factors nuclear factor κB (NF-κB), CCAAT/enhancer binding protein-α (C/EBPα), PU.1, and IFN regulatory factor 5 (IRF5) participate in M1 activation, while STAT6, peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer binding protein-β (C/EBPβ), and Kruppel-like factor 4 (KLF4) are involved in the polarization of M2 phenotype.⁴⁻⁶

Macrophages are recruited into tumor site by multiple cytokines expressed in the tumor microenvironment and become tumor-associated macrophages (TAMs).⁷ TAMs coexist in tumors and function as an accomplice to promote tumor progression and metastasis, especially once polarized into M2 phenotype by the tumor microenvironment.⁸⁻¹⁰ TAMs stimulate cancer cell invasion, motility, and migration, and that these effects can be impaired by inhibiting expression of epidermal growth factor.¹¹ The shift of the TAMs phenotype from M2 toward M1 lead to a shift of plasma chemokine profiles toward tumor-attacking chemokines.¹² The histidine-rich glycoprotein inhibits tumor growth and metastasis, while improving chemotherapy by skewing TAM polarization away from the M2- to a tumor-inhibiting M1-like phenotype.¹³ Thus regulating TAM polarization is a new perspective for the design of new and more efficient therapeutic strategies to counteract cancer cell invasion. However, the molecular mechanisms underlying TAM polarization are unclear.

miRNAs are a class of noncoding small RNAs, which induce gene silencing by modulating gene expression at the post-transcriptional level. miRNAs processing machinery is expressed in most eukaryotic cells, including ~30–90% human genes, thereby indicating that miRNA regulation of gene expression is a widespread phenomenon. It plays essential roles in many cellular and developmental processes, including cell proliferation, apoptosis, and differentiation, as well as organ morphogenesis. Increasing evidence suggested that miRNAs are involved in inflammatory responses and regulate differential activation of macrophages.¹⁴⁻¹⁶ miR-155 was reported to promote inflammatory reaction and induce M1 phenotype by targeting C/EBPβ and interleukin

The first two authors contributed equally to this work.
¹Department of Pharmacology, School of Pharmacy, China Medical University, Shenyang, Liaoning, China; ²Department of Immunology, School of Basic Medical Science, China Medical university, Shenyang, Liaoning, China. Correspondence: Minjie Wei, Department of Pharmacology, School of Pharmacy, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang City, 110122, Liaoning, China. E-mail: weiminjiecmu@163.com

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miR-181a was expressed at a higher level in M2 than in M1. Firstly, we detected the differential expression level of miR-181a between M1 and M2 by real-time polymerase chain reaction (PCR). We found that miR-181a levels are significantly up-regulated in H-M2 than in H-M1 (Figure 2c). This differential expression suggested that miR-181a may be a negative regulator of classical activation of M1 macrophages. To examine whether miR-181a contributes to the plasticity of macrophage polarization, we attempted to convert one population into another by culturing H-M1 macrophages with IL-4 and H-M2 macrophages with LPS and IFN-γ. As expected, the M2 macrophage marker, CCL17, CCL22, and CD163 were increased in M1-to-M2 conversion (Figure 2a), however, decreased in M2-to-M1 conversion (Figure 2b). M1-to-M2 conversion resulted in increased miR-181a (Figure 2d), whereas M2-to-M1 conversion led to miR-181a expression decreased (Figure 2e). These results explain that miR-181a was dynamically changing with macrophage phenotype: miR-181a decrease when M2 phenotype transform to M1, however, increase in higher M2 polarization degree. These data suggest that miR-181a may participate in macrophage polarization.

Knockdown of miR-181a in M2 diminishes the expression of M2 phenotypes
To further determine the role of miR-181a in macrophage polarization, H-M2 was transfected with miR-181a inhibitors or control inhibitors. miR-181a knockdown by miR-181a inhibitors enhanced the mRNA expression of TNFα, IL-1β, and HLA-DR, diminished expression of CCL17, CCL22, and CD163 in human macrophages (Figure 3a). Western blot analysis also revealed the higher level of IL-1β and HLA-DR and the lower level of CCL17 and CD163 after knockdowning miR-181a in M2 (Figure 3b). Besides, miR-181a knockdown in M2 substantially enhanced the percentage of CD68 positive macrophages and diminished the percentage of CD206 positive macrophages (Figure 3c). miR-181a inhibition in M2 resulted in the fluorescence intensity of CD86 significantly increased, however, fluorescence intensity of CD206 was declined (Figure 3d). Knockdown of miR-181a in M2 also diminished the release of TNFα and IL-1β, simultaneously, induced the release of CCL17 and CCL22 (Figure 3e).

As miR-181a is broadly conserved among vertebrates, an investigation was conducted to determine whether its functions are similar to that of murine macrophage cells. This was done by detecting the expression of M1/M2 markers in RAW264.7 derived M2 macrophage (M-M2) and mouse bone marrow-derived M2 macrophages (B-M2). As expected, the expressions of Arg-1, YM-1, TGFβ, and IL-10, which are M2 macrophage markers, were significantly lower after miR-181a knockdown, and the expressions of M1 macrophage marker, TNFα, IL-1β, and inducible nitric oxide synthase (iNOS) were higher (Figure 1a–e).

These data further suggest that miR-181a participates in sustaining the M2 macrophage phenotype. Inhibition of miR-181a in M2 reduced the expression of M2 marker, transformed macrophages phenotype to M1.

Overexpression of miR-181a promotes M1 transition to the M2 phenotype
Because our experiments showed that knockdown of miR-181a in M2 reduces the expression of M2 phenotypes, we next determined if miR-181a overexpression in M1 also participates in macrophage plasticity by promoting the transition of M1 to M2 phenotype. In these experiments, M1 (H-M1, M-M1, and B-M1) was transfected with miR-181a mimics to induce overexpression of miR-181a. We found that expressions of TNFβ, IL-1β, HLA-DR, CD86, and iNOS in M1 transfected with miR-181a mimics was significantly less than that in M1 cells transfected with control mimics (Figure 4a–e). Meanwhile, miR-181a overexpression in M1 reduced the expressions M2 markers: CCL17, CCL22, CD163, CD206, Arg-1, YM-1, TGFβ, and IL-10 (Figure 4a–e). Collectively, these results indicate that miR-181a is a negative regulator of M1 macrophage phenotypes. Overexpression of miR-181a increased M2 markers, drive the transition of M1 toward the M2 phenotype.
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Figure 1  Characterization of the M1 and M2 macrophages. (a-e) THP-1 cells were cultured in 200 ng/ml phorbol-12-myristate-13-acetate (PMA) for 24 hours to establish H-MØ. H-MØ cells were cultured in 100 ng/ml LPS and 100 ng/ml IFN-γ for 24 hours to establish H-M1. H-MØ cells were cultured in 20 ng/ml IL-4 for 24 hours to establish H-M2. (a) RNA was isolated and mRNA levels of IL-1β, TNF-α, HLA-DR, CCL17, CCL22, and CD163 were determined by real-time polymerase chain reaction (PCR). β-actin was used as an internal control. (b) IL-1β, HLA-DR, CCL17, and CD163 protein expression were detected by Western blot on total cell lysates. β-actin protein levels were determined in parallel for loading control purposes. Densitometric analysis for the protein expression of IL-1β, HLA-DR, CCL17, and CD163. (c) The surface levels of CD86 and CD206 in MΦ, M1, and M2 were determined by flow cytometry analysis. Representative results are shown. (d) The double-staining of fluorescent images of H-MΦ, H-M1, and H-M2 with anti-CD86 (red) combined with anti-CD206 (green) using a laser scanning confocal microscope. The double-staining cells were incubated with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (blue) to mark cell nucleus. (e) IL-1β, TNF-α, CCL17, and CCL22 levels released from H-MΦ, H-M1 or H-M2 were determined by enzyme-linked immunosorbent assay (ELISA) method. n = 3; mean ± SD.
miR-181a downregulates C/EBP-α and KLF6 and their downstream pathway genes

To delineate the mechanism by which miR-181a regulates macrophage polarization, we searched predicted targets of human miR-181a through miRBase and TargetScan. We found that the 3'UTR of KLF6 and C/EBP-α gene containing potential miR-181a binding sites, the two genes participate in macrophages polarization (Figure 5a). Through RNAfold software, we found that the minimum free energy between human miR-181a and the putative binding sites in the 3'-UTR of KLF6 and C/EBP-α mRNA were <−20, which suggested that miR-181a may target KLF6 and C/EBP-α via binding these putative sites (Figure 5b). KLF6 has been shown to promote inflammatory in macrophage polarization by inhibiting PPARγ, an important transcriptional factor in M2 polarization.23 C/EBP-α has been reported to induce M1 macrophage activation.24,25 To determine whether KLF6 and C/EBP-α could participate in macrophage polarization, we evaluate KLF6 and C/EBP-α mRNA expression in M1 and M2. We found that KLF6 and C/EBP-α both have a higher expression in M1 (Figure 5c). To confirm whether miR-181a targets KLF6 and C/EBP-α, we respectively cloned the 3'UTR of KLF6 including the positions 314–938 bp (KLF6-1) and 2,288–3,080 bp (KLF6-2), meanwhile, we cloned the 3'UTR of C/EBP-α into luciferase reporter and cotransfected it with control or miR-181a mimics. As shown in Figure 5d, compared with the negative control, miR-181a downregulated luciferase activity of the reporter that contained the 3'UTR of KLF6 or C/EBP-α. Conversely, luciferase activity has little change in cells transfected with miR-181a and mutant 3'UTR reporter plasmids. These results suggest that miR-181a directly targets KLF6 and C/EBP-α.

To confirm whether miR-181a represses the expression of KLF6 and C/EBP-α in macrophages, we transfected H-M1 with miR-181a mimics and transfected H-M2 with miR-181a inhibitors and found that KLF6 and C/EBP-α expressions were significantly suppressed by the miR-181a mimic at 24 hours (Figure 6a), while the transfection of miR-181a inhibitors led to a significant increase in expression of KLF6 and C/EBP-α (Figure 6b). We next explore whether miR-181a could regulate the downstream pathway genes of KLF6 and C/EBP-α. PPARγ was downstream pathway gene of KLF6, which promote H-M2 polarization. KLF6 was found to reduce PPARγ expression and activity. C/EBP-α was reported to cooperate with PPARγ to promote M1 polarization.24 We transfected miR-181a mimic into H-M1, and found that the protein expression of PPARγ was increased and PU.1 was suppressed (Figure 6c). In agreement with the above results, knockdown of miR-181a in M2 resulted in a significantly higher level of PU.1 and lower level of PPARγ (Figure 6d).

To gain further evidence that miR-181a regulates macrophage polarization through targeting KLF6 and C/EBP-α, we used siRNA against KLF6 and C/EBP-α to assay its function. As shown in Figure 6e, transfection of siRNA KLF6 into H-M1 effectively downregulated KLF6 in protein levels. Meanwhile, the protein expression of IL-1β was significantly suppressed and CD163 was upregulated. When we transfected siRNA C/EBP-α into H-M1, the protein expression of C/EBP-α and IL-1β was significantly downregulated and CD163 was increased. When the two siRNAs were used to transfet H-M1, we found that the inhibition of KLF6 and C/EBP-α significantly blocked the IL-1β expression and induced the CD163.
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Figure 3 Knockdown of miR-181a diminishes the expression of M2-polarized makers in M2. (a-e) M2 (H-M2, M-M2, and B-M2) was transfected with 20 nmol/l negative control inhibitors (NC-i) or miR-181a inhibitors (181a-i) for 24 hours. (a) Real-time polymerase chain reaction (PCR) showing the mRNA expressions of IL-1β, TNF-α, HLA-DR, CCL17, CCL22, and CD163 in H-M2; IL-1β, iNOS, ARG-1, and YM-1 in M-M2; or B-M2. (b) IL-1β, HLA-DR, CCL17, CD163, TNF-α, and TGFβ protein expression were detected by Western blot on total cell lysates. β-actin protein levels were determined in parallel for loading control purposes. Densitometric analysis for the protein expression of IL-1β, HLA-DR, CCL17, CD163, TNF-α, and TGFβ. (c) The proportion of CD86+ cells and CD206+ cells were measured by flow cytometry. (d) Expressions of CD86 and CD206 in M2 were detected after transfection with miR-181a inhibitors by immunofluorescence staining. (e) IL-1β, TNF-α, CCL17, CCL22, and IL-10 secretion levels were determined by enzyme-linked immunosorbent assay (ELISA). n = 3; mean ± SD.
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IL-1β, IL-10, TNFα, CCL17, CCL22, CD163, β-actin

CD86, CD206

Relative mRNA expression (fold)

Relative protein expression (fold)

Concentration of cytokines (ng/ml)

Relative mRNA expression (fold)

Concentration of cytokines (ng/ml)

Counts

Relative staining (%)

Merged
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We have shown that miR-181a suppresses M1 macrophage polarization and promotes M2 macrophage activation. Next, we investigated whether miR-181a regulates cellular functions associated with the M1 and M2 phenotypes. It was previously shown that M2 macrophages possess greater activity to promote cancer cells invasion, metastasis and Epithelial–Mesenchymal Transition (EMT) than do M1 macrophages.26–30 We assessed cancer cells invasion and metastasis abilities and expression of EMT markers when conditional culture with M2 transfected with miR-181a inhibitor. To this end, three human tumor cell lines, colorectal carcinoma cell line (HCT116), breast adenocarcinoma cell line (MCF-7), and ovarian carcinoma cell line (OVCAR3), were used. Transwell migration assays indicated that miR-181a promote tumor cell migration by inducing M2 polarization. The migration property of MCF-7, OVCAR3, and HCT116 cells was suppressed 31.5 ± 4.5, 37.0 ± 4.5, and 29.5 ± 6.3% when conditional cultured with M2 after being transfected with miR-181a inhibitor (Figure 7a). Meanwhile, transwell invasion assays clearly showed that the invasion capacity of HCT116, MCF-7, and OVCAR3 was also attenuated when inhibiting miR-181a expression in M2 (Figure 7b). The invasion property of MCF-7, OVCAR3, and HCT116 cells was suppressed 25.9 ± 8.2, 30.0 ± 2.4, and 26.0 ± 8.6%. It indicates that transfection of miR-181a inhibitor into M2 inhibits the invasion and metastatic properties of tumor cells. Figure 7c showed that knockdown of miR-181a in M2 enhanced E-cadherin protein expression, whereas decreased vimentin protein expression in three tumor cell lines, compared with transfecting with NC-I in M2.

TAMs mediated production of matrix metalloproteinase (MMP)2, MMP9, and uPA is a major phenotype of invasion of tumor cells. To examine whether those recognized soluble factors were regulated by miR-181a in the conditional media of M2 macrophages, we examine MMP2, MMP9, and uPA expression by enzyme-linked immunosorbent assay (ELISA). We found that MMP2, MMP9, and uPA levels are significantly down-regulated in M2 which knockdowned miR-181a (Figure 7d).

These data are consistent with the findings that miR-181a attenuated M2 activation of macrophages. Furthermore, knockdown miR-181a inhibits M2 mediated production of invasion associated cytokines.

Discussion

The biological role of miR-181a in the macrophage has not been clearly described. In these experiments, we for the first time found that miR-181a promotes M2 macrophage polarization and suppresses M1 polarization. miR-181a is at a higher level in M2 than in M1. Furthermore, when M2 were converted to M1, the levels of miR-181a were significantly decreased. In contrast, when M1 were converted to M2, the levels of miR-181a were significantly increased. Transfection of macrophages with miR-181a mimic resulted in up-regulation of markers and cytokines associated with the phenotype of the M2 macrophages, whereas cytokines and markers associated with the phenotype of M1 macrophages were downregulated. These data suggest that miR-181a skews their polarization from an M1 toward an M2 phenotype. Although many miRNAs can regulate inflammatory response in macrophages, only a few miRNAs are shown to participate in both M1 and M2 macrophage polarization. Previously, there was an effort to systematically identify miRNAs that change of expression in differentially polarized macrophages (miR-181a, miR-155-5p, miR-204-5p, miR-451, miR-125-5p, miR-146a-3p, miR-143-3p, and miR-145-5p).29 Among these miRNAs, some of them have been identified to have regulatory roles on macrophages polarization. miR-155 was found to promote M1 phenotype by targeted suppressing key genes of M2 phenotype, C/EBPα and interleukin 1 receptor alpha1.19,20 miR-125-5p and miR-146a were found to promote M2 polarization.30,31 This study also showed that miR-181a promotes macrophage conversion from M1 to M2. Therefore, miRNAs play an important role in regulating macrophage polarization.

Previous studies have reported that miR-181a regulate inflammation responses through inhibiting inflammatory factors levels. miR-181a directly targeted the 3’UTR of IL-1α and down-regulated IL-1α levels in monocytes and macrophages.21 In addition, miR-181a targeted inhibits TNF expression.32 It has been reported to regulate TLR-NF-κB signaling in monocytes.33 Those above-mentioned inflammatory cytokines are all M1 markers.

Transcriptional regulation is an important approach to regulate polarization of macrophages. PU.1 is a transcription factor and activated by TLRs to induce inflammatory.34 C/EBPα is the upstream of PU.1, which directly activates PU.1 gene transcription. C/EBPα and NF-κB cooperatively induce numerous genes during the inflammatory response.35 At the same time, C/EBPα and PU.1 have been found to participate in TLR ligand induced M1 activation.36 PPARγ is a key transcription factor involved in the polarization of M2 macrophages. PPARγ activation significantly increases expression of the M2 marker ARG-1, IL-10, and MR.36 PPARγ also inhibits inflammatory by suppressing the activity of NF-κB.37
miR-181a has been shown to promote inflammatory macrophage polarization by inhibiting PPAR\(\gamma\). Mechanistically, we further demonstrate that miR-181a regulates macrophage phenotype through combinatorial targeting of C/EBP\(\alpha\) and KLF6 genes. Several lines of evidence in our study support a direct regulation of C/EBP\(\alpha\) and KLF6 by miR-181a: first, overexpression of miR-181a downregulates C/EBP\(\alpha\) and KLF6 in macrophages at both mRNA and protein levels; second, the 3′UTR in C/EBP\(\alpha\) and KLF6 transcripts contains a miR-181a binding site; and third, the C/EBP\(\alpha\) and KLF6 3′UTR is responsive to miR-181a regulation. In our data, knockdown of miR-181a was found to induce KLF6 expression while PPAR\(\gamma\) was reduced. Furthermore, miR-181a knockdown promotes both C/EBP\(\alpha\) and PU.1 expression. Collectively, these findings raise the possibility that miR-181a regulates macrophage polarization by targeting KLF6 and C/EBP\(\alpha\) and regulating PPAR\(\gamma\) and PU.1 expression. miR-181a has stronger binding capacity with C/EBP\(\alpha\) than KLF6 and has stronger inhibitory effect on C/EBP\(\alpha\). However, which one has a stronger ability on macrophage polarization needs further research.

EMT plays an important role in tumor invasion and metastasis. In addition to EMT, MMP2, MMP9, and uPA are perceived to play a key role in cancer cell invasion and metastasis. A high level of infiltration of TAMs was associated with EMT-related proteins in human GC tissues. TAMs mediated production of MMP2, MMP9, and uPA are major phenotype of invasion of tumor cells. These studies suggest that TAMs are recruited to the tumor site, promoting tumor metastasis and invasion. There is increasing evidence that TAMs show M2-like macrophage and the transformation from M1 to the M2 phenotype in TAMs is a critical event for tumor promotion. Therefore, the phenotypical reversion of M2-like TAMs may be used as a therapeutic target in tumors.

Our experiments demonstrated that miR-181a not only regulates the expression of M2 macrophage markers, but also controls macrophage functions associated with the M2 states. When miR-181a was inhibited in M2, the facilitated capacity of the migration and invasion of cancer cells (HCT116, MCF-7, and OVCAR3) was attenuated to varying degrees. In addition, knockdown miR-181a in M2 suppressed the EMT and the expressions of MMP2, MMP9, and uPA of tumor cells. All of these data suggest that regulation of miR-181a could impair the ability of M2 macrophages to induce metastasis and invasion of tumor cells. The aberrant expression of human miR-181a has been implicated in the pathogenesis of various cancers, serving as an oncogene or tumor suppressor. miR-181a is associated with tumor progression and metastasis. High miR-181a expression is correlated to shorter time to recurrence and poor outcome in many cancers, such as epithelial ovarian cancer, breast cancer, and colorectal cancer. MiR-181a as a TGF-β-regulated target that enhanced the metastatic potential of cancers by promoting EMT, migratory, and invasive phenotypes. The results obtained here...
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suggest that, at least in part, miR-181a exerted promoting migratory, and invasive phenotypes effect by regulating macrophages polarization. Therefore, targeting miR-181a in M2 or cancer cell may have potential utility in the treatment of cancer.

In summary, we provide a new understanding of the roles miR-181a has in macrophages by regulating the phenotype of macrophages through targeting the KLF6 and C/EBPα and other genes in their downstream signaling pathway. The suppression of miR-181a promotes transformation of M2 to M1 and results in the inhibition of migration and invasion of tumor cells. As polarization has been shown to be important in macrophages’ functions, any dysregulation of macrophages differentiation state will lead to pathologic conditions. Thus, miR-181a can potentially be used in cancer immunotherapy and for other threatening diseases through modulation of macrophage activity.

Figure 6 miR-181a inhibits the expression of Kruppel-like factor 6 (KLF6) and CCAAT enhancer binding protein alpha (C/EBPα) and their downstream proteins. (a) C/EBPα and KLF6 mRNA expressions were detected in H-M1 transfected with the miR-181a mimics by real-time polymerase chain reaction (PCR). (b) C/EBPα, PU.1, KLF6, and peroxisome proliferator–activated receptor-γ (PPARγ) protein expression were detected in H-M1 transfected with the miR-181a inhibitors by real-time PCR. (c) C/EBPα, PU.1, KLF6, and PPARγ protein expression were detected in H-M2 transfected with the miR-181a mimics by Western blot. Densitometric analysis for the protein expression of IL-1β, C/EBPα, PU.1, KLF6, and PPARγ. (d) C/EBPα, PU.1, KLF6, and PPARγ protein expression were detected in H-M2 transfected with the miR-181a inhibitors by Western blot. (e) H-M1 was transfected with 50 nmol/l negative control inhibitors (NC) or C/EBPα siRNA and/or KLF6 siRNA for 48 hours. KLF6, C/EBPα, IL-1β, and CD163 protein expression were detected by Western blot on total cell lysates. β-actin protein levels were determined in parallel for loading control purposes. Densitometric analysis for the protein expression of KLF6, C/EBPα, IL-1β, and CD163. n = 3; mean ± SD.
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Figure 7 miR-181a diminishes cellular functions of M2 associated with promoting tumor invasion and metastasis. (a) The M2 culture supernatant were collected after H-M2 cells were transfected with miR-181a inhibitors (181a-i), control inhibitors (NC-i) or 1640 medium (blank control (BC)). MCF-7, OVCAR3, or HCT116 cells were cocultured with M2 conditional medium for 24 hours, and then subjected to a cell migration analysis. (magnification ×200). (b) The M2 culture supernatant were collected after M2 cells were transfected with miR-181a inhibitors (181a-i), control inhibitors (NC-i) or 1640 medium (BC). MCF-7, OVCAR3, or HCT116 cells were cocultured with M2 conditional medium for 24 hours, and then subjected to a cell invasion analysis. (magnification ×200). (c) UP: Western blot analysis of expression levels of the EMT inhibitor, E-cadherin, and EMT inducer, vimentin, in tumor cell lines (MCF-7, OVCAR3, and HCT116). DOWN: Densitometric analysis for the protein expression of E-cadherin and vimentin. (d) Matrix metalloproteinase (MMP)2, MMP9, and uPA levels released from M2 culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) method. n = 3, mean ± SD.
Materials and methods

Cell lines. Human monocyte cell lines (THP-1), mouse macrophage cell lines (RAW264.7), human breast cancer cell lines (MCF-7), human ovarian cancer cell lines (OVCAR3), and human colorectal cancer cell lines HCT116 were purchased from Cell Resource Center of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, China. THP-1 was grown in suspension cultures and cultured in RPMI (Roswell Park Memorial Institute) 1640 (HyClone, Logan, UT) supplemented with 10% FBS and antibiotics. MCF-7, OVCAR3, and HCT116 were adherent cultured in RPMI media (HyClone) containing 10% FBS and antibiotics. MCF-7, OVCAR3, and HCT116 were adherent cultured in RPMI 1640 medium with 10% FBS, and then cultured in RPMI 1640 without FBS for 24 hours. H-M2 macrophages without transfection were cultured for 24 hours in RPMI 1640 with 10% FBS, and then cultured in RPMI 1640 without FBS for another 24 hours to be blank control. The supernatant was centrifuged at 2,000xg for 15 minutes and collected to be used as conditional medium in the following experiments.

Preparation of conditional medium. After transfected with miR-181a inhibitors (181a-i) or control inhibitors (NC-i) for 24 hours, the H-M2 macrophages were further cultured in RPMI 1640 without FBS for 24 hours. H-M2 macrophages without transfection were cultured for 24 hours in RPMI 1640 with 10% FBS, and then cultured in RPMI 1640 without FBS for another 24 hours to be blank control. The supernatant was centrifuged at 2,000xg for 15 minutes and collected to be used as conditional medium in the following experiments.

Quantitative real-time PCR. Total RNA was extracted using the Trizol reagent kit (CWBio, Beijing, China) according to the manufacturer’s directions and converted to complementary DNA (cDNA) using the PrimeScript RT-PCR Kit (Takara, Dalian, China) primed with oligo (dT). The reaction condition was as follows: 37°C, 15 minutes; 85°C, 5 seconds; and 95°C, 5 minutes. miRNA was extracted from cells with miRNA Purification Kit (CWBio). miRNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Takara). The reaction condition was as follows: 16°C, 30 minutes, 42°C, 1 hour, and 85°C, 5 minutes. The PCR primers of U6 and miR-181a were purchased from RIBOBIO. Quantitative real-time PCR was carried out using the SYBR Green PCR Mix Kit (Takara). The reaction condition was followed by 95°C, 10 seconds, 56°C, 45 seconds, and 72°C, 20 seconds for 40 cycles of amplification. Primer sequences are detailed in Table 1 and Table 2. Assays were made in triplicates, and results were normalized according to the expression levels of β-actin mRNA or U6 miRNA. Results were expressed using the ΔΔCT (cycle threshold) method for quantification.

Western blotting. Cells were lysed in Radio Immunoprecipitation Assay (RIPA) lysis buffer, and the cell lysates were incubated on ice for 30 minutes and centrifuged at 13,000xg at 4°C for 15 minutes before the supernatant was collected. All above cell supernatant was collected and protein concentrations were determined using a bicinchoninic acid (BCA) protein quantitation kit (Beyotime, Jiangsu, China). Proteins (50 μg) from each cell lysate were subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes. Membranes were blocked with 1% BCA Fraction V for 1 hour.

Table 1 List of polymerase chain reaction (PCR) primers of human used in the study

| Gene names | Forward primer (5'-3') | Reverse primer (5'-3') |
|------------|------------------------|-----------------------|
| IL-1β      | CTCGCCAGTGAAGATGGCT    | GTCCGAGATTCGATGCTGAT  |
| TNFα       | CCACCTGGAGAGGGTGA      | AGGGTTCGGGAAAGGTTG   |
| HLA-DR     | GCCCTCTTCCTAAGCCTGAGA  | CCACCGGAGCCGACAGTGA  |
| CCL17      | CCTTCTCAGCACATCACAC    | AGATCTCAGCGCAGC-ACCTCC|
| CCL22      | TGCCGGTATAGCCGAGTTTA   | AAGGTAGACCAAGCAGGCC  |
| CD163      | CAATGGGG-TGGACTTACCTG  | AAACCTGCTGGTTCCCTG   |

Table 2 List of polymerase chain reaction (PCR) primers of murine used in the study

| Gene names | Forward primer (5'-3') | Reverse primer (5'-3') |
|------------|------------------------|-----------------------|
| IL-1β      | CCTGCACTGGAAGATGGCT    | GTGGCTCTGCTTGAGGCTGCT |
| iNOS       | CCTTGTTGAAGGACTGAGC    | CAAGCTCCTGCTGTCCTTCG  |
| YM-1       | ACCCTGTGGTGACTACCTC    | CACTGAGCGGGGAGGTCAAAA |
| Arg-1      | TTAAGCCGAAGGTGCTTGCCC  | TACCATGCGCTGAGGTTTC   |

KLF6 siRNA, C/EBPα siRNA and Control siRNA were purchased from RIBOBIO (Guangzhou).

M1 were transfected with 2 nmol/l (final concentration) miR-181a mimics (181a-m) or negative control mimics (NC-m). M2 were transfected with 20 nmol/l miR-181a inhibitors (181a-i) or control inhibitors (NC-i) for 24 hours. KLF6 siRNA, C/EBPα siRNA or control siRNA were transfected into H-M1 with 50 nmol/l (final concentration) for 48 hours.

Preparation of conditional medium. After transfected with miR-181a inhibitors (181a-i) or control inhibitors (NC-i) for 24 hours, the H-M2 macrophages were further cultured in RPMI 1640 without FBS for 24 hours. H-M2 macrophages without transfection were cultured for 24 hours in RPMI 1640 with 10% FBS, and then cultured in RPMI 1640 without FBS for another 24 hours to be blank control. The supernatant was centrifuged at 2,000xg for 15 minutes and collected to be used as conditional medium in the following experiments.

Quantitative real-time PCR. Total RNA was extracted using the Trizol reagent kit (CWBio, Beijing, China) according to the manufacturer's directions and converted to complementary DNA (cDNA) using the PrimeScript RT-PCR Kit (Takara, Dalian, China) primed with oligo (dT). The reaction condition was as follows: 37°C, 15 minutes; 85°C, 5 seconds; and 95°C, 5 minutes. miRNA was extracted from cells with miRNA Purification Kit (CWBio). miRNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Takara). The reaction condition was as follows: 16°C, 30 minutes, 42°C, 1 hour, and 85°C, 5 minutes. The PCR primers of U6 and miR-181a were purchased from RIBOBIO. Quantitative real-time PCR was carried out using the SYBR Green PCR Mix Kit (Takara). The reaction condition was followed by 95°C, 10 seconds, 56°C, 45 seconds, and 72°C, 20 seconds for 40 cycles of amplification. Primer sequences are detailed in Table 1 and Table 2. Assays were made in triplicates, and results were normalized according to the expression levels of β-actin mRNA or U6 miRNA. Results were expressed using the ΔΔCT (cycle threshold) method for quantification.

Western blotting. Cells were lysed in Radio Immunoprecipitation Assay (RIPA) lysis buffer, and the cell lysates were incubated on ice for 30 minutes and centrifuged at 13,000xg at 4°C for 15 minutes before the supernatant was collected. All above cell supernatant was collected and protein concentrations were determined using a bicinchoninic acid (BCA) protein quantitation kit (Beyotime, Jiangsu, China). Proteins (50 μg) from each cell lysate were subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes. Membranes were blocked with 1% BCA Fraction V for 1 hour.

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ELISA. Macrophage supernatants were tested for the presence of cytokines and chemokines using commercially available ELISA for human IL-1β, TNFα, CCL22, CCL17, MMP2, MMP9, and uPA, for mouse IL-10 and TNFα (all from BOSTER, Wuhan, China). Membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies or goat antimouse secondary antibodies (1:4,000 dilution, Santa Cruz, CA) at room temperature for 2 hours, and detected with a chemiluminescent detecting system (Amersham, Freiburg, Germany).

Flow cytometry assay. Macrophages were trypsinized and suspended in phosphate-buffered saline, followed by incubation with 1 μg/ml fluorescein isothiocyanate-conjugated human anti-CD206 (BD Bioscience, San Jose, CA) or phycoerythrin-conjugated human anti-CD86 (BD Bioscience) monoclonal antibody for 30 minutes at 4°C in the dark. The samples were subjected to flow cytometry analysis within 1 hour.

Immunofluorescence. Macrophages were 4% formaldehyde fixed (10 minutes) and then incubated in 10% normal goat serum for 1 hour. The cells were then incubated with the primary antibodies: rabbit antihuman CD206 (1:1,000 dilution, abcam, Cambridge, UK) and mouse antihuman CD86 (1:100 dilution, Santa Cruz, CA) overnight at 4°C. The secondary antibodies DyLight 488 goat antirabbit IgG (H + L) and Alexa Fluor 594 WGA were used at a 1:200 dilution for 1 hour. 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was used to stain the nucleus at a concentration of 100ng/ml.

Luciferase reporter assay. The 3’-UTR of human KLF6 gene contains two potential miR-181a-targeting sites at the positions 403–409bp and 2,955–2,962bp. Luciferase reporter vector pmirGlo-REPORT plasmid (RIBOBIO) contains the positions 314–938bp and 2,288–3,080bp respectively. The resulting construct was designated as pmirGlo-REPORT-KLF6-1 and pmirGlo-REPORT-KLF6-2. The 3’UTR of human C/EBPα gene (997–1,003bp) was cloned into pmirGlo-REPORT plasmid (RIBOBIO). Site-directed mutagenesis plasmid of the miR-181a target-sites in the 3’-UTR of KLF6-1 (314–938bp), KLF6-2 (2,288–3,080bp) and C/EBPα (703–1,331bp) were purchased from Genechem (Shanghai, China). 293 T-cells were cotransfected with 100ng (final concentration) wt or mutant reporter plasmid and 20 nmol/l miR-181a mimic (final concentration) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Firefly and Renilla luciferase activities were measured using Dual Luciferase Reporter Gene Assay kit (Promega, WI). Relative luciferase activity normalized to the negative control was used for comparison among groups.

In vitro migration assays and invasion assays. Assays were performed using 8-μm pore size FalconTM cell culture inserts (BD Biosciences) in a 24-well format according to the vendor’s instructions. In the migration assay, MCF-7, OVCAR3, and HCT-116 cells (104 cells/well) were seeded onto membranes of the upper chambers with 0.5ml serum-free medium, which had been inserted into wells of 24-well plates containing 10% FBS-supplemented conditional medium. After 24 hours, cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 30 minutes. Non-invasive cells remaining on the upper surface of the membranes were removed by wiping the top of the insert membranes with a damp cotton swab, which left only those cells that had migrated to the underside of the membranes. The membranes were mounted on glass slides, and numbers of cells in three randomly chosen high-power fields were counted. For the invasion assay, MCF-7, OVCAR3, and HCT-116 (104 cells/well) were seeded onto Matrigel-coated membranes (BD Biosciences) of the upper chambers and incubated at 37°C. The lower chambers contained the same amount of berberine in 10% FBS conditional medium. After 48 hours, noninvasive cells remaining on the upper surface of the membranes were removed with a cotton swab. Cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet for 30 minutes. Membranes were mounted on glass slides, and numbers of cells in three randomly chosen high-power fields were counted. All experiments were performed three times and photographed under a phase-contrast microscope.

Statistical analysis. Statistical analysis was performed using SPSS16.0 statistical software (SPSS, Chicago). Values were expressed as mean ± SD. One-way analysis of variance was performed for multiple group comparisons. A value of P < 0.05 was used as the criterion for statistical significance.

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