BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-18-232

Title: Ets-1 enhances tumor migration through regulation of CCR7 expression

Article Type: Article

Keywords: Ets-1; CCR7; tumor migration; PNT domain

Corresponding Author: Tzong-Shyuan Tai

Authors: Li-Wen Fang[2], Ying-Hsien Kao[1,4], Ya-Ting Chuang[3], Huey-Lan Huang[5], Tzong-Shyuan Tai[1,4,*]

Institution: [1]Department of Medical Research, E-Da Hospital,
[2]Department of Nutrition, I-Shou University,
[3]Department of Medical Research, National Taiwan University Hospital,
[4]I-Shou University,
[5]Department of Bioscience Technology, Chang Jung Christian University,

Manuscript Type: Article

Title: Ets-1 enhances tumor migration through regulation of CCR7 expression

Authors: Li-Wen Fang[2], Ying-Hsien Kao[1], Ya-Ting Chuang[3], Huey-Lan Huang[4] and Tzong-Shyuan Tai[1,*]

Affiliations:

1 Department of Medical Research, E-Da Hospital, I-Shou University, Kaohsiung, Taiwan

2 Department of Nutrition, I-Shou University, Kaohsiung, Taiwan

3 Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan

4 Department of Bioscience Technology, College of Health Science, Chang Jung Christian University, Tainan, Taiwan

Running Title: Ets-1 regulates CCR7 expression

Keywords: Ets-1, CCR7, tumor migration, PNT domain

Corresponding Author Information: Tzong-Shyuan Tai; Tel: +886-7-6151100 ext. 5069; No 1. Yi-Da Road, Jiao-Su Village, Yan-Chao District, Kaohsiung City 824, Taiwan. Email: ed107299@edah.org.tw

ABSTRACT

Ets-1 is a prototype of the ETS protein family, the members of which contain a unique ETS domain. Ets-1 is associated with cancer progression and metastasis in many types of cancer. In many studies, the elevated expression of Ets-1 in cancer biopsies has been linked to poor survival. CCR7 is a chemokine that binds to the specific ligand CCL21/CCL19. CCR7 expression is associated with tumor metastasis and infiltration into lymph nodes. In this study, we tested whether Ets-1 could regulate CCR7 expression and enhance tumor metastasis. Our data show that CCR7 expression is downregulated in Ets-1-deficient T cells upon T-cell stimulation. The overexpression of Ets-1 increases CCR7 expression in breast cancer cell lines. In contrast, the knockdown of Ets-1 reduces CCR7 expression. Ets-1 directly binds to the CCR7 promoter and mediates CCR7 expression in luciferase reporter assays and chromatin immunoprecipitation assays. The transactivation activity of Ets-1 is independent of the Pointed domain of Ets-1. Ets-1 also enhances the NF-κB and CBP transactivation of the CCR7 promoter. Finally, we show that Ets-1 can modulate cancer cell transmigration by altering CCR7 expression in a transwell assay and wound healing assay. Taken together, our data suggest that Ets-1 enhances CCR7 expression and contributes to tumor cell migration.

INTRODUCTION

Chemokines are a family of small chemotactic cytokines that direct the homing and control of homeostasis of immune cells (1). ~~They are well known to play a role in the control of homeostasis and the homing of immune cells.~~ In addition to ~~being involved in the~~ immune system, chemokines have been implicated in the pathogenesis of cancer. Several studies have shown that the elevated expression of chemokines is correlated with cancer progression and metastasis (2, 3).

CCR7 is a CC chemokine family member, the ligand of which is CCL21/CCL19. In the immune system, CCL19/CCL21 expressed on fibroblastic reticular cells (FRCs) in the secondary lymphoid organs can help CCR7-expressing cells move to the lymphoid organs. Several studies, including those on breast cancer, melanoma, gastric cancer, and head and neck cancer, have shown that tumor cells also use this interaction to establish lymph node metastasis (4-7). In a mouse model, anti-CCR7 treatment was found to cause a significant delay in tumor growth and metastasis (8). CCR7 expression is controlled by transcription factors, miRNAs or epigenetic mechanisms. Transcription factors such as NF-AT1, AP-1, NF-κB, and SP-1 bind to the CCR7 promoter region and control its mRNA transcription (4) (9, 10). However, whether these transcription factors are involved in tumor metastasis has not been elucidated.

Ets-1, as a transcription factor, contains a unique ETS domain with DNA binding activity. The ETS domain is a winged helix-turn-helix structure that recognizes the DNA GGAA/T core motif. Ets-1 has been shown to play a critical role in regulating the differentiation and function of T helper (Th) cells. Deficiency in Ets-1 has a profound impact on Th1 immune responses (11). The Pointed (PNT) domain located on the N-terminus of Ets-1 can serve as a protein-interacting domain; among the interacting proteins, ERK2 has been shown to bind to the PNT domain and phosphorylate the threonine residue (T38) at the N-

terminus. Phosphorylation of T38 increases the ability of Ets-1 to induce reporter gene expression in transient expression assays, and this effect is closely related to the recruitment of the coactivator CBP/p300 (10) (12). Ets-1 has been linked to cancer progression in many types of cancer, and elevated expression of Ets-1 in cancer biopsies has been connected to poor survival in many studies (13). However, how Ets-1 enhances tumor metastasis is still unknown.

In this study, we tested whether Ets-1 could regulate CCR7 expression and enhance tumor metastasis. We found that Ets-1 could bind to the CCR7 promoter and enhance CCR7 expression in a concentration-dependent manner. Ets-1 increased CCR7 expression in a PNT domain-independent manner. In addition, Ets-1 cooperated with NF-κB and CBP to enhance CCR7 expression, and the overexpression of Ets-1 enhanced tumor cell migration through a CCR7/CCL21 interaction.

RESULTS

CCR7 expression is upregulated during T-cell activation.

Previous studies have shown that CCR7 expression is upregulated in T cells upon T-cell stimulation (14). To determine whether Ets-1 regulated CCR7 expression, we first verified the expression of CCR7 upon T-cell activation. Splenocytes from wild-type (WT) mice were activated with CD3 and CD28, and CCR7 expression was measured with quantitative RT-PCR (Q-PCR). Similar to previous results, the CCR7 expression levels increased by at least 10-fold after the cells were stimulated for 5 hours (Figure 1A). To examine whether Ets-1 regulated CCR7 expression during T-cell activation, we examined CCR7 expression in Ets-1-deficient cells. Splenocytes from Ets-1 heterozygous (Het) and knockout (KO) mice were activated with CD3 and CD28 for 5 hours, and CCR7 expression was measured with Q-PCR.

Ets-1 protein deficiency did not affect CCR7 expression in splenocytes without stimulation, suggesting that Ets-1 does not control CCR7 expression under resting conditions (Figure 1B). However, CCR7 expression was upregulated in Het splenocytes, but not in Ets-1-deficient splenocytes, upon stimulation (Figure 1B). The expression level of CCR7 in KO cells was approximately 50% of that in Het cells after activation. The data suggest that Ets-1 controls CCR7 expression upon T-cell activation.

Ets-1 enhanced CCR7 expression through promoter binding and upregulated transcriptional activity in cancer cells

~~Our data from T cells suggest that Ets-1 controls CCR7 expression upon T cell activation. In addition to its role in T cells,~~ The expression of CCR7 in some tumor cells plays a role in tumor metastasis to lymph nodes. The overexpression of Ets-1 in basal-like tumor cells has been linked to tumor metastasis (15). We hypothesized that Ets-1 may promote tumor cell metastasis by upregulating CCR7 expression. To test this hypothesis, we overexpressed Ets-1 in MDA-MB-231 breast cancer cells and examined CCR7 expression. The overexpression of Ets-1 increased CCR7 mRNA expression (Figure 1C). CCR7 protein expression in MDA-MB-231 cells was also increased upon Ets-1 overexpression, and the expression was increased further at higher levels of Ets-1 transfection (Figure 1D). These data suggest that Ets-1 can upregulate CCR7 expression.

To test whether Ets-1 could bind to the CCR7 promoter and regulate CCR7 expression, we searched for putative Ets-1 binding sites in the 1000 bp CCR7 promoter region using multiTF and Mulan software. There were at least 2 potential Ets-1 binding sites identified in the CCR7 promoter region. The two putative Ets-1 binding sites were located at -72 bp and -32 bp upstream of the transcription start site of the CCR7 gene. The sequences of both of these 2 potential binding sites showed conservation in both the human and mouse CCR7 promoter

regions. The human CCR7 promoter region was then cloned into a luciferase reporter plasmid and cotransfected with different concentrations of Ets-1 into MDA-MB-231 cells. Transfection of the 1 kb CCR7 promoter region caused a 50-fold increase in promoter activity compared to that caused by the control plasmid. The promoter activity was further increased by cotransfection with different concentrations of Ets-1. The promoter activity increased by up to 120-fold when cells were transfected with 3 μg of Ets-1 expression plasmid (Figure 2A). These results showed that CCR7 is constitutively expressed in MDA-MB-231 cells and that overexpression of Ets-1 further upregulates CCR7 promoter activity.

We then mutated these 2 binding sites through site-directed mutagenesis and tested the Ets-1 transactivation activity. While Ets-1 cotransfected with the WT promoter increased CCR7 promoter activity by 5-fold, cotransfection with Ets-1 with a mutation in the binding site at the -72 region did not affect luciferase activity (Figure 2B). The Ets-1 transcriptional activity decreased by approximately 30% when cells were transfected with Ets-1 with a mutation in the binding site at the −32 region (Figure 2B). The transcriptional activity also showed a 30% reduction when both binding sites were mutated. These data suggest that Ets-1 binds to the -32 region of the CCR7 promoter region and enhances CCR7 promoter activity. To further confirm that Ets-1 binds to the CCR7 promoter region in vivo, we performed a chromatin immunoprecipitation (ChIP) assay to immunoprecipitate the CCR7 promoter region with an Ets-1 antibody using normal rabbit Ig as a background control. The samples were subjected to Q-PCR using primers to specifically amplify the -32 Ets-1 binding regions. The results showed an enrichment in anti-Ets-1-immunoprecipitated DNA containing the -32 Ets-1 binding site (Figure 2C).

To confirm the role of Ets-1 in contributing to CCR7 expression, we knocked down Ets-1 expression in MDA-MB-231 cells with siRNA. Ets-1 expression in the cells was completely

abolished upon transfection with 25 nM Ets-1 siRNA. However, CCR7 expression was reduced by approximately 70% after transfection with 100 nM Ets-1 siRNA, which also caused a complete knockdown of Ets-1 expression. The data suggest that a complete knockdown of Ets-1 expression does not abolish CCR7 expression (Figure 2D). Taken together, these data suggest that Ets-1 is sufficient but not necessary for CCR7 expression.

Ets-1 regulated CCR7 expression through a PNT domain-independent mechanism.

To examine the role of the PNT domain in CCR7 promoter activity, we deleted the PNT domain of Ets-1 (dp51 Ets-1) and tested its transcriptional activity. Similar to the effect of full-length Ets-1, cotransfection of dp51 Ets-1 enhanced CCR7 promoter activity. The enhancement of CCR7 promoter activity by dp51 Ets-1 was also concentration dependent (Figure 3A). These data suggest that Ets-1 enhances the CCR7 promoter through a PNT domain-independent mechanism.

~~Many proteins have been shown to play a role in CCR7 expression.~~ Among proteins that involve in CCR7 expression, Ets-1 has been shown to interact with the NF-κB protein. We then tested whether Ets-1, together with NF-κB, can enhance the transcriptional activity of the CCR7 promoter. Similar to the results of a previous report, transfection with NF-κB enhanced CCR7 promoter activity by 1.5-fold. Cotransfection of NF-κB with Ets-1 enhanced CCR7 promoter activity in a concentration-dependent manner. The promoter activity increased by up to 2-fold after transfection with 1 μg of Ets-1 (Figure 3B). These data indicate that Ets-1 interacts with NF-κB to regulate CCR7 expression.

Previous studies have shown that CBP interacts with and enhances Ets-1 transcriptional activity. We tested whether CBP could enhance the Ets-1 transcriptional activity on the CCR7 promoter. Transfection with 1 μg of CBP expression vector with Ets-1 resulted in 2.5-

fold enhancement of the transactivation of the CCR7 promoter. Furthermore, the coexpression of 2 μg of CBP led to a 2.8-fold enhancement in CCR7 promoter activity (Figure 3C). These data suggest that CBP can further enhance Ets-1 transcriptional activity on the CCR7 promoter.

Ets-1 modulates tumor cell migration.

~~CCR7 expression in tumor cells can aid in tumor cell metastasis to lymph nodes. The enhancement of CCR7 expression by Ets-1 could lead to tumor cell migration,~~ We cotransfected MDA-MB-231 cells with Ets-1 and GFP expression plasmids and measured the migration activity of the MDA-MB-231 cells. We used GFP to mark the Ets-1-overexpressing cells. We placed the transfected cells in the upper chamber of a transwell plate and counted the GFP+ cells in the lower chamber, which represented the migrating cells. The ~~results showed that the~~ number of GFP+ cells increased by 4-fold in the lower chamber when the cells were transfected with the Ets-1 expression plasmid. ~~We then tested whether a CCR7 ligand could enhance the migration of Ets-1 transfected cells.~~ The number of migrating cells increased when the CCR7 ligand CCL21 was added to the lower chamber, and the number of GFP+ cells increased 10-fold when 3 μg of Ets-1 was transfected (Figure 4A). We also examined the migration activity in a wound healing assay. The migration activity of MDA-MD-231 cells in CCL21 environment was decreased while knocking down Ets-1 expression (Figure 4B). These data clearly suggest that the expression level of Ets-1 affects tumor cell migration in a CCL21-containing environment.

DISCUSSION

In this study, we examined the role of Ets-1 in the regulation of CCR7 expression~~, which may lead to tumor metastasis~~. We found that Ets-1 directly binds to and transactivates CCR7

promoter activity. The mutation of Ets-1 binding sites on the CCR7 promoter or knockdown of Ets-1 expression in a tumor cell line altered CCR7 expression. Functionally, overexpression of Ets-1 enhanced cancer cell transmigration activity in a tumor cell migration assay. Taken together, our data suggest that Ets-1 may enhance CCR7 expression in tumor cells and help tumor cells infiltrate the lymph nodes.

We demonstrated that Ets-1 directly activated CCR7 promoter activity through a luciferase promoter assay. However, mutations in 2 conserved Ets-1 binding sites in the CCR7 promoter reduced CCR7 expression only by approximately 30%. The reduced activity was similar while we mutated the -32 Ets-1 binding site (B site in Figure 2B). The data suggested the -32 is the major Ets-1 binding site in controlling CCR7 expression. These data are consistent with the results of the Ets-1 knockdown experiment, which showed that the complete knockdown of Ets-1 expression only partially affected CCR7 protein expression. We also found a similar regulation of CCR7 expression in T cells. CCR7 expression was unaffected in unstimulated Ets-1-deficient T cells. However, CCR7 was downregulated in Ets-1-deficient T cells upon T-cell stimulation (Figure 1). Collectively, these data suggest that Ets-1 may not play a role in basal CCR7 expression.

~~Our data show that the deletion of the PNT domain of Ets-1 does not reduce the transcriptional activity of Ets-1 on the CCR7 promoter. The PNT domain of Ets-1 can interact with ERK and enhance Ets-1 activity. While CCR7 expression in thymocytes requires ERK activity, we showed that Ets-1 could still enhance CCR7 even if the PNT domain was deleted. These data suggest not only that Ets-1 enhances CCR7 expression through a PNT domain-independent pathway but also that ERK is not involved in the enhancement of CCR7 expression by Ets-1.~~

~~Cotransfection with NF-κB also enhanced the Ets-1 transactivation of the CCR7 promoter.~~

~~A previous study has shown that Ets-1 interacts with NF-κB through the DNA binding domain. Such data suggest that Ets-1 enhances CCR7 expression through the DNA binding domain along with other transcription factors. Other transcription factors, such as NFAT and AP-1, have been shown to enhance CCR7 expression. Whether these transcription factors regulate CCR7 expression with Ets-1 requires further investigation.~~

Our data show that Ets-1 controls CCR7 expression in MDA-MB-231 cells. MDA-MDA-MB-231 is a highly metastatic and triple negative (ER-, PR- and HER2-negative) basal type breast cancer cell line (16, 17). The constitutive expression of Ets-1 in this cell line is well correlated with CCR7 expression and a highly metastatic phenotype. Our data also show that the overexpression of Ets-1 in MDA-MB-231 cells further enhanced CCR7 expression and cell migration activity (Figure 1D and Figure 4). Ets-1 is an oncogene and plays a role in the epithelial-to-mesenchymal transition (EMT) (18, 19). The transwell migration assay showed that the overexpression of Ets-1 in tumor cells also enhanced tumor migration to an environment containing CCL21. In summary, our data suggest that Ets-1 plays multiple roles during cancer development. The overexpression of Ets-1 during cancer development may contribute to the EMT and to CCR7 overexpression, which aids in tumor cell migration. The downregulation of Ets-1 in tumor therapy may be a promising target to block the EMT and tumor metastasis.

MATERIALS AND METHODS

Mice

Ets-1 KO mice were described previously (11). Heterozygous littermates were used as controls. All animals were housed under specific pathogen-free conditions, and experiments

were performed in accordance with the institutional guidelines for animal care at E-Da University under approved protocols (IACUC-105001).

Quantitative RNA analysis

Total RNA was purified using a TRIzol Plus RNA Purification Kit (Invitrogen). First-strand cDNA synthesis was performed on 200 ng of total RNA using a QuantiTect Reverse Transcription Kit (QIAGEN). Gene expression levels were determined by real-time PCR analysis performed using a PowerUp SYBR Green Q-PCR Kit (Applied Biosystem) according to the manufacturer's protocol on an 7500 Real Time PCR system (Applied Biosystem) using the following cycling conditions: denaturation at 95°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 30 s. Primer sets were designed using the Primer3 web utility. The mRNA levels were adjusted for differences in actb (β-actin) or RPII (RNA polymerase II) expression.

Transwell migration assay

A CytoSelect^{TM} 24-well cell migration assay (Cell Biolab, San Diego, CA, USA) was used to examine cell migration activity. A total of 1 x 10^5 MDA-MB-231 cells transfected with pcDNA4/GFP or Est-1/GFP vectors in 200 μL of serum-free medium were placed in the upper chamber, and the insert was placed on the lower well, which held 500 μL of complete medium containing 10% fetal bovine serum with or without 100 ng/mL CCL21. After incubation for 48 hours, the nonmigratory cells were removed from the upper surface of the membrane by scrubbing with wet cotton-tipped swabs 2-3 times. GFP-positive cells that migrated through the polycarbonate membrane and those climbing the bottom side were photographed. Four random images of each insert were acquired using fluorescence microscopy at 40x magnification. The migrated GFP-positive cells in each image were counted. The experiments were performed in triplicate.

Wound Healing assay

The migratory capacity of MDA-MB-231 cells were examined using the Culture-Insert 2 Well (80209; Ibidi) according to the manufacturer's instructions. 2.1 x 10^4 cells were suspended in 70ul medium and incubated in each chamber overnight. After cell attachment, the culture insert was gently removed by tweezers, leaving a cell-free 500 µm gap. Fresh DMEM medium with CCL-21 (100ng/ml) was added. Migration of cells were monitored and pictures were acquired after 40 hours. Pictures were analyzed and migration area was calculated with MRI Wound Healing Tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool) in ImageJ.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed according to the protocol of the Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (#17-10085, EMD Millipore, Billerica, MA, USA). For each sample, 1 × 10^7 cells were fixed in 1% freshly prepared formaldehyde for 10 min at room temperature, and glycine (125 mM) was added to quench the unreacted formaldehyde for 5 min. Then, the cells were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.2) and lysed in cell lysis buffer (5 mM PIPES, pH 8.0; 85 mM KCl; 0.5% NP-40) for 15 min on ice. After centrifugation, the cell pellet was resuspended in nuclear lysis buffer (50 mM Tris-Cl, pH 8.1; 10 mM EDTA; 1% SDS). Cross-linked DNA was sheared with a Misonix Sonicator® 3000 (Qsonica, Newtown, CT, USA) in six 15 s pulses with 50 s rest between pulses and a power setting of 6. The sheared chromatin was separated from debris by centrifugation at 10,000 x g for 10 min at 4°C. A total of 1 x 10^6 cell equivalents of chromatin were used for immunoprecipitation. The chromatin was well mixed with 1 µg of the indicated antibody and protein A/G magnetic beads and incubated for 4 hours at 4°C with rotation. The chromatin-antibody-protein A/G complex was separated with a magnetic separator and

washed. The complex was then digested with proteinase K at 62°C for 2 hours, and the beads were separated with a magnetic separation device. The DNA was purified with a spin column in preparation for PCR.

Plasmids, transfection, and luciferase assay

An approximately 2 kb genomic fragment containing nucleotides +28 to -1964 in relation to the transcriptional start site of the human CCR7 gene was amplified and cloned into the BglII site of the pGL3-Basic Vector (Promega). In all luciferase assays, cells were transfected with 2 μg of luciferase reporter and 0.5 μg of pTK-Renilla with Lipofectamine 2000 (Invitrogen). Luciferase activity was determined in duplicate with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The firefly luciferase activity obtained from each sample was normalized to the Renilla luciferase activity from the same sample.

Western blot analysis and antibodies

In each sample, cells were lysed in freshly prepared whole-cell lysis buffer. Cell lysate was separated from debris by centrifugation at 13,000 rpm for 10 min. The lysate was loaded onto 10% polyacrylamide gels and transferred onto PVDF membranes (PolyScreen, PerkinElmer). The membranes were subsequently blocked in 5% milk and probed with an anti-Ets-1 or anti-CCR7 antibody. Proteins were visualized using an enhanced chemiluminescence (ECL) kit (PerkinElmer).

Statistical analysis

Statistical analyses were performed with Student's t test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

ACKNOWLEDGMENTS

This study was supported by research grants from E-Da Hospital (EDAHT-103030, EDAHT-104046, EDPJ-106084 and EDAHI-106001), Taiwan.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1 CCR7 expression is regulated by Ets-1. (A) Splenocytes from B6 mice were activated with CD3/CD28 for the indicated time periods. The cells were harvested, and the CCR7 expression level was examined. **(B)** Splenocytes from Ets-1 heterozygous (Het) or knockout (KO) mice were left untreated (white bar) or were activated with CD3/CD28 (black bar) for 5 hours. The cells were harvested, and the CCR7 expression level was examined. MDA-MB-231 cells were transfected with the indicated amounts of Ets-1 DNA. The cells were harvested after 24 hours, and the CCR7 mRNA **(C)** and protein **(D)** expression are shown.

Figure 2 Ets-1 binds to the CCR7 promoter and controls its expression. (A) MDA-MB-231 cells were transfected with the indicated plasmids. The cells were harvested after 24 hours, and the promoter activity was examined by luciferase assay. **(B)** The 2 Ets-1 binding sites on the CCR7 promoter region are shown. The sites were either mutated separately (MuA or MuB) or were both mutated (MuA/B). The promoter activity was examined by luciferase assay and is shown in the lower panel. **(C)** MDA-MD-231 cells were subjected to ChIP analysis. Chromatin was precipitated with an anti-Ets-1 antibody or an IgG antibody as a negative control. The precipitated DNA was amplified using primers (Figure 2B, arrows) specific for a 220 bp fragment of the CCR7 promoter spanning the -32 Ets-1 binding sites. As a positive control, total DNA was diluted 1:100 and subjected to PCR (input). (D) MDA-MB-231 cells were transfected with the indicated amounts of Ets-1 siRNA and harvested after 24 hours. Whole-cell lysates were prepared and subjected to Western blot analyses with the indicated antibodies.

Figure 3 Control of CCR7 expression by Ets-1. (A) MDA-MB-231 cells were transfected with a reporter plasmid containing the wild-type human CCR7 promoter or an

empty vector plasmid (pGL3). The cells were cotransfected with various concentrations of plasmid containing Ets-1 with the Pointed domain deleted (dp51). The promoter activity was examined by luciferase assay. **(B)** MDA-MB-231 cells were transfected with a reporter plasmid containing the wild-type human CCR7 promoter and a NF-κB expression plasmid (p65). Additionally, the cells were transfected with various amounts of Ets-1 expression plasmids. The promoter activity was examined by luciferase assay. **(C)** MDA-MB-231 cells were transfected with a reporter plasmid containing the wild-type human CCR7 promoter and an Ets-1 expression plasmid. Additionally, the cells were transfected with various amounts of CBP expression plasmids. The promoter activity was examined by luciferase assay.

Figure 4 Ets-1 modulates tumor metastasis. (A) MDA-MB-231 cells were cotransfected with Ets-1 and GFP. After transfection, the cells were added to the upper chamber of a transwell plate. The lower chamber was filled with medium without CCL21 (white bar) or with CCL21 (black bar). After 48 hours, the GFP+ cells in the lower chamber were quantified. **(B)** MDA-MB-231 cells were transfected with indicated siRNA. Cells were then treated with CCL21 and subjected to wound healing assay. Pictures were acquired at 40 hours. Migration area was calculated with MRI wound healing tool of Image J software and expressed as percentage change of the Ets-1 siRNA vs Non-target control (NT-SiRNA).

REFERENCES

1. Vela M, Aris M, Llorente M, Garcia-Sanz JA and Kremer L (2015) Chemokine receptor-specific antibodies in cancer immunotherapy: achievements and challenges. Front Immunol 6, 12
2. Zlotnik A (2004) Chemokines in neoplastic progression. Semin Cancer Biol 14, 181-185
3. Chen K, Bao Z, Tang P, Gong W, Yoshimura T and Wang JM (2018) Chemokines in homeostasis and diseases. Cell Mol Immunol 15, 324-334
4. Al Akoum C, Akl I, Rouas R et al (2015) NFAT-1, Sp-1, Sp-3, and miR-21: New regulators of chemokine C receptor 7 expression in mature human dendritic cells. Hum Immunol 76, 307-317
5. Muller A, Homey B, Soto H et al (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410, 50-56
6. Wang J, Xi L, Hunt JL et al (2004) Expression pattern of chemokine receptor 6 (CCR6) and CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype. Cancer Res 64, 1861-1866
7. Ma H, Gao L, Li S et al (2015) CCR7 enhances TGF-beta1-induced epithelial-mesenchymal transition and is associated with lymph node metastasis and poor overall survival in gastric cancer. Oncotarget 6, 24348-24360
8. Somovilla-Crespo B, Alfonso-Pérez M, Cuesta-Mateos C et al (2013) Anti-CCR7 therapy exerts a potent anti-tumor activity in a xenograft model of human mantle cell lymphoma. J Hematol Oncol 6, 89
9. Moran TP, Nakano H, Kondilis-Mangum HD, Wade PA and Cook DN (2014) Epigenetic Control of Ccr7 Expression in Distinct Lineages of Lung Dendritic Cells. The Journal of Immunology 193, 4904-4913

10. Mburu YK, Egloff AM, Walker WH et al (2012) Chemokine receptor 7 (CCR7) gene expression is regulated by NF-kappaB and activator protein 1 (AP1) in metastatic squamous cell carcinoma of head and neck (SCCHN). J Biol Chem 287, 3581-3590

11. Grenningloh R, Kang BY and Ho IC (2005) Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. J Exp Med 201, 615-626

12. Garrett-Sinha LA (2013) Review of Ets1 structure, function, and roles in immunity. Cell Mol Life Sci 70, 3375-3390

13. Dittmer J (2015) The role of the transcription factor Ets1 in carcinoma. Semin Cancer Biol 35, 20-38

14. Schwendemann J, Choi C, Schirrmacher V and Beckhove P (2005) Dynamic differentiation of activated human peripheral blood CD8+ and CD4+ effector memory T cells. J Immunol 175, 1433-1439

15. Bosman JD, Yehiely F, Evans JR and Cryns VL (2010) Regulation of alphaB-crystallin gene expression by the transcription factor Ets1 in breast cancer. Breast Cancer Res Treat 119, 63-70

16. Betapudi V, Licate LS and Egelhoff TT (2006) Distinct roles of nonmuscle myosin II isoforms in the regulation of MDA-MB-231 breast cancer cell spreading and migration. Cancer Res 66, 4725-4733

17. Neve RM, Chin K, Fridlyand J et al (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10, 515-527

18. Li C, Wang Z, Chen Y et al (2015) Transcriptional silencing of ETS-1 abrogates epithelial-mesenchymal transition resulting in reduced motility of pancreatic cancer cells. Oncol Rep 33, 559-565

19. Gilles C, Polette M, Birembaut P, Brunner N and Thompson EW (1997) Expression of c-ets-1 mRNA is associated with an invasive, EMT-derived phenotype in breast

carcinoma cell lines. Clin Exp Metastasis 15, 519-526

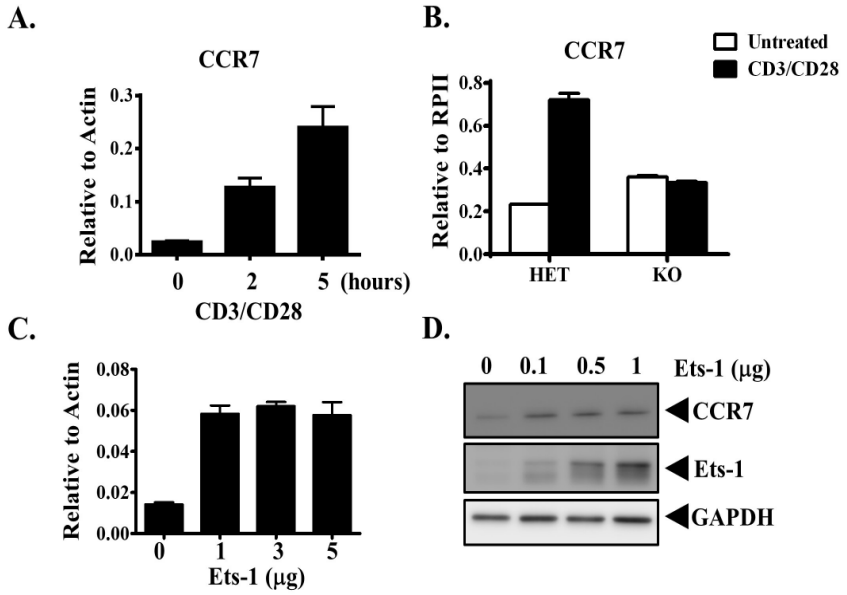

Figure 1

Fig. 1.

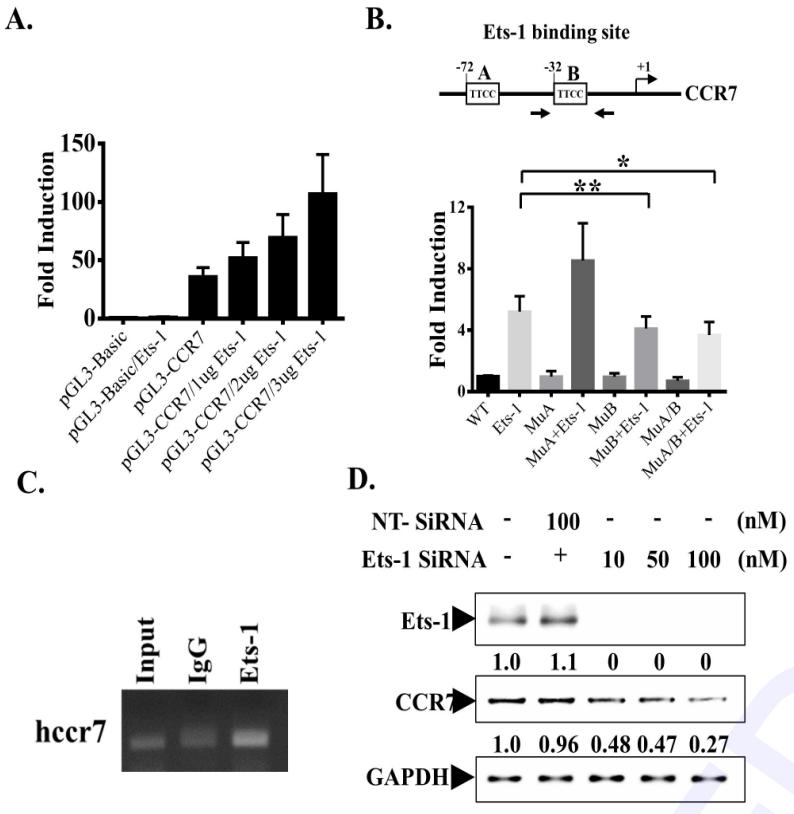

Figure 2

Fig. 2.

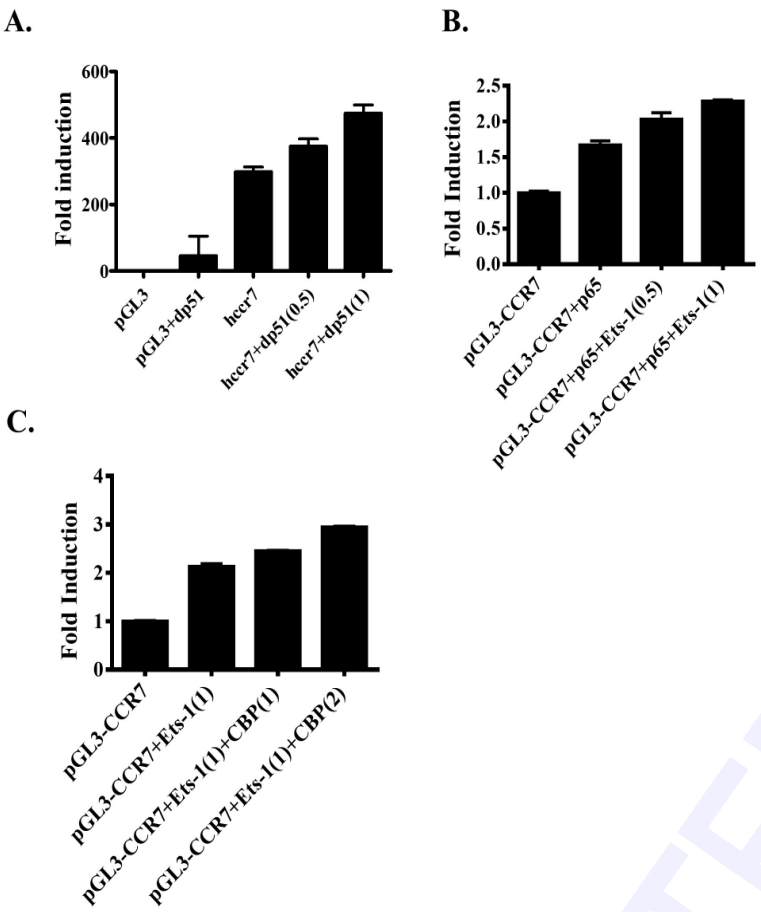

Figure 3

Fig. 3.

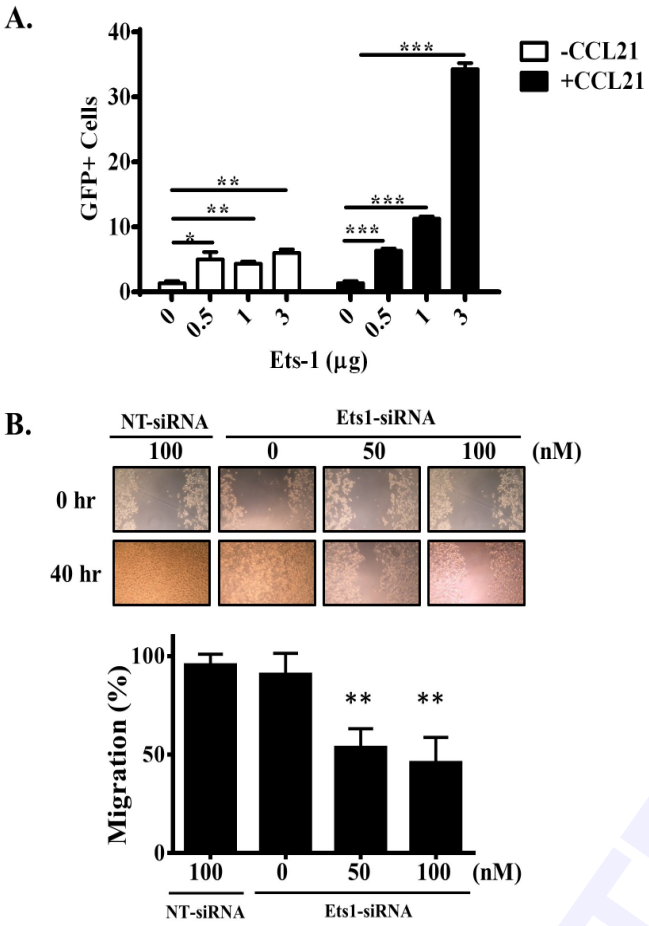

Figure 4

Fig. 4.