Cellular Signalling 63 (2019) 109363

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Platelet-secreted CCL3 and its receptor CCR5 promote invasive and migratory abilities of anaplastic thyroid carcinoma cells via MMP-1

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ARTICLE INFO

Keywords:
Anaplastic thyroid carcinoma
Platelet
CCL3
CCR5
MMP-1

ABSTRACT

Platelet counts have been reported to be closely related to distant metastasis of many malignant tumors. Our previous study showed that elevated peripheral blood platelet counts may be an adverse prognostic factor of anaplastic thyroid carcinoma (ATC) patients, indicating that platelets may promote ATC progression. In the present study, we aimed to identify the role of platelets in ATC cell invasion and migration and to explore the underlying mechanisms. We found that platelets can promote the invasive and migratory of ATC cells, which may be related to the interaction between activated platelet-secreted chemokine (C-C motif) ligand 3 (CCL3) and its receptor CCR5. The interaction was shown to induce the upregulation of matrix metalloproteinase (MMP)-1 via NF-κB pathway. These findings could provide a new idea for the research of targeted platelets to inhibit tumor metastasis.

1. Introduction

Thyroid cancers include papillary, follicular, medullary and anaplastic carcinomas. Anaplastic thyroid carcinoma (ATC) is one of the most aggressive malignant tumors in humans, accounting for 1.6–5% of all thyroid cancers, and is characterized by high levels of rapid proliferation, local invasion and distant metastases. In addition, it is the major cause of all thyroid carcinoma-related deaths [1–4]. Moreover, it has been reported that the high lethality of ATC is closely related to high distant metastases rate [1,3]. ATC is resistant to conventional treatments, including surgery, chemotherapy and isotopic interventions [3–5]. Therefore, it is quite vital and urgent to uncover the potential mechanisms underlying ATC metastasis and to identify new therapeutic targets.

In 1878, Billroth accidentally discovered that tumor metastasis was accompanied by platelet thrombosis, suggesting that metastases originate from tumor cell-platelet emboli formed by thrombus disintegration [6]. Subsequently, an increasing number of researchers confirmed that platelets promote tumor metastasis, while reduced platelet counts or weakened function can significantly inhibit tumor metastasis [7–14]. Our previous study also found that elevated peripheral platelet counts are an adverse prognostic factor of ATC patients, and elevated platelet counts are associated with distant metastases in patients with ATC [1]. However, the mechanism of platelet involvement in tumor metastasis has not been studied clearly.

Platelets are anucleate and the vesicle-like, whose average diameter is 2 to 5 μm and are shed from the cytoplasm of megakaryocytes in the bone marrow [15,16]. Platelets also contain three types of granules: α granules, dense granules and lysosomal granules, which synthesize and secrete various proteins and compounds that are necessary for platelet functions [15–17]. It has been long established that thrombosis and hemostasis are common functions of platelets [15,17]. However, recent discoveries have found that platelets also play an important role in other diverse pathophysiological processes, such as inflammation, immunity, atherosclerosis, cancer progression and cancer metastasis [17–20].

Studies have demonstrated that tumor cells can induce platelet aggregation and activation [21,22]. As a result, activated platelets can adhere to tumor cells to form platelet-tumor loops, which protect tumor cells from the body's immune system and increase the survival rate of tumor cells [21–23]. In addition, activated platelets can also secrete various growth factors, cytokines and proteases to promote tumor cell

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https://doi.org/10.1016/j.cellsig.2019.109363
Received 18 January 2019; Received in revised form 19 July 2019; Accepted 19 July 2019
Available online 22 July 2019

growth, invasion and metastasis [15–18,23].

Chemokine (C-C motif) ligand 3 (CCL3), also known as macrophage inflammatory protein-1α (MIP-1α), is a pro-inflammatory cytokine and a ligand for CCR1 and CCR5, which stimulates chemotactic activities in many immune cells such as monocytes, lymphocytes, macrophages and natural killer cells [24]. CCL3 also contributes to tumor growth, angiogenesis and metastasis in a variety of malignant tumors, such as renal cell carcinoma [25], melanoma [26], colorectal cancer [27] and chondrosarcoma [28].

In the present study, we found that activated platelets can secrete the chemokine CCL3, which binds its receptor CCR5 to upregulate matrix metalloproteinase (MMP)-1 via NF-κB pathway and finally promotes the invasive and migratory abilities of ATC cells. This discovery could lead to the identification of possible drug targets in ATC progression.

2. Materials and methods

2.1. Cell lines and cell culture

Human ATC cell line SW579 and human embryonic kidney cell line 293 T were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human ATC cell line ARO was provided by George G. Chen (The Chinese University of Hong Kong, Hong Kong SAR, China). The SW579 and 293 T cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and ARO cells were cultured in RPMI-1640 supplemented with 5% fetal bovine serum (FBS, Gibco, USA). All cell lines were cultured with penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in a humidified 5% CO_2 incubator.

2.2. Reagents and antibodies

Recombinant human CCL3, CCL4, CCL5, CXCL7, IL-8, TIMP-1 and EGF were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). A C1000 Human Cytokine Antibody Array was purchased from RayBiotech, Inc. (Norcross, GA, USA). Anti-CCR1, CCR5, MMP-1, MMP-2, MMP-3 and MMP-10 antibodies were purchased from Abcam (Cambridge, MA, USA). Mouse anti-beta-actin was obtained from Cell Signaling Technology (Beverly, MA, USA). Goat anti-mouse and goat anti-rabbit peroxidase-conjugated secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL, USA). APC Mouse Anti-CCR1 and CCR5 were purchased from BD biosciences (San Jose, CA, USA). Human CCL3 Quantikine ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA). Cell MMP-1 in situ zymography fluorescence staining kit was obtained from Genmed Scientifics Inc. (Shanghai, China). Unless otherwise indicated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Platelet isolation

Whole blood samples were collected from healthy volunteers. A citric acid-citrate-dextrose solution (85 mM trisodium citrate, 67 mM citric acid and 111.5 mM glucose, pH 4.5; 1:6 blood vol/vol) was added for anti-coagulation. Blood was centrifuged at 180*g* at room temperature for 15 min to obtain platelet-rich plasma (PRP), which was centrifuged at 1500*g* for 10 min to obtain platelet pellets. The platelets were washed twice in CGS buffer (13 mM trisodium citrate, 30 mM dextrose and 120 mM NaCl, pH 7.0) in the presence of 1 μg/ml PGE_1. Washed platelets were resuspended at a concentration of 2×10^8 cells/ml in Tyrode's buffer (138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) containing 0.2% BSA.

2.4. Flow cytometry

Flow cytometry was used to detect whether thrombin could activate platelets by analyzing the expression of CD62P (P-selectin). First, platelets were incubated with 1 U/ml thrombin (Sigma-Aldrich) for 15 min at 37 °C. Next, platelets were incubated with APC mouse anti-human CD62P (P-selectin) antibody at room temperature for 15 min. Then, the platelets were centrifuged and washed. CD62P (P-selectin) expression was detected using a Moflo-XDP flow cytometer (Beckman-Coulter).

2.5. Human cytokine antibody array

A total of 3×10^5 ARO cells per well were plated in 12-well plates and incubated overnight. A total of 2×10^8 activated platelets per ml were added to cells for coculturing for 24 h. Cell supernatants were collected after 24 h and were analyzed with a human cytokine antibody array according to the manufacturer's instructions. Briefly, membranes were blocked with blocking buffer for 30 min at room temperature and incubated with 1 ml of the supernatants overnight at 4 °C. After washing, the biotinylated antibody cocktail was added for incubation overnight at 4 °C. Next, 2 ml HRP-streptavidin was pipetted into each well and incubated for 2 h at room temperature. The results were obtained using XAR films. Chemiluminescence signal intensity was quantified using Quantity One software (Bio-Rad).

2.6. RNA interference

Effective siRNA sequences that specifically target CCR1, CCR5 and MMP-1 were purchased from Guangzhou Ribobio Company (Guangzhou, China). A total of 1×10^5 cells/well were seeded into 12-well plates, incubated overnight and transfected with 50 nM siRNA using Lipofectamine RNAiMax (Invitrogen) the following day according to the manufacturer's instructions. The three siRNA sequences against CCR1, CCR5 and MMP-1 are listed as follows: siCCR1-1#, 5′-AGACGA CCAAATGAGAAGA-3′; 2#, 5′-TCCTCACGAAAGCCTACGA-3′; 3#, 5′-TGACGATTGACAGGTACCT-3′; siCCR5-1#, 5′-CGGGAATCCTAAAA ACTCT-3′; 2#, 5′-CTCTCATTTTCCATACAGT-3′; 3#, 5′-GAGCGAGCAA GCTCAGTTT-3′; siMMP-1-1#, 5′-GCTTGAAGCTGCTTACGAA-3′; 2#, 5′-GGACCATGCCATTGAGAAA-3′; 3#, 5′-GCACATGACTTTCCTG GAA-3′.

2.7. Gene transfection

The generation of CCR5-overexpressing ATC cell lines was based on molecular cloning technology. The retrovirus packaging expression plasmids were cotransfected into 293 T cells. Then, the supernatants containing viruses were collected and filtered using 0.45 μm filters, which were used to infect the ATC cell lines for 48 h. Then, stable clones of these cells were selected with 1 μg/ml puromycin (Sigma-Aldrich, USA) for 3 days after infection. The expression levels of CCR5 were identified by quantitative RT-PCR and western blotting assays.

2.8. Quantitative RT-PCR

Total RNA was isolated from ATC cells using TRIzol Reagent (Sigma-Aldrich, USA). We used M-MLV reverse transcriptase (Promega, USA) to reverse transcribe RNA to cDNA according to the manufacturer's instructions. mRNA expression levels were detected by quantitative RT-PCR (qRT-PCR) using LightCycler 480 SYBR Green I Master (Roche) and analyzed on a CFX96 Touch sequence detection system (Bio-Rad, USA). β-actin was used as an internal control to normalize gene expression levels. The experiments were run in triplicate independently. The primer sequences were as follows:

CCR5 sense 5′-GTTGGACCAAGCTATGCAGGT-3′;

CCR5 anti-sense 5′-GCAGAAGCGTTTGGCAATGT-3′;

MMP-1 sense 5′-GGGAGATCATCGGGACAACTC-3′;

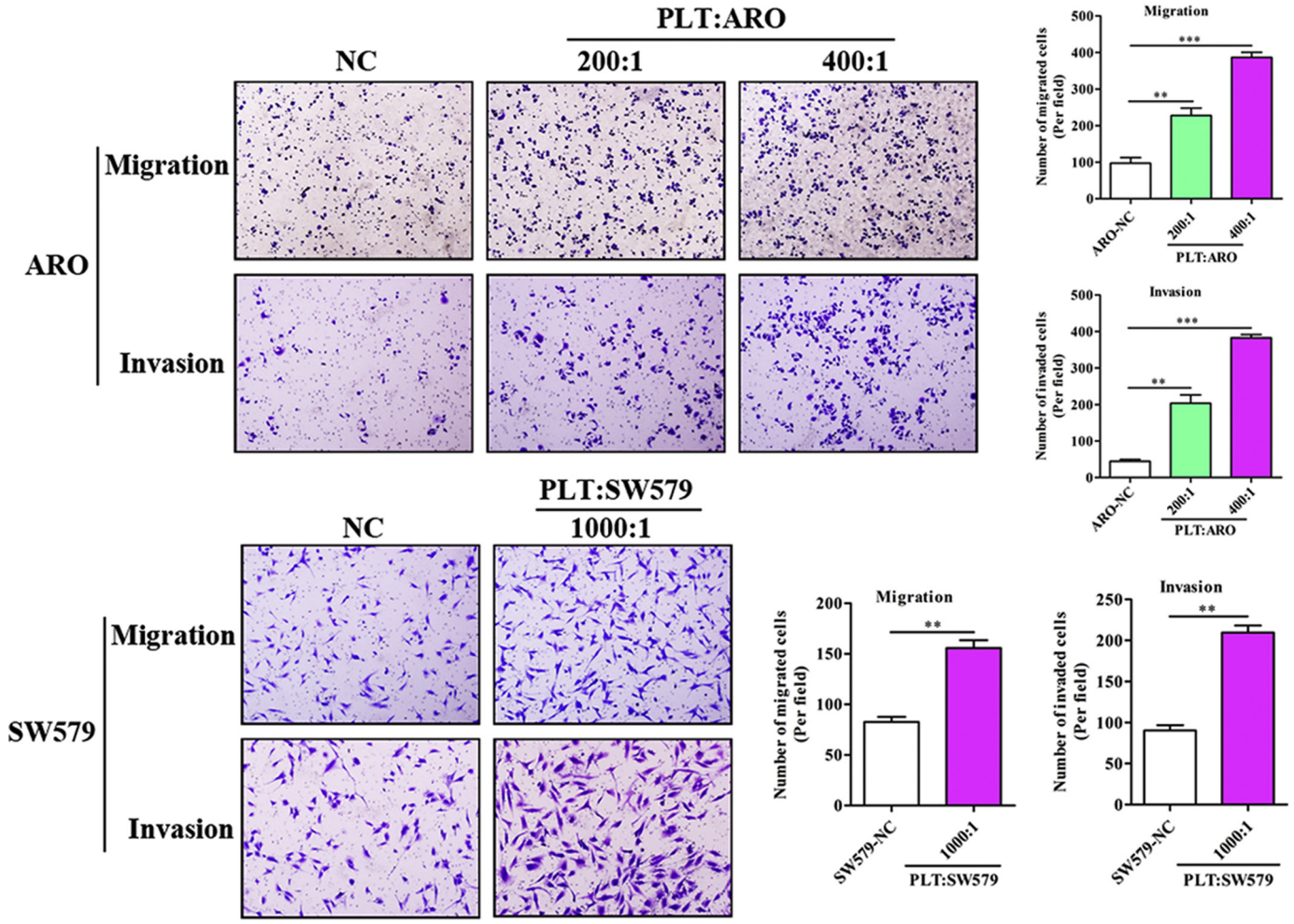

Fig. 1. Platelets promote ATC cell invasion and migration. Representative images of Transwell invasion andmigration assays in ARO and SW579 cells treated either with or without platelets. Histograms show the mean ± SD of the number of migrated or invaded cells from three independent experiments. Photomicrographs were obtained at 100× magnification. *P < 0.05, **P < 0.01 and ***P < 0.001.

MMP-1 anti-sense 5′-GGGCCTGGTTGAAAAGCAT-3′;
β-actin sense 5′-GTGAAGGTGACAGCAGTCGGT-3′; and.
β-actin anti-sense 5′-AAGTGGGGTGGCTTTTAGGAT-3′.

2.9. Western blotting

To obtain cell proteins, cells were lysed in 1× sodium dodecyl sulfate (SDS) sample buffer, and protein concentration was measured by BCA protein assays. Protein extracts were separated on 9–12% SDS-polyacrylamide gels by electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore, USA) and blocked with 5% skim milk or bovine serum albumin for 1 h. Then, the membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation with secondary antibodies at room temperature for 45 min. Next, bound antibodies were visualized via enhanced chemiluminescence and captured by XAR films. In addition, β-actin acted as a loading control for all samples.

2.10. Transwell invasion and migration assays

Transwell 24-well plates and chambers with 8 μm pore sizes (Corning Costar Corp, Cambridge, MA, USA) were used for the invasion and migration assays. For invasion assays, the chambers were precoated with Matrigel (200–300 μg/ml) for 30 min at 37 °C. In brief, 5×10^4 ARO cells or 1×10^4 SW579 cells were suspended in 200 μl of serum-free medium and plated into the upper chambers and were either left untreated or treated with 2×10^8 platelets/ml. The lower chambers contained 600 μl of medium supplemented with 5% FBS. The plates were incubated for 24 h (migration) or 36 h (invasion) at 37 °C in 5% CO_2, and cells were then fixed with methanol for 15 min and stained with 0.5% crystal violet (Sigma-Aldrich) for 30 min. Cells on the upper side of the filters were removed with cotton swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each experiment was repeated at least 3 times independently.

2.11. Enzyme-linked immunosorbent assay (ELISA)

ATC cells were incubated in serum-free basal medium in the presence or absence of platelets. Supernatant was collected and stored at −80 °C until further use. CCL3 secretion in the cell culture supernatants was assayed using a human CCL3 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Total protein in the cells was estimated using the Bradford method.

2.12. Fluorescent in situ zymography

According to the manufacturer's instructions. Briefly, 50 μl of 37 °C preheated GENMED staining solution (Reagent B) was mixed with 400 μl GENMED colloidal liquid (Reagent A). 40 μl of the mixed liquid was added to the cell sample on the unfixed glass slide. Cover the coverslips with mixed liquid (avoid air bubbles). Cells were incubated

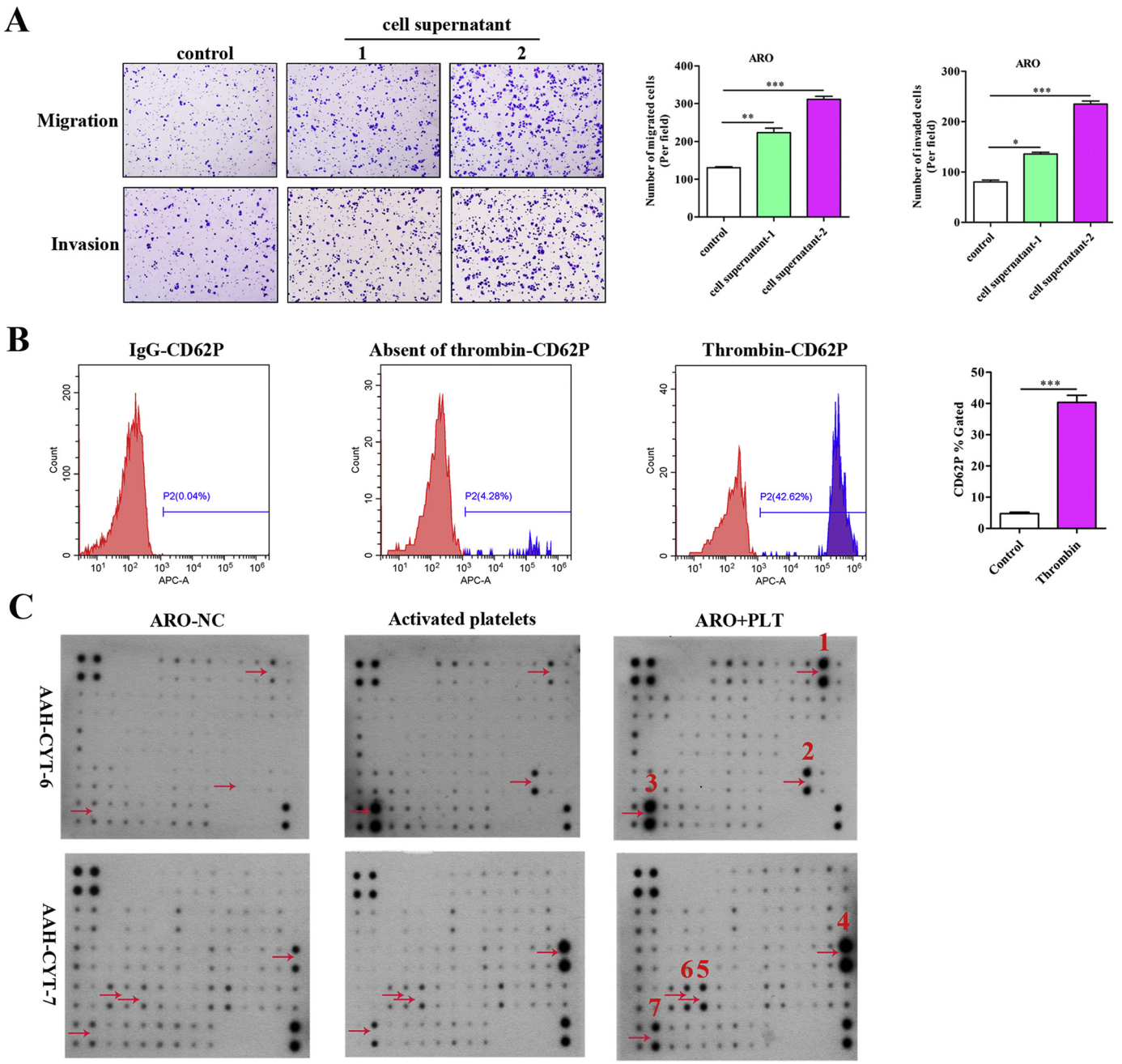

Fig. 2. Activated platelets secrete cytokines to promote ATC cell invasive and migratory abilities. (A) Transwell assays of ARO cells either in the presence or absence of cell supernatants that were cocultured with platelets after 24 and 48 h (marked as cell supernatants 1 and 2, respectively). (B) CD62P (P-selectin) expression of platelets activated by thrombin were analyzed by flow cytometry. (C) A human cytokine antibody array revealed that CCL5, EGF, CXCL7, IL-8, CCL4, CCL3, and TIMP-1 (From 1 to 7 in order) were increased significantly in platelet-treated ARO cells.

in 4 °C for 10 min and avoid light. Next day incubated cells in a 37°C for 60 min and avoid light. Fluorescence was assessed under a fluorescence microscope (the excitation wavelength was 480 nm, and the emission wavelength was 530 nm). Green fluorescence indicates high MMP activity.

2.13. Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Student's *t*-test was used to compare the differences between two independent groups. An ANOVA was performed to compare the differences between 3 or more groups. *P < 0.05, **P < 0.01, ***P < 0.001 were indicative of statistically significant. The results are representative of three independent experiments performed in triplicate and are presented as the mean ± SD.

3. Results

3.1. Platelets promote the invasive and migratory abilities of ATC cells

To identify the role of platelets in the invasion and migration of ATC cells, we first isolated platelets from the whole blood of healthy volunteers as described previously [29]. Then, we attempted different ratios between the number of ATC cells and platelets to perform Transwell invasion and migration assays. The results revealed that platelets can improve the invasive and migratory capacities of ATC

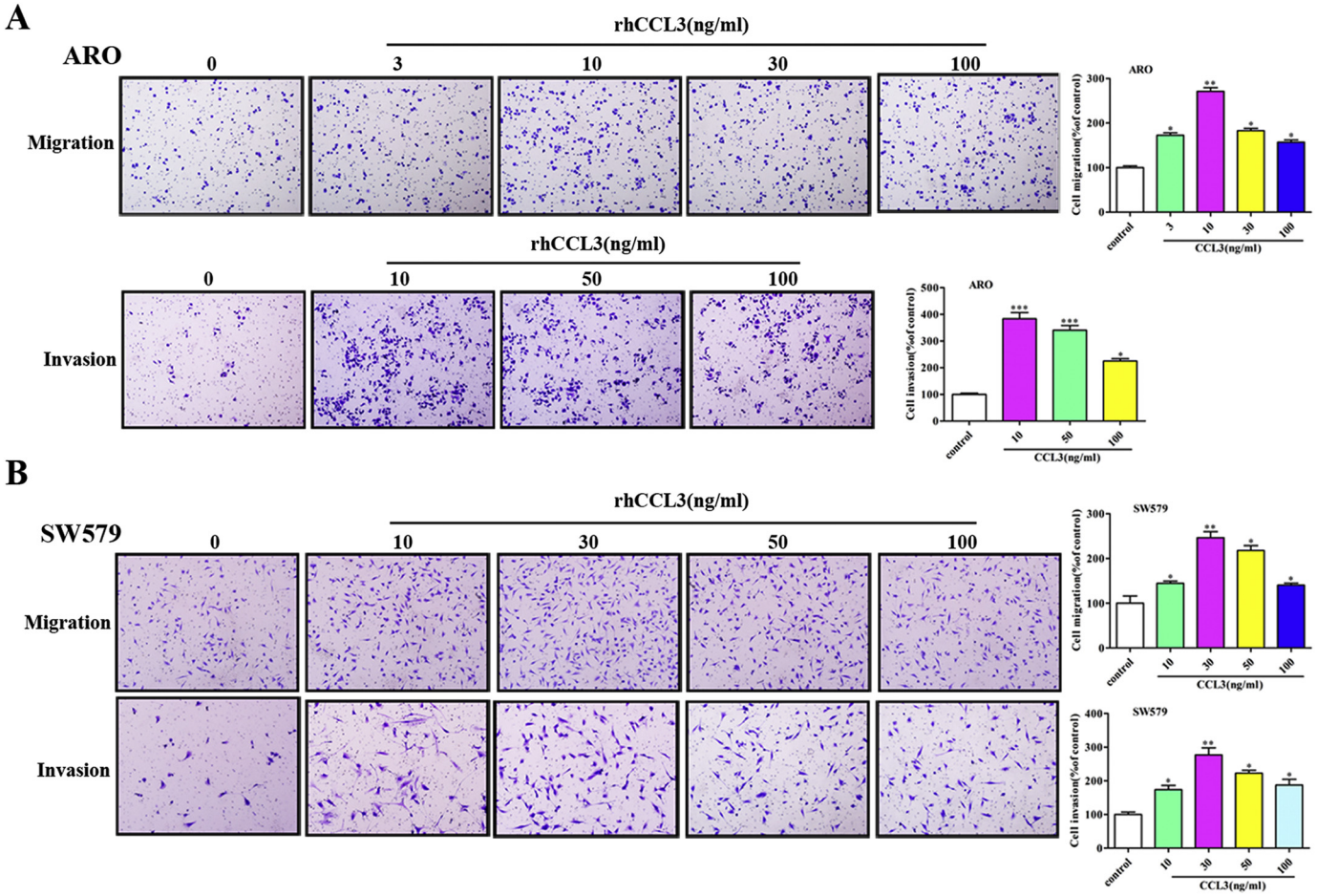

Fig. 3. CCL3 promotes the invasive and migratory potentials of ATC cells. (A), (B) ARO and SW579 cells were incubated with rhCCL3 at increasing concentrations (0–100 ng/ml) for 24 h, and invasive and migratory abilities were measured by Transwell assays. Histograms show the mean ± SD of the number of migrated or invaded cells from three independent experiments. Photomicrographs were obtained at 100× magnification. *P < 0.05, **P < 0.01, ***P < 0.001.

cells. Furthermore, with increasing platelet numbers, the ability of platelets to promote invasion also increased (Fig. 1).

3.2. Activated platelet-secreted CCL3 promotes the invasive and migratory abilities of ATC cells

It has been reported that various growth factors and cytokines secreted by activated platelets are associated with tumor invasion and migration [23]. To verify this result in ATC cells, we collected ARO cell supernatants after coculture with platelets after 24 h and 48 h, and we found that the invasive and migratory capacities were markedly increased in the cocultured ATC cell group compared with the platelet-free control group by Transwell assays (Fig. 2A).

To explore which cytokines are secreted by activated platelets, human cytokine antibody arrays were used. First, flow cytometry was performed to examine CD62P (P-selectin) expression in the platelet membrane surface after thrombin stimulation to detect whether platelets were activated. The results showed that thrombin can activate platelets (Fig. 2B). Then, we collected the supernatants from the platelet-free ARO cells control group, ARO cells cocultured with platelets group and activated platelets group, and then detected the differences in relative protein expression of 120 specific human cytokines by a human cytokine antibody array. We found that the cytokines CCL5, EGF, CXCL7, IL-8, CCL4, CCL3 and TIMP-1 (from 1 to 7 in order) were markedly increased in ARO cells cocultured with platelets compared with the platelet-free ARO cells control group (Fig. 2C). Furthermore, Transwell assays were used to identify whether these cytokines could promote ATC cell invasion and migration. The results showed that only CCL3 and CCL4 can promote the invasive and migratory capacities of ATC cells, and the role of CCL3 was greater than that of CCL4. Furthermore, we found that ARO and SW579 cells had the greatest invasive and migratory abilities in the presence of 10 ng/ml rhCCL3 and 30 ng/ml rhCCL3 (0–100 ng/ml), respectively (Fig. 3A, B and Supplementary Fig. S1). To assess the source of CCL3, we conducted a series of ELISA assays. As seen in Supplementary Fig. S2, CCL3 expression level in cell supernatant of platelets co-culture with ATC cells was higher than that of ATC cells alone, and knockdown CCL3 in ATC cells did not significantly reduce the CCL3 expression level in cell supernatants. These results suggested that CCL3 protein in supernatant of ATC cells co-cultured with platelets was largely derived from activated platelets.

3.3. Knockdown of CCR5 suppresses the ability of CCL3 to promote ATC cell invasion and migration

It has been shown that CCR1 and CCR5 are CCL3 receptors [24]. To verify their roles on ATC cell invasive and migratory abilities, we first examined the expression of CCR1 and CCR5 in ATC cells. The results suggested that both CCR1 and CCR5 were expressed in ATC cells (Fig. 4A, B and Supplementary Fig. S3). Then, we knocked down the expression of CCR1 or CCR5 by siRNA (Fig. 4C). Using Transwell assays, we found that the numbers of migrated cells in CCR5 knockdown cells were significantly decreased compared with CCR5 control cells (Fig. 4D). However, no significant difference was observed in cell migration with or without CCL3 after CCR5 knockdown (Fig. 4E) or

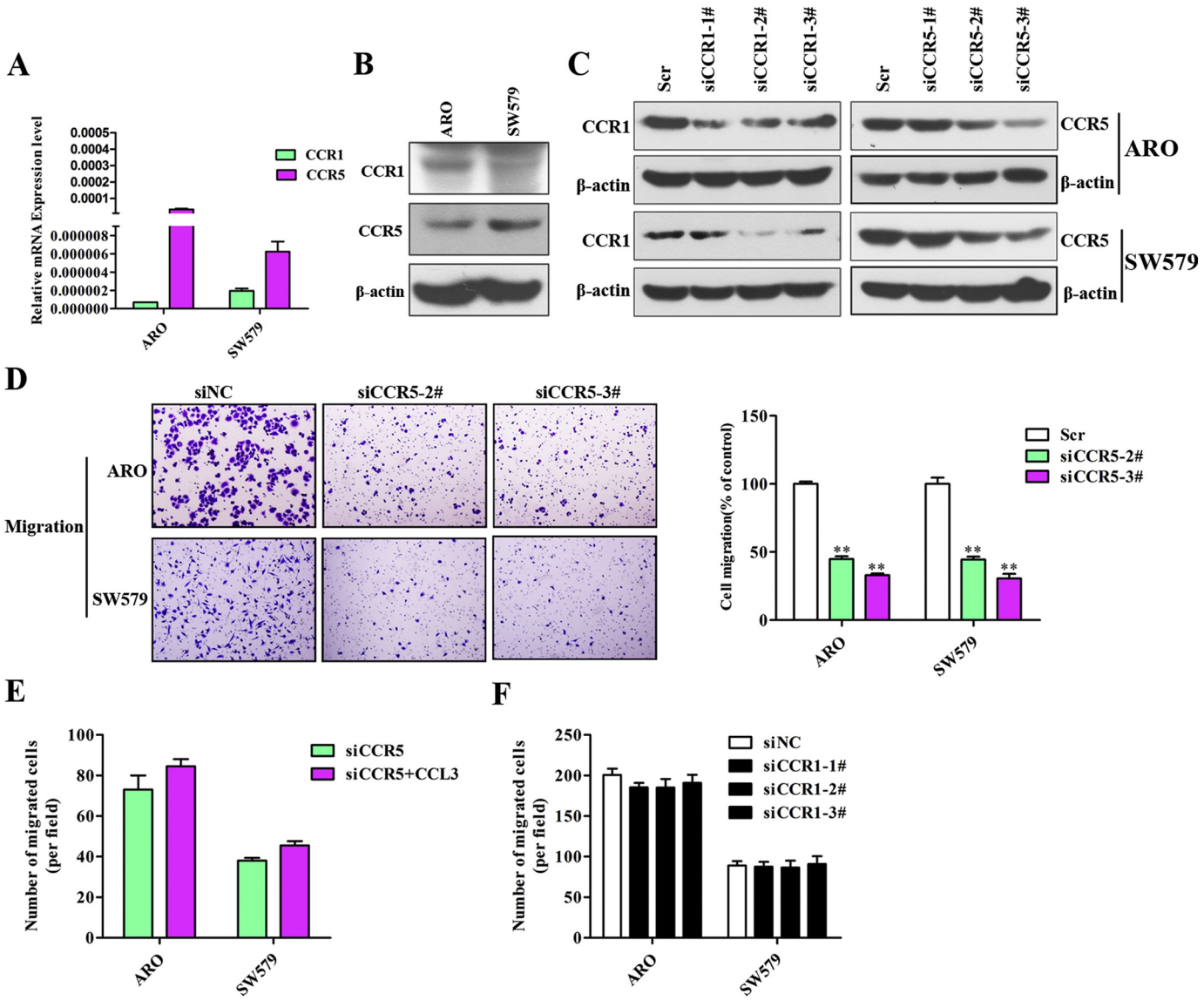

Fig. 4. CCR5 silencing reduced the ability of CCL3 to promote ATC cell migration. CCR1 and CCR5 mRNA (A) and protein (B) expression were measured by qRT-PCR assays and western blotting assays, respectively, in ARO and SW579 cells. (C) Knockdown of CCR1 or CCR5 proteins with specialized siRNAs in ARO and SW579 cells were identified by western blotting. β-actin was used as a loading control. (D) Transwell assays for ATC parental cells and siCCR5 transfected cells showed that siCCR5 significantly inhibited the migration of ATC cells. Photomicrographs were obtained at 100× magnification. *P < 0.05, **P < 0.01, ***P < 0.001. (E) The number of migrated cells did not significantly change in siCCR5 cells with or without CCL3 treatment. (F) Transwell assays showed that knockdown of CCR1 had no significant effect on the migratory abilities of ATC cells.

between control cells and CCR1 knockdown cells (Fig. 4F).

3.4. Overexpression of CCR5 increases the ability of CCL3 to promote ATC cell invasion and migration

To further clarify the role of CCR5 in ATC cells, we generated stable cell lines expressing the CCR5 vector and the control vector (pMSCV-CCR5 and pMSCV-vector, respectively) in ATC cells (Fig. 5A). Using Transwell assays, we found that overexpressed CCR5 significantly enhanced the migratory capability of ATC cells (Fig. 5B). Therefore, we concluded that CCL3 promotes ATC cell invasion and migration by binding with its receptor CCR5, but not CCR1.

3.5. CCL3/CCR5 promotes ATC cell invasion and migration abilities by upregulating MMP-1

MMPs play an important role in cancer metastasis [19]. To detect the effect of CCL3 on MMP expression in ARO cells, we used qRT-PCR to compare mRNA expression levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-14 between control and CCL3-treated cells. As demonstrated in Fig. 6A, the mRNA levels of MMP-1, MMP-2, MMP-3 and MMP-10 were upregulated in the CCL3-treated cells, and the expression levels of MMP-7, MMP-9, and MMP-14 did not change significantly. However, only the MMP-1 protein level was increased, as indicated by western blotting (Fig. 6A and Supplementary Fig. S4). In addition, Fluorescent in situ zymography results showed that rhCCL3 upregulated MMP-1 activity in ATC cells (Supplementary Fig. S5). Furthermore, we found that pretreatment of cells with CCR5 mAb or siCCR5 significantly inhibited CCL3-induced MMP-1 expression. In contrast, overexpression of CCR5 significantly enhanced MMP-1 expression (Fig. 6B).

To examine whether MMP-1 was involved in CCL3-induced cell invasion and migration, Transwell assays were performed. We found that MMP-1 knockdown significantly inhibited CCL3-induced cell

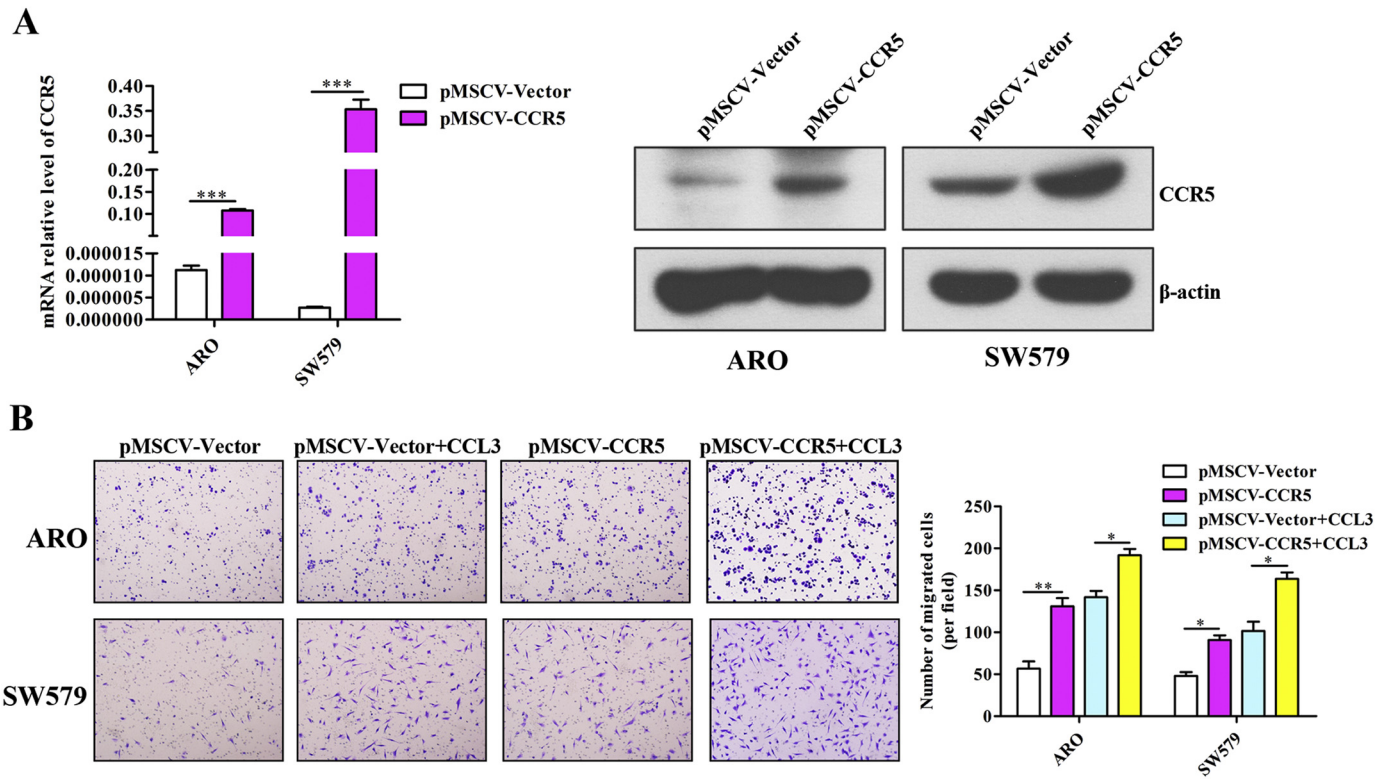

Fig. 5. Overexpression of CCR5 enhances the ability of CCL3 to promote ATC cell migration. (A) CCR5 mRNA and protein expression levels in ATC cells with stable overexpression of CCR5 were measured by qRT-PCR assays and western blotting assays, respectively. (B) Transwell assays showed that stable overexpression of CCR5 in ATC cells had greater migratory ability than vector control cells. Representative and summary images are shown. Photomicrographs were obtained at 100× magnification. *P < 0.05, **P < 0.01, ***P < 0.001.

invasion and migration (Fig. 6C, D, and E). These results suggested that CCL3/CCR5 promotes ATC cell invasive and migratory abilities by upregulating MMP-1 expression.

3.6. CCL3 may activate the NF-κB pathway to upregulate MMP-1

CCL3 has been reported to induce tumor cell invasion and migration through NF-κB activation [28,30]. Similarly, we found that ARO cells treated with CCL3 resulted in NF-κB p65 phosphorylation (Fig. 7A). Furthermore, we found that PDTC, an NF-κB inhibitor, significantly inhibited CCL3-induced MMP-1 expression (Fig. 7B), and CCR5 mAb also significantly reduced CCL3-induced p65 phosphorylation in ARO cells (Fig. 7C).

4. Discussion

ATC is the most malignant thyroid cancer, and its prognosis is extremely poor. Distant metastasis is the leading cause of death in ATC patients [1,2]. Although treatments such as surgery, chemotherapy, radiotherapy, and targeted treatment are increasing development, it is still extremely difficult to control its progress [3–5]. Therefore, uncovering the mechanism of the highly invasive and migratory abilities of ATC in patients is the key to develop new therapies.

In recent decades, some studies have found that platelets can promote tumor metastasis in gastrointestinal, liver, ovarian, cervical, breast and lung cancer patients and that reducing platelet count or inhibiting platelet function can significantly prevent tumor metastasis [7–14]. Our previous clinical study also found that the elevated peripheral platelet count may be an adverse prognostic factor of ATC patients and that platelet counts are associated with distant metastasis of ATC in patients [1]. However, the distinct mechanism remains unclear.

In the present study, for the first time, we revealed that platelets can promote ATC invasion and migration in vitro. Because platelets can secrete various growth factors and cytokines [23,30], we collected cell supernatants from cells cocultured with platelets and found that invasive and migratory capacities were markedly increased compared with the platelet-free control group. Furthermore, we found that EGF, CXCL7, CCL5, IL-8, CCL3, CCL4 and TIMP-1 were significantly increased in platelet-treated ARO cells when compared with the platelet-free control group. However, only CCL3 and CCL4 have been shown to promote ATC cell invasion and migration, and the former is more potent than the latter, which is similar to the results in breast carcinoma reported by Kitamura et al. [31], their studies found that CCL3 promoted metastasis in breast cancer by binding with the CCR1. However, Sasaki et al. [32] reported that CCL3 and its another receptor, CCR5, mediated fibroblast accumulation to induce carcinogenesis. In this study, we found that both CCR1 and CCR5 receptors were present on the surface of ATC cells. SiRNA against CCR5 or CCR5 mAb significantly inhibited CCL3-induced cell invasion and migration, but CCR1-specific siRNA did not play a role. These results indicated that CCL3/CCR5 interactions are responsible for platelet-induced promotion of invasive and migratory abilities in ATC cells.

MMPs are a large family of proteolytic enzymes, which play a key role in cancer invasion and metastasis [33]. In human cancer cells, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-14 have been found to be correlated with many malignant metastases [19,25,28,34,35]. In this study, we found that CCL3 could induce MMP-1 expression and secretion in ATC cells via NF-κB pathway. We also found that CCR5 knockdown inhibited CCL3-induced MMP-1 upregulation. Overall, these results suggested that CCL3/CCR5 increased the invasive and migratory abilities in ATC cells via upregulation of MMP-1.

Despite these findings, there are still several apparent limitations in this study. First, it has been reported that platelets have multiple

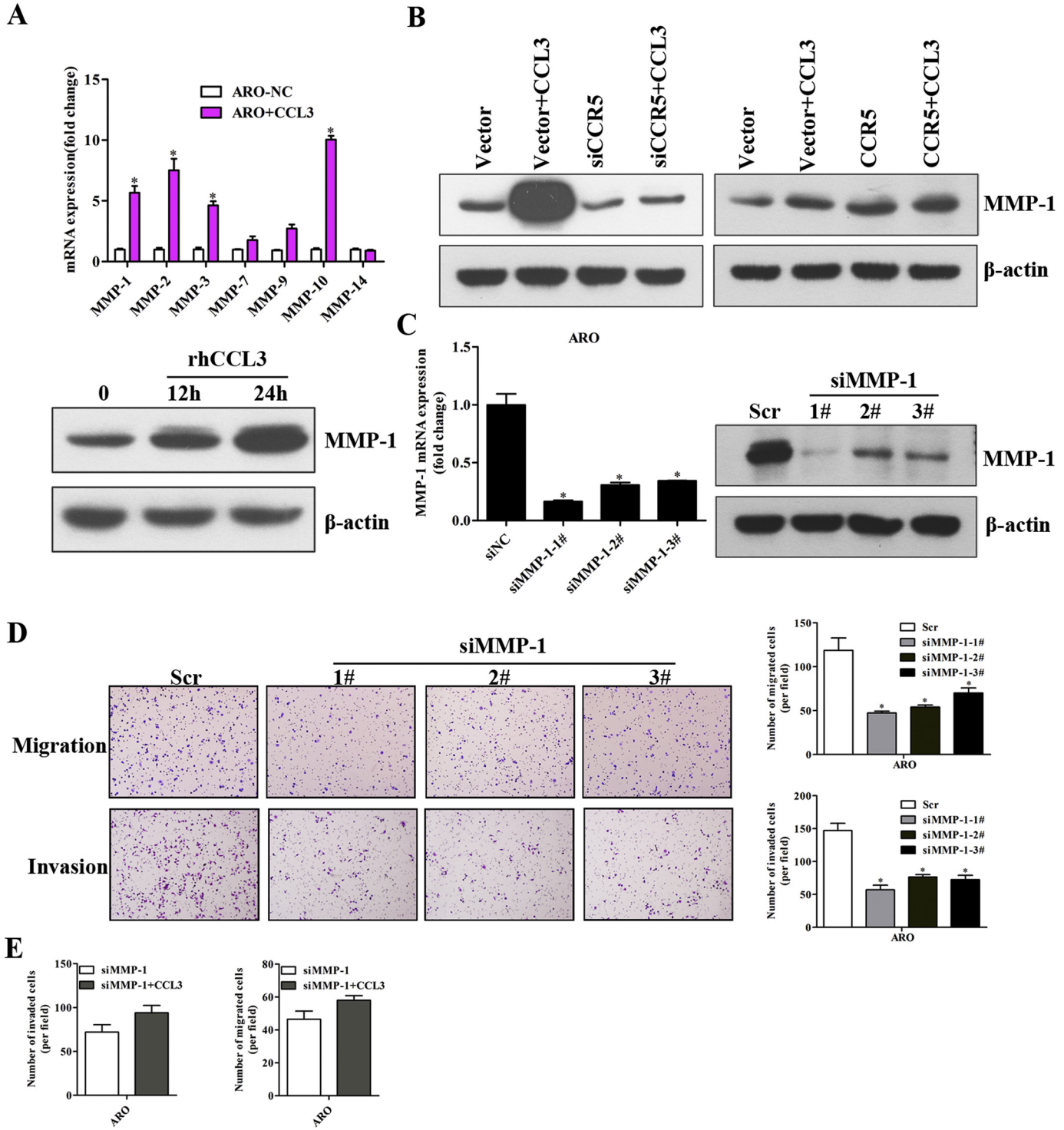

Fig. 6. CCL3-increased invasive and migratory capabilities of ATC cells involve upregulation of MMP-1. (A) ARO cells were incubated with CCL3 for 24 h, and MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-14 mRNA levels were determined using qRT-PCR assays. ARO cells were treated with CCL3 for the indicated periods of time, and cell lysates were collected. MMP-1 protein levels were detected by western blotting. (B) Western blotting showed that knockdown or overexpression of CCR5 could reduce or increase the expression of MMP-1, respectively. (C) Knockdown of MMP-1 with three different siRNAs in ARO cells was evidenced by qRT-PCR and western blotting. (D) ARO cells were transfected with MMP-1 siRNA, and the invasive and migratory abilities were measured with Transwell assays. The values are expressed as the mean ± SD of three independent experiments. *P < 0.05. (E) Cell invasion and migration were not significantly different in siMMP-1 cells with or without CCL3 treatment.

mechanisms to promote tumor metastasis, but we only investigated the mechanism of cytokines and chemokines secreted from activated platelets. Second, it is very important to reveal the distinct mechanism by which platelets synthesize and secrete CCL3 when cocultured with ATC cells, but this mechanistic investigation was not completed in the present study. All of these questions need to be elucidated in future research.

Despite these limitations, to the best of our knowledge, this is the first report showing that platelets promote the invasive and migratory abilities of ATC cells via CCL3 secretion. Furthermore, we revealed that

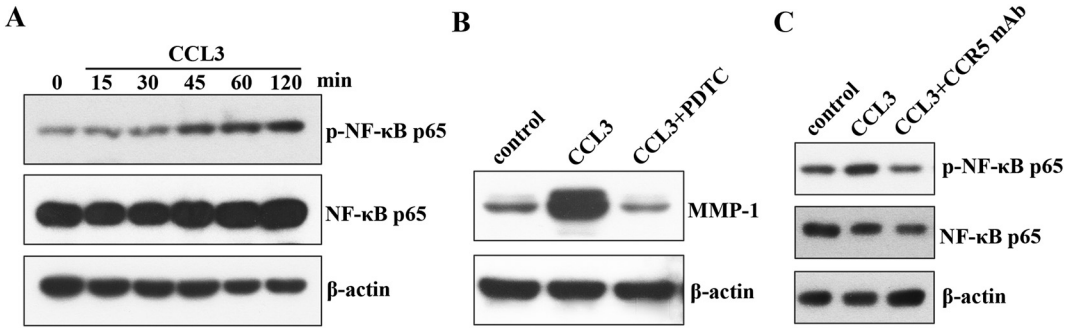

Fig. 7. CCL3-CCR5 axis may induce MMP-1 upregulation through the NF-κB pathway. (A) ARO cells were incubated with CCL3 for the indicated time intervals, and p-p65 expression was examined by western blotting. (B) ARO cells were pretreated with PDTC (10 μM) for 30 min following treatment with CCL3 (10 ng/ml) for 24 h, and MMP-1 protein expression was detected by western blotting. (C) ARO cells were pretreated with CCR5 mAb for 30 min and subsequently stimulated with CCL3 for 24 h. p-p65 expression was measured by western blotting. β-actin was used as a loading control for western blotting.

CCL3 interacts with the CCR5 receptor and upregulates MMP-1via NF-κB pathway, which could be one of the mechanisms by which platelets promote the invasive and migratory abilities of ATC cells. These observations may lead to the development of effective therapies for ATC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109363.

Declaration of Competing Interests

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81560470 and 81773127), the Special Foundation of Young and Middle-Aged Academic Leaders Reserve Talent in Yunnan Province (2015HB086), Special Funds for High-Level Medical Leaders in Yunnan Province (L-2017025), and Yunnan Province Basic Research Program (2018FE001-058/246).

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