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Original Articles

Tumor-associated macrophages promote lung metastasis and induce epithelial-mesenchymal transition in osteosarcoma by activating the COX-2/STAT3 axis

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ABSTRACT

Osteosarcoma (OS) is a common, malignant musculoskeletal tumor in young people. Neoadjuvant chemotherapy has improved the survival of osteosarcoma patients but with limited benefit due to metastasis. Tumor-associated macrophages (TAMs) are involved in various mechanisms of tumor biology, which include oncogenesis, drug resistance, and tumor immune escape, as well as tumor metastasis. In this study, we proved that TAMs possess the ability to promote OS cell migration and invasion by upregulating COX-2, MMP9, and phosphorylated STAT3 and to induce the epithelial-mesenchymal transition (EMT). This evidence has also been verified in a tumor-bearing animal model, and in OS patients. Furthermore, we observed the anti-metastasis effect of COX-2 inhibition by repressing COX-2 expression, EMT-activating transcription factors and the STAT3 pathway, both in vitro and in vivo. We propose that TAMs promote OS metastasis and invasion by activating the COX-2/STAT3 axis and EMT. These findings suggest that TAMs and COX-2 may be potential targets for future anti-metastasis therapy.

1. Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor and is most prevalent in children, adolescents and young adults. Approximately 15%–20% of patients show clinically detectable metastases at presentation [1]. Pulmonary metastasis is the most common type, accounting for more than 85% of metastatic osteosarcomas [2]. It is noteworthy that the overwhelming majority of OS patients die of distant metastasis. Despite the fact that neoadjuvant chemotherapy plus surgery has greatly raised the 5-year survival rate of patients with primary osteosarcoma, more than 80% of OS patients who receive surgical treatment with or without neoadjuvant chemotherapy will develop metastasis [3].

The metastatic progression of tumors consists of complicated mechanisms including the initial driving oncogenic mutation, enhanced epithelial-mesenchymal transition (EMT), local invasion, intravasation, survival in circulation, increased cancer stemness and angiogenesis [4,5]. The communication between tumor cells and the corresponding microenvironment is critical for tumor growth and metastasis [6]. Furthermore, inflammatory microenvironments are now recognized to be an integral factor contributing to carcinogenesis, tumor metastasis and treatment resistance [6–8].

In these tumor microenvironments, tumor-infiltrating inflammatory cells, particularly tumor-associated macrophages (TAMs) play an important role in metastatic processes [9]. It has been confirmed that there is a strong correlation between poor patient prognosis and infiltrating macrophage density in lung, thyroid, and hepatocellular cancers [9–13]. Macrophages have diverse functions and show plasticity in response to microenvironments [14]. According to their functions, macrophages can be classified into immunosuppressive, angiogenic, metastasis-associated, and phagocytic. TAMs can be either antineoplastic (M1) or tumor-promoting (M2). The evidences from transcriptome analysis indicate that TAMs have a mixed phenotype expressing both M1 and M2 markers [15–17].

Abbreviations: TAMs, tumor-associated macrophages; EMT, epithelial-mesenchymal transition; COX-2, Cyclooxygenase-2; MMP, matrix metalloproteinases; OS, osteosarcoma; iNOS, inducible nitric oxide synthase; CM, conditioned medium; PBMC, peripheral blood mononuclear cell

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Cyclooxygenase-2 (COX-2), an essential molecule in the inflammatory microenvironment, has been demonstrated by numerous studies to have close associations with oncogenesis, progression and metastasis [18–21]. TAMs are considered one of the major sources of COX-2 in various tumors [22–25]. Although previous studies have demonstrated that COX-2 affects tumor migration and invasion, the pro-metastatic effect of TAMs on OS cells remains unclear.

To investigate the role of TAMs in OS metastasis, we examined the amounts of TAMs in both primary and metastatic OS lesions. A co-culture model of OS cells and TAMs was established in vitro to explore the pro-metastatic effect of TAMs and the underlying mechanism. In addition, we studied the effect of COX-2 inhibition in vitro and in vivo to verify potential therapeutic targets.

2. Methods and materials

2.1. Patient and tissue microarray construction

Eighteen pairs of primary osteosarcomas with corresponding lung metastases and one set of tissue microarray slides were used in this study for immunohistochemical analysis. All tumor specimens mentioned in this study were acquired from the Musculoskeletal Tumor Center, Peking University People's Hospital (Beijing, China). Informed consent was obtained from each patient and their guardians if patients were under 18 years old, and the study was approved by the ethics committee of Peking University People's Hospital. Clinical data were assembled from medical records or clinical follow-up.

2.2. Cell culture

MG63, KHOS, 143B, HOS, SAOS2, U2OS and THP-1 cells were obtained from the American Type Culture Collection (ATCC). The MG63, KHOS, 143B, HOS, SAOS2, U2OS cell lines used in this study have been authenticated by the Beijing Microread Genetics Co., Ltd., with STR analysis. The MG63, KHOS, HOS, U2OS and THP-1 cells were maintained in RPMI 1640 medium (HyClone, SH30027.01) containing 10% fetal bovine serum (FBS, Gibco, 10100147), while the 143B and SAOS2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) with 10% FBS. All cell lines were cultured at 37 °C with 5% CO_2.

2.3. Macrophage induction and conditioned medium preparation

To induce monocyte-differentiated macrophages, the human monocytic cell line THP-1 was treated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA, Abcam, ab120297) followed by incubation in RPMI 1640 medium for 24 h [26]. Blood samples were collected from healthy volunteers with written informed consent, and the study was approved by the Institutional Review Board of Peking University People's Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated using the Human Buffy Coat CD14 Positive Selection Kit (Stemcell, #18088) following the manufacturer's protocol. M1 polarization was stimulated by incubation with 20 ng/ml of recombinant human interferon-γ (Pepro tech, #300–02) and 100 pg/ml of LPS (Sigma, #8630) for 18 h, whereas M2 polarization was stimulated by incubation with 20 ng/ml of human interleukin 4 (Pepro tech, #200–04) for 72 h. The different macrophages were incubated in serum-free medium for 24 h, and the culture supernatants were collected as conditioned medium (CM).

2.4. Flow cytometry and qRT-PCR assays

The differentiated THP-1 and PBMC macrophages and subtypes were analyzed for CD86, CD68, HLA-DR, CD80, CD206, and CD163 expression by flow cytometry and qRT-PCR assays. GAPDH was used as an internal control for gene expression. The antibodies and primers used in this study are listed in Supplementary Tables 1 and 2

2.5. Western blotting

The procedure for Western blotting was same as our previously study [27]. The antibodies for Western blotting are listed in Supplementary Table 2.

2.6. Immunohistochemistry

After deparaffinating, rehydration, and heat-induced antigen retrieval with citrate solution, paraffin sections were incubated with the corresponding antibodies overnight at 4 °C. For CD68 and F4/80 staining, the average numbers of positive cells were calculated from at least 6 fields (× 200 magnification). The other staining scores were the sum of the staining area (0: no staining or staining in < 10%, 1: staining in 10%–40%, 2: staining in 40%–70%, 3: staining in ≥70%) and the staining intensity (0: no staining, 1: yellow, 2: brown, 3: maroon). The immunohistochemistry (DAB) was assessed by two independent pathologists without any previous information of the clinical characteristics and outcomes. The antibodies are listed in Supplementary Table 2.

2.7. siRNA knockdown and inhibitor reagent

siRNAs were obtained from GenePharma (Suzhou, Jiangsu, China), and the sequences of the siRNAs are listed in Supplementary Table 3. The COX-2 selective inhibitor celecoxib (S1261) was purchased from Selleck (Houston, TX, USA).

2.8. Transwell and matrigel invasion assays

A total of 6×10^4 osteosarcoma cells were seeded into the upper chambers of non-coated (3422, Corning) or matrigel-coated (354480, Corning) Transwell plates with membranes containing 8.0-μm pores. The average numbers of cells were calculated from at least 6 fields (× 200 magnifications) by two independent researchers.

2.9. Wound-healing assay

Osteosarcoma cells were seeded in 6-well plates and cultured in different CM for 48 h. When the confluency reached 80%, a 200-μl pipette tip was used to make a straight artificial wound. Image acquisition was performed using a phase-contrast microscope (Leica, Leica Microsystems). Image analysis was conducted with Image-Pro Plus software.

2.10. RNA-sequencing

The total RNA was extracted using TRIzol reagent (15596018, Invitrogen), and the RNA sample quantification, qualification, library preparation and subsequent RNA-sequencing were conducted by Novogene Co., LTD (Beijing, China). Differential expression analysis of the two conditions was performed using the edgeR R package (3.12.1). Corrected P-values of 0.05 and absolute fold-changes of 2 were set as the threshold for significantly differential expression.

2.11. Tumor xenografts

Female BALB/c nude mice were purchased from the Experimental Animal Center of the Vitalriver Company (Beijing, China) and housed in SPF conditions. All animal care and handling procedures were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. 5×10^6 KHOS cells and 143B cells were separately seeded into the right flanks of mice via subcutaneous injection. For COX-2 inhibition in vivo, aspirin was

Table 1
Demographics and clinical variable.

Variables	Case	COX-2 expression		
		High	Low	P value (χ^2test)
Gender				0.389
male	42	19	23	
female	45	16	29	
Age				1.000
≤20	49	20	29	
>20	38	15	23	
Tumor location				0.482
femur	38	14	24	
tibia	8	4	4	
humerus	10	5	5	
others	31	12	19	
Histological types				0.837
osteoblastic	37	14	23	
chondroblastic	36	16	20	
others	14	5	9	
Metastasis				**< 0.0001**
present	28	19	9	
absent	59	16	43	

administered in the drinking water (600 μg/ml), and the water was replaced every 2 days. The tumor-bearing mice were sacrificed at the sixth week, and the primary tumor and lungs were collected for further investigation. At humane endpoints, all mice were euthanized by CO2 inhalation followed by cervical dislocation.

3. Results

3.1. Tumor-associated macrophages and COX-2 expression are closely associated with osteosarcoma metastasis

To investigate the infiltration of the tumor-associated macrophages and COX-2 expression in OS, we first examined the expression of the CD68 and COX-2 proteins in human primary OS tissues (n = 87) by immunohistochemistry (Table 1). The human OS tissues were grouped according to the status of lung metastases at the time of resection (presented with radiographic evidence of the presence or absence of pulmonary metastasis). Both CD68 and COX-2 were significantly higher in OS tissues with detectable metastasis (Fig. 1B). We further examined CD68, COX-2, MMP-9, CD163, and iNOS levels in human primary OS tissue and corresponding lung metastases (n = 18 pairs). TAMs infiltration into the lung metastases was significantly higher than that into the primary lesions, and the expression of COX-2 was more intense (Fig. 1A). Although some evidences indicated that M2 macrophages play an important role in tumor metastasis, no significant difference was detected between the OS primary lesions and metastases in this study (Fig. 1A). iNOS, a biomarker of M1 macrophages, was expressed more strongly in primary lesions, which may be ascribed to the tissue necrosis caused by chemotherapy (Fig. 1A). Some evidences have confirmed that COX-2 can upregulate the expression level of MMP9, and we found similar results in OS metastases and primary lesions (Fig. 1A). The outcomes of IHC showed a significant correlation between pulmonary metastasis with TAMs infiltration and COX-2 expression in OS patients.

3.2. Differentiation of THP-1 and human peripheral blood mononuclear cells into macrophages

THP-1 cells and PBMCs were isolated and induced according to the procedure mentioned in the Materials and Methods section. The expression of the recognized macrophage markers CD86 and CD68 was analyzed by flow cytometry to confirm monocyte-to-macrophage differentiation (Supplementary Fig. 1A and B). Macrophage polarization into classical (M1) and alternative (M2) macrophages were studied by flow cytometry and qRT-PCR to detect the expression of CD80 and HLA-DR for M1 and CD206 and CD163 for M2 (Supplementary Fig. 1 A-C).

3.3. TAMs increase the migration and invasion of osteosarcoma cells in vitro

Both the THP-1- and PBMC-derived macrophages could promote the migration of MG63 and KHOS cells in vitro (Fig. 2). The THP-1-derived M0 and M2 macrophages promoted the migration of MG63 and KHOS cells more significantly than the M1 macrophages, and the same results were found in the corresponding matrigel invasion experiment (Fig. 2A, C). PBMC-derived macrophages promoted the migration and invasion of OS cells, and there was no significant difference between the different polarizations (Fig. 2 B, D).

Epithelial-to-mesenchymal transition-activating transcription factors (EMT-TFs) are considered to be closely related to tumor migration and invasion. Even partially activated EMT-TFs can promote tumor cell motility, thereby favoring invasion and dissemination. With Western blot, we found that macrophage-conditioned medium could invoke changes in EMT-TFs and other EMT markers in OS cells (Fig. 2E and F). One of the most important epithelial markers, E-cadherin, was downregulated in MG63 and KHOS cells co-cultured with CM from THP-1- and PBMC-derived macrophages. Mesenchymal markers, such as N-cadherin and vimentin, were also upregulated in MG63 and KHOS cells by the macrophage CM. Moreover, the expression levels of EMT-TFs, such as ZEB-1 and SNAIL, were also upregulated significantly in the CM co-cultured group (Fig. 3E and F). We further observed the upregulation of MMP9 in OS cells co-cultured with macrophages. These in vitro results were consistent with the outcomes of the IHC study. These data demonstrate that TAMs increase the migration, invasion and EMT transition of OS cells.

3.4. Macrophages promote COX-2 expression and phosphorylation of STAT3 in OS cells

We identified differences in gene expression by analyzing the RNA extracted from KHOS cells co-cultured in THP-1-derived macrophage CM and compared the gene expression patterns to those of control KHOS cells. A total of 3823 differentially expressed genes (DEGs, upregulated: 1716; downregulated: 2107) were found by transcriptome analysis (Fig. 3A). The 3823 DEGs were classified according to gene ontology (GO) analysis and Gene Set Enrichment Analysis (GSEA) analysis (Fig. 3B–C). As expected, nearly 161 DGEs were associated with the regulation of cell metastasis, including matrix metallopeptidase (MMP) 9, MMP2, MMP14, and prostaglandin G/H synthase (2PTGS2, alias: COX-2) (Fig. 3D). The transcriptome sequencing results were validated by Western blot. Compared with the control group, COX-2 and MMP9 protein levels were upregulated in KHOS and MG63 cells cultured with TAMs CM (Fig. 3E). We also found evidence that increased phosphorylation of Signal Transducers and Activators of Transcription 3 (p-STAT3) occurred in OS cells co-cultured with TAMs (Fig. 3E). STAT protein phosphorylation leads to STAT dimerization and translocation into the nucleus, where the STAT dimers can activate or repress transcription. Given that tyrosine phosphorylation of STAT3 is a common observation in a variety of solid tumors, we hypothesized that the STAT3 pathway plays an important role in the promotion of tumor metastasis and invasion by TAMs.

3.5. By blocking STAT3, COX-2 inhibition can reverse the TAM-induced migration and invasion in OS cells

A specific siRNA to COX-2 and the highly selective COX-2 inhibitor celecoxib were used to inhibit the expression of COX-2 in OS cells after co-culture with macrophage CM (Fig. 4C). The outcomes of the wound healing and Transwell assays with or without matrigel indicated that

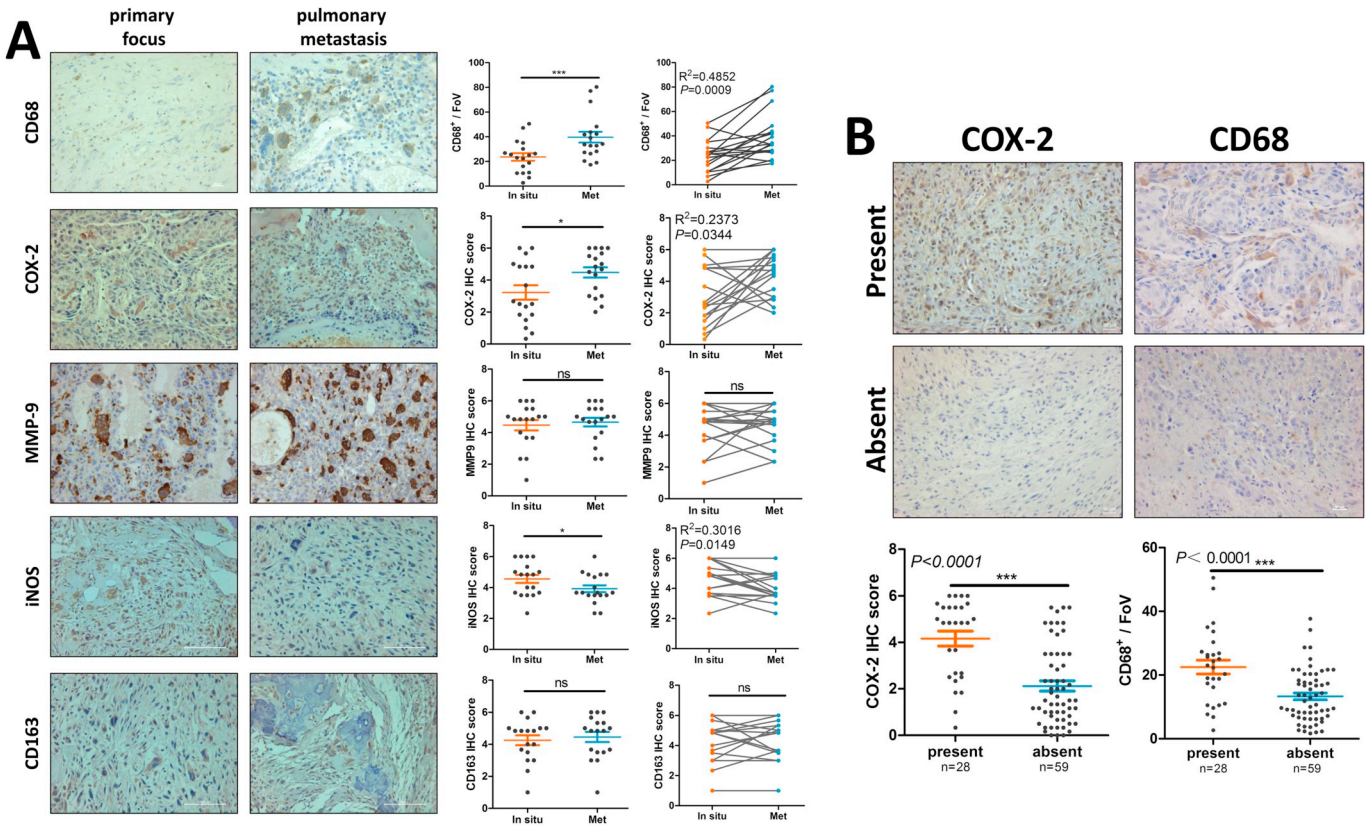

Fig. 1. Tumor-associated macrophages and COX-2 expression are closely associated with osteosarcoma metastasis. (A) Representative images of Immunostaining of paired specimens of OS lung metastases and primary lesions were shown at 200 × magnification. The IHC results indicated that significantly higher levels of COX2, CD68 are expressed in pulmonary metastasis focuses. While the M1 macrophages biomarker, iNOS, was expressed relatively higher in the primary tumor. There was no significant difference in the expression levels of MMP9 and CD163 between lung metastases and primary lesions. **(B)** Compared with those without lung metastases, OS patients with radiographic evidences of lung metastasis at the time of diagnosis had higher expression levels of COX2 and CD68 in the primary tumor.

suppressing COX-2 expression in the co-culture models reduced the migration and invasion of KHOS and MG63 cells (Fig. 4A–B). Furthermore, COX-2 inhibition led to less phosphorylated STAT3 and STAT3 in OS cells (Fig. 4C). The application of specific siRNA to STAT3 could also significantly reverse the TAM-induced OS cell migration and invasion (Fig. 4A–B). In addition, COX-2 inhibition induced downregulation of MMP9 and EMT-TFs, such as ZEB1 and SNAIL (Fig. 4D). Therefore, the mechanism by which TAMs promote OS cell metastasis and invasion is to increase the expression of COX-2 in OS cells, which then leads to the activation of the STAT3 pathway to cause upregulation of downstream EMT-TFs and MMP9.

3.6. Aspirin, a cox-2 inhibitor, can significantly lower the risk of pulmonary metastasis in vivo

We first measured the basal expression of COX-2 in different OS cell lines by Western blot (Fig. 5A). The 143B cell line (with high COX-2 expression) and the KHOS cell line (with low COX-2 expression) were selected for in vivo study. Lung metastases in the 143B group presented with larger volumes and greater numbers than those in the KHOS group (Fig. 5B and C). We found that COX-2 and MMP9, which had low expression in KHOS cells in vitro, were significantly increased in metastases but not in primary lesions in the in vivo experiment (Fig. 5E). Furthermore, IHC outcomes showed a massive infiltration of F4/80 + cells around the metastases of both groups. Consistent with the results of the in vitro study, elevated COX-2 was correlated with the occurrence of lung metastases. At the same time, the phosphorylation of STAT3 and the expression of MMP-9 were also significantly increased in metastases (Fig. 5D–E).

Aspirin blocks both COX-1 and COX-2 and can be administered to mice in drinking water. Regular use of aspirin induced a significant reduction in the amount and volume of lung metastases in both groups (Fig. 5B–C). In addition, the levels of COX-2, MMP9 and phosphorylated STAT3 were lowered by aspirin (Fig. 5D–E). These results suggested that COX-2 plays an important role in OS cell metastasis. By blocking the phosphorylation of STAT3, COX-2 inhibitors can downregulate MMP9, thereby reducing OS cell metastasis.

4. Discussion

In the present study, we observed that OS patients with detectable metastasis at diagnosis have more TAMs in the primary site. Further experiments showed that there were more TAMs in OS lung metastases than in the corresponding primary lesions. These results indicated that TAMs contribute to metastasis and invasion in OS patients; however, some previous studies have reached different conclusions. A study by Buddingh et al. demonstrated that TAMs were related to metastasis suppression in high-grade OS [28]. Compared with patients who developed metastases during follow-up, the expression of TAM-related genes was upregulated in OS biopsies of patients without detectable metastases. Another immunohistochemistry analysis of 124 diagnostic biopsies revealed that high CD163 levels (a marker of M2 macrophages) were correlated with longer metastasis progression-free survival (MPFS), and CD68 had the same trend [29]. This difference can be attributed to the following factors. The specimens in this study were surgically resected after chemotherapy, while the other studied diagnostic biopsies were without chemotherapy. In addition, TAMs are a well-known double agent in the tumor microenvironment [30]. TAMs

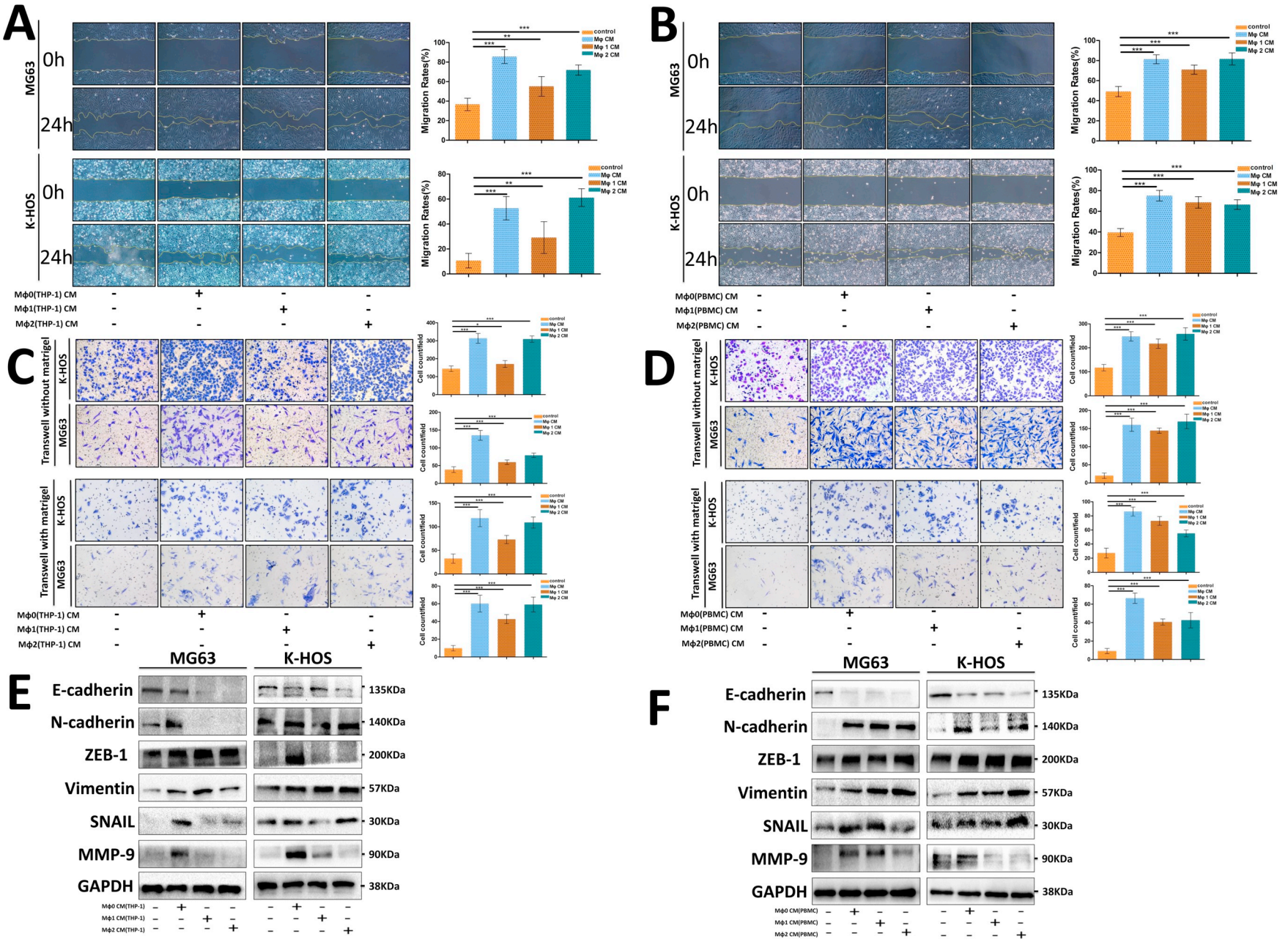

Fig. 2. TAMs increase the migration and invasion of osteosarcoma cells in vitro. (A, B) Wound healing assays of MG63 and KHOS cells. Co-cultured with THP-1- or PBMCs-derived macrophages conditioned medium (CM) for 48 h, the migration of MG63 and KHOS cells was significantly increased. The representative images were shown at magnification of 100 × . **(C, D)** Transwell assays with or without matrigel were used to analyzed the differences between the macrophages CM effects on migration and invasion of MG63 and KHOS cells. Migrated cells were stained and counted, and the average cell number is compared between groups (right panel). The representative images were shown at magnification of 100 × . **(E, F)** Epithelial to mesenchymal transition activating transcription factors and markers expression levels in MG63 and KHOS cells after co-culture with different CMs for 48 h.

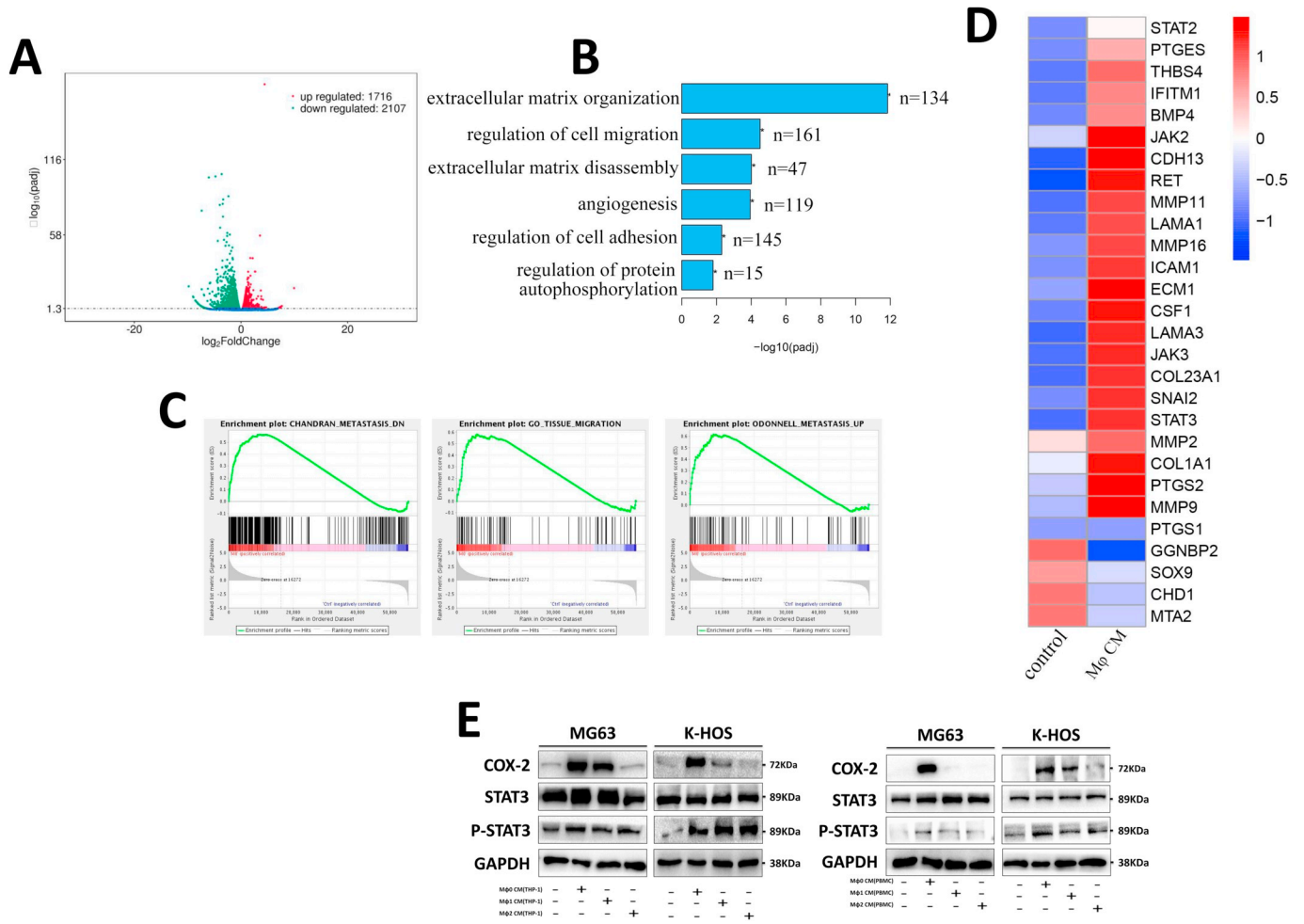

Fig. 3. Macrophages promote COX2 expression and phosphorylation of STAT3 in OS cells. (A) Volcano-plot showing fold changes for genes differentially expressed between KHOS cells co-cultured with macrophages CM for 48 h and negative control. **(B)** GO enrichment analysis of DEGs demonstrated those candidates have a strong relationship with cell migration. **(C)** Gene set enrichment analysis (GSEA) validated enrichment of 'tumor metastasis' after co-cultured with THP-1-derived macrophages CM. (NES, normalized enrichment score). **(D)** A heatmap showing changes in representative cell migration related genes, including PTGS2 (encoding COX2), and pathways (STAT3). **(E)** By western blot, it was found that both THP-1- and PBMCs-derived macrophages CM can promote the expression of COX2 in MG63 and KHOS cells, as well as phosphorylate STAT3.

can have both supportive and inhibitory influences on tumors [31,32]. As the polarization and infiltration of TAMs dynamically change in tumors, the current studies of TAMs can hardly present the real story in tumor microenvironments, which might lead to inconsistent results.

To explore the pro-metastatic function of TAMs more specifically, we investigated the TAMs in paired metastases and their primary sites. In addition, we confirmed that TAMs promoted the migration and invasion of OS cells in vitro. Consistently, Zhou et al. reported that specific depletion of macrophages using clodronate-encapsulated liposomes reduces the number of OS lung metastases of mice transplanted with K7M2 cells [33]. The neutralizing antibody to tumor-induced C—C chemokine ligand 2 (CCL2), an essential mediator of TAM recruitment, has been confirmed to prevent TAMs infiltration in primary lesions and to reduce the lung metastasis of human breast cancer cells [34,35]. Of note, the direct contact of macrophages, endothelial cells, and tumor cells has been described as a tumor microenvironment of metastasis (TMEM), which can predict the risk of metastatic potential in primary human breast cancers [36]. Accumulative evidence indicates that TAMs enhance tumor cell migration and invasion, while the underlying mechanism remains unclear. Some studies have found that TAMs can directly promote tumor cell migration and invasion in the process of tumor cell intravasation into circulation. Furthermore, TAMs also secrete several other molecules, such as osteonectin [37,38], cathepsin proteases [39,40] and TGF-β [41,42], to stimulate tumor cell migration and invasion. In this study, we not only report that TAMs are capable of promoting OS cell migration and invasion but also confirm their regulatory functions in the expression of COX-2 and EMT-TFs in OS cells and a tumor-bearing mouse model.

This study demonstrated that TAMs increase OS cell migration and invasion via the activation of COX-2 in tumor cells. Furthermore, COX-2 can promote EMT-TFs or upregulate downstream MMP9 through activation of the STAT3 pathway. These findings were not only obtained in vitro but also confirmed by animal experiments and patient tissue analysis. Emerging evidence has indicated that COX-2 is an important inflammatory factor for the progression of various tumors. In a study on human breast cancer, C. Bocca et al. reported that COX-2 overexpression in MCF-7 cells was accompanied by characteristic EMT-related changes, augmented secretion of MMPs, and overall invasive capability [43]. Moreover, COX-2 inhibition apparently restored the epithelial phenotype in MCF-7 cells and inhibited tumor invasion. Another study reported that TAMs enhanced the aggressiveness of HCC1954 cells via IL-1β-dependent upregulation of COX-2 in breast cancer [43]. Moreover, the same conclusions have been reached in studies on pancreatic cancer [44], gastric cancer [45], and nasopharyngeal carcinoma [46]. Similarly, this study found that OS cells acquire greater metastatic and invasive capabilities following co-culture with

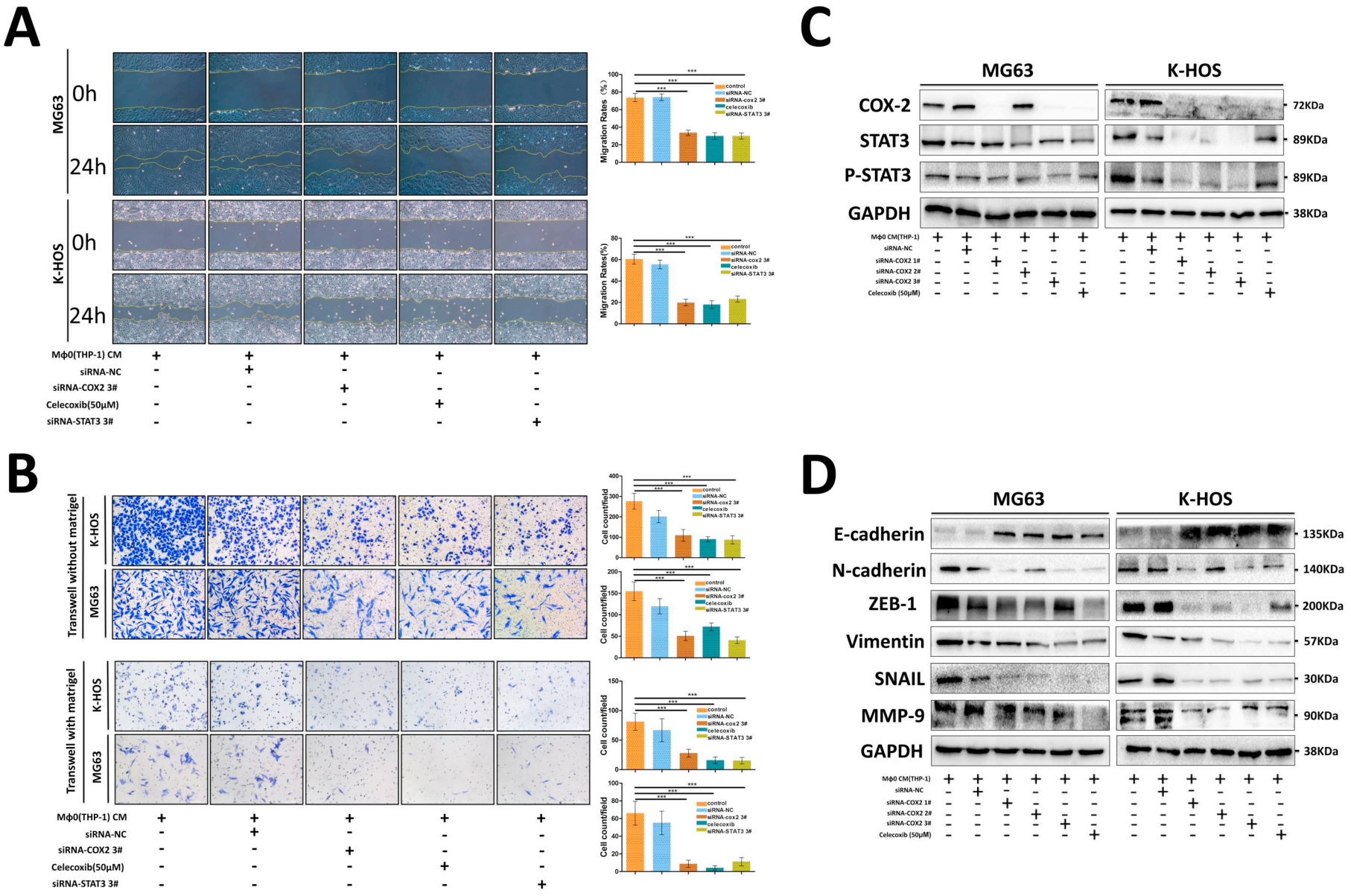

Fig. 4. By blocking STAT3, COX2 inhibition can reverse the TAM-induced migration and invasion in OS cells. **(A)** Wound healing assays indicated the promoting effect of TAMs on the migration of KHOS and MG63 cells was attenuated by treatments of COX2-siRNA, STAT3-siRNA, and Celecoxib (50 μM). **(B)** Transwell assays with or without matrigel demonstrated that COX2-siRNA, STAT3-siRNA, and Celecoxib (50 μM) inhibit the migration and invasion of MG63 and KHOS cells in co-culture with macrophages CM. Migrated cells were stained and counted, and the average cell number is compared between groups (right panel). The representative images were shown at magnification of 100 × . **(C)** Treatment with COX2-siRNA or Celecoxib could block the STAT3 pathway and inhibited STAT3 phosphorylation in MG63 and KHOS cells. **(D)** Inhibition of COX2 reversed the macrophages-induced EMT process in OS cells, and inhibited the expression of EMT-TFs and MMP9.

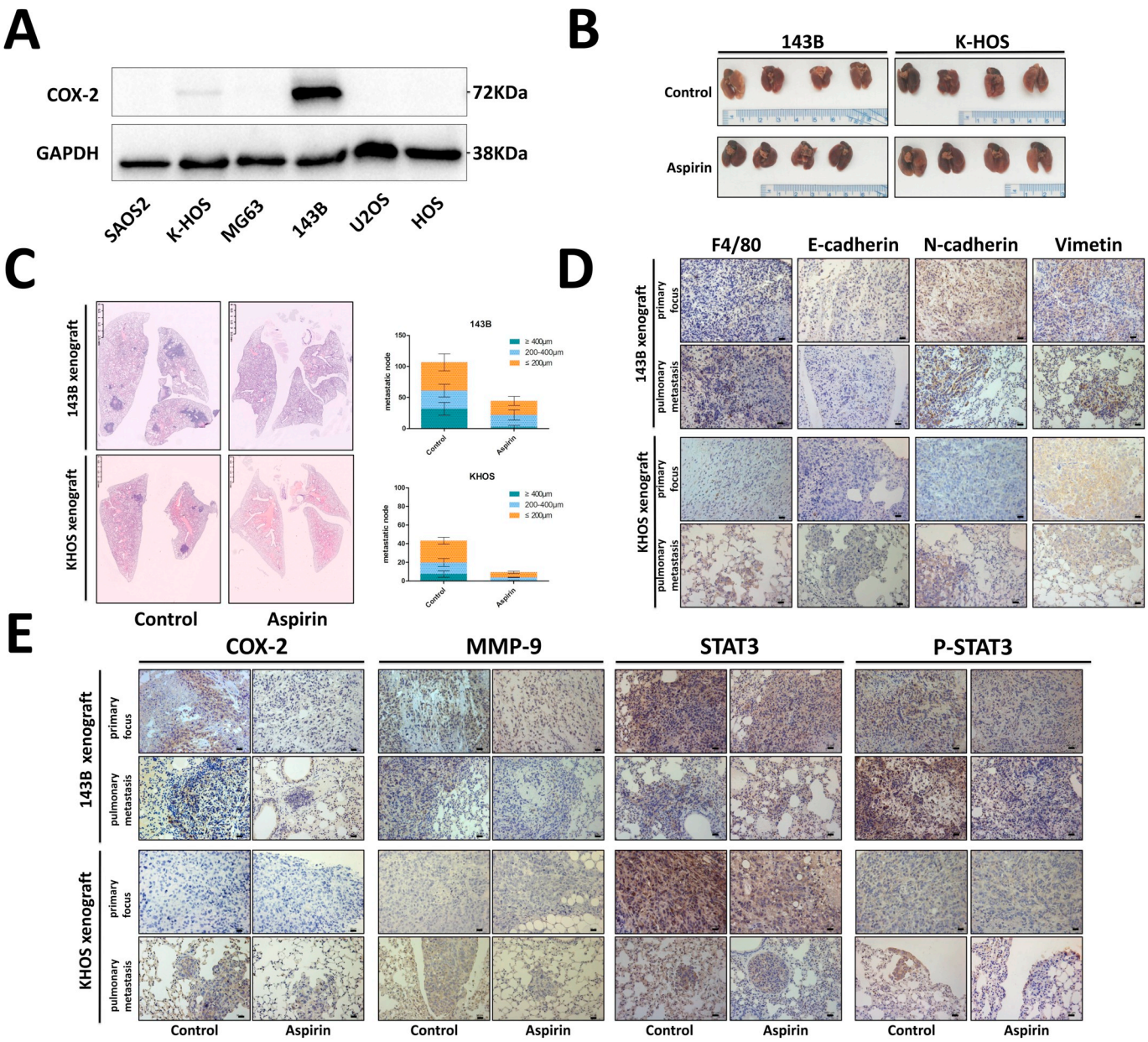

Fig. 5. Aspirin, a cox-2 inhibitor, can significantly lower the risk of pulmonary metastasis in vivo. **(A)** The basal expression of COX2 in different osteosarcoma cell lines was detected by western blot. **(B)** Representative pictures of lungs of Aspirin treated and control group. **(C)** Different size lung metastases (large: ≥ 400 μm; medium: 200–400 μm; small: < 200 μm, right penal) were counted according to the results of H&E staining (left penal). **(D)** IHC results indicated the differences in the expression of EMT markers (E-cadherin, N-cadherin, and Vimentin) and macrophage infiltration (F4/80 positive cell) between orthotopic lesions and corresponding lung metastases. Representative images were shown at 200 × magnification. Scale bars, 50 μm **(E)** Effects of Aspirin on COX2, MMP9, and STAT3 Pathway in Orthotopic Foci and Metastases. Representative images were shown at 200 × magnification. Scale bars, 50 μm.

TAMs, and this effect can be ascribed to the up-regulation of COX-2. In addition, OS cell lines with high basal expression of COX-2, such as 143b cells, exhibit stronger invasiveness both in vitro and in vivo. Finally, COX-2-induced invasiveness, EMT-related changes, and MMPS release can be reversed by the administration of COX-2 inhibitors or specific siRNA.

The activation of STAT3 is a recognized pathway for tumor metastasis and EMT. A variety of molecules have been reported to promote tumor cell invasion and metastasis by regulating the activity of the STAT3 pathway, which increases the expression of EMT-TFs, such as zeb-1, snail, and twist-1 [47,48]. A small-molecule inhibitor targeting STAT3, InS3-54A18, has been confirmed to effectively inhibit tumor growth and metastasis in a lung cancer xenograft model [49]. Consistently, we found that STAT3 knockdown with specific siRNA can inhibit the TAM-induced migration and invasion of OS cells.

Although COX-2 inhibitors (aspirin, celecoxib) have been used primarily as anti-inflammatory drugs, the results from numerous clinical trials suggest that COX-2 inhibitors also possess antineoplastic effects through different mechanisms [50,51]. In a randomized breast cancer phase-II clinical trial, Shaashua et al. [51] reported that perioperative administration of a COX-2 inhibitor induces changes in genes associated with tumor metastasis, reduces EMT, inhibits STAT3 transcription and reduces TAM infiltration. Additionally, metformin was reported to have the same anti-metastasis effect by repressing the COX-2/PGE2/STAT3 axis [52]. In this study, we demonstrated that COX-2 inhibitors (aspirin, celecoxib) are capable of preventing OS cell migration and invasion in vitro and in vivo. In addition, we revealed the regulatory roles of COX-2 inhibition in the expression of EMT-TFs and the STAT3 pathway. Taken

together, these findings indicate that TAMs and COX-2 are vital to tumor metastasis and suggest that they are potential therapeutic targets for preventing OS metastases.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.011.

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