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ORIGINAL RESEARCH

Down-regulation of CCL17 in cancer-associated fibroblasts inhibits cell migration and invasion of breast cancer through ERK1/2 pathway

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Objective: Cancer-associated fibroblasts (CAFs) in the tumor microenvironment are involved in cancer development and progression including breast cancer (BC). Up-regulation of CCL17 was observed in BC and predicted a decrease in overall survival, suggesting an important role of CCL17 in BC development. Nonetheless, little is known about the role of CCL17 in the interaction between CAFs and BC.

Materials and methods: Real-time quantitative PCR, Western blot, and enzyme-linked immunosorbent assay were performed to examine C-C motif chemokine ligand 17 (CCL17) and C-C motif chemokine receptor 4 (CCR4) levels in BC tissues and CAFs. Cell proliferation, migration, and invasion of CAFs co-cultured with or without BC cell lines were measured by Cell Counting Kit-8 and Transwell analysis. Expression of CCL17, CCR4, dual specificity phosphatase 6 (DUSP6), matrix metallopeptidase 13 (MMP13), extracellular signal-regulated kinase (ERK1/2), and phosphor-ERK1/2 (p-ERK1/2) in BC cell lines co-cultured with or without CAFs was measured by Western blotting.

Results: We found that BC tissues and CAFs demonstrated higher levels of CCL17 compared with adjacent-normal breast tissues and adjacent-normal fibroblasts (NFs), respectively. CCL17 expression is correlated with lymph nodes, TNM stage and tumor size of BC patients. CCL17 knockdown significantly inhibited CCL17 release, CCR4 expression, and the cell proliferation of CAFs, while CCL17 overexpression demonstrated an inverse effect in NFs. Co-culture with CAFs induced the increases in cell proliferation, migration, invasion, and the expression of CCL17, CCR4, MMP13, and p-ERK1/2 in MCF-7 and MDA-MB-231 cells were markedly reversed by CCL17 knockdown in CAFs. Meanwhile, co-culture with NFs induced the malignant phenotype of MCF-7 cells was markedly enhanced by CCL17 overexpression in NFs. Moreover, DUSP6, a negative regulator of ERK1/2, was dose-dependent decrease in response to recombinant CCL17 and inhibited cell migration, invasion, MMP13 expression, and ERK1/2 activation in MCF-7 cells.

Conclusion: The findings of this study suggest that CCL17 may function as a novel biomarker as well as potential therapeutic target against BC and CAF-secreted CCL17 promotes BC cell migration and invasion through the DUSP6-dependent ERK1/2 pathway.

Keywords: cancer-associated fibroblasts, metastasis, CCL17, DUSP6, ERK1/2

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Introduction

Breast cancer (BC) is the second most common malignant cancers diagnosed in women and found as the second cause of cancer-associated death among women,[1] with the highest mortality in less developed countries and with a higher incidence in developed countries, supporting the need for identification of novel diagnostic

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biomarkers.[2] Clinically, BC has different histological forms, which may lead to different treatment regimens and prognosis.[3,4] Although early diagnosis and treatment strategies, such as endocrine agents, surgery, radiotherapy, chemotherapy, and biological targeting agents, have been improved in recent decades, the prognosis of BC remains poor, especially of patients with recurrence and metastasis.[5,6] Therefore, establishing molecular biomarkers that can detect early metastasis of BC is necessary for BC patients.

It has been shown that the interaction between cancer cells and tumor microenvironment plays an important role in the development and progression of cancer. The interactions between cancer cells and tumor microenvironment are mediated through direct or indirect mechanisms, which involve in deposited substances, stromal cells, or secretion of proteins.[7] The activated cancer-associated fibroblasts (CAFs) are believed to be the most enriched cells in tumor stroma and promote tumor growth and metastasis by secreting a variety of soluble factors, directly interacting with tumor cells, regulating immune-response, and remodeling the extracellular matrix (ECM).[8] Studies comparing co-culture of BC cells with or without fibroblasts showed increased proliferation, anti-apoptosis, metastasis, metabolism, and chemoresistance when they were co-cultured.[9,10] Interestingly, not only CAFs within tumor but also CAFs immediately adjacent to tumor promoted cancer progression.[11]

Chemokines are a family of cytokines or signaling proteins exerting their biological effects by interacting with G-protein-coupled receptors, initiating an intracellular signal cascade that facilitates migration to chemokine source.[12] There are increasing evidences that chemokines and chemokine receptors directly affect the survival, proliferation, angiogenesis, migration, and metastasis of many cancer cells, including BC cells which express chemokine receptors and respond to specific chemokines.[2,4] C-C motif chemokine receptor 4 (CCR4) is an important chemokine receptor for BC progression that is expressed in BC and correlated with a poor survival outcome of BC patients.[4] The ability of CAFs to influence tumor growth was partly dependent on their ability to induce angiogenesis by CAF derived C-X-C motif chemokine ligand 12 (CXCL12), a ligand of CCR4.[13] C-C motif chemokine ligand 17 (CCL17) is another ligand of CCR4 that is highly expressed by thymus and other cells such as fibroblasts,[14] and CCL17/CCR4 signaling promotes lung metastasis of BC.[15] Nevertheless, the mechanisms underlying the CCL17/CCR4 signaling mediated by CAFs in BC progression remain elusive.

The mitogen-activated protein kinase (MAPK) signaling pathway, including the well-known mediator extracellular signal-regulated kinase 1/2 (ERK1/2), regulates matrix metallopeptidase (MMP) expression.[16] MMPs are a family of zinc-dependent endopeptidases that play a role in cell proliferation, migration, differentiation, apoptosis, angiogenesis, tissue repair, and immune-response and affect bioactive molecules on the cell surface,[17] suggesting that they play important roles inside and outside of ECM degradation. MMP13 is known to promote ECM cell invasion and metastasis and is associated with overall survival of BC patients.[18] ERK1/2 as well as its downstream signaling effector MMP13, is some of the mechanism by which CXCL12/CXCR4 or CCL17/CCR4 regulates cell invasion and migration of laryngeal and hypopharyngeal squamous cell carcinoma and bladder cancer.[19,20] However, how CCL17 regulates ERK1/2 pathway directly or indirectly remains unclear. MAP kinase phosphatase enzymes (MKPs) belong to the family of dual-specificity phosphatases (DUSPs), inactivating different MAPK proteins, including ERK1/2.[21] Among these, DUSP6 displays a high specificity for ERK1/2 and has been found to regulate BC cell migration and invasion.[22]

Hence, the correlation of clinicopathological characteristics with CCL17 expression, the functional effect of CAFs on BC progression, and the potential mechanisms by which CCL17 promotes tumor were clarified. In the present study, we found that CCL17 expression is increased in BC tissues and CAFs and associates with lymph nodes, TNM stage, and tumor size of BC patients. CCL17 produced by CAFs promotes migration and invasion of BC cells through DUSP6-dependent ERK1/2 signaling pathway. Therefore, our findings emphasize an important role for CCL17 in controlling BC development, which is helpful to identify potential therapeutic targets for its treatment.

Materials and methods

Tissue samples and isolation of primary fibroblasts

Human BC tissues and the corresponding adjacent-normal breast tissues as well as peripheral blood samples were obtained from 140 BC patients who underwent surgery at Sichuan Cancer Hospital between 2016 and 2018. These adjacent-normal breast tissues were resected within at least

5 cm of the tumor margin when the patients underwent definitive surgery. The healthy control groups included 140 healthy women who underwent mammary gland examination prior to peripheral blood sample collection. Primary fibroblasts were isolated as described previously.[23] Briefly, tumor and adjacent-normal tissues were digested with 2% collagenase II (1 mg/mL; Thermo Fisher Scientific, Waltham, MA, USA) for 30 mins at 37°C, then cultured in DMEM containing 1% penicillin-streptomycin mixture (Solarbio, Beijing, China) and 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) at 37°C in an incubator, and the fibroblasts derived from tumor and adjacent-normal tissues were defined as CAFs and NFs, respectively. The medium was changed every 2 days. In the present study, low-passage-number primary fibroblasts (<4) were used and characterized by strong immunohistochemical staining for vimentin and negative immunohistochemical staining for CK19 (Figure S1), using anti-CK19 (Abcam, Cambridge, MA, USA; ab52625) or anti-vimentin antibody (Abcam; ab8978).

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Sichuan Cancer Hospital and with the 1964 Helsinki declaration. This study protocol was approved by the Ethics Committee of Sichuan Cancer Hospital. Written informed consents were provided prior to enrollment of patients.

Cell culture

Human BC cell lines MCF-7 (Michigan Cancer Foundation, Rochester, MI, USA) and MDA-MB-231 (EG&G Mason Research Institute, Worcester, MA, USA) were maintained at 37°C with 5% CO_2 in a humidified air atmosphere in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin mixture (Solarbio).

Transfection and lentivirus infection

The RNAi (RNA interference) sequences targeting position 227–245 (shRNA-1; 5′-GCTGCCTGGAGTACTTCAA-3′), position 366–384 (shRNA-2; 5′-GCTGCCTGGAGTACTTCAA-3′) or position 379–397 (shRNA-3; 5′-GCAGTTAAATACCTGCAAA-3′) of the human CCL17 gene were cloned into the pLKO.1 lentiviral vector (Addgene, Watertown, MA, USA). For lentiviral production, HEK-293T cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were transfected with 1 μg of the lentiviral vectors for 4 hrs. After incubation for 48 hrs, viral particles in cell culture medium were collected and infected CAFs. CCL17 or DUSP6 overexpression was constructed by cloning full-length human CCL17 or DUSP6 into the pLVX-Puro lentiviral expression vector (Clontech, Palo Alto, CA, USA), and then transfected into 293T cells as above described and used to infect NFs or MCF-7 cells. Cells transduced with pLKO.1-scramble shRNA (shNC) or blank pLVX-Puro (Vector) were used as negative controls.

Cafs/nfs and MCF-7/MDA-MB-231 cells co-culture

CAFs with pLKO.1-CCL17-shRNA or shNC infection and NFs with pLVX-Puro-CCL17 or blank vector infection were digested with trypsin and resuspended in DMEM with the cell density of 5×10^4/mL. 0.1 mL cell suspension (5×10^3 cells per well) in each group was added into the upper well of the transwell inserts containing polycarbonate filters with 3-μm pores (Corning, Madrid, Spain). The lower chamber was inoculated with MCF-7 or MDA-MB-231 cells at a density of 5×10^3 cells per well and cultured in the incubator of 5% CO_2 at 37°C. After co-culture for 48 hrs, the MDA-MB-231 and MCF-7 cells were collected for the following experiments.

Cell proliferation

Cells (3×10^3/well) were seeded into 96-well plate and maintained overnight prior to conduction of experiments. After 12, 24, and 48 hrs, cells were incubated with 10% Cell Counting Kit-8 (CCK-8) solution (SAB Biotech., College Park, MD, USA) at 37°C for 1 hr. Optical density at wavelength 450 nm (OD450) was determined using a microplate reader.

Transwell assay

Cells (3×10^5/well) were seeded onto 24-well plates and maintained overnight prior to conduction of experiments. After treatment, cells were serum-starved for 24 hrs, following which 3×10^5 cells/well in 300 μL serum-free DMEM were placed in the upper chamber at 37°C. The DMEM medium containing 10% FBS (700 μL) was added into the lower chamber. After 24 hrs incubation, the cell invasion and migration were determined in Transwell inserts (Millipore) containing polycarbonate filters with 8 μm pores coated with or without Matrigel (Becton Dickinson, Bedford, MA, USA) as detailed previously.[24]

RNA extraction and real-time quantitative PCR

RNA was extracted from BC cell lines or tissues using the RNeasy Mini kit (Qiagen, Hilden, Germany), and reverse transcription reaction on RNA was carried out using PrimeScript reagent kit (Takara Biomedical Technology (Beijing) Co., Ltd, Beijing, China) according to the manufacturer's protocols. Real-time Quantitative PCR for CCL17, CCR4, and DUSP6 was performed using an ABI Prism 7,500 sequence detection PCR system (Applied Biosystems, Shanghai, China). The primer sequences were shown subsequently: CCL17 (Homo sapiens), 5′-GTCTTGAAGCCTCCTCAC-3′ (forward), 5′-CATGGCTCCAGTTCAGAC-3′ (reverse); CCR4 (Homo sapiens), 5′-CCTTCCTGGCTTTCTGTTC-3′ (forward), 5′-CATCTTCACCGCCTTGTTC-3′ (reverse); GAPDH (Homo sapiens), 5′-AATCCCATCACCATCTTC-3′ (forward), 5′-AGGCTGTTGTCATACTTC-3′ (reverse). Gene expression was determined by normalizing to GAPDH and calculating by the $2^{-\Delta\Delta CT}$ method.

Enzyme-linked immunosorbent assay (ELISA)

CCL17 content in peripheral blood or fibroblast supernatant was measured by Human TARC ELISA Kit (CCL17) (Abcam; ab183366) and MMP13 content in BC cell supernatants was measured by MMP13 Human ELISA Kit (Abcam; ab100605), following the manufacturer's instruction.

Western blot

Western blotting was conducted as previously described.[25] Proteins were analyzed with the following antibodies: anti-CCL17 (Abcam; ab182793; 1:500), anti-CCR4 (Abcam; ab83250; 1:1,000), anti-DUSP6 (Abcam; ab181088; 1:1,000), anti-MMP13 (Abcam; ab84594; 1:1,000), anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA; 9102; 1:1000), anti-p-ERK1/2 (Cell Signaling Technology; 9,101; 1:1,000), anti-GAPDH (Cell Signaling Technology; 5,174; 1:2,000) and the secondary antibodies (Beyotime Institute of Biotechnology, Haimen, China; A0208; 1:1000).

Statistical analyses

All experiments were performed in triplicates and repeated at least three times. The results represent the mean ± SD where applicable. The numerical data were statistically analyzed with GraphPad Prism 6.0 (GraphPad Software, Inc., CA, USA). Differences between the groups were determined with one-way ANOVA analyses followed by Dunnett's test, two-way ANOVA analyses followed by Tukey post-test or Student's *t*-test when only two groups were compared. Correlations between CCL17 and DUSP6 expression levels in BC tissues were assessed by Pearson correlation. Values of $P<0.05$ were considered to be of statistical significance.

Results

CCL17 expression is up-regulated and correlates with tumor progression in BC patients

We first investigate CCL17 expression by Real-time Quantitative PCR and ELISA in tumor tissues and peripheral blood from BC patients (n=140). CCL17 expression was increased in tumor BC tissues compared with corresponding adjacent normal tissues as well as in peripheral blood from BC patients compared with healthy controls (Figure 1A and B). Moreover, CAFs in the microenvironment are essential for tumor progression in BC.[7,9] To know the role of CCL17 in CAFs, CCL17 expression is also measured by Real-time Quantitative PCR and Western blot analysis. As shown in Figure 1C and D, CCL17 expression was also increased in CAFs compared with NFs. To further analyze the correlation between clinicopathological characteristics and CCL17 expression, the 140 BC patients were divided into two groups according to the mRNA level of CCL17 measured by Real-time Quantitative PCR comprising a CCL17 high expression group with fold change >1.5 (n=98, 70%) and CCL17 low expression group with fold change <1.5 (n=42, 30%) (Table 1). The age, histologic grade, histological type, ER status, PR status, and HER2 status of the patients were not significantly associated with the expression of CCL17. A statistically significant association of CCL17 was observed with tumor size, TNM stage, and lymph nodes. These data suggest that high CCL17 expression is closely correlated with tumor progression in BC patients.

CCL17 regulates CCR4 expression and the proliferation of fibroblasts

To investigate the pivotal role of CCL17/CCR4 signaling in fibroblasts, CCL17 was knockdown and overexpression in CAFs and NFs, respectively, and CCL17 and CCR4 expression level in cell lysate and cell supernatant was measured by Real-time Quantitative PCR, Western blot and ELISA analysis. As shown in Figure S2A and B, CCL17 shRNA-1, shRNA-2, and shRNA-3 significantly decreased the mRNA levels of CCL17 by 74.6%, 81.6%, and 65.6% and the protein

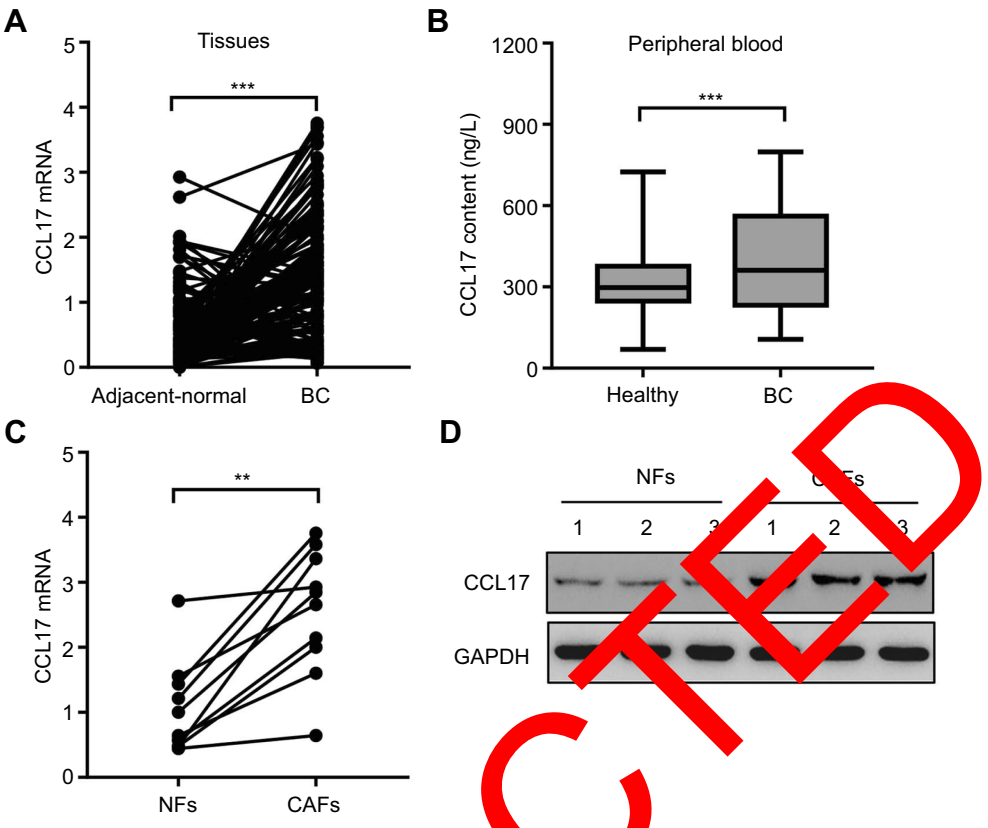

Figure 1 CCL17 expression was increased in BC patients. (**A**) CCL17 mRNA levels in BC tissues (n=140) and corresponding adjacent-normal tissues (n=140) were measured by Real-time Quantitative PCR. (**B**) CCL17 contents in peripheral blood of BC patients (n=140) and healthy controls (n=140) were measured by ELISA. (**C, D**) CCL17 mRNA levels in CAFs (n=10) and NFs (n=10) were measured by Real-time Quantitative PCR and Western blotting. *P<0.05, **P<0.01, ***P<0.001.
Abbreviations: BC, breast cancer; CAFs, cancer-associated fibroblasts; NFs, normal fibroblasts; ELISA, enzyme-linked immunosorbent assay; CCL17, C-C motif chemokine ligand 17; PCR, polymerase chain reaction.

levels of CCL17 by 75.3%, 70.7%, and 69.4% in CAFs compared with shNC, respectively. CCL17 overexpression significantly increased the mRNA and protein levels of CCL17 by 4.3-fold and 3.9-fold in NFs compared with blank vector, respectively (Figure S2C and D). Meanwhile, CCL17 shRNA-1 and shRNA-2 also significantly decreased the CCL17 release and CCR4 expression in CAFs (Figure 2A and B), while CCL17 overexpression markedly increased the CCL17 release and CCR4 expression in NFs (Figure 2C and D). Moreover, our data showed that CCL17 knockdown markedly decreased the cell proliferation of CAFs by approximately 17% at 48 hrs, while CCL17 overexpression markedly increased the cell proliferation of NFs by 15.6% at 48 hrs (Figure 2E and F). These data suggest that CCL17/CCR4 signaling involves in the proliferation of fibroblasts.

CCL17 knockdown in CAFs inhibits BC cell invasion and migration

To explore the important role of CAFs in invasion and migration of BC cells, MCF-7 or MDA-MB-231 cells were co-cultured with CAFs with CCL17 knockdown, and the CCK-8 and Transwell assay were performed. As shown in Figure 3A and B, CAFs could increase the proliferation of MDA-MB-231 and MCF-7 cells by 19.8% and 21.7% at 48 hrs, which were significantly inhibited by CCL17 knockdown in CAFs. Moreover, CAFs also increased the migration and invasion of MCF-7 by 2.04-fold and 2.0-fold and that of MDA-MB-231 cells by 2.78-fold and 3.02-fold, which were significantly inhibited by CCL17 knockdown in CAFs (Figure 3C–E).

To further determine the importance of CCL17/CCR4 signaling in BC cell invasion and migration, the expression of p-ERK1/2, ERK1/2, DUSP6, and MMP13 was measured by Western blotting. We found that CAFs not only induced the expression of CCL17 and CCR4 in MCF-7 and MDA-MB-231 cells, but also increased the expression of MMP13 and p-ERK1/2 and decreased the DUSP6 expression. However, these CAFs-mediated protein expressions were significantly inhibited by CCL17 knockdown in CAFs (Figure 4A–H). These data suggested that CAFs with CCL17 knockdown suppressed BC cell migration and invasion.

Table 1 Correlation of CCL17 expression with clinicopathological features of BC

Clinicopathological parameter	CCL17 expression		*P*-value
	High (n=98)	Low (n=42)	
Age (years)			0.2445
<57	55	28	
≥57	43	14	
Tumor size (cm)			0.0333
<3	51	30	
≥3	47	12	
TNM stage			0.0299
I/II	61	34	
III/IV	37	8	
Lymph nodes			0.0004
Positive	62	13	
Negative	36	29	
Histologic grade			0.7158
I	29	11	
II	31	12	
III	38	19	
Histological type			0.0756
Ductal	58	18	
Non-ductal	40	24	
ER status			0.7122
Positive	48	22	
Negative	50	20	
PR status			0.1526
Positive	55	29	
Negative	43	13	
HER2 status			0.4980
Positive	57	27	
Negative	41	15	

Note: Differences between group[illegible]one by the chi-square test.
Abbreviations: BC, b[illegible]cancer; [illegible] estroge[illegible]receptor; PR, progesterone receptor; HER2, epi[illegible]mal gr[illegible]th facto[illegible]cep[illegible]type 2.

CCL17 overexpression in NFs promotes BC cell invasion and migration

We further examined the effect of NFs with CCL17 overexpression on BC cell migration and invasion. We found that co-culture with NFs increased MCF-7 cell proliferation, invasion, migration, and the expression of CCL17, CCR4, MMP13, and p-ERK1/2 and decreased the DUSP6 expression, which was significantly enhanced by CCL17 overexpression in NFs (Figure 5A–G).

DUSP6 overexpression inhibited BC cell invasion and migration

To further examine the function of CCL17 in BC cell invasion and migration, MCF-7 cells were treated with different concentrations of recombinant CCL17 protein (50, 100, and 200 ng/mL), and the expression of CCR4 and DUSP6 was measured by Real-time Quantitative PCR and Western blot. It was found that recombinant CCL17 protein significantly increased the CCR4 expression and decreased the DUSP6 expression in a dose-dependent manner (Figure 6A–C). These [illegible]suggest that DUSP6 may involve in CCL17-m[illegible]diated BC [illegible]ogression. To confirm our hypothesis, MC[illegible]7 cells wi[illegible] DUSP6 overexpression were treate[illegible]with reco[illegible]binant [illegible]CL17 protein (100 ng/mL), and t[illegible] cell in[illegible]sion, m[illegible]ation, and the expression of p-ERK1/2, [illegible]RK1/2, and MMP13 were measured. The res[illegible]s showe[illegible]hat D[illegible]SP6 overexpression inhibited rec[illegible]bi[illegible]nt CCL17 [illegible]tein-induced cell migration, invasion, and th[illegible]xpression of MMP13 and p-ERK1/2 in [illegible]CF-7 cells (Figu[illegible]6D–G). Previous study demonstra[illegible]d that DUSP6 is a key ERK-specific phosphatase that indu[illegible]es dephospl[illegible]rylation of ERK1/2, negatively regulating t[illegible]ERK1/[illegible] pathway.[26] Therefore, it suggests that [illegible]RK1/2 signaling may associate with CCL17-induced B[illegible] cel[illegible]igration and invasion.

Correlation analyses in BC tissues

We next investigate DUSP6 expression by Real-time Quantitative PCR in tumor tissues from BC patients. DUSP6 expression was decreased in tumor tissues compared with their corresponding adjacent-normal controls (Figure 7A). Pearson correlation analysis showed that DUSP6 mRNA expression was negatively correlated with the mRNA expression of CCL17 in BC tissues (Figure 7B). These data further supported the findings in BC cell lines.

Discussion

There are reports shown that development of cancer is not only dependent on tumor cells, but also co-mediated by the tumor microenvironment. CAFs, the most common stromal cells in the tumor microenvironment, significantly contribute to tumor initiation, proliferation, invasion, migration, metastasis, and drug resistance in various types of cancers, including BC.[9,10,27] It has been well established that chemokines and their receptors are participated in the cross-talk of fibroblasts and cancer cells.[10,12] Here, we focused on the function of CAF-associated

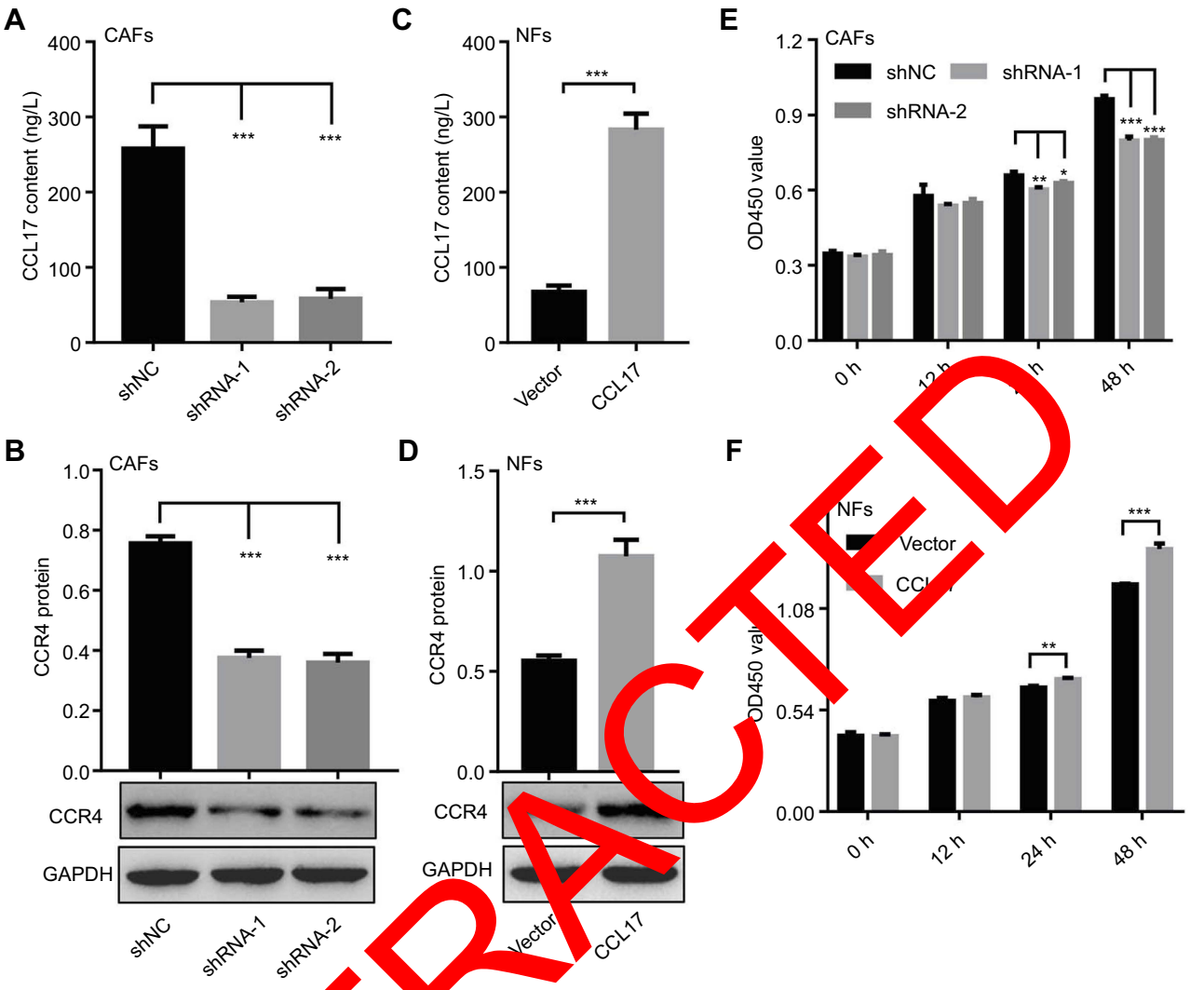

Figure 2 CCL17 expression regulated the proliferation of fibroblasts. CAFs were transduced with pLKO.1-CCL17 shRNA or shNC, and NFs were transduced with pLVX-Puro-CCL17 or blank pLVX-Puro (vector). (**A, C**) CCL17 content was measured by ELISA; (**B, D**) CCR4 expression was measured by Western blot; and (**E, F**) cell proliferation was measured by CCK-8 assay. *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: CAFs, cancer-associated fibroblasts; NFs, normal fibroblasts; ELISA: enzyme-linked immunosorbent assay.

CCL17 in cell invasion and migration of BC via a clinical exploration and in vitro investigation. In this study, we showed that CCL17 levels were significantly up-regulated in BC tissues, peripheral blood, and CAFs compared with their corresponding controls, respectively. CCL17 expression was correlated with lymph nodes, TNM stage and tumor size of BC patients. CAFs or NFs-derived CCL17 may promote BC cell invasion and migration through DUSP6-dependent ERK1/2 pathway.

It is worth noting that the CCL17 plays an important role in promoting cancer progression.[11,14] In fact, our clinicopathological analysis demonstrated that high CCL17 expression was correlated with advanced TNM stage, larger tumor size and increased lymph node metastasis, indicating that CCL17 is associated with the aggressiveness of BC. Consistent with our findings, CCL17 expression was found increased in BC tissues in comparison to normal tissues.[28] CCL17 recruits CCR4 positive regulatory T cells and promotes lung metastasis of BC.[4,15] Moreover, CCR4 positively correlates with lymph node metastasis in BC patients,[4] supporting our result of higher expression of CCL17 in BC patients with positive lymph nodes. However, CCL17 mRNA expression was correlated with races and HER2 status, but age and stage were not found to be correlated with CCL17 expression in BC.[28] Weide et al, reported that serum CCL17 level was lower in melanoma patients compared with healthy controls.[29] Similar finding in our study was

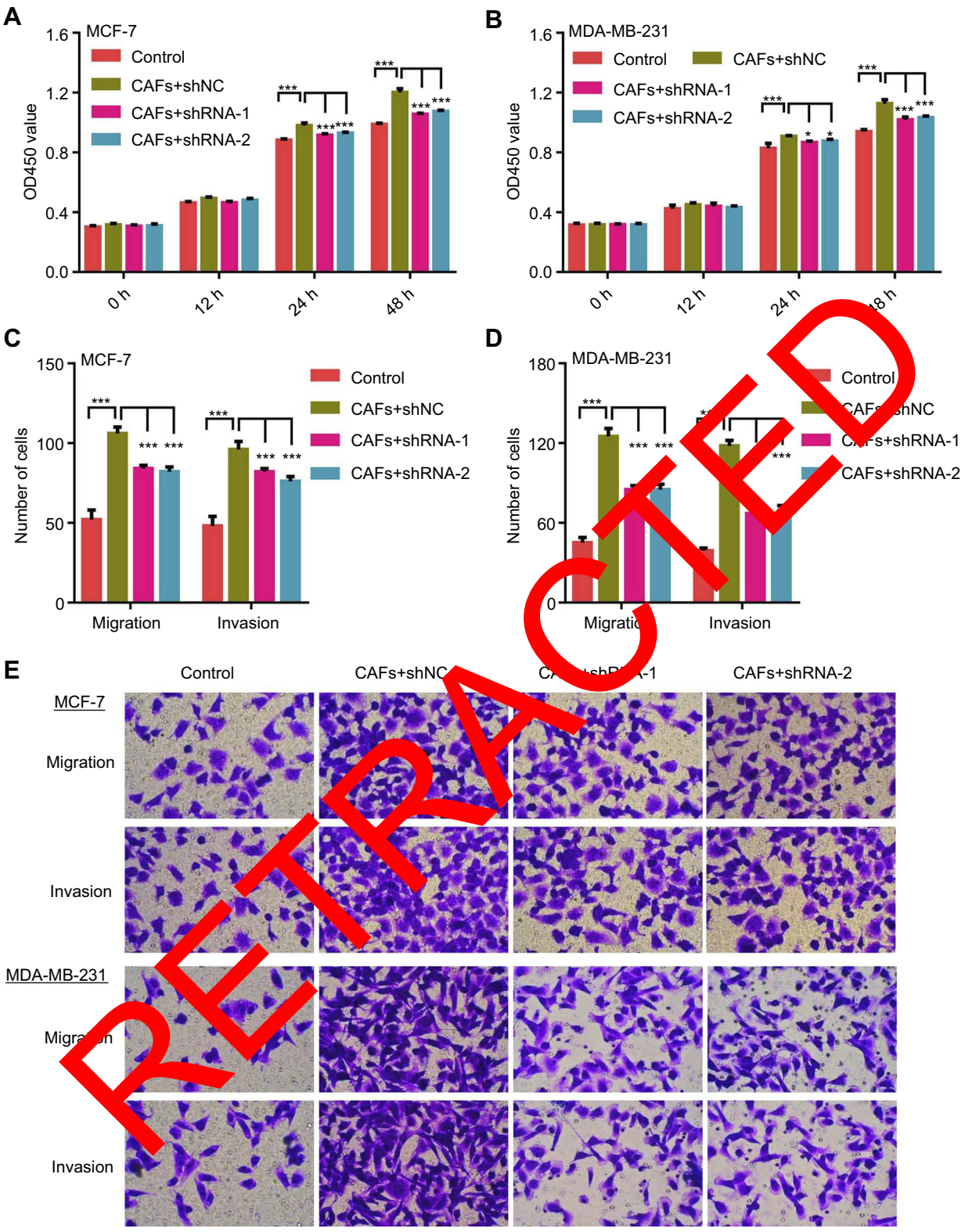

Figure 3 CCL17 knockdown in CAFs inhibited BC cell proliferation, migration, and invasion. MDA-MB-231 and MCF-7 cells were co-cultured with CCL17 knockdown-induced CAFs. (**A, B**) Cell proliferation was measured by CCK-8, and (**C–E**) invasion and migration were measured by Transwell assay. *P<0.05, ***P<0.001.
Abbreviations: CAFs, cancer-associated fibroblasts; BC, breast cancer.

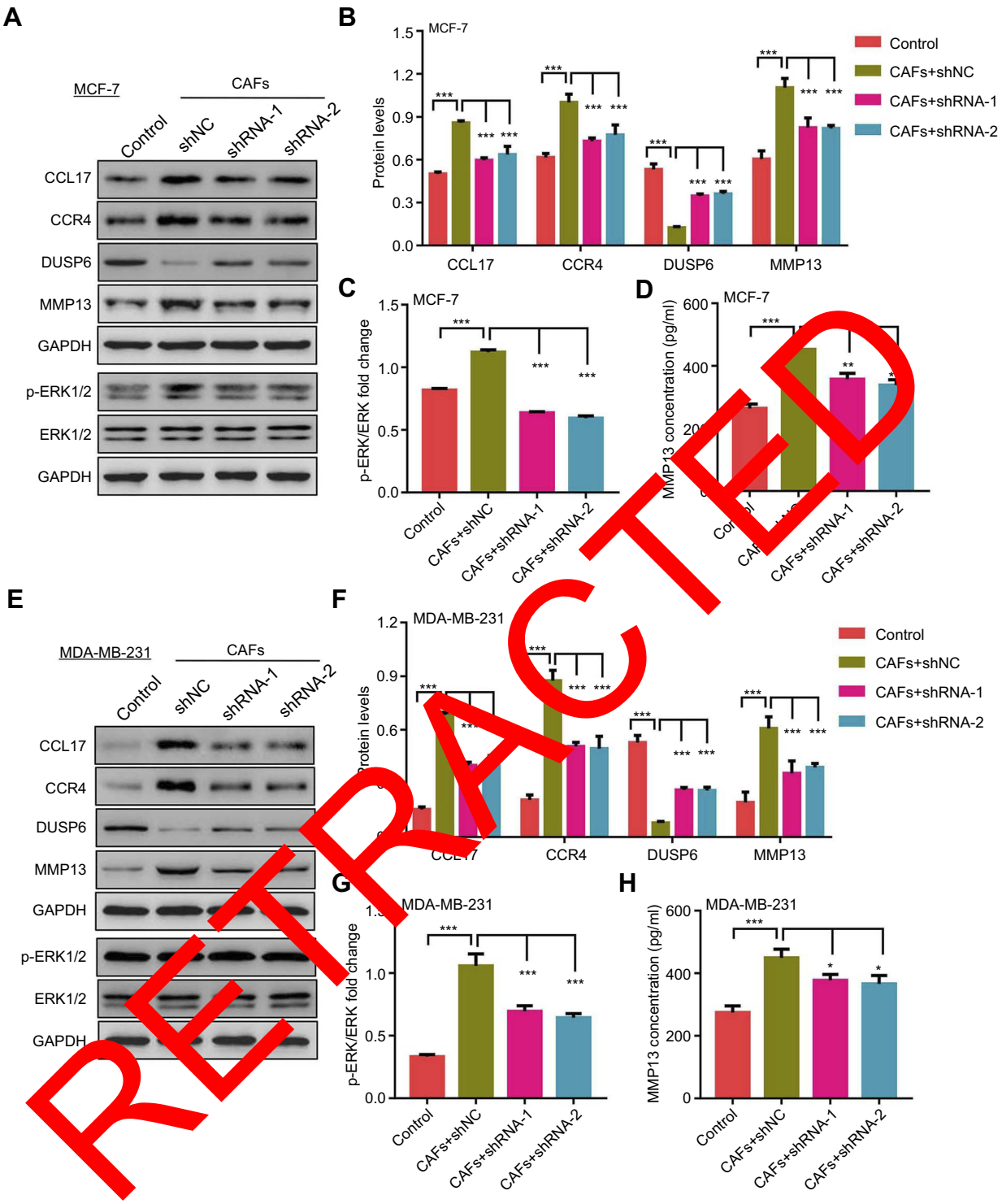

Figure 4 CCL17 knockdown in CAFs inhibited CCL17, CCR4, MMP13, and p-ERK1/2 but promoted DUSP6 expression in BC cell lines. MCF-7 cells were co-cultured with CCL17 knockdown-induced CAFs, and the expression of (**A, B**) CCL17, CCR4, DUSP6, MMP13, (**A, C**) p-ERK1/2, and ERK1/2 was measured by Western blotting. MDA-MB-231 cells were co-cultured with CCL17 knockdown-induced CAFs, and the expression of (**E, F**) CCL17, CCR4, DUSP6, MMP13, (**E, G**) p-ERK1/2, and ERK1/2 was measured by Western blotting. MCF-7 cells were co-cultured with CCL17 knockdown-induced CAFs, whereas MDA-MB-231 cells were co-cultured with CCL17 knockdown-induced CAFs. (**D, H**) The MMP13 concentration was measured by ELISA. *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: CAFs, cancer-associated fibroblasts; BC, breast cancer.

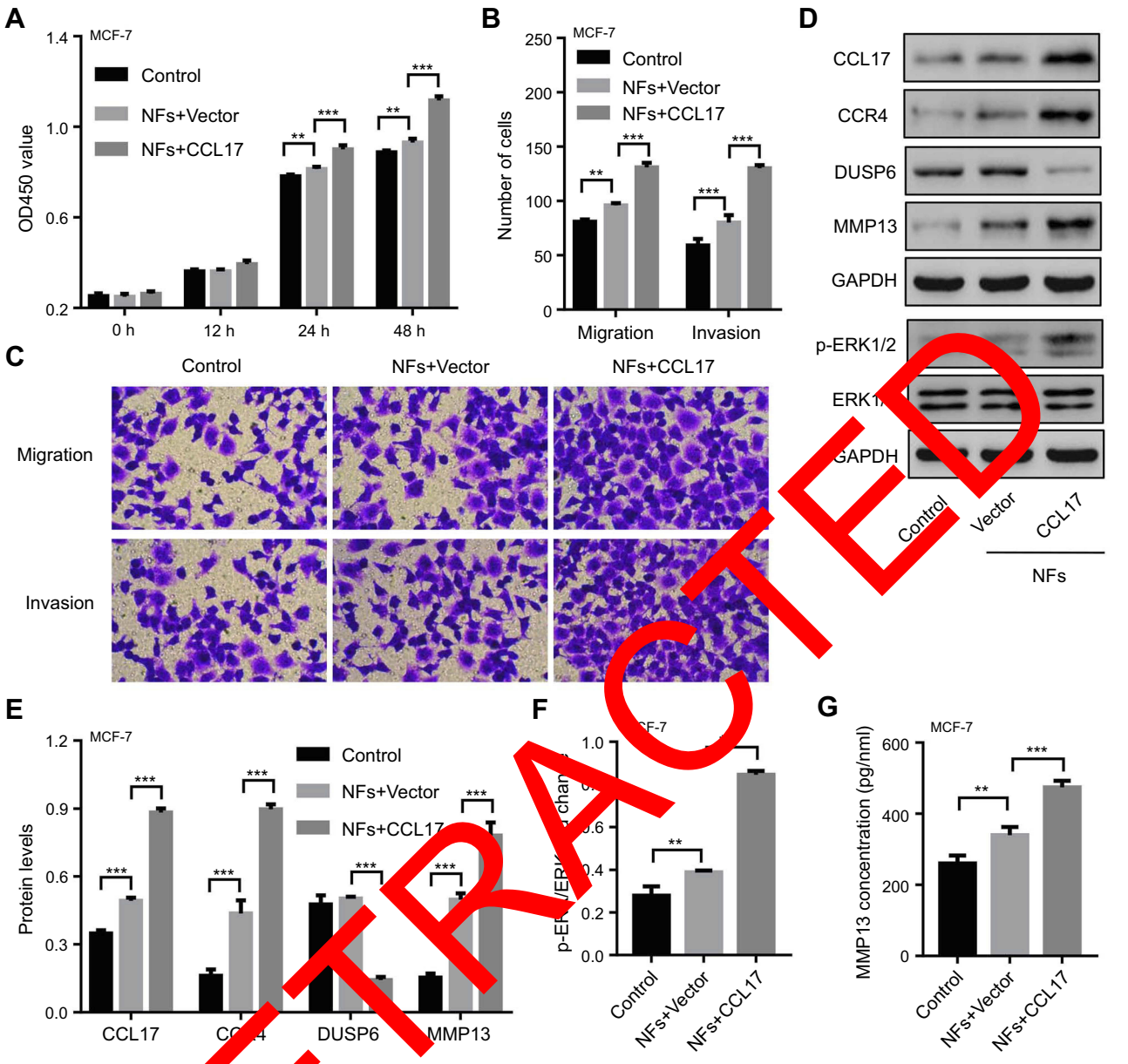

Figure 5 CCL17 overexpression NFs promotes BC cell proliferation, migration, and invasion. MCF-7 cells were co-cultured with CCL17 overexpression-induced NFs. (**A**) Cell proliferation was measured by CCK-8; (**B, C**) migration and invasion were measured by Transwell; (**D–F**) expression of CCL17, CCR4, DUSP6, MMP13, p-ERK1/2, and ERK1/2 was measured by Western blotting; and (**G**) MMP13 concentration was measured by ELISA. ***P*<0.01, ****P*<0.001.
Abbreviations: NFs, normal fibroblasts; BC, breast cancer; ELISA: enzyme-linked immunosorbent assay.

that of a statistically significant increase of CCL17 content in peripheral blood in BC patients compared with healthy controls, and CCL17 may therefore as differentiation maker between BC and healthy women.

Many groups have identified that CAFs significantly contributed to cancer progression. For example, CAFs-derived interleukin 32 (IL32) promotes BC cell invasion and metastasis via integrin β3-p38 MAPK signaling.[7] CAFs promoted hepatocellular carcinoma metastasis by CCL2, CCL5, CCL7, and CXCL16 through hedgehog and TGF-β pathways.[30] CAFs promoted integrin β1 clustering and invasiveness in gastric cancer through activating CXCL12/CXCR4 axis.[12] Our study showed that CAFs-induced BC cell proliferation, invasion and migration, and CCR4 expression were significantly reversed by CCL17 down-regulation in CAFs, while the tumor-promoting effects of NFs on BC were markedly strengthened by CCL17 up-regulation in NFs. Since CCL17 activates ERK1/2 signaling and CCR4 acts as a downstream target of ERK1/2/NF-κB pathway, we indicate that CCL17

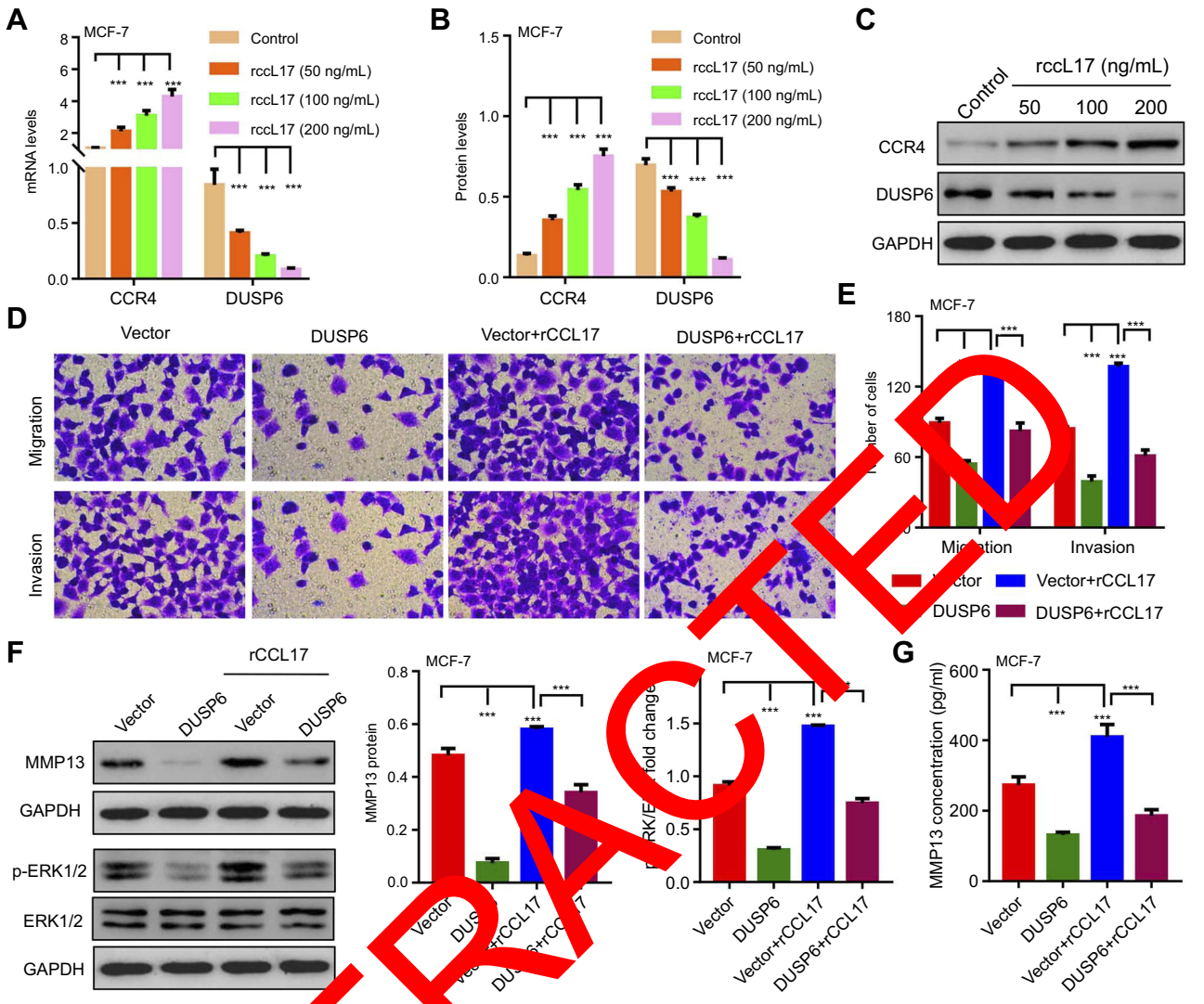

Figure 6 DUSP6 overexpression inhibits BC cell proliferation, migration, and invasion. (**A–C**) MCF-7 cells were treated with different concentrations of recombinant CCL17 protein (rCCL17; 50, 100, and 200 ng/mL), and the expression of CCR4 and DUSP6 was measured by Real-time Quantitative PCR and Western blot. MCF-7 cells were transduced with pLVX-Puro-CCL17 or blank pLVX-Puro (vector) in the presence or absence of recombinant CCL17 (100 ng/mL). (**D, E**) Cell migration and invasion were measured by Transwell; (**F**) expression of MMP13, p-ERK1/2, and ERK1/2 was measured by Transwell, Western blot; and (**G**) MMP13 concentration was measured by ELISA. ***P<0.001.
Abbreviation: ELISA: enzyme-linked immunosorbent assay.

positively regulates CCR4 protein levels through activating ERK1/2 signaling.[31] CCL17/CCR4 signaling is of great importance in CAFs/NFs-induced BC metastasis. In line with our findings, CCL17 originating from CXCL14-activated fibroblasts that display phenotypes similar to CAFs can markedly increase migration and invasion abilities of BC cells and is potentially associated with EMT, which is markedly inhibited by CCR4 down-regulation.[32] Moreover, CCR4 enhances the chemotactic response of BC cells to CCL17, promotes BC cell growth and metastasis in vivo, but has no effect on BC cell proliferation in vitro.[4] It is acknowledged that normal fibroblasts are often considered to be tumor suppressive.[7,9] Meanwhile, NFs also have no effect on regulating cancer cells in the co-culture system.[33] Therefore, the role of NFs in tumorigenesis needs to be further investigated.

Previous study also showed that CXCL14 secreted by BC cells activated mammary fibroblasts through the ERK1/2 pathway, and activated CAFs promoted the cell migration and metastasis of BC through the CCL17/CCR4 axis.[32] In the present study, CAFs or NFs-induced ERK1/2 activation and its downstream signaling effector MMP13 in BC cells were inhibited by CCL17 knockdown in CAFs and strengthened by CCL17 overexpression in NFs,

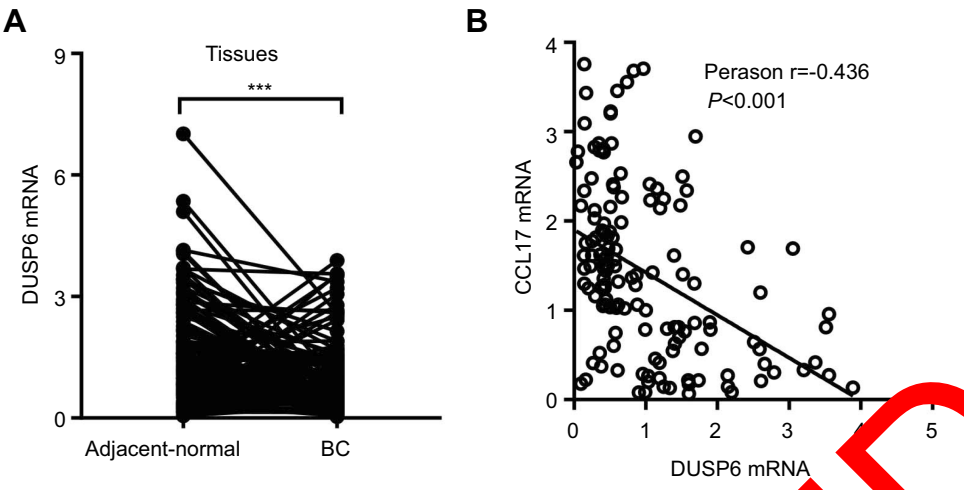

Figure 7 Correlation analysis between DUSP6 and CCL17 in BC tissues. (**A**) DUSP6 mRNA levels in BC tissues (n=14[illegible] and correspond[illegible] adjac[illegible]-normal tissues (n=140) were measured by Real-time Quantitative PCR. (**B**) Pearson correlation scatter plots in BC tissues (n=140). ***[illegible]0.001.
Abbreviation: BC, breast cancer.

respectively. DUSP6 is an effective phosphatase that reverses ERK1/2 phosphorylation and is therefore a negative regulator of MAPK activity,[26] suggesting a tumor-suppressive role of DUSP6 via pivotal negative feedback regulation of the ERK1/2 in diverse cancers.[34,35] Treatment of BC cells with recombinant CCL17 significantly increased CCR4 while decreased DUSP6 expression in a dose-dependent manner. DUSP6 overexpression markedly inhibited cell invasion, migration, MMP13 expression and the ERK1/2 activation in MCF-7 cells with recombinant CCL17. These findings suggest DUSP6-dependent ERK1/2 pathway may involve in BC invasion and migration induced by CAFs-derived CCL17. MMP13 is known to promote BC cell invasion and metastasis and be associated with overall survival of BC patients.[18] Stimulation of regulatory T cells with CCL17/CCR4 can phosphorylate ERK1/2,[36] and CCR4 up-regulates MMP13 expression through ERK1/2 in colorectal cancer.[37] Similarly, Zhao et al reported that CCL17/CCR4 axis can induce the metastasis of bladder cancer cells by promoting ERK1/2 signaling and MMP13 expression.[19] CXCL12/CXCR4 promoted laryngeal and hypopharyngeal squamous cell carcinoma metastasis through MMP-13-dependent invasion via the ERK1/2 signaling pathway.[20] Since DUSP6 undergoes highly specific interaction with ERK1/2, which leads to the DUSP6 inactivation,[38] we suggest that CCL17 may regulate the DUSP6 levels through the ERK1/2 signaling pathway. However, contrary to a tumor-suppressive role of DUSP6 in cancers, DUSP6 knockdown in MDA-MB-231 cells demonstrated decreased cell migration and invasion.[22] Therefore, DUSP6 may play [illegible]functions depending on the histological subtypes of the cells.

Conclusion

In conclusion, our study elucidated that regulation of CCL17 expression in CAFs promoted cell invasion and migration of BC through DUSP6-dependent ERK1/2 pathway. These findings suggest that CAFs-derived CCL17 could be potential therapeutic targets for BC metastasis, although the further mechanistic study is warranted.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

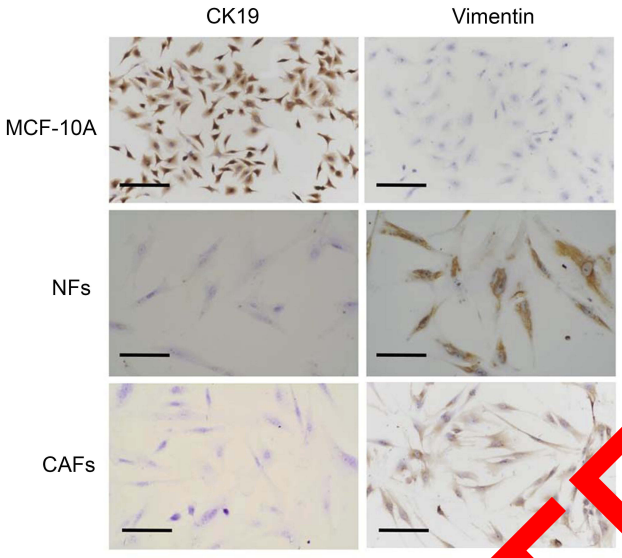

Figure S1 Fibroblasts were interstitially derived. Low-passage-number primary fibroblasts (<4) and normal breast epithelial cell line MCF-10A were characterized by immunohistochemical staining for vimentin and CK19. Scale bars: 100 μm.

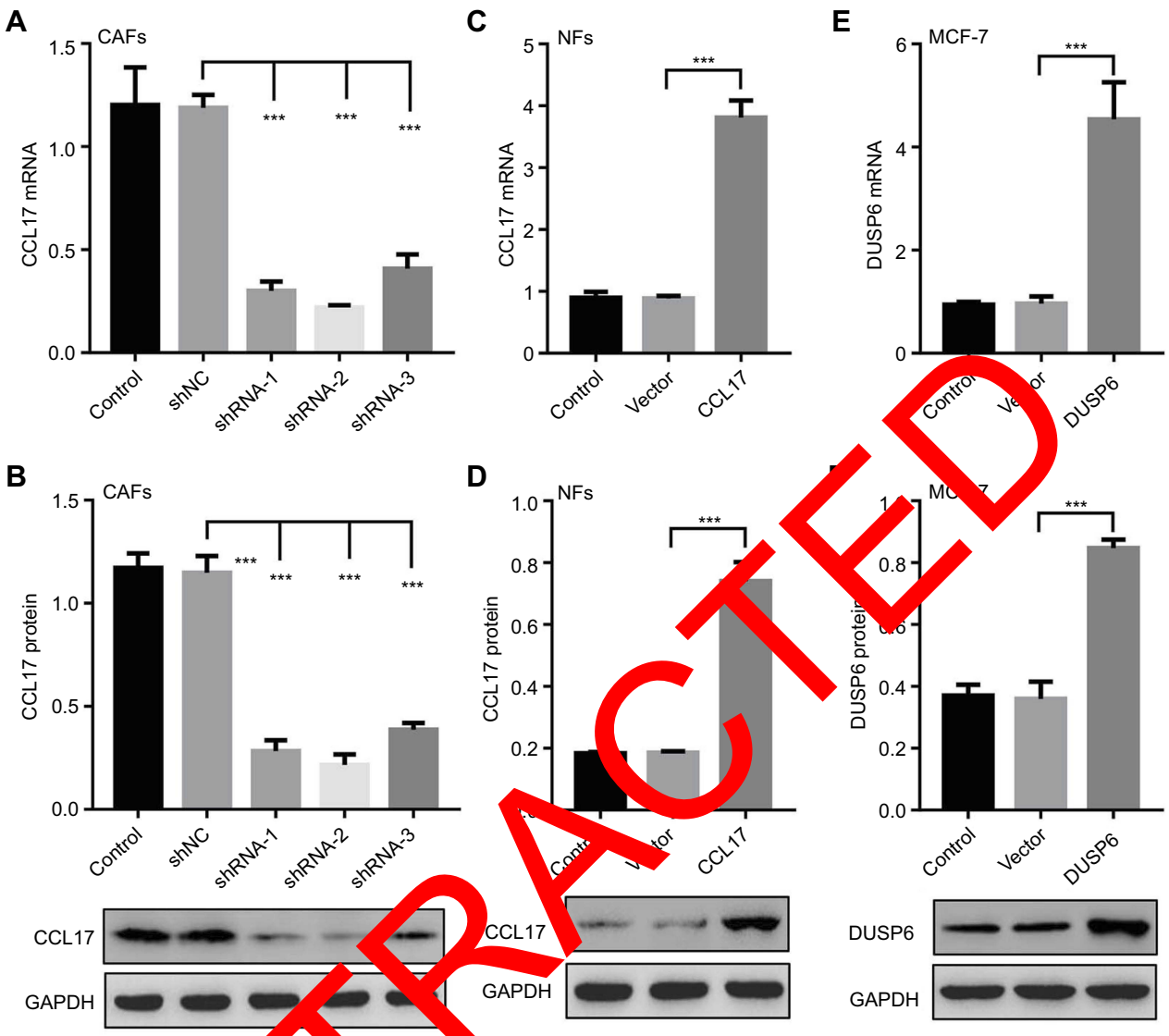

Figure S2 CCL17 and DUSP6 expression in BC cell lines and fibroblasts. (**A, B**) CAFs were transduced with pLKO.1-CCL17 shRNA or shNC and (**C, D**) NFs were transduced with pLVX-Puro-CCL17 or blank pLVX-Puro (vector), and CCL17 expression was measured by Real-time Quantitative PCR and Western blot. (**E, F**) MCF-7 cells were transduced with pLVX-Puro-DUSP6 or blank pLVX-Puro (vector), and DUSP6 expression was measured by Real-time Quantitative PCR and Western blot. ***$P<0.001$.

Abbreviations: BC, breast cancer; CAFs, cancer-associated fibroblasts; NFs, normalfibroblasts; CCL17, C-C motif chemokine ligand 17; DUSP6, dual specificity phosphatase 6; PCR, polymerase chain reaction.