miR-155–Deficient Bone Marrow Promotes Tumor Metastasis

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Abstract

Infiltration of immune cells in primary tumors and metastatic sites is known to influence tumor progression and metastasis. Macrophages represent the most abundant immune cells in the tumor microenvironment, and evidence has shown that macrophages promote seeding, extravasation, and persistent growth of tumor cells at metastatic sites. miR-155 plays an essential role in immune cell development/function, and its aberrant expression is associated with lymphomas and several solid tumor types. However, it is unknown how miR-155 expression in immune cells affects solid tumor growth and metastasis. To this end, bone marrow transplantation was performed using miR-155–deficient mice as bone marrow donors and wild-type (WT) mice as recipients, and the chimeric mice were inoculated with tumor cells. We demonstrate that bone marrow lacking miR-155 significantly enhanced lung metastasis without a substantial effect on primary tumor growth. Relative to mice with WT bone marrow, miR-155–deficient bone marrow accumulated more macrophages in the spleen and lungs. Further analysis revealed that miR-155–deficient macrophages in metastatic sites exhibited a tumor-promoting M2 phenotype. In vitro study suggested that miR-155–null macrophages were prone to M2 polarization upon incubation with tumor cell–conditioned medium, due to elevated expression of C/EBPβ, an identified miR-155 target. These data, for the first time, demonstrate that miR-155 in host immune cells plays a vital role in modulating solid tumor metastasis by affecting the recruitment and polarization of bone marrow–derived macrophages.

Implications: Targeted inhibition of miR-155 delays tumor development but inhibition in host immune cells may encourage metastasis. Mol Cancer Res; 11(8); 923–36. ©2013 AACR.

Introduction

Tumor progression and metastasis positively correlate with the infiltration of immune cells, including myeloid-derived suppressor cells (MDSCs), macrophages, neutrophils, dendritic cells, granulocytes, T cells, and B cells (1–7). Among them, macrophage is the most abundant leukocyte type present in neoplastic stroma (5, 8). Accumulating clinical and experimental evidences have shown that tumor-associated macrophages (TAM) promote tumor progression at the primary tumor sites (5, 9), and enhance tumor metastasis in distant organs (4, 10, 11). In addition, metastasis-associated macrophage is another population that is identified in experimental models of tumor metastasis (4, 5). Recent ex vivo imaging studies of metastatic lungs showed that macrophages were recruited toward extravasating tumor cells and that depletion of these macrophages dramatically reduced the seeding and extravasation efficiency and the subsequent survival of tumor cells (4).

miRNA-155 (miR-155) was among the first miRNAs that have been shown to play roles in immunity and inflammation. Its expression is significantly increased in activated B cells (12), T cells (13), macrophages, and dendritic cells (14–16), and is also upregulated in multiple immune cell lineages, including macrophages, in inflammatory responses induced by Toll-like receptor (TLR) ligands, cytokines, and specific antigens (13–15, 17). miR-155–deficient mice showed impaired immune responses (17, 18); whereas miR-155 overexpression in CD34+ hematopoietic progenitor cells resulted in a defective differentiation of these cells into mature myeloid and erythroid cells (19). Altogether, these observations strongly indicate miR-155 as a central regulator of the immune system.

In addition to its essential role in immunity, dysregulation of miR-155 is closely related to cancer. miR-155 transgenic mice develop B-cell malignancy and elevated miR-155
expression was observed in several types of human B-cell lymphomas (12, 20, 21). Altered miR-155 expression has also been found in pediatric Burkitt’s lymphoma and chronic lymphocytic leukemia (21–23). Moreover, elevated miR-155 levels were also found associated with several types of solid tumors including colon cancer, breast cancer, hepatocellular carcinoma, and lung cancer (24–26). Therefore, targeting miR-155 has been proposed to be a promising approach to combat cancers of hematopoietic source as well as several solid tumors (25). However, it has not been examined whether miR-155 expression in host immune cells influences growth and metastasis of solid tumors. To test this, we conducted a bone marrow transplantation (BMT) study by using wild-type (WT) and miR-155−/− mice as bone marrow donors and using WT mice as recipients, and examined the effects of miR-155 deficiency in bone marrow on solid tumor growth and metastasis. Our data show that miR-155 deficiency in bone marrow cells enhanced lung metastasis through increasing macrophage infiltration, which exhibited a M2 phenotype, in metastatic lungs of tumor-bearing mice, and that miR-155−/− macrophages were prone to M2 polarization in the presence of tumor-derived factors due to elevated CCAAT/enhancer binding protein β (C/EBPβ) expression.

Materials and Methods

Cell culture

Lewis lung carcinoma (LLC) and B16–F10 melanoma cell lines (both syngeneic to C57BL/6 mice) were obtained from the American Type Culture Collection and cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen Life Technologies) with 10% FBS (Invitrogen) and glucose. Lewis tumours (both syngeneic to C57BL/6 mice) were obtained from the American Type Culture Collection and cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen) with 10% FBS (Invitrogen) and penicillin/streptomycin at 37°C.

Animals

C57BL/6 (WT) and bic/mir-155 knockout mouse (miR-155−/−) were obtained from the Jackson Laboratory. Experimental animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at University of South Carolina (Columbia, SC). Mice were maintained at the University of South Carolina according to NIH guidelines.

Bone marrow transplantation

BMT was conducted as described previously (27). Briefly, recipient WT mice (6–8 weeks old, female) were lethally irradiated (900 rad). Bone marrow cells were harvested from WT and miR-155−/− donor mice by flushing the femurs and tibias with PBS supplemented with 2% FBS. The flushed bone marrow cells were resuspended in PBS, and 5 × 10^6 nucleated cells were injected retro-orbitally into irradiated WT mice (8 mice/group) within 6 hours after irradiation.

Tumor cell injection

For tumor growth experiments, 1 × 10^7 LLC cells (in 200 μL PBS) were subcutaneously implanted in the back of mice 4 weeks after BMT. Tumor growth was monitored by measurement of tumor size with a caliper every 3 days. Tumor volume was determined by the formula: length × width^2/2. Primary tumors were surgically resected when they reached a length of 10 mm (14 days after subcutaneous injection of tumor cells). To promote lung metastasis, 2 × 10^3 LLC cells (in 200 μL PBS) were intravenously injected 10 days after subcutaneous implantation of LLC cells.

Immunohistochemistry

The lungs were perfused with PBS to eliminate circulating tumor and blood cells. Primary tumor tissues and whole lungs were fixed in 4% paraformaldehyde at 4°C for 12 hours, dehydrated in 30% sucrose overnight, and embedded in OCT. Serial sections (8-μm thick) were cut throughout the entire tumor tissues and lungs. For F4/80 staining, frozen sections were fixed in aceto, incubated with 5% BSA/PBS, and incubated with fluorescein isothiocyanate (FITC)—conjugated rat anti-F4/80 antibody (1:100; eBioscience) overnight at 4°C. For Ym1 staining, frozen sections were incubated with anti-mouse Ym1 rabbit polyclonal antibodies (1:200; StemCell Technologies). The Alexa Fluor 488—conjugated secondary antibodies (Invitrogen) were used at a 1:100 dilution and incubated for 1 hour at room temperature. Slides were mounted in ProLong Gold Mounting Medium containing 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen), and the tissue sections were visualized using a Nikon ECLIPSE E600 microscope (Nikon Inc.). For quantitative analysis of F4/80 and Ym1 staining in lungs, we counted the number of positively stained cells in 6 to 12 random fields of indicated magnification per section. For F4/80 staining in tumor tissues, we determined the area occupied by immunostained cells normalized by area of DAPI-stained cells using Image-Pro Plus 6.0 analysis software (28). For von Willebrand factor (vWF) staining, the tumor tissue sections were fixed in 4% paraformaldehyde, rinsed with PBS, and incubated with 0.3% H_2O_2 in methanol for 10 minutes. After washing with PBS, anti-mouse vWF antibody (1:2,000; Abcam) was applied. Immunocomplexes were detected with biotin-conjugated secondary antibodies and AEC chromogen/HRP substrate kit (GeneTex). The sections were counterstained with hematoxylin and mounted with a permanent mounting medium. Microvessel density was assessed with vWF staining and counted on ×200 magnification fields. vWF-positive endothelial cell or cell cluster clearly separate from adjacent structures was considered a single vessel. For hematoxylin and eosin (H&E) staining, sections were stained using standard procedures. H&E–stained lung sections were analyzed for tumor microscopically under ×4 magnification.

Conditioned medium collection

To obtain conditioned medium, LLC and B16–F10 cells were seeded at 5 × 10^6 cells per dish of 75 cm^2 and cultured till 90% confluence. The media were then replaced with serum-free DMEM. After 24 hours, the supernatants were collected and filtered through a 0.22-μm filter.

In vitro macrophage treatment

Mice were injected with 3 mL of 3% thioglycollate in sterile PBS intraperitoneally. Three days later, mice were euthanized.
and peritoneal macrophages were harvested by lavaging the peritoneal cavity with 2 × 10 mL of PBS. Cells were suspended with DMEM media containing 10% FBS and plated in 6-well or 12-well plates. After 2 hours, the nonadherent cells were removed by PBS, and the adhered macrophages were further cultured in serum-free DMEM overnight, followed by treatment with control medium, tumor cell conditioned medium, or interleukin (IL)-4 (20 ng/mL; AASN BioAbChem Inc.) for indicated period of time.

**Bone marrow–derived macrophages**

Bone marrow cells were harvested from WT or miR-155−/− mice by flushing the femurs and tibias with PBS supplemented with 2% FBS, and resuspended in bone marrow differentiation media (DMEM supplemented with 10% FBS, 20% L929 cell-conditioned medium (LCCM), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L L-glutamine) at 6 × 10⁶ cells/mL. Ten milliliters of cells were seeded on each 10-cm tissue culture plate (BD Biosciences). Four days after seeding, an extra 10 mL of fresh media were added to each plate and incubated for an additional 3 days. To obtain bone marrow–derived macrophages (BMDM), cells were trypsinized, centrifuged at 200 × g for 5 minutes and then resuspended in 10 mL of BMDM cultivation media, which is composed of DMEM, 10% FBS, 5% LCCM, and 2 mmol/L L-glutamine. The cells were counted, seeded, and cultivated in tissue culture plates 12 hours before any further experimental procedure.

**Quantitative real-time PCR**

Total RNA was extracted using the TRIzol reagent (Invitrogen). RNA (1 μg) was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Life Science). Quantitative real-time PCR (qRT-PCR) was conducted on a CFX96 system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). All primers used for qRT-PCR analysis were synthesized by Integrated DNA Technologies. All assays were conducted following the manufacturer’s instructions. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18S. PCR thermal cycling conditions contained 3 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 58 seconds at 60°C. Samples were run in triplicate. The primer sequences were listed in Supplementary Table S1.

**Western blot analysis**

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Pierce) supplemented with protease inhibitor cocktail (Sigma). Total cellular extracts (30 μg) were separated in 10% SDS-PAGE precast gels (Bio-Rad) and transferred onto nitrocellulose membranes (Millipore Corp.). Membranes were first probed with C/EBPβ (1:1,000; Santa Cruz Biotechnology, Inc.), SOCS-1 (1:1,000; Abcam), or β-actin (1:1,000; Sigma) antibodies, followed by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP; Millipore). The Protein detection was conducted using Pierce ECL Western Blotting Substrate (Pierce). Image-Pro Plus 6.0 analysis software was used to quantify signal intensities.

**Flow cytometry**

Bone marrow cells were harvested from mice by flushing the femurs and tibias with PBS supplemented with 2% FBS. Splenocytes were prepared by mechanical dissociation. Cells were stained with anti-CD11b phycoerythrin (PE) monoclional antibody (mAb), anti-F4/80 FITC mAb, anti-Gr-1 FITC mAb, anti-ly6C FITC mAb, anti-ly6C FITC mAb, anti-CD3 PE mAb, anti-CD4 FITC mAb, anti-CD8 FITC mAb, or anti-CD19 FITC mAb (all from eBioscience) in staining buffer (PBS containing 2% FBS) for 30 minutes on ice in the dark. Samples were washed twice in staining buffer, analyzed by flow cytometry using a Cytoflex FC 500 flow cytometer and CXP software version 2.2 (Beckman Coulter). Data were collected for 10,000 live events per sample.

**Bio-Plex cytokine/chemokine assay**

Sera from WT and miR-155−/− chimeric mice were diluted 1:4. Concentrations of 23 mouse cytokines/chemokines were measured by Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad; M60-009RD) following the manufacturer’s instructions.

**miR-155 inhibitor transfection**

mirVana miR-155 inhibitor (MH4464084) and negative control (MH4464076) were purchased from Invitrogen. LLC cells (2 × 10⁵ cells) were seeded into 6-well plates. Twelve hours later, LLC cells were transfected with miR-155 inhibitor or negative control using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instruction. Forty-eight hours after transfection, total RNAs were extracted and cDNAs were prepared to confirm the miR-155 inhibition of the cells were used for further experiments. Gene expression of miR-155 targets [hypoxia-inducible factor-1α (HIF-1α), Bcl-6, c-Maf, and SOCS1] was measured by RT-PCR.

**[³H] thymidine incorporation assay**

LLC (5 × 10⁴ cells) or B16-F10 (4 × 10⁵ cells) were seeded into the 24-well plates and transfected with negative control inhibitor or miR-155 inhibitor. Twenty-four hours later, peritoneal macrophages (5 × 10⁵ cells) from WT and miR-155−/− mice were added into LLC and B16-F10 cells. After 48 hours of coculture, [³H] thymidine (1 μCi/well) was added and further incubated for 4 hours. Cultures were harvested and thymidine incorporation was measured by scintillation counting (PerkinElmer). Data are expressed as Counts Per Minute (CPM) (mean ± SEM) of 6 cultures. Three independent experiments were carried out.

**Transwell migration assay**

Peritoneal macrophages (1 × 10⁶ cells) from WT and miR-155−/− mice were seeded into 24-well plates. Two hours after seeding, the nonadherent cells were removed by washing with PBS, and the adhered macrophages were treated with serum-free DMEM and LLC- or B16-F10-derived conditioned medium. LLC or B16-F10 cells (2 × 10⁵) were seeded onto the top chamber of Transwell insert with 8-μm pore size (Corning Incorporated Life Sciences). Migrated tumor cells were stained with Calcein (1 μg/mL; Invitrogen) and counted.
under an inverted widefield fluorescence microscope at ×40 magnification (20 fields/well, triplicate for each experimental group).

**Statistical analyses**

Data were presented as mean ± SEM. Statistical significance was calculated by use of Student t test (two-group comparison) or one-way ANOVA (multi-group comparison) using the GraphPad Prism statistical program (GraphPad Prism; GraphPad Software, Inc.). P < 0.05 was considered significant.

**Results**

**miR-155 deficiency in bone marrow enhanced tumor metastasis in the lungs**

To examine whether miR-155 deficiency in bone marrow affects solid tumor growth and metastasis, BMT was conducted. WT or miR-155−/− bone marrow cells were transplanted into lethally irradiated WT mice. Four weeks after BMT, WT and miR-155−/− chimeric mice (referred as WT-BMT and miR-155−/−-BMT hereafter, respectively) were inoculated with LLC cells in the back. We began to measure tumor size after the xenografts became palpable. We found that both WT-BMT and miR-155−/−-BMT mice showed a similar tumor growth rate (Fig. 1A). In addition, the tumor size showed no difference between WT-BMT and miR-155−/−-BMT mice when tumors were removed 2 weeks after inoculation (Fig. 1B). However, miR-155−/−-BMT mice had significantly more tumor nodules in lungs compared with WT-BMT mice (Fig. 1C). Further analysis showed that the number of micro-metastases but not macro-metastases was remarkably increased in miR-155−/−-BMT mice (Fig. 1C and E). Consistently, there was a larger total metastatic area in lungs of miR-155−/−-BMT mice than in lungs of WT-BMT counterparts (Fig. 1D). Several studies showed that LLC cells metastasize to the lungs and occasionally the liver (2, 29, 30). However, in our current study, LLC tumor metastases were observed only in lung but not in liver or other organs.

qRT-PCR showed that the miR-155 level in spleen of miR-155−/−-BMT mice was only one sixth of that of WT-BMT mice. A, growth rate of LLC primary tumors in WT and miR-155−/− chimeric mice. A total of 1 × 10⁷ LLC cells were subcutaneously implanted in the back of WT and miR-155−/− chimeric mice and tumor size was measured with a caliper. Tumor volume was shown as mm³. B, average tumor weight at day 14 after LLC inoculation. C, quantification of average number of nodules in lung of WT and miR-155−/− chimeric mice at day 28 after LLC cell implantation. Nodules smaller than 70 μm are defined as micro-metastases (micro-). Nodules larger than 70 μm are defined as macro-metastases (macro-). D, the percentage of metastatic area in lung tissues was calculated (n = 8 mice/group). E, representative H&E staining sections of the lungs from WT and miR-155−/− chimeric mice (n = 8) carrying LLC tumors. The red arrows point to the metastatic nodules in the lungs. Magnification, ×4. Data are presented as the mean ± SEM of 8 mice. *, P < 0.05 by Student t test.
WT-BMT mice (Supplementary Fig. S1), confirming the successful bone marrow reconstitution.

**miR-155−/− chimeric mice produced higher levels of tumor-promoting factors**

Cytokines and chemokines derived from inflammatory cells as well as tumor cells can promote tumor growth and metastasis in a variety of tumor models (31, 32). In light of a higher frequency of metastases in miR-155−/−-BMT mice, we postulated that these mice may produce more tumor-promoting cytokines and chemokines. To test this, a Bio-Plex assay (23-plex) was conducted in tumor-bearing mice. The concentrations of IL-1β, IL-6, and IL-10 in sera were dramatically increased in miR-155−/−-BMT mice than in WT-BMT mice (Fig. 2A). Moreover, a higher amount of CCL3, a chemokine for macrophage infiltration (33), was also detected in miR-155−/−-BMT mice than in WT-BM mice (Fig. 2A). IL-17 and granulocyte colony-stimulating factor (G-CSF) levels in miR-155−/−-BMT mice were greatly increased as well (Fig. 2A). Our data indicate that miR-155−/−-BMT mice produced more tumor-promoting factors, which may create a favorable microenvironment for tumor cell seeding and survival in metastatic sites. However, other cytokine (Fig. 2B and C) or chemokine (Fig. 2D) levels did not show statistical difference between these 2 groups.

**miR-155 deficiency in bone marrow resulted in more macrophage mobilization in tumor-bearing mice**

Bone marrow–derived cells (BMDC) play an indispensable role in the establishment of tumor microenvironment, primarily through producing and secreting protumor factors, including cytokines, chemokines, and matrix-degrading enzymes (2, 30, 34). Because miR-155 is critical for the host immune system (17, 18), we aimed to investigate whether the increases in serum cytokine and chemokine levels and lung metastasis in miR-155−/−-BMT mice were due to the mobilization and accumulation of BMDCs in peripheral tissues. We thus examined bone marrow cell mobilization in tumor-bearing mice using flow cytometrical analyses. The subpopulation of cells in bone marrow, including CD11b+ Gr-1+, F4/80+, CD11b+/Ly6G+, CD11b+/Ly6C+, CD3+ T cells, and CD19+ B cells did not show any statistical difference between WT-BMT and miR-155−/−-BMT mice (Supplementary Fig. S2); however, we found that the absolute splenocyte number in miR-155−/−-BMT mice was increased by 1.67-fold compared with that in WT-BMT counterparts although the splenic weight showed no significant difference between the 2 groups (Fig. 3A and B). Strikingly, not only the percentage but also the absolute number of F4/80+ macrophages in spleen of miR-155−/−-BMT mice were greatly increased compared with those in WT-BMT mice (Fig. 3C–E). Although there were increases in the percentage and the absolute number of CD11b−/Ly6G− and CD11b+ /Ly6C+, both have been shown to promote tumor progression, in miR-155−/−-BMT mice compared with WT-BMT counterparts, the differences were not statistically significant (Fig. 3C and D). In addition, there were more CD3+ /CD4+ and CD19+ B cells in tumor-bearing miR-155−/−-BMT mice compared with WT-BMT counterparts (Supplementary Fig. S3).
miR-155 deficiency in bone marrow did not affect macrophage infiltration in primary tumor

Because the spleen serves as a reservoir for circulating leukocytes, we next examined whether increased splenic macrophages resulted in enhanced recruitment of macrophages to the primary tumor. TAMs have been known to support tumor-associated angiogenesis, promote tumor cell invasion, migration, and intravasation, as well as suppress antitumor immune responses (5). To our surprise, there seemed to be no significant difference of F4/80+ macrophage accumulation in primary tumors from WT and miR-155−/− chimeric mice (Fig. 4A and B). Moreover, the numbers of infiltrating Ym1 (a M2 macrophage marker) staining positive cells also showed no difference between WT-BMT and miR-155−/−-BMT mice (Fig. 4A and B). Furthermore, we conducted immunohistochemistry to detect the expression of mouse endothelial cell antigen vWF, a specific marker of angiogenesis, and found no difference in the density of intratumoral microvessels in these 2 groups (Fig. 4C and D). Moreover, qRT-PCR was conducted to detect the expression of proangiogenic and growth factors in tumor tissues. In line with the earlier observations, there were no significant differences in matrix metalloproteinase (MMP)9 and Placental growth factor (PIGF) expression in primary tumors from WT-BMT and miR-155−/−-BMT mice (Supplementary Fig. S4), albeit MMP2 expression was decreased in the tumors of miR-155−/−-BMT mice.

miR-155 deficiency in bone marrow increased macrophage infiltration in the lungs

Although the recruitment of macrophages to tumors has been well documented in many tumor models (5, 8, 31, 35), the role of macrophages in promoting metastatic cell seeding, enhancing tumor cell extravasation, and subsequent growth of metastatic lesions in distant sites has only recently started...
to be appreciated. We thus examined whether macrophages were recruited to the lungs in our tumor model and contributed to the increased lung metastasis. We found that the infiltration of F4/80+ macrophages into lung tissues was increased by 3.2-fold in miR-155−/−BMT mice compared with that in WT-BMT counterparts (Fig. 5A and C, left). In response to microenvironmental signals, macrophages undergo different activation, including "classic" activation to produce a proinflammatory phenotype (also called M1) and the "alternative" activation to yield an anti-inflammatory phenotype (also called M2; refs. 5, 36). We therefore investigated whether macrophages in the lungs of miR-155−/−BMT mice exhibited more of a "M2" phenotype than those in the lungs of WT-BMT counterparts. Immunostaining was carried out in lung tissues using an antibody against Ym1, a M2 macrophage marker. Interestingly, the lung tissues of miR-155−/−BMT mice contained significantly more Ym1+ cells than those of WT-BMT mice (Fig. 5B and C, right). These results suggest that bone marrow miR-155 deficiency promoted infiltration and M2 polarization of macrophages in metastatic lungs under tumor burden.

miR-155−/− macrophages were prone to M2 polarization in vitro

To test whether miR-155 deficiency promotes M2 polarization of macrophages, peritoneal macrophages from WT or miR-155−/− mice were stimulated with LLC cell conditioned medium (LCM) and the expression of Arg1, a potent M2 marker gene, was measured by qRT-PCR. We found that, upon LCM treatment, mRNA level of Arg1 was significantly increased in miR-155−/− macrophages compared with WT macrophages (Fig. 6A). We also treated WT and miR-155−/− macrophages with IL-4 or B16-F10 cell
conditioned medium (B16-CM). We found that both IL-4 and B16-CM treatment resulted in higher levels of Arg1 expression in miR-155<sup>−/−</sup> macrophages than in WT ones (Fig. 6B). Furthermore, we replicated the experiments using BMDMs and obtained similar results (Fig. 6C and D).

The polarization of M2 macrophage is modulated primarily through transcription factors and their effector molecules, such as C/EBPβ, SOCS-1, and STAT6 (37–39). To identify the signaling pathways responsible for the M2 polarization of miR-155<sup>−/−</sup> macrophages, we evaluated the protein levels of C/EBPβ, SOCS-1, and STAT6. C/EBPβ has been identified as a direct target of miR-155 in B cells and macrophages (40, 41). As expected, the basal level of C/EBPβ protein in miR-155<sup>−/−</sup> macrophages was higher than that in WT macrophages; LCM did not increase C/EBPβ levels in both WT and miR-155<sup>−/−</sup> macrophages at 30 minutes of incubation; however, C/EBPβ protein level was significantly increased 8 hours after LCM treatment (Fig. 6E). Importantly, LCM-treated miR-155<sup>−/−</sup> macrophages exhibited a significantly elevated level of C/EBPβ than...
LCM-treated WT macrophages (Fig. 6E). LCM treatment for 48 hours resulted in much higher level of C/EBPβ expression in miR-155−/− macrophages than in WT ones (Fig. 6E). Increased expression of SOCS1, another confirmed target of miR-155, has been shown to repolarize proinflammatory M1 macrophages to anti-inflammatory M2 macrophages (42). However, we did not observe an increase of SOCS1 expression in miR-155−/− macrophages compared with WT macrophages at any of the 30 minutes, 8, and 48 hours time points (Fig. 6E). In addition, the levels of phosphorylated and total STAT6 did not show apparent difference between WT and miR-155−/− macrophages with or without LCM treatment (data not shown). Our data suggest that the enhanced M2 polarization of miR-155−/− macrophages compared with WT macrophages may be mainly due to increased C/EBPβ signaling pathway resulted from the loss of repression of C/EBPβ by miR-155.

miR-155 expression was not increased in LLC and B16-F10 cells and their conditioned medium reduced macrophage miR-155 expression

It has been reported that miR-155 may function as an onco-gene in several solid tumors. We examined the miR-155 expression in cultured LLC and B16-F10 cells using qRT-PCR, and we found that, compared with that in lung tissue of WT mice, miR-155 expression was not increased in both of the cell types. Instead, miR-155 expression was slightly but not significantly reduced in LLC cells, whereas it was significantly reduced in B16-F10 cells (Supplementary Fig. S5A). To investigate whether tumor cells may influence miR-155 expression in macrophages, we measured the expression level of miR-155 in BMDMs upon treatment with tumor cell–derived conditioned medium. As shown in Supplementary Fig. S5B, both LLC-CM and B16-CM resulted in a significant reduction of miR-155 expression in macrophages (99% and 77%, respectively), indicating a reciprocal regulation of tumor cells and macrophages.
miR-155–deficient MΦ promoted tumor cell migration and proliferation in vitro

Macrophages in tumor microenvironment may directly modify tumor cell behavior, such as migration and proliferation, which will facilitate tumor development (5, 31, 43). First, to test whether miR-155−/− macrophages promote tumor cell migration, Transwell migration assays were conducted. As shown in Fig. 7A and B, migration of LLC cells toward serum-free medium–treated miR-155−/− macrophages was significantly enhanced compared with that toward WT macrophages, and LLC cells migrating toward LCM–treated miR-155−/− macrophages was significantly increased than that toward LCM–treated WT macrophages, indicating that miR-155−/− macrophages possess much more potent chemotactic ability for tumor cells. Similar results were obtained when B16-F10 cells were used for the assays (Supplementary Fig. S6). The promoting effect of miR-155−/− macrophages on tumor cell migration might at least partially explain the increased lung metastasis in bone marrow miR-155−/− deficient mice.

We next tested whether miR-155−/− macrophages promote tumor cell proliferation. When LLC cells were cocultured with either WT or miR-155−/− macrophages, we did not find difference in the proliferation of LLC cells (data not shown). Then we sought to examine whether miR-155 expression level in LLC cells influences their proliferative response to macrophages. LLC cells were transfected with miR-155 inhibitor or control inhibitor and the miR-155 inhibition efficiency was confirmed by qRT-PCR measurement of miR-155 target gene expression, such as HIF-1α, Bcl-6, c-Maf, and SOCS1 (Supplementary Fig. S7A). [3H] thymidine incorporation assay was conducted in the following groups: tumor cells transfected with control inhibitor cocultured with WT MΦ and miR-155−/− MΦ for another 48 hours. Data are represented as the mean ± SEM (n = 6/group), **, P < 0.001 by Student t test.
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There was no significant difference in LLC cell proliferation between con inhibitor+/WT MΦ group and con inhibitor+/miR-155<sup><sub>/</sub></sup>-/- MΦ group (Fig. 7C, column 1 and 2). However, in miR-155 inhibitor transfected LLC cells, addition of miR-155<sup><sub>/</sub></sup>-/- MΦ significantly promoted LLC proliferation by 54% as compared with WT MΦ (Fig. 7C, column 3 and 4). Our data indicated that lower expression level of miR-155 in tumor cells may sensitize tumor cells to miR-155<sup><sub>/</sub></sup>-/- MΦ. Interestingly, the LLC proliferation was significantly even though not dramatically inhibited by 19% in miR-155 inhibitor transfected cells compared with con inhibitor group in the presence of WT macrophages (Fig. 7C, column 1 and 3). This phenomenon may be correlated with the oncogenic role of miR-155 in solid tumor cells. We also conducted proliferation assays using B16-F10 cells, and found that even without miR-155 inhibition, the proliferation of B16-F10 cells was enhanced by miR-155<sup><sub>/</sub></sup>-/- macrophages (Supplementary Fig. S7B).

Discussion

miRNAs have emerged as a class of gene expression regulators that are involved in many pathophysiology processes including cancer development. miR-155 is one of miRNAs that have attracted much attention recently. Aberrant expression of miR-155 in hematopoietic cells drives development of several leukocyte-derived tumors. In addition, miR-155 expressed in certain types of solid tumor cells are increasingly implicated in the regulation of tumor growth and metastasis. Therefore, targeting miR-155 has been proposed as a promising strategy for treatment of cancers (25). However, how host hematopoietic miR-155 expression affects solid tumor development and metastasis remains unexplored. Here, we showed that miR-155 deficiency in hematopoietic cells in tumor-bearing mice promoted macrophage infiltration in lungs and enhanced lung metastasis, although without obvious influence on primary tumor progression (Figs. 1 and 5). Therefore, our data uncover an important role for hematopoietic miR-155 in modulating the establishment of prometastatic microenvironment in the lungs, raising the concern that targeting miR-155 in host immune cells may enhance the metastasis of established cancer.

Metastasis is responsible for 90% of death in patients with cancer. Metastasis requires not only the release of cells from the primary sites, but also tumor cell seeding, extravasation, and subsequent survival at distant sites as well as metastatic colonization, which are the major rate-limiting events for tumor metastasis. Hence, a truly effective antimetastatic therapy must be capable of interfering multiple steps of the invasion–metastasis cascade. Unfortunately, many antitumor agents currently in preclinical or clinical development stages can only block the dissemination of neoplastic cells from primary tumors but cannot sufficiently suppress metastasis formation in distant organs once tumor cells escape the primary tumors (44). For this reason, the development of new therapeutic agents that alter the microenvironment where the neoplastic cells proliferate and survive in the distant tissues represents an intriguing strategy to suppress tumor metastasis.

It is well recognized that metastasis of solid tumors requires collaborative interactions between malignant cells and a variety of "activated" stromal cells at both primary and metastatic sites (2, 3, 6, 30). Among them, macrophages are the most abundant leukocytes that are present in neoplastic stroma (5, 8, 45). Emerging evidence has shown that macrophages potentiate the seeding and establishment of metastatic cells in distant tissues in addition to its well-defined functions in primary tumors (4, 11, 45, 46). Said and colleagues showed that tumor endothelin-1 (ET-1) expression in bladder cancer cells was necessary for metastatic lung colonization, which was dependent on macrophage infiltration in the lungs (46). Gil-Bernabe and colleagues found that tissue factor expression by tumor cells enhanced tumor cell survival in the lungs by recruiting macrophages (45). Although there are only few studies focused on metastasis-associated macrophages, existing data indicate that infiltration of macrophages in metastatic sites is essential for effective metastasis. Our current study suggests that miR-155 expression in metastasis-associated macrophages may serve as a defense against cancer metastasis through halting macrophage polarization toward a protumor M2 phenotype.

Macrophages in tumor microenvironment may directly modify tumor cell behavior, such as migration and proliferation, which will facilitate tumor development (5, 31, 43). Because tumor cell migration is a critical step of the metastatic cascade, we investigated the ability of WT and miR-155<sup><sub>/</sub></sup>-/- macrophages to attract tumor cells by a Transwell migration assay. We verified that miR-155<sup><sub>/</sub></sup>-/- macrophages exerted a stronger chemotactic effect than WT macrophages on both LLC (Fig. 7A and B) and B16-F10 cells (Supplementary Fig. S6), which may partially explain the increased lung metastasis in bone marrow miR-155-deficient mice. We also found that miR-155<sup><sub>/</sub></sup>-/- macrophages affected proliferation of LLC cells and B16-F10 cells. Interestingly, inhibition of miR-155 expression in LLC cells reduced proliferation by 19% under the influence of WT macrophages (Fig. 7C column 3 vs. column 1), indicating inhibition of LLC miR-155 expression could be beneficial; however, when these cells were incubated with miR-155<sup><sub>/</sub></sup>-/- macrophages, the proliferation was increased by 54% (Fig. 7C column 4 vs. column 3). Given the fact that LCM suppressed macrophage miR-155 expression by 99% (Supplementary Fig. S5B), it is conceivable that universal inhibition of miR-155 expression in both tumor cells and macrophages will more likely favor metastasis of LLC. In the case of B16-F10 cells, even without miR-155 inhibition, miR-155<sup><sub>/</sub></sup>-/- macrophages promoted their proliferation (Supplementary Fig. S7B). The difference between LLC cells and B16-F10 cells may be due to their expression levels of endogenous miR-155; B16-F10 cells express much lower level of miR-155 than LLC cells (Supplementary Fig. S5A).

By flow cytometry analysis, we found that macrophage was the only cell type that the percentage was increased in miR-155<sup><sub>/</sub></sup>-/-→BMT mice, which indicated that macrophages might be recruited into primary tumor sites and metastatic lungs. However, in our study, macrophages specifically appeared in the lungs but not the primary tumors. We postulate that the
expression level of chemokines that are capable of recruiting macrophages are specifically increased in lungs but not in tumor sites. During tumor development, tumor cell–secreted factors educate distant metastatic organs through activating stromal cells, such as resident macrophages, fibroblasts, and endothelial cells, which create a favorable microenvironment for the recruitment of BMDCs (2, 29, 30, 32, 47). A variety of inflammatory factors, such as fibronectin, SDF1, MMP9, S100A8, and S100A9, upregulated in lung stromal cells, are responsible for the recruitment of macrophages and BMDCs from circulation into metastatic sites in tumor-bearing mice. Hiratsuka and colleagues showed that S100A8 and S100A9 from circulation into metastatic sites in tumor-bearing mice. S100A8, and S100A9, upregulated in lung stromal cells, are responsible for the recruitment of macrophages and BMDCs for the recruitment of macrophages into lung tissues. In addition, Kaplan and colleagues showed that fibronectin expression in resident fibroblasts was significantly increased in lungs of tumor-bearing mice compared with tumor-free mice (2). Increased fibronectin expression attracts VLA-4–expressing BMDCs migrating into lung tissues. In our model, we also observed an increase of fibronectin expression in lung of tumor-bearing mice.

Several genes that are involved in the regulation of macrophage phenotype have been identified or predicted as targets of miR-155, such as C/EBPβ, SOCS1, and c-Maf (18, 42, 48). C/EBPβ plays a critical role in regulation of Arg1 expression and M2 macrophage polarization (39). It has been shown that miR-155 reduces the endogenous expression of C/EBPβ in macrophages through interacting with the 3′-untranslated region of the C/EBPβ mRNA (40, 48). Downregulation of miR-155 in Akt2/+/− macrophages caused an increase of C/EBPβ expression, which resulted in M2 polarization (49). In addition, SOCS1 is upregulated in M2 macrophages and its rapid increase is critical for sustaining the anti-inflammatory phenotype and function of M2 macrophages (42). c-Maf, an identified target of miR-155 in T-helper cell 2 (Th2) cells (18), strongly activates IL-10 and IL-4 expression in macrophages (50, 51), potentially contributing to the state of anti-inflammatory function of macrophages. All these evidences indicate that miR-155 may modulate macrophage polarization. In our current study, we not only confirmed the high responsiveness of miR-155+/− macrophages to IL-4 stimulation (a potent M2 macrophage inducer; Fig. 6), but further showed that miR-155−/−deficient macrophages were prone to M2 polarization under the treatment with tumor-conditioned medium and that C/EBPβ played a crucial role in the increased M2 polarization of miR-155+/− macrophage (Fig. 6E). However, although the basal level of SOCS1 was slightly higher in miR-155+/− macrophages than in WT ones, tumor cell–conditioned medium failed to induce a further increase of SOCS1 level (Fig. 6E), indicating that SOCS1 may not be involved in the miR-155 modulation of macrophage polarization induced by tumor-derived factors. Our data are in agreement with 2 recent in vitro studies. Cai and colleagues reported that overexpression of miR-155 could reprogram anti-inflammatory, protumor M2 TAMs to proinflammatory, antitumor M1 macrophages (52). He and colleagues showed that induction of miR-155 suppressed C/EBPβ protein expression as well as cytokine production in tumor-activated monocytes (40). Importantly, our current work provides the first evidence that the modulation of macrophage polarization by miR-155 may operate in vivo in cancer metastasis.

Recent studies highlighted the oncogenic function of tumor cell–expressed miR-155, as miR-155 was found upregulated in multiple types of human cancers and ectopic expression of miR-155 significantly promoted tumor development in many experimental mouse models (24–26). These findings suggested that therapeutic targeting of miR-155 in malignant cells may provide a novel strategy for delaying tumor development. However, our current study suggests that universal inhibition of miR-155 in patients with cancer may backfire. On the one hand, suppression of miR-155 in tumor cells may inhibit tumor cell growth; on the other hand, impaired expression of miR-155 in host macrophages may promote tumor metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: F. Yu, W. Ai, D. Fan
Development of methodology: F. Yu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Yu, X. Jia, F. Du, J. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Yu, X. Jia, F. Du, J. Wang, W. Ai, D. Fan
Writing, review, and/or revision of the manuscript: F. Yu, W. Ai, D. Fan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Yu, Y. Wang, D. Fan
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