Positive feedback loop of HMGB1 and tumor-associated macrophages plays a key role in osteosarcoma metastasis

Changhe Hou
The Third Affiliated Hospital of Southern Medical University

Ming Lu
The Third Affiliated Hospital of Southern Medical University

Zixiong Lei
The Third Affiliated Hospital of Southern Medical University

Shuangwu Dai
The Third Affiliated Hospital of Southern Medical University

Wei Chen
The Third Affiliated Hospital of Southern Medical University

Shaohua Du
The Third Affiliated Hospital of Southern Medical University

Qinglin Jin
The Third Affiliated Hospital of Southern Medical University

Zhongxin Zhou
The Third Affiliated Hospital of Southern Medical University

Haomiao Li (lihaom@mail.sysu.edu.cn)
The Third Affiliated Hospital of Southern Medical University

Research Article

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Abstract

**Background**

Numerous studies have demonstrated the important roles of tumor-associated macrophages (TAMs) in osteosarcoma metastasis. In osteosarcoma, higher levels of HMGB1 correlate with osteosarcoma progression. However, whether HMGB1 is involved in the polarization of M2 macrophages into M1 macrophages in osteosarcoma still remains largely unknown.

**Methods**

HMGB1 and CD206 mRNA expression was measured by qRT-PCR in osteosarcoma tissues and cells. HMGB1 and RAGE protein expression was measured by western blotting. Osteosarcoma migration was measured using a Transwell and wound-healing assay. Osteosarcoma invasion was measured using a Transwell assay. Macrophage subtypes were detected using flow cytometry.

**Results**

HMGB1 is aberrantly overexpressed in osteosarcoma, and positively correlates with the TNM III & IV stages, lymph node metastasis, and distant metastasis. Silencing HMGB1 inhibits migration, invasion, and metastasis-related proteins in osteosarcoma cells. Furthermore, the reduced HMGB1 expression in the conditioned media derived from osteosarcoma cells also induces the polarization of M2 TAMs to M1 TAMs. In addition, silencing HMGB1 inhibits the liver and lung metastases of osteosarcoma and reduces the expression of HMGB1, CD163, and CD206 in vivo experiments. HMGB1 regulates macrophage polarization through RAGE. Interestingly, the polarized M2 macrophages could induce osteosarcoma migration and invasion, which in turn results in activation of HMGB1 expression in osteosarcoma cells to form a positive feedback loop.

**Conclusions**

HMGB1 and M2 macrophages enhance osteosarcoma migration, invasion, and metastasis capability through positive feedback regulation. These findings reveal the significance of tumor cell and TAM interaction in the metastatic microenvironment.

1. **Background**

Osteosarcoma, one type of sarcoma with highest incidence rate among primary bone malignancies in children, youth, and adolescents, severely affects the patients' physical and mental health and causes a heavy burden on the patients' families [1]. The pathological characteristics of osteosarcoma are rapid progression, early metastasis, and high mortality. The combination of surgical resection and systemic
multi-drug chemotherapy is currently the mainly adopted treatment strategy for patients with osteosarcoma [2, 3]. After treatment, the 5-year overall survival (OS) rate of osteosarcoma patients with and without metastasis is about 30% and 50-70% respectively, of which there is still much space for further improvement [3–5]. Therefore, exploring the biological mechanism of osteosarcoma metastasis is necessary to develop new treatment options for improving the survival rate of the osteosarcoma patients.

In recent years, tumor-associated macrophages (TAMs), which are present in the vicinity of tumor cells and involved in inflammatory responses, have garnered extensive interest as means of intervention in osteosarcoma progression. TAMs are composed of M1- and M2-polarized macrophages, which are classified as anti-tumor cells and pro-tumor regulators respectively [6]. Indeed, M1 and M2 macrophages coexist in osteosarcoma patients and that M1 macrophages are associated with better survival, whereas the M2-phenotype is associated with poor prognosis [7, 8]. M2-type macrophages contribute to tumor growth, vascularization, lung metastatic dissemination, and exacerbate intra-tumor T-lymphocyte immunosuppression in osteosarcoma cells [9–12]. These results suggest that the dysregulation of the balance between M1 and M2 TAMs regulates osteosarcoma progression. Modulation of TAM-polarization from an M2 to M1 phenotype may minimize the metastatic potential for patients with osteosarcoma and improve the prognosis of current therapies. However, drugs that can regulate TAM-polarization from an M2 to M1 phenotype (e.g., mifamurtide, all-trans retinoic acid) in osteosarcoma treatment require additional investigation [10]. Therefore, further understanding of the mechanism of TAM polarization in osteosarcoma will help to identify new targets that regulate the polarization of M2 macrophages to M1 macrophages.

High mobility group box 1 (HMGB1) is a nonhistone chromosome-binding protein that localizes in the nucleus to help stabilize nuclear homeostasis [13]. HMGB1, which is localized in cytoplasm, can maintain the cell homeostasis. However, the HMGB1 which is localized in the microenvironment, can bind to the Toll-like receptors (TLRs) and drive inflammatory responses to boost the immune system [14, 15]. Notably, the excessive accumulation of HMGB1 can induce abnormal pathophysiological events leading to many diseases, such as cancer [16, 17]. HMGB1 favors tumor development, migration and invasion, angiogenesis, and lymphatic metastasis, and reduce tumor chemo- and radio-therapy sensitivities [18, 19]. Moreover, HMGB1 could increase the recruitment of immunosuppressive cells such as TAMs, Tregs, and myeloid-derived suppressor cells; thereby further facilitating immunosuppression, tumorigenesis, and metastasis [20, 21]. In osteosarcoma, HMGB1 expression is elevated in osteosarcoma tissues and higher HMGB1 promotes osteosarcoma progression and drug resistance [22, 23]. However, the role of HMGB1 in regulating the polarization of M2 macrophages to M1 macrophages in osteosarcoma remains unclear. Accordingly, the expression and effect of HMGB1 in osteosarcoma were studied. Furthermore, the effect of HMGB1 on the polarization of M2 macrophages to M1 macrophages was elucidated. Lastly, we also established the role of TAMs in regulating the expression of HMGB1 in osteosarcoma cells.

2. Materials And Methods

2.1 Collection of clinical samples
This study was approved by the Ethics Committee of the third affiliated hospital of Southern Medical University. Informed consent forms were obtained from all participants. From January 2020 to May 2020, a total of 16 osteosarcoma tissue samples and the adjacent normal tissues were obtained from patients at the third affiliated hospital of Southern Medical University. All samples were immediately transferred to and stored at -80°C until further quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

2.2 Cell culture and stable cell lines generation

The human fetal osteoblastic cell line hFOB1.19 and human osteosarcoma cell lines MG-63 and 143B (Institutes for Biological Sciences, Shanghai, China) were cultured in a humidified incubator with 95% air and 5% CO$_2$ according to a previous study [24]. After culturing for 48 h, HMGB1 expression was measured by qRT-PCR. To create stable HMGB1 knockdown and control cells, short hairpin RNA targeting HMGB1 (sh-HMGB1) and the negative control of short hairpin RNA (sh-NC) were packaged into lentivirus. MG-63 and 143B cells were infected with sh-HMGB1 and sh-NC lentiviral particles to construct HMGB1 knockdown and control stable cell lines. Stable cell lines were then collected used for further studies.

2.3 qRT-PCR

Total RNA was extracted from osteosarcoma samples and cells using TRIzol reagent (Invitrogen). cDNA synthesis was performed using the ImProm-IIITM Reverse Transcription System (Promega, Madison, WI, USA) and then analyzed using qRT-PCR and the SYBR Premix ExTaQ Kit (Takara Bio) on an ABI 7500 RT-PCR system (Applied Biosystems). 18srRNA was used as an internal control for HMGB1 and CD163 expression. The relative expression level data were analyzed using the $2^{-\Delta\Delta C_T}$ method. All reactions were performed in triplicates. The sequences of the primers used in this study were as follows: HMGB1 F: 5’-ATATGGACTGCTCAGGAAAC-3’; HMGB1 R: 5’-GGCAAACCGTAATATAGGAA-3’. CD206 F: 5’-TTCAGTGGACCATCGAGGAAGAGG-3’; CD206 R: 5’-ATGGCAACACACCCTGGCTTTC-3’. CD163 F: 5’-GCCACAAACAGTCGCTCATCC-3’; CD163 R: 5’-GCAAGCGCGCTGTCTCTGCTT-3’. 18srRNA F: 5’-CCTGGATACCGCAGCTAGGA-3’; 18srRNA R: 5’-GCGGCGCAATACGAATGCCC-3’.

2.4 Western blotting assay

For the assay, 30 µg of total protein extracted from cells using RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China) was separated by 10% SDS polyacrylamide gel electrophoresis and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked and incubated with rabbit anti-HMGB1 (1:10000, No. 79823, Abcam, Cambridge, MA, USA), mouse anti-E-cadherin (1:1000, No. 76055, Abcam), rabbit anti-N-cadherin (1:5000, No. 76011, Abcam), rabbit anti-vimentin (1:2000, No. 92547, Abcam), rabbit anti-Snail (1:1000, No. 180714, Abcam), mouse anti-Twist (1:1000, No. 175430, Abcam), rabbit anti-MMP-2(1:2000, No. 92536, Abcam), rabbit anti-MMP-9 (1:1000, No. 38898, Abcam), and rabbit anti-RAGE (1:1000, No. 216329, Abcam) for 1 h at 37°C. Subsequently, membranes were washed and incubated with HRP-conjugated secondary antibody at 37°C for 40 min. After washing, membranes were assayed via enhanced chemiluminescence (ECL) and quantified using ImageJ 6.0. GAPDH served as the loading control.
2.5 Transwell migration and invasion and wound-healing assay

Cell migration (the top chamber of wells coated without Matrigel) and invasion (the top chamber of wells coated with Matrigel) assays were performed using a BD 24-well Transwell chamber (BD Biosciences, San Jose, CA, USA). For wound-healing assay, 143B and MG-63 stable cell lines were seeded into six-well plates at a density of $2 \times 10^5$ cells/well and cultured for 24 h. An artificial wound in the middle of the plates was made using a sterile pipette tip. After culturing for 48 h, the areas migrated by the cells were evaluated. The scraped areas were captured under a microscope (Olympus, Tokyo, Japan) at 40X magnification at 0 and 48 h. Wound closure is reported as a percentage of migrated area as compared to the area of the initial scratch. All assays were performed in triplicates.

2.6 Macrophage polarization assays

The study the effect of osteosarcoma cells on the migration and polarization of macrophages, co-culture assays were performed using Transwell chambers. THP-1 cells were treated with 100 nM phorbol-12-myristate-13-acetate (PMA) for 24 h to induce macrophage polarization. For the polarization assay, the macrophages were added to the lower chamber and the 143B or MG-63 stable cell lines were added to the upper chamber. The upper chamber containing PBS and anti-HMGB1 neutralizing antibody (0.1 µg/mL) was used as the control group. After induction for 48 h, the relative mRNA levels of CD163 and CD206 in the macrophages were detected using qRT-PCR. The percentage of CD11b$^+$CD68$^+$ and CD163$^+$CD206$^+$ cells was detected using flow cytometry.

2.7 Tail vein injection assay

Animal experiments were approved by the third affiliated hospital of Southern Medical University (2020-022), and conducted according to National Institutes of Health guidelines. For tail vein injection assay, $1 \times 10^6$ cells of sh-HMGB1 and sh-NC stable osteosarcoma cell was injected into the tail vein of 6-week-old female BALB/c nude mice. Six weeks post-injection, all mice were sacrificed and lungs and liver were isolated for HE staining to evaluate the degree of lung and liver metastasis.

2.7 Statistical analysis

All data were analyzed using SPSS version 19.0 statistical analysis package (SPSS Inc., Chicago, IL, USA). For independent two-group analyses, Student’s t-tests were used. Differences in P-values < 0.05 were considered statistically significant.

3. Results

3.1 HMGB1 and CD206 are highly expressed and positively correlated in osteosarcoma tissues.
To understand the relationship between HMGB1 and M2 TAMs, HMGB1 and CD206 (M2 TAM marker) expression was analyzed in osteosarcoma clinical samples by qRT-PCR. Our results revealed that, compared to the adjacent normal tissues, HMGB1 expression was remarkably enhanced in osteosarcoma tissues (Figure 1A). Additionally, HMGB1 expression was not significantly different between the clinical samples in patient cohorts categorized with respect to age (≤ 20 and > 20 years), sex (male and female), and tumor size (≤ 7.5 and > 7.5 cm) (Figure 1B-1D). HMGB1 expression was higher in patients with TNM III and IV stages, lymph node metastasis, and distant metastasis than in patients with TNM I and II stage, no lymph node metastasis, and no distant metastasis, respectively (Figure 1E-1G). Compared with adjacent normal tissues, CD206 expression in osteosarcoma tissue was remarkably elevated (Figure 1H). Furthermore, the expression of HMGB1 and CD206 was positively correlated in patients with osteosarcoma (Figure 1I).

3.2 Silenced HMGB1 knockdown inhibits migration, invasion, and metastasis capability in osteosarcoma cells.

To assess if there is differential expression of HMGB1 in human osteosarcoma and normal osteoblastic cell lines, qRT-PCR and western blotting was performed. The results revealed that the expression of HMGB1 is higher in human osteosarcoma cell lines MG-63 and 143B than in the human fetal osteoblastic cell line hFOB1.19 (Figure 2). To further study the effect of HMGB1 on the migration, invasion, and EMT of osteosarcoma cells, HMGB1 knockdown (sh-HMGB1 group) and control (sh-NC group) stable cell lines were generated. They were further validated by qRT-PCR and Western blotting assays. The results revealed that HMGB1 expression was significantly reduced in the sh-HMGB1 group compared to the sh-NC group (Figure 3A and 3B). Furthermore, the migration and invasion in the sh-HMGB1 group was significantly inhibited than in the sh-NC group (Figure 3C and 3D). Analysis of metastasis-related proteins and invasion markers by immunoblotting revealed that, compared to the sh-NC group, E-cadherin expression was increased while the expression of N-cadherin, Vimentin, Snail, Twist, MMP-2, and MMP-9 was evidently decreased in the sh-HMGB1 group (Figure 4). In vivo experiments proved that compared with the sh-NC group, the liver and lung metastasis of tumors was significantly inhibited in the sh-HMGB1 group (Figure 5A). In addition, the expression of HMGB1, CD163, and CD206 was significantly reduced (Figure 5B).

3.3 Silencing HMGB1 promotes the polarization of M2 TAMs to M1 TAMs.

To elucidate the effect of HMGB1 on the polarization of TAMs, the expression of HMGB1 in the conditioned media derived from osteosarcoma cells was measured by qRT-PCR. Our results showed that HMGB1 expression was indeed higher in the conditioned media of MG-63 and 143B cells than in that of hFOB1.19 cells (Figure 6A). Additionally, the expression of HMGB1 was found to be significantly reduced in the conditioned media derived from sh-HMGB1 group compared to the sh-NC group (Figure 6A). After co-culturing of osteosarcoma cells (143B and MG-63) with macrophages, it was found that 143B and MG-63 osteosarcoma cells increased the expression of CD206 and CD163 in macrophages, and
enhanced the percentage of CD163⁺CD206⁺ cells (Figure 6B-6E). Furthermore, silencing of HMGB1 in 143B and MG-63 and the addition of HMGB1-neutralizing antibody in the conditioned media resulted in decreased expression of CD206 and CD163 in macrophages, and reduction in the percentage of CD163⁺CD206⁺ cells (Figure 6B-6E). These data indicate that HMGB1 present in the conditioned media derived from osteosarcoma cells promotes the M2 polarization of macrophages.

### 3.4 HMGB1 regulates macrophage polarization through RAGE

In order to study the mechanism of HMGB1 regulating the polarization of macrophages, we studied the receptor proteins regulated by HMGB1. Previous studies have shown that HMGB1 can bind to the surface receptors RAGE in macrophage [25], hence, we investigated the role of RAGE in the HMGB1 regulation of macrophage polarization. First, we analyzed the expression of RAGE protein. Studies have shown that compared with the normal cell culture group, the expression of RAGE protein in macrophages cultured with tumors was significantly increased; compared with the sh-NC and PBS groups, the expression of RAGE mRNA and protein in the sh-HMGB1 and anti-HMGB1 groups was significantly reduced, respectively (Figure 7A and 7B). Then further analyze whether the addition of RAGE antibody can prevent the effect of HMGB1 on the migration and differentiation of macrophages. In this project, PBS and RAGE antibodies were added to the tumor culture supernatant to analyze its effect on the polarization of macrophages. The study showed that compared with the PBS group, the proportion of M2 macrophages in the RAGE antibody group was significantly reduced (Figure 7C); Finally, recombinant human HMGB1 and RAGE antibodies were added to the macrophage culture medium to analyze its effect on the polarization of macrophages. The results showed that compared with the HMGB1+PBS co-treatment group, the proportion of M2 macrophages in the HMGB1+RAGE antibody co-treatment group was significantly reduced (Figure 7C). The above results indicate that HMGB1 in the supernatant of tumor culture media promotes the polarization of macrophages to M2 through RAGE.

### 3.5 Macrophages educated by HMGB1 knockdown stable cell lines inhibit the migration and invasion of osteosarcoma cells

In order to study the effect of macrophage on HMGB1 knockdown stable osteosarcoma cells, co-culture of the medium supernatant of macrophages was performed with osteosarcoma cells. The medium supernatant which came from sh-NC and sh-HMGB1 osteosarcoma cells co-cultured macrophages were added to the lower chamber and the 143B and MG-63 cells were added to the upper chamber, and co-culture for 48 h. Compared with sh-NC group, HMGB1 expression and the migration and invasion of 143B and MG-63 cell were significantly decreased the sh-HMGB1 co-culture group (Figure 8). These results suggested that the medium supernatant which came from sh-HMGB1 osteosarcoma cells co-cultured macrophages can inhibit the ability of migration and invasion in osteosarcoma cells.
4. Discussion

The metastasis of osteosarcoma is a major factor responsible for unsatisfactory 5-year overall survival (OS) rates in osteosarcoma patients [3–5]. Modulation of TAM-polarization from M2 to M1 phenotype can decrease the metastatic potential for patients with osteosarcoma and improve the prognosis and OS with current therapies [7, 8]. In this study, we have revealed that knockdown of HMGB1 results in inhibition of the migration, invasion, and metastasis capability in osteosarcoma cells. Furthermore, HMGB1 silencing also diminishes M2 TAM-polarization. These data indicate that HMGB1 functions to promote osteosarcoma progression.

A previous study had shown that increased HMGB1 was found in tumor tissues and serum in patients with osteosarcoma, which predicted a worse prognosis [26]. Dysregulation of HMGB1-assisted osteosarcoma tumorigenesis and its crucial role in metastasis is evident by inhibition of cell migration and invasion in osteosarcoma cells upon microRNA-mediated silencing of HMGB1 expression [27]. In this study, we further revealed that HMGB1 expression is elevated in osteosarcoma tissues. Moreover, the elevated HMGB1 levels are associated with higher TNM stage, lymph node metastasis, and distant metastasis. In addition, HMGB1 knockdown also resulted in inhibition of the migration and invasion of osteosarcoma cells. E-cadherin is a membrane protein belonging to the cadherin family that contributes to cell adhesion, while N-cadherin breaks cell–cell adhesion to promote cancer metastasis [28]. During the process of cancer metastasis, the switch from E-cadherin to N-cadherin contributes prominently [29, 30]. Vimentin, Snail, and Twist are markers which enhance the metastasis of tumor cells [29, 30]. In osteosarcoma, elevation of those markers also correlates with tumor metastasis [31, 32]. In this study, we found that E-cadherin expression increased while N-cadherin, Vimentin, Snail, Twist, MMP-2, and MMP-9 expression decreased after silencing HMGB1 in osteosarcoma cells. These results suggest that HMGB1 is capable of promoting osteosarcoma metastasis.

TAMs in the tumor microenvironment are associated with tumor progression and metastasis [7, 8]. Many clinical and epidemiological studies have found a positive association between TAM density and poor prognosis in osteosarcoma [9–12]. Importantly, tumor-derived cytokines recruit TAMs, which contribute to tumor progression and metastasis by directly affecting the extracellular matrix remodeling, and neo-angiogenesis [33]. In this study, we have found that CD206 and CD163, which are M2 polarization markers of macrophages, are significantly elevated in osteosarcoma tissues and positively correlate with HMGB1 expression. Moreover, HMGB1 in the conditioned medium derived from osteosarcoma cells promotes M2 polarization of macrophages. Neutralizing HMGB1 with anti-HMGB1 antibody and reduced HMGB1 in the conditioned medium results in decreased M2 polarization of macrophages. RAGE exists on the membrane of macrophages and is the surface receptor of HMGB1, which can bind by HMGB1 to regulate macrophage polarization [34, 35]. In this study, we found that anti-REAG can prevent M2 polarization of macrophages which induced by HMGB1. These results suggest that HMGB1 came from osteosarcoma cells promoted M2 polarization of macrophages via binding with RAGE. In addition, the macrophages induced by HMGB1 knockdown stable osteosarcoma cell lines inhibit the HMGB1 expression and the migration and invasion of osteosarcoma cells. Overall, these findings indicate that the
positive feedback loop formed by HMGB1 and TAMs contributes significantly to the progression of osteosarcoma.

5. Conclusions

The present study demonstrates that HMGB1 is overexpressed in osteosarcoma and contributes to the migration, invasion, and metastasis capability in vitro in osteosarcoma cells by forming a positive feedback loop with TAMs. These findings suggest that HMGB1 may act as a potential target for developing therapeutic strategies for the treatment of osteosarcoma. However, the novel mechanism should be further validated via in vivo experiments.

Abbreviations

TAMs
tumor-associated macrophages
HMGB1
high mobility group box 1
RAGE
receptor for advanced glycation end products
TLRs
toll-like receptors
qRT-PCR
quantitative real-time polymerase chain reaction
EMT
epithelial-mesenchymal transition

Declarations

Ethics approval and consent to participate: This study was approved by the Ethics Committee of the third affiliated hospital of Southern Medical University.

Consent for publication: Not applicable

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare no potential conflicts of interest.

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Authors' contributions: HL designed the research; CH, ML, ZL, SD, and WC performed research and analyzed data; SD and QL contributed patient material; CH and ML wrote the manuscript and ZZ and HL reviewed the manuscript. All authors read and approved the final manuscript.

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**Figures**

**Figure 1**

HMGB1 and CD206 expression are highly elevated and positively correlated in osteosarcoma tissues. (A) HMGB1 expression in osteosarcoma tissues and adjacent normal tissues was analyzed by qRT-PCR. (B-G) HMGB1 expression in different clinical feature groups was analyzed. (H) CD206 expression in osteosarcoma tissues and adjacent normal tissues was analyzed by qRT-PCR. (I) The relationship between HMGB1 and CD206 was analyzed by Spearman correlation analysis. *P<0.05

**Figure 2**

HMGB1 expression was enhanced in human osteosarcoma cell lines. HMGB1 expression in human osteosarcoma cell lines MG-63 and 143B and human fetal osteoblastic cell line hFOB1.19 were analyzed
Silencing HMGB1 inhibits migration and invasion in osteosarcoma cell. (A and B) HMGB1 expression in human osteosarcoma cell lines MG-63 and 143B stable cell were analyzed by qRT-PCR (A) and western blot (B) after transduction with negative control (sh-NC) and sh-HMGB1 lentiviral particles. (C) Migration...
potential was analyzed by wound-healing assay. (D) Migratory and invasive cells were analyzed using Transwell chambers (Magnification: 200×). ***$P<0.001$.

#### Figure 4

Silencing HMGB1 inhibits EMT-related proteins in osteosarcoma cell. E-cadherin, N-cadherin, Vimentin, Snail, Twist, MMP-2, and MMP-9 expression was measured by western blot.

|        | 143B |                  | MG63 |                  |
|--------|------|-----------------|------|-----------------|
|        | sh-NC | sh-HMGB1 | sh-NC | sh-HMGB1 |
| E-cadherin | ![Image](E-cadherin.png) | ![Image](E-cadherin.png) | ![Image](E-cadherin.png) | ![Image](E-cadherin.png) |
| N-cadherin | ![Image](N-cadherin.png) | ![Image](N-cadherin.png) | ![Image](N-cadherin.png) | ![Image](N-cadherin.png) |
| Vimentin | ![Image](Vimentin.png) | ![Image](Vimentin.png) | ![Image](Vimentin.png) | ![Image](Vimentin.png) |
| Snail | ![Image](Snail.png) | ![Image](Snail.png) | ![Image](Snail.png) | ![Image](Snail.png) |
| Twist | ![Image](Twist.png) | ![Image](Twist.png) | ![Image](Twist.png) | ![Image](Twist.png) |
| MMP2 | ![Image](MMP2.png) | ![Image](MMP2.png) | ![Image](MMP2.png) | ![Image](MMP2.png) |
| MMP9 | ![Image](MMP9.png) | ![Image](MMP9.png) | ![Image](MMP9.png) | ![Image](MMP9.png) |
| GAPDH | ![Image](GAPDH.png) | ![Image](GAPDH.png) | ![Image](GAPDH.png) | ![Image](GAPDH.png) |
Figure 5

Tail vein injection assay results showed that HMGB1 knockdown inhibited osteosarcoma cell lung and liver metastasis. (A) The lung and liver metastasis were analyzed by tail vein injection assay. (B) HMGB1, CD163, and CD206 expression in lung and liver tissues was measured by qRT-PCR.
Figure 6

HMGB1 in the conditioned media derived from osteosarcoma cells regulates the polarization of M2 TAMs to M1 TAMs. (A) HMGB1 expression in the medium supernatant was measured by qRT-PCR. (B and C) the expression of CD206 and CD163 in macrophages was measured by qRT-PCR after co-culturing osteosarcoma cells (143B and MG-63) with macrophages for 48 h. (D and E) the percentage of CD163⁺CD206⁺ cells were measured by flow cytometry after co-culturing osteosarcoma cells (143B and MG-63) with macrophages for 48 h. ***P<0.001.
Figure 7

HMGB1 in the supernatant of tumor culture media promotes the polarization of macrophages to M2 through RAGE. (A) RAGE mRNA expression in macrophages was measured by qRT-PCR after treatment medium supernatant. (B) RAGE protein in macrophages was measured by qRT-PCR after treatment medium supernatant. (C) The percentage of CD163⁺CD206⁺ cells were measured by flow cytometry after macrophages co-culturing the culture media of osteosarcoma cells (143B and MG-63) and anti-RAGE for 48 h. *** \( P < 0.001 \).
The medium supernatant which came from sh-HMGB1 osteosarcoma cells co-cultured macrophages can inhibit the ability of migration and invasion in osteosarcoma cells. The macrophages were co-cultured with sh-NC and sh-HMGB1 stable osteosarcoma cells at 48 h. Then collected the macrophages and cultured at 48 h. After culture, the medium supernatant of macrophages was collected and added to added to the lower chamber and the 143B and MG-63 cells were added to the upper chamber, and co-
culture at 48 h for further study. (A) HMGB1 expression were measured by western blot after co-culturing macrophages. (B and C) The migratory and invasive cells were analyzed by Transwell chamber assays after co-culture for 48 h (Magnification: 200×).