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**Autonomous and intercellular chemokine signaling elicited from mesenchymal stem cells regulates migration of undifferentiated gastric cancer cells**

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**Key words:** Gastric cancer, Tumor microenvironment, Mesenchymal stem cell, Ror2-mediated signaling, CXCL16-CXCR6 signaling, CCL5-CCR1/3 signaling

## **Abstract**

Accumulating evidence demonstrates that bone marrow (BM)-derived mesenchymal stem cells (MSCs) play critical roles in regulating progression of various types of cancer. We have previously shown that Wnt5a-Ror2 signaling in MSCs induces expression of CXCL16, and that CXCL16 secreted from MSCs then binds to its cognate receptor CXCR6 on the surface of an undifferentiated gastric cancer cell line MKN45 cells, eventually leading to proliferation and migration of MKN45 cells. However, it remains unclear about a possible involvement of another (other) cytokine (s) in regulating progression of gastric cancer. Here, we show that CXCL16-CXCR6 signaling is also activated in MSCs through cell-autonomous machinery, leading to up-regulated expression of CCL5. We further show that CCR1 and CCR3, receptors of CCL5, are expressed on the surface of MKN45 cells, and that CCL5 secreted from MSCs promotes migration of MKN45 cells presumably via its binding to CCR1/CCR3. These data indicate that cell-autonomous CXCL16-CXCR6 signaling activated in MSCs up-regulates expression of CCL5, and that subsequent activation of CCL5-CCR1/3 signaling in MKN45 cells through intercellular machinery can promote migration of MKN45 cells. Collectively, these findings postulate the presence of orchestrated chemokine signaling emanated from MSCs to regulate progression of undifferentiated gastric cancer cells.

## **Introduction**

Gastric cancer is third leading cause of cancer-related death worldwide (Bray *et al.* 2018), and thus it is important to understand how progression of gastric cancer is regulated. Growing evidence demonstrates that tumor microenvironment (TME) plays critical roles in progression of various types of cancers, including gastric cancer (Hanahan & Weinberg 2011; Yang *et al.* 2021). In addition to cancer cells, TME consists of other types of cells, including tumor associated macrophages (TAMs), cancer associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs), and these cells secrete

chemokines thereby contributing to proliferation, migration, survival and metastasis of cancer cells (Reyes *et al.* 2020). Chemokines bind to their cognate receptors in cell-autonomous and/or intercellular manners, thereby regulating various signaling pathways mediated by PI3K, MAPK and Rho family of signaling molecules, eventually leading to progression of cancer cells (Nagarsheth *et al.* 2017; Reyes *et al.* 2020). Therefore, it is of importance to understand the molecular mechanisms how chemokine-mediated signaling promotes progression of gastric cancer.

Receptor tyrosine kinase-like orphan receptor 2 (Ror2) acts as a receptor for Wnt5a to elicit non-canonical Wnt signaling ( $\beta$ -catenin-independent Wnt signaling), thereby regulating cell polarity, migration, proliferation, and differentiation (Endo *et al.* 2015; Kamizaki *et al.* 2021). It has been shown that Wnt5a and Ror2 are highly expressed in various types of cancer cells, resulting in activation of Wnt5a-Ror2 signaling, eventually leading to progression of cancers (Enomoto *et al.* 2009; Nishita *et al.* 2010; O'Connell *et al.* 2013). On the other hand, it has been reported that Ror2 is also expressed in stromal cells in pancreatic and ovarian cancers, and that higher expressions of Ror2 in stromal cells are associated with poor prognosis (Huang *et al.* 2015; Henry *et al.* 2017). We have previously shown that constitutive Wnt5a-Ror2 signaling in bone marrow (BM)-derived primary human MSCs induces expression of CXCL16, and subsequently CXCL16 secreted from MSCs binds to its cognate receptor CXCR6 on the surface of an undifferentiated gastric cancer cell line, MKN45 cells, resulting in the promotion of proliferation of MKN45 cells (Takiguchi *et al.* 2016). We have further shown that CXCL16, secreted from MSCs, promotes proliferation and migration of MKN45 cells by inducing expression of Ror1, another member of Ror-family of receptor, via activation of STAT3 (Ikeda *et al.* 2020). These data indicate CXCL16-CXCR6 signaling, activated through an intercellular molecular machinery, in gastric cancer cells might be critical for the progression of at least some types of gastric cancers. On the other hand, in prostate and breast cancers, CXCL16 secreted from cancer cells binds to CXCR6 on the surface of MSCs, leading to progression of these cancer cells through up-regulated

expression of CXCL12 and CXCL10 in MSCs, respectively (Jung *et al.* 2013; Chaturvedi *et al.* 2014). Therefore, it can be envisaged that CXCL16-CXCR6 signaling might also be activated in MSCs and contribute to the progression of gastric cancer cells through an intercellular molecular machinery. However, it remains uncovered whether or not CXCL16-CXCR6 signaling in MSCs can also promote progression of gastric cancer cells.

Here, we show that, like CXCL16, expression of another chemokine CCL5 is also up-regulated by Ror2-mediated signaling in BM-derived MSCs. Interestingly, it was found that constitutive CXCL16-CXCR6 signaling in MSCs is required for up-regulated expression of CCL5. We further show that CCR1 and CCR3, receptors for CCL5, are expressed on the surface of MKN45 cells, and CCL5 secreted from MSCs can promote migration of MKN45 cells through its binding to CCR1 and CCR3. These data indicate that cell-autonomous CXCL16-CXCR6 signaling activated by Ror2-mediated signaling up-regulates expression of CCL5 in MSCs, and subsequent activation of CCL5-CCR1/3 signaling in MKN45 cells through an intercellular machinery can promote migration of MKN45 cells.

## **Results and Discussion**

### **Ror2-mediated signaling is required for up-regulated expression of CCL5 and CXCL16 in bone marrow-derived human MSCs and UE6E7T-12 cells.**

We have previously shown that endogenous Wnt5a-Ror2 signaling in BM-derived primary human MSCs induces expression of CXCL16, and CXCL16 secreted from MSCs binds to CXCR6 on surface of MKN45 cells, undifferentiated gastric cancer cell line, thereby promoting proliferation of MKN45 cells (Takiguchi *et al.* 2016). We have further shown that CXCL16-CXCR6 signaling in MKN45 cells induces expression of Ror1 through activation of STAT3, eventually leading to proliferation and/or progression of MKN45 cells as assessed by *in vitro* and *in vivo* analyses (Ikeda *et al.* 2020). However, it remains unclear whether other chemokines are also up-regulated by endogenous Wnt5a-Ror2

signaling in BM-derived MSCs and can promote progression of MKN45 cells.

To clarify these issues, we carried out a human chemokine array analysis using conditioned media from MSCs treated with either control siRNA (si-Ctrl) or *Ror2* siRNA (si-*Ror2* #3) as described in Experimental procedures. We first assessed knockdown efficiency of si-*Ror2* #3 (Figure 1a), and then conditioned media from MSCs treated with si-Ctrl or si-*Ror2* #3 were subjected to chemokine array analysis. Under our experimental setting where *Ror2* expression was suppressed significantly (~60%), protein expression levels of CCL5 (orange boxed spots in Figure 1b) and CXCL16 (blue boxed spots in Figure 1b) were found to be decreased apparently (Figure 1b-d). Since BM-derived primary human MSCs are somewhat difficult to handle with due to their functional alterations depending on their passages, we decided to use immortalized human MSCs UE6E7T-12 cells (Mori *et al.* 2005) in our following experiments. Consistent with our findings using BM-derived primary human MSCs, *Ror2* knockdown in UE6E7T-12 cells resulted in suppressed expression of *CCL5* and *CXCL16* (Figure 1e). The findings indicate that *Ror2*-mediated signaling is required for up-regulated expression of *CCL5* and *CXCL16* in both BM-derived primary human MSCs and UE6E7T-12 cells.

#### **CXCL16-CXCR6 signaling is required for up-regulated expression of *CCL5* in UE6E7T-12 cells.**

It has been shown that chemokines elicit various cellular responses through intercellular and/or cell-autonomous chemokine signaling (Tokunaga *et al.* 2018). Although CXCL16, secreted from MSCs, has been shown to promote proliferation and migration of MKN45 cells by acting on its cognate receptor CXCR6 expressed on the cell surface of MKN45 cells (Takiguchi *et al.* 2016; Ikeda *et al.* 2020), it remains unclear whether or not CXCL16, secreted from MSCs, can also act on MSCs in an autocrine manner, thereby contributing to proliferation and/or migration of MKN45 cells. We thus examined whether secreted CCL5 and/or CXCL16, induced by *Ror2*-mediated signaling in UE6E7T-12 cells, can also exert on UE6E7T-12 cells in an autocrine manner. For this purpose, we first examined

whether or not chemokine receptors for CCL5 and CXCL16 are expressed apparently on the cell surface of UE6E7T-12 cells by flow cytometric analysis. As shown in Figure 2a & b, expression of CXCR6 (a receptor for CXCL16) was clearly detectable on UE6E7T-12 cells, while we failed to detect any apparent expression of receptors for CCL5, CCR1, CCR3 and CCR5, on UE6E7T-12 cells.

Therefore, it can be envisaged that constitutive CXCL16-CXCR6 signaling in UE6E7T-12 cells might affect cellular function of UE6E7T-12 cells. As an attempt to test this possibility, we examined the effect of inhibition of CXCL16-CXCR6 signaling on expression of *CCL5* in UE6E7T-12 cells. Interestingly, suppressed expression of *CXCL16* in UE6E7T-12 cells by transfection with *CXCL16* siRNAs (si-*CXCL16* #1 and #2) resulted in the inhibition of *CCL5* expression in the cells (Figure 2c). We have previously shown that Wnt5a and Ror2 are expressed in UE6E7T-12 cells, and as a result constitutively Wnt5a-Ror2 signaling, elicited by cell-autonomous (or autocrine) machinery, can induce expression of CXCL16 in UE6E7T-12 cells (Takiguchi *et al.* 2016; Ikeda *et al.* 2020). We thus examined whether or not secreted CXCL16 from UE6E7T-12 cells can affect expression of Wnt5a or Ror2 in the cells. It was found that suppressed expression of *CXCL16* failed to affect expression of Wnt5a and Ror2, respectively, in UE6E7T-12 cells (Figure S1). These findings indicate that CXCL16-CXCR6 signaling induces expression of *CCL5* in UE6E7T-12 cells without affecting Wnt5a-Ror2 signaling (or expression of Wnt5a and Ror2). At present, it remains unclear about the mechanism how CXCL16-CXCR6 signaling can induce expression of *CCL5*. Future study will be required to clarify this issue.

#### **CCL5-CCR1/3 signaling promotes migration of MKN45 cells.**

Although accumulating evidence has demonstrated that CCL5 plays a crucial role in progression of various types of cancer cells (Aldinucci & Colombatti 2014), it is not fully understood whether CCL5 secreted from UE6E7T-12 cells is responsible for proliferation and/or migration of MKN45 cells. Thus,

we first examined that expression levels of chemokine receptors for CCL5, CCR1, CCR3, and CCR5, on the surface of MKN45 cells by flow cytometric analysis. It was found that expression of CCR1 and CCR3, but not CCR5, can be detected apparently on the surface of MKN45 cells (Figure 3a, b). We next examined whether or not proliferation of MKN45 cells can be promoted by stimulation with recombinant CCL5. As shown, stimulation of MKN45 cells with CCL5 failed to promote growth rate of MKN45 cells (Figure S2). We further investigated whether or not migration of MKN45 cells can be promoted by stimulation with recombinant CCL5. It was found that CCL5 can promote migration of MKN45 cells (Figure 3c). Since CCR1 and CCR3 are expressed on the surface of MKN45 cells (Figure 3a, b), we next examined whether or not suppressed expression of either *CCR1* or *CCR3* can inhibit CCL5-induced migration of MKN45 cells. It was found that suppressed expression of either *CCR1* or *CCR3* can inhibit almost completely CCL5-induced migration of MKN45 cells (Figure 3d). With this respect, we also noticed that suppressed expression of *CCR1* or *CCR3* in MKN45 cells resulted in decreased expression of CCR3 or CCR1, respectively (Figure S3). Although CCR1 and CCR3 are related gene products, siRNA sequences against *CCR1* and *CCR3* genes are obviously distinct and thus off-target effects of these siRNAs are unlikely, Further study will be required to clarify the molecular basis explaining the effects of si-*CCR1* or si-*CCR3* on CCR3 or CCR1, respectively. Nevertheless, these findings clearly indicate that CCL5 secreted from UE6E7T-12 cells can promote migration of MKN45 cells through CCR1 and CCR3.

We have previously shown that CXCL16 secreted from MSCs binds to CXCR6 expressed on the surface of MKN45 cells, thereby promoting migration and proliferation of MKN45 cells (Figure S4 Upper panel) (Takiguchi *et al.* 2016; Ikeda *et al.* 2020). Our present study shows that cell-autonomous CXCL16-CXCR6 signaling regulated by Ror2-mediated signaling up-regulates expression of CCL5 in BM-derived MSCs, and CCL5 secreted from MSCs then promotes migration of MKN45 cells through CCR1 and CCR3 (Figure S4 Lower panel). Collectively, these data indicate that cell-



autonomous and intercellular CXCL16-CXCR6 signaling elicited from MSCs promotes progression of MKN45 cells (Figure S4). However, it remains unclear whether or not bidirectional chemokine signaling exists between MSCs and gastric cancer cells, i.e. another (other) chemokine(s) secreted from gastric cancer cells might regulate functions of MSCs, affecting progression of gastric cancer. Further study will be required to elucidate an entire view of chemokine signaling between MSCs and gastric cancer cells.

## Experimental procedures

### Cell culture and transfection

MKN45 cells, which express luciferase stably, were purchased from JCRB cell bank (Osaka, Japan) and cultured in RPMI-1640 medium (Nacalai Tesque, Tokyo, Japan) containing 10% fetal bovine serum (FBS). The short-tandem repeat profile of MKN45 cells was analyzed (BEX CO., LTD, Tokyo, Japan), and we confirmed that MKN45 cells were not contaminated. Primary human MSCs derived from BM were obtained from Lonza (Basel, Switzerland) and maintained in MSCGM (Lonza) and used up to 5 passages. UE6E7T-12 cells, human BM-derived MSCs that were immortalized by infection with recombinant retroviruses expressing the E6, E7 and hTERT, were kindly provided by Dr. H. Yokozaki (Kobe University) and maintained in RPMI-1640 medium containing 10% FBS. All cells were incubated at 37 °C with 5% CO<sub>2</sub> and 90% humidity.

Cells were transfected with the respective siRNAs by using Lipofectamine RNAiMax (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Briefly, 30 nmol/L siRNAs were mixed with the RNAiMax reagent diluted in Opti-MEM (Thermo Fisher Scientific), incubated for 20 minutes at room temperature, and added to cultured cells. The sequences of the siRNAs used were as follows: si-*Ror2* #1, 5'-GCAACCUUCCAACUACAATT-3' (sense) and 5'-UUGUAGUUGGAAAGGUUGCTT-3' (anti-sense); si-*Ror2* #2, 5'-

GCAAUGUGCUAGUGUACGAUU-3' (sense) and 5'-UCGUACACUAGCACAUUGCUU-3' (anti-sense) (Sigma-Aldrich, MO, USA); si-*Ror2* #3, 5'-AACAGGUGACCUUUGUAGACUUUCC-3' (sense) and 5'-GGAAAGUCUACAAAGGUCACCUUU-3' (anti-sense) (Invitrogen, MA, USA); si-*CXCL16* #1, 5'-CUCACUCGUCCCAAUGAAATT-3' (sense) and 5'-UUUCAUUGGGACGAGUGAGTT-3' (anti-sense); si-*CXCL16* #2, 5'-GAGCUUACCAUCGGUGUCUTT-3' (sense) and 5'-AGACACCGAUGGUAAGCUCTT-3' (anti-sense); si-*CCR1* #1, 5'-GCUGUUUCAGGCUCUGAAA-3' (sense) and 5'-UUUCAGAGCCUGAAACAGC-3' (anti-sense); si-*CCR1* #2, 5'-CAAAGCUGUCCGUUUGAUU-3' (sense) and 5'-AAUCAACGGACAGCUUUG-3' (anti-sense); si-*CCR3* #1, 5'-CUCCGAAUUAUGACCAACA-3' (sense) and 5'-UGUUGGUCAUAAUUCGGAG-3' (anti-sense); si-*CCR3* #2, 5'-GGAGAUGAAGCAAACACAU-3' (sense) and 5'-AUGUGUUUGCUUCAUCUCC-3' (anti-sense) (Sigma-Aldrich).

### **Human cytokine array**

Conditioned media obtained from MSCs, treated with either control siRNA or *Ror2* siRNA (#3), were analyzed by using the Proteome Profiler Human Cytokine Array Kit (ARY017; R&D Systems, MN, USA), according to the manufacturer's instructions. Briefly, array membranes, bound with antibodies against a series of specific target cytokines, were incubated with respective conditioned media pretreated with biotinylated detection antibodies for 24 hours at 4 °C. Membranes were subsequently washed and incubated with streptavidin-HRP for 30 minutes at room temperature. After washing the membranes, captured proteins on the membranes were detected by using chemiluminescent detection reagents. The relative band intensities were determined by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### **Proliferation assay**

Proliferation assay was carried out using 96 well plates (Corning, NY, USA). MKN45 cells ( $1.0 \times 10^3$  cells in 100  $\mu$ L medium) were seeded in the respective wells, using RPMI-1640 medium containing 10% FBS. MKN45 cells were treated with recombinant human CCL5 (at a final concentration of 20 ng/mL; Peprotech, NJ, USA). After 3, 5, and 10 days of culture, cells in the well were incubated with fresh medium containing 10% WST-8 (Dojindo, Kumamoto, Japan) for 90 minutes at 37°C. Subsequently, the absorbance of the culture supernatant from each well was measured at 450 nm using the EnSpire Multimode Plate Reader (Perkin Elmer, MA, USA).

### **Migration assay**

Migration assay was conducted as described previously (Enomoto *et al.* 2009). Transwells with 8- $\mu$ m  $\phi$  pore size membranes were coated with 10  $\mu$ g/mL fibronectin (Sigma-Aldrich). 600  $\mu$ L of RPMI-1640 medium containing recombinant human CCL5 was loaded in the lower chamber. MKN45 cells ( $2.0 \times 10^4$  cells) were suspended in 100  $\mu$ L RPMI-1640 medium and loaded in the upper chamber. After 24 hours incubation, cells migrated to the lower surface of the membrane were stained with DAPI and observed by using fluorescence microscope BZ-X710 (Keyence, Osaka, Japan). The number of DAPI-stained cells was counted by using ImageJ software.

### **Flow cytometric analysis**

MKN45 cells and UE6E7T-12 cells were collected and resuspended in PBS containing 2% FBS, and incubated with the respective antibodies for 1 hour on ice. The antibodies used were as follows: anti-CCR1 antibody (PE-conjugated; cat: 362903; clone: 5F10B29; BioLegend, CA, USA); anti-CCR3 antibody (PE-conjugated; cat: 561746; clone: 5E8; BD Biosciences, NJ, USA); anti-CCR5 antibody (PE-conjugated; cat: 561747; clone: 2D7; BD Biosciences); anti-CXCR4 antibody (PE-conjugated;

cat: FAB170P; clone: 12G5; R&D Systems); anti-CXCR6 antibody (unconjugated; cat: MAB699; clone: 56811; R&D Systems); anti-CXCR7 antibody (PE-conjugated; cat: 391403; clone: 10D1-J16; BioLegend). In case of anti-CXCR6 staining, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Invitrogen). Flow cytometric analysis was performed by using the BD LSRFortessa™ X-20 (BD Biosciences).

### **RNA isolation and quantitative RT-PCR**

RNA isolation, reverse transcription, and quantitative RT-PCR analysis were carried out as described previously (Avincsal *et al.* 2021). Total RNAs were isolated by using Sepasol-RNA (Nacalai Tesque, Tokyo, Japan). Isolated RNAs were reverse transcribed by using PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Expression levels of the respective genes of interest were analyzed using LightCycler 480 II system (Roche Diagnostics, Basel, Switzerland). The amounts of mRNAs were normalized relative to that of *18S ribosomal RNA*. The following primers were used: *Ror2*, 5'-CAATTCCACTGGTCATCGCT-3' (forward) and 5'-TGAGGGGCATTTCCATGTC-3'(reverse); *CCL5*, 5'-CAGTCGTCTTTGTCACCCGA-3' (forward) and 5'-CGGGTGGGGTAGGATAGTGA-3' (reverse); *CXCL16*, 5'-TCTCCAGATCTGCCGGTTCA-3' (forward) and 5'-CCTACCAATGTTGTCAGGGGT-3' (reverse); *Wnt5a*, 5'-TAAGCCCAGGAGTTGCTTTG-3' (forward) and 5'-GCAGAGAGGCTGTGCTCCTA-3' (reverse) (Invitrogen); *18S ribosomal RNA*, 5'-ATGGCCGTTCTTAGTTGGTG-3' (forward) and 5'-CGCTGAGCCAGTCAGTGTAG-3' (reverse) (Invitrogen).

### **Statistical analysis**

Experimental data were analyzed by using GraphPad Prism 5.0 (GraphPad Software, CA, USA). Statistical significance was determined as \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  using Student's *t* test

when two groups were compared, or using one-way ANOVA followed by Dunnett's comparison test or Tukey's honest significance difference (HSD) test when more than three groups were analyzed.

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## Figure legends

**Figure 1. Ror2-mediated signaling is required for up-regulated expression of CCL5 and CXCL16 in bone marrow-derived human MSCs and UE6E7T-12 cells.** (a-d) Bone marrow (BM)-derived primary human MSCs were transfected with either control (si-Ctrl) or *Ror2* siRNA (si-*Ror2* #3). Knockdown efficiency of *Ror2* was evaluated by quantitative RT-PCR (a). Six days after transfection, conditioned media were collected and subjected to cytokine array analysis as described in Experimental procedures. Protein expression levels of CCL5 (orange box), CXCL16 (blue box) and positive control (green box) in (b) were indicated by surrounding with square, respectively, following manufacturer's instruction (Human chemokine array, ARY017, R&D systems). High magnification images of the boxed spots were shown in (c). Left four and right four panels in (c) were shown as

different contrast images adjusted within a linear range. Band intensities of *CCL5* and *CXCL16*, respectively, normalized by positive control were determined and the relative band intensities, defining the expression level in si-Ctrl as 1, were shown in (d). (e) UE6E7T-12 cells, an immortalized cell line of BM-derived human MSCs, were transfected with either control (si-Ctrl) or *Ror2* siRNA (si-*Ror2* #1, #2). Three days after transfection, expression levels of *Ror2*, *CCL5* and *CXCL16* were analyzed by quantitative RT-PCR analyses. Relative expression levels were determined by defining the expression level in control (si-Ctrl) as 1. Data are expressed as mean  $\pm$  SD (n = 6). \**P* < 0.05, \*\**P* < 0.01, Dunnett's multiple comparisons test.

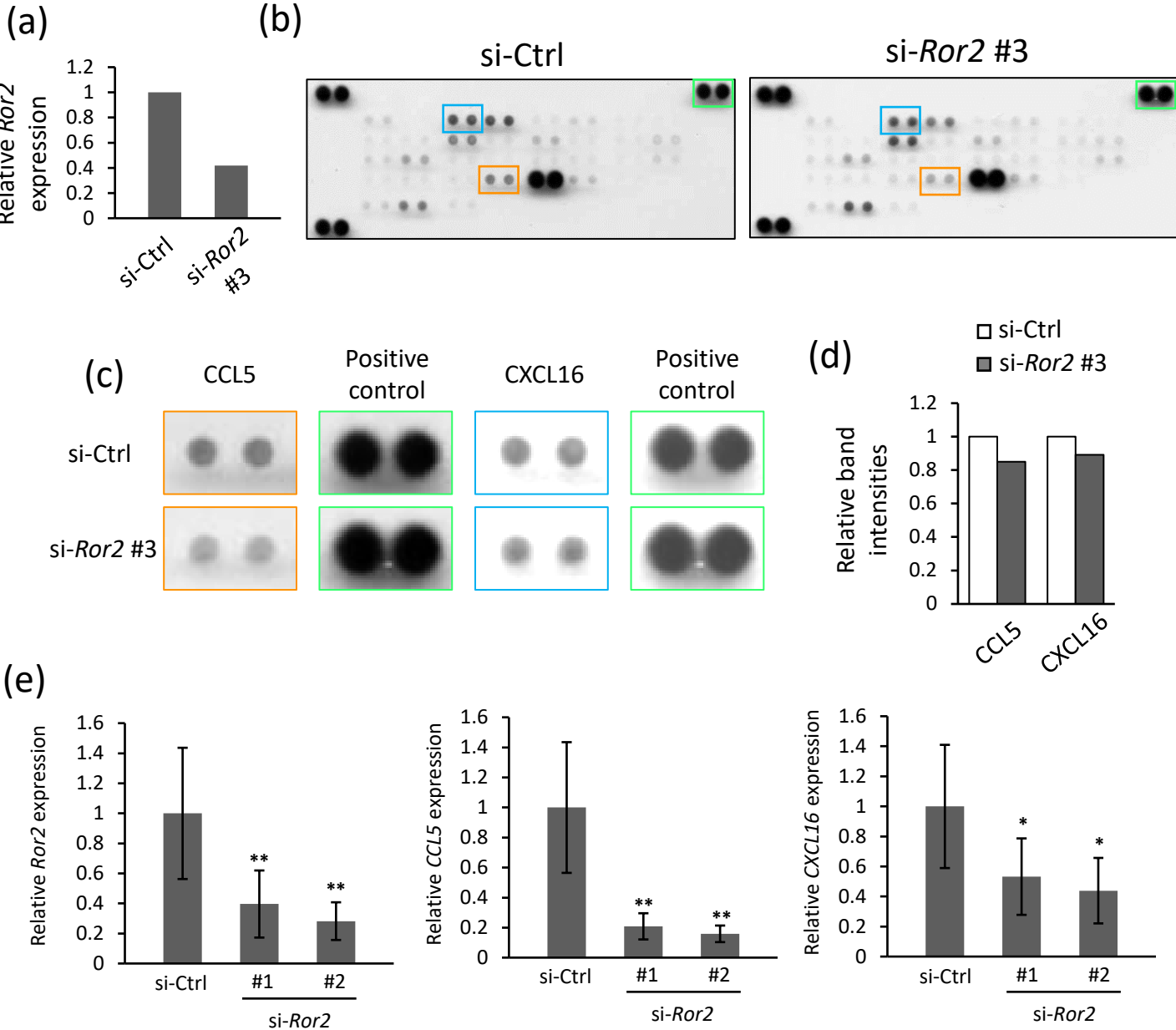
**Figure 2. CXCL16-CXCR6 signaling is required for up-regulated expression of *CCL5* in UE6E7T-12 cells.** (a) Expression levels of the respective chemokine receptors for *CCL5* and *CXCL16* on the surface of UE6E7T-12 cells were analyzed by flow cytometric analysis. CCR1, CCR3, and CCR5 act as receptors for *CCL5*, while CXCR6 act as a receptor for *CXCL16*. (b) The mean fluorescence intensities of the indicated chemokine receptors were measured. Relative mean fluorescence intensities were determined by defining the mean fluorescence intensity of control IgG (Ctrl-IgG) as 1. Data are expressed as mean  $\pm$  SD (n = 3). \**P* < 0.05, n.s.; not significant, Dunnett's multiple comparisons test. (c) UE6E7T-12 cells were transfected with either control (si-Ctrl) or *CXCL16* siRNA (si-*CXCL16* #1, #2). Three days after transfection, expression levels of *CXCL16* and *CCL5* were analyzed by quantitative RT-PCR analyses, respectively. Relative expression levels were determined by defining the expression level in control (si-Ctrl) as 1. Data are expressed as mean  $\pm$  SD (n = 8). \**P* < 0.05, \*\**P* < 0.01, Dunnett's multiple comparisons test.

**Figure 3. CCL5-CCR1/3 signaling is required for enhanced migration of MKN45 cells.** (a) Expression levels of CCR1 and CCR3, chemokine receptors for *CCL5* on the surface of an

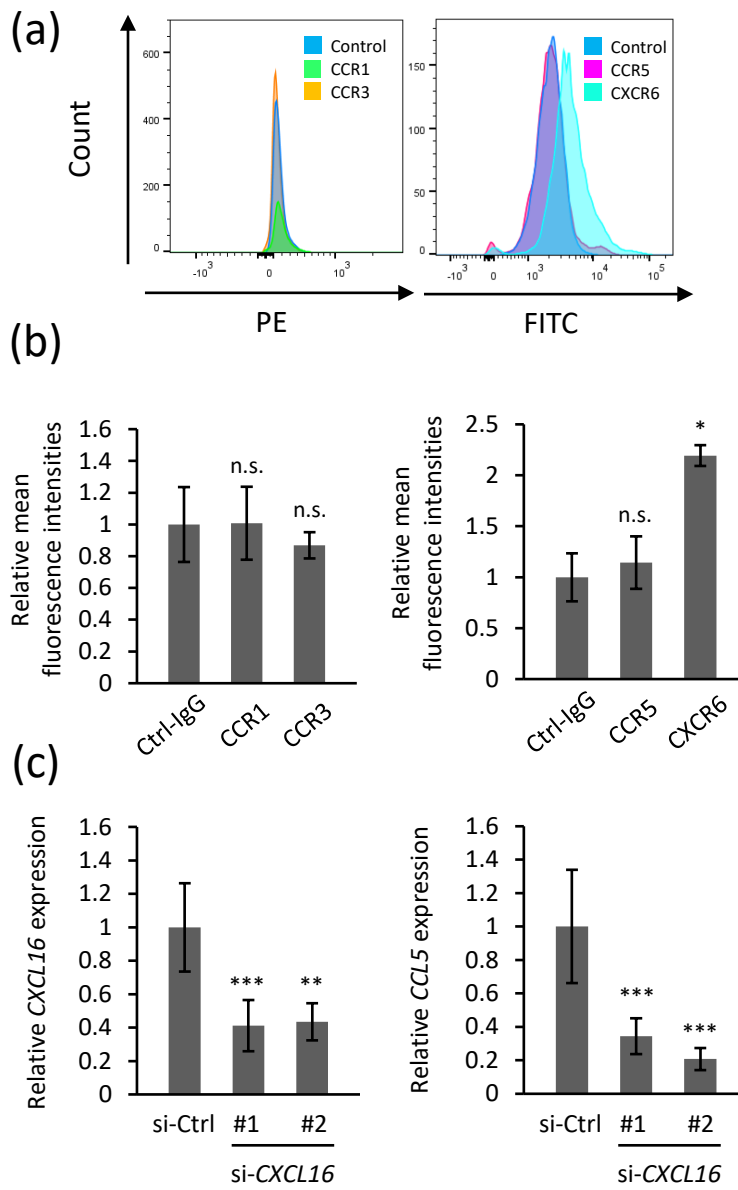


undifferentiated gastric cancer cell line, MKN45 cells were analyzed by flow cytometric analysis. (b) The mean fluorescence intensities of the indicated chemokine receptors were measured. Relative mean fluorescence intensities were determined by defining the mean fluorescence intensity of control IgG (Ctrl-IgG) as 1. Data are expressed as mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, n.s.; not significant, Dunnett's multiple comparisons test (left graph) or Student's  $t$ -test (right graph). (c) Migration of MKN45 cells following stimulation with CCL5 was evaluated as described in Experimental procedures. Relative number of migrated MKN45 cells upon stimulation with CCL5 was determined by defining that of vehicle (0.1% BSA solution) treated cells (indicated as (-)) as 1. Data are expressed as mean  $\pm$  SD (n = 3). \* $P$  < 0.05, Student's  $t$ -test. (d) MKN45 cells were transfected with control siRNA (si-Ctrl), *CCR1* siRNA (si-*CCR1* #1, #2) or *CCR3* siRNA (si-*CCR3* #1, #2). Three days after transfection, migration of MKN45 cells induced upon stimulation with CCL5 was evaluated as described in Experimental procedures. Relative number of migrated MKN45 cells following stimulation with CCL5 was determined by defining that of vehicle treated cells (indicated as (-)) as 1. Data are expressed as mean  $\pm$  SD (n = 6). \* $P$  < 0.05, Tukey's honest significance difference (HSD) test.

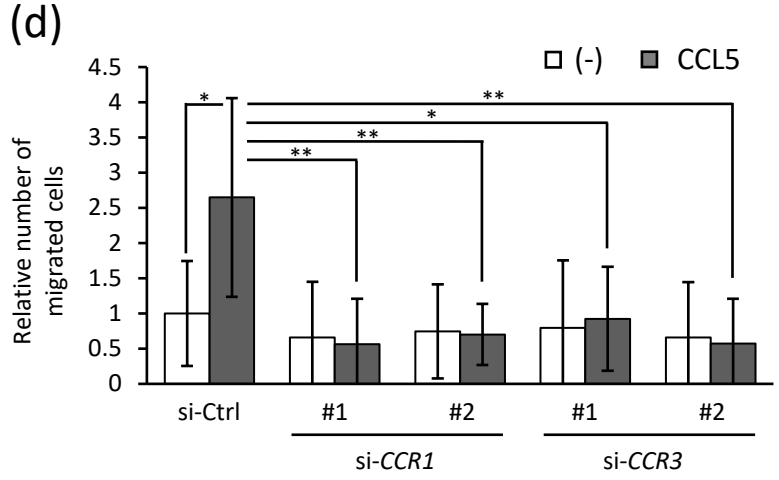
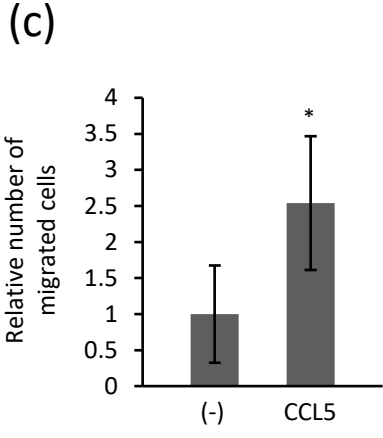
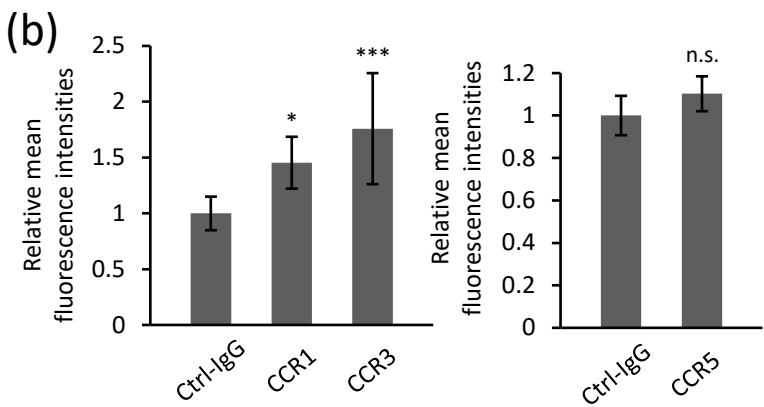
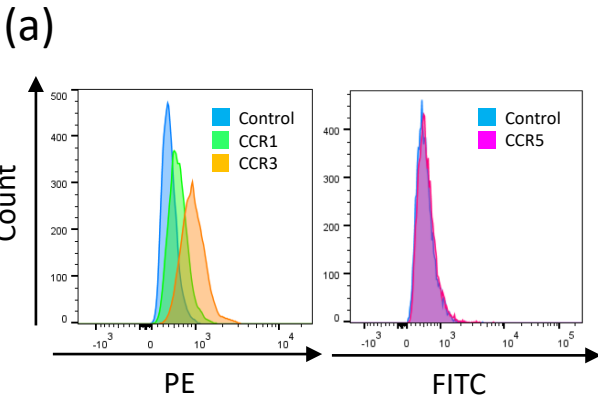
# Figure 1

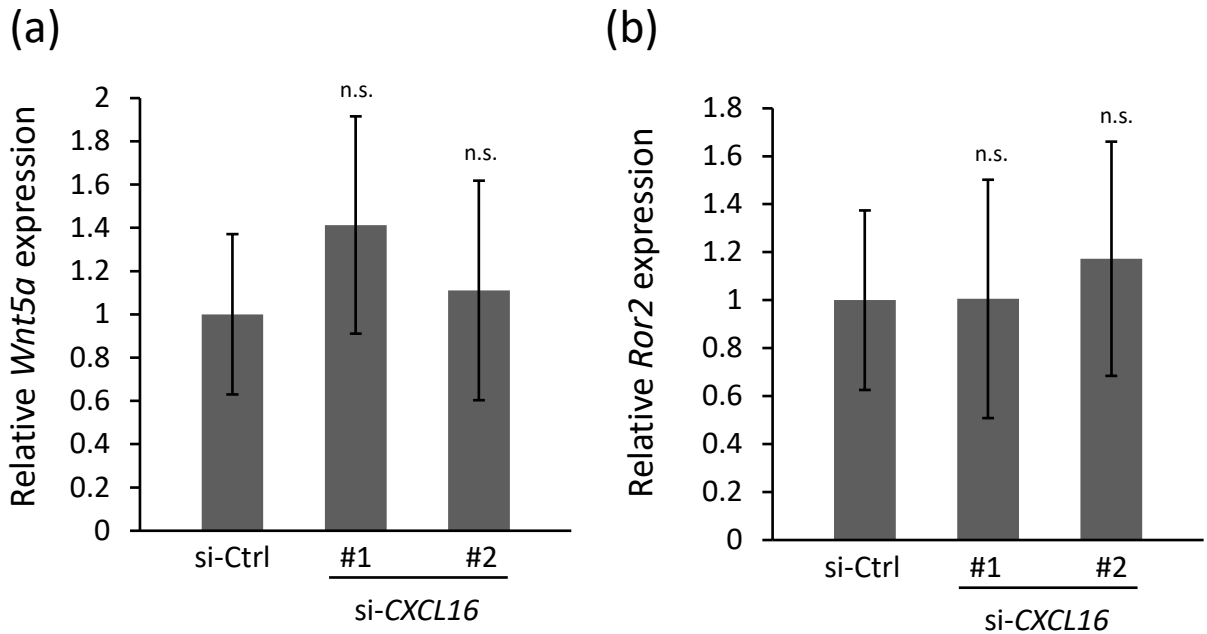


# Figure 2

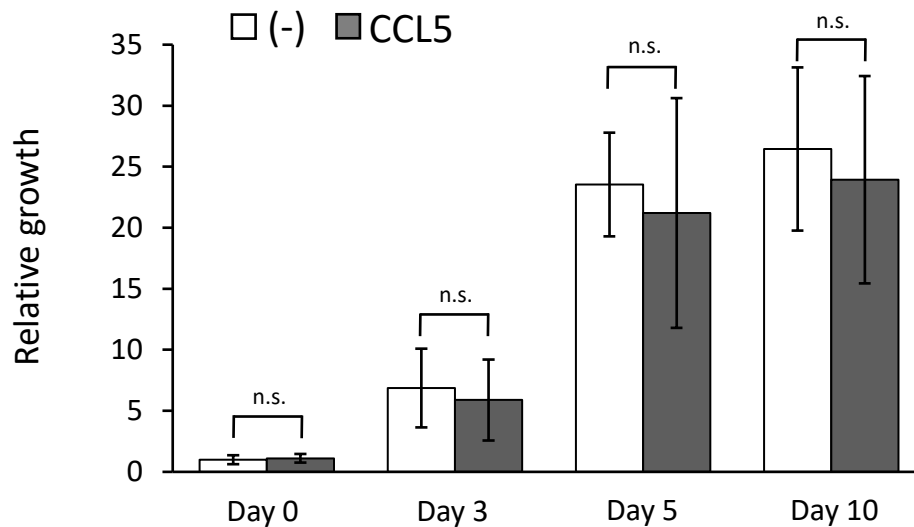


# Figure 3

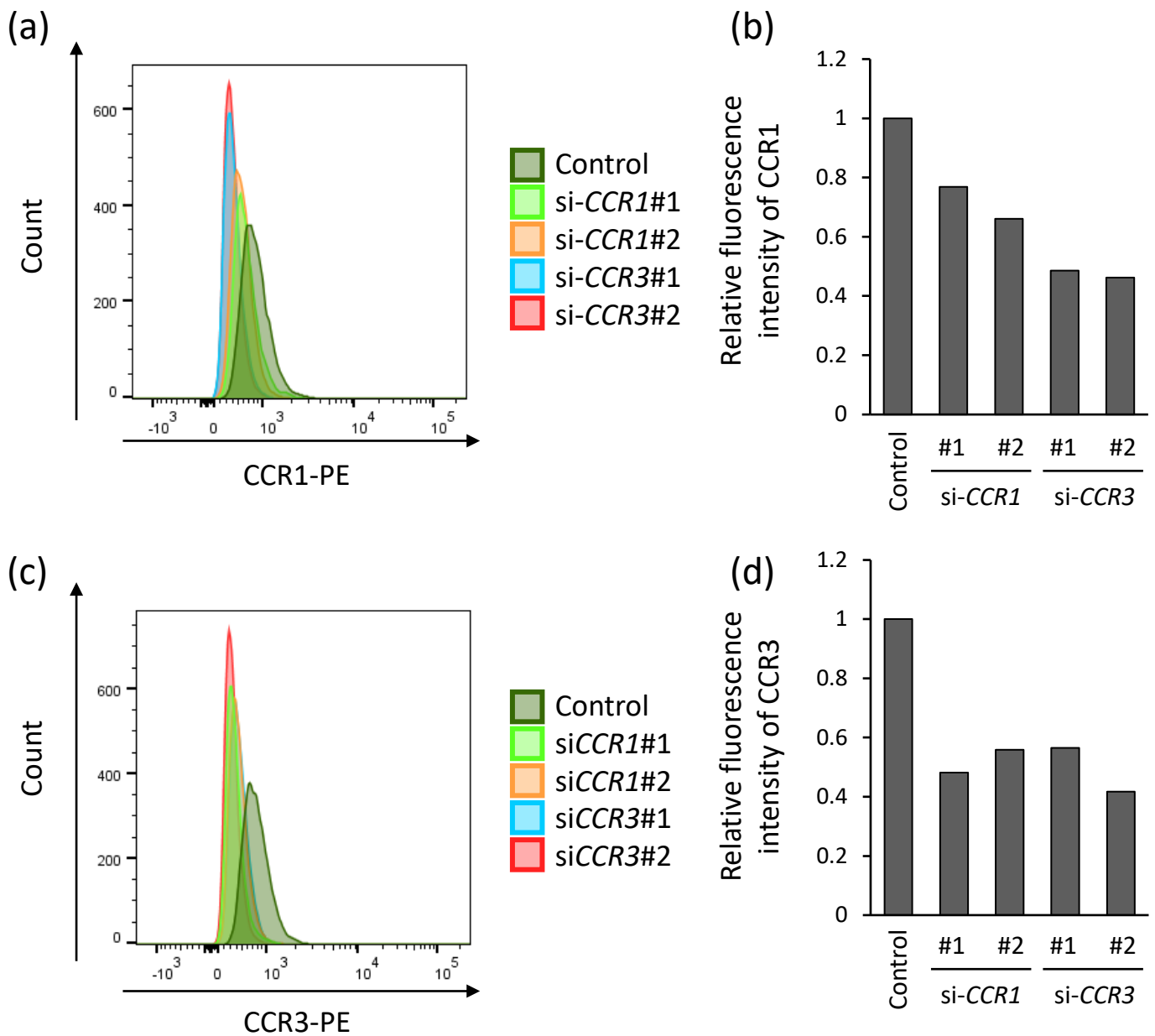




**Figure S1. Expression levels of *Wnt5a* and *Ror2* in UE6E7T-12 cells are irrespective of that of CXCL16.** (a) UE6E7T-12 cells were transfected with either control siRNA (si-Ctrl) or *CXCL16* siRNA (si-*CXCL16* #1, #2). Three days after transfection, expression level of *Wnt5a* was analyzed by quantitative RT-PCR analyses. Knockdown efficiency of *CXCL16* was shown in Figure 2c. Relative expression levels of *Wnt5a* were determined by defining the expression level in control (si-Ctrl) as 1. Data are expressed as mean  $\pm$  SD (n = 3). n.s.; not significant, Dunnett's multiple comparisons test. (b) UE6E7T-12 cells were transfected with either control siRNA (si-Ctrl) or *CXCL16* siRNA (si-*CXCL16* #1, #2). Three days after transfection, expression level of *Ror2* was analyzed by quantitative RT-PCR analyses. Knockdown efficiency of *CXCL16* was shown in Figure 2c. Relative expression levels of *Ror2* were determined by defining the expression level in control (si-Ctrl) as 1. Data are expressed as mean  $\pm$  SD (n = 3). n.s.; not significant, Dunnett's multiple comparisons test.

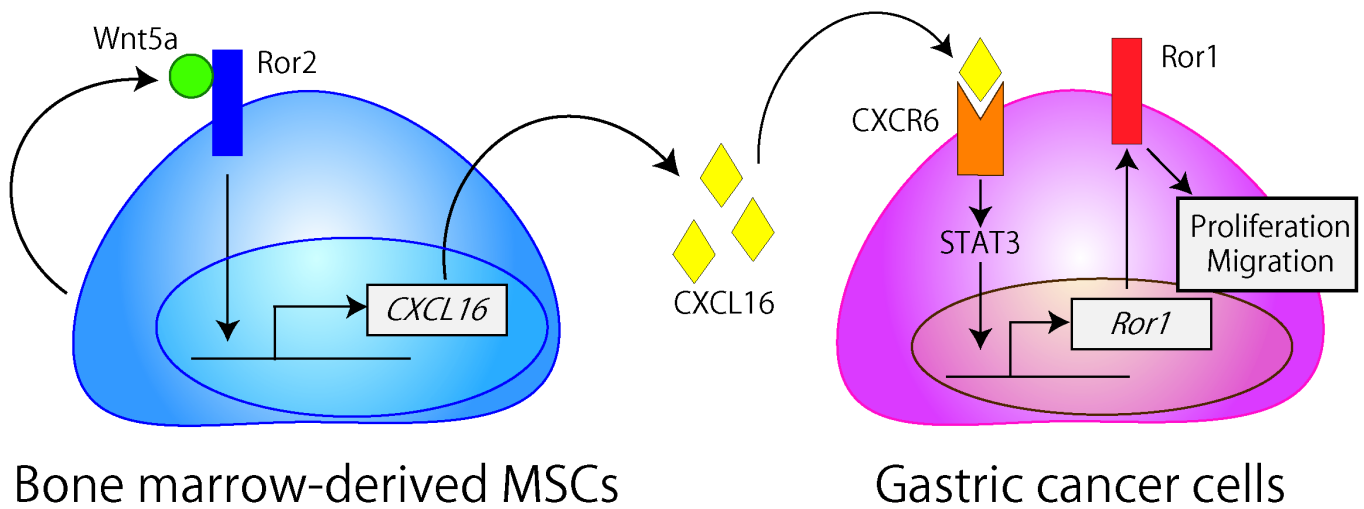


**Figure S2. Proliferation of MKN45 cells is unaffected by stimulation with CCL5.** Proliferation of MKN45 cells following stimulation with CCL5 was evaluated as described in Experimental procedures. Relative growth of MKN45 cells upon stimulation with CCL5 was determined by defining viable cell number of vehicle (0.1% BSA solution) treated cells (indicated as (-)) at day 0 as 1. Data are expressed as mean  $\pm$  SD (n = 3). n.s.; not significant, Student's *t*-test.

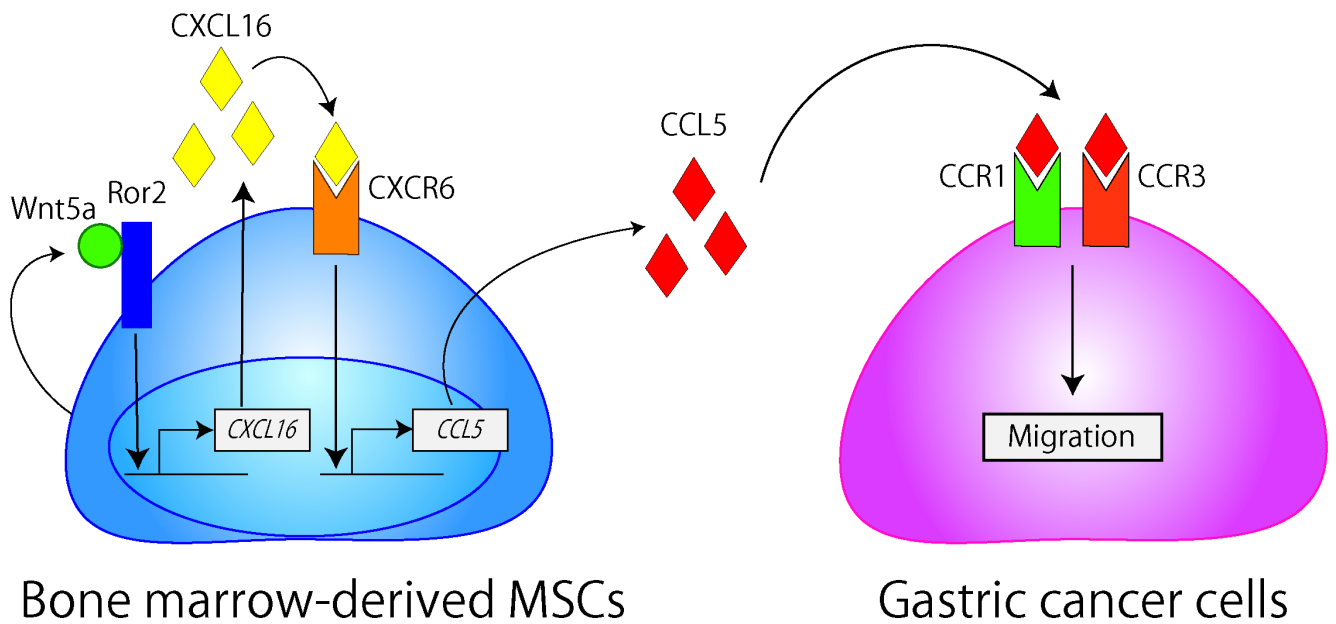


**Figure S3. Suppressed expression of either *CCR1* or *CCR3* in MKN45 cells decrease expression of both *CCR1* and *CCR3*.** (a, b) Expression level of *CCR1* on the surface of MKN45 cells was analyzed by flow cytometric analysis. Representative image of histogram is shown in (a). The mean fluorescence intensities of the cells treated with indicated siRNAs were measured. Relative mean fluorescence intensities were determined by defining the mean fluorescence intensity of control siRNA treated cells (Control) as 1 (b). (c, d) Expression level of *CCR3* on the surface of MKN45 cells was analyzed by flow cytometric analysis. Representative image of histogram is shown in (c). The mean fluorescence intensities of cells treated with indicated siRNAs were measured. Relative mean fluorescence intensities were determined by defining the mean fluorescence intensity of control siRNA treated cells (Control) as 1 (d).

## Intercellular CXCL16-CXCR6 signaling



## Cell-autonomous CXCL16-CXCR6 signaling



**Figure S4. Bone marrow-derived MSCs promotes proliferation and migration of gastric cancer cells through cell-autonomous and intercellular chemokine-chemokine receptor signaling.** A schematic representation of our model showing mechanisms underlying progression of gastric cancer regulated by BM-derived MSCs. It has previously been shown that expression of *CXCL16* is induced by endogenous Wnt5a-Ror2 signaling in MSCs and CXCL16 secreted from MSCs can bind to CXCR6 expressed on MKN45 cells (Takiguchi *et al.* 2016). Subsequently, CXCL16-CXCR6 signaling activated in MKN45 cells can induce expression of *Ror1* via STAT3 activation, eventually leading to proliferation and migration of MKN45 cells through intercellular signaling machinery (Ikeda *et al.* 2020) (Upper panel). On the other hand, in this study it has been found that CXCL16-CXCR6 signaling activated in MSCs might induce expression of *CCL5* through intracellular signaling machinery. As a result, CCL5 secreted from MSCs binds to CCR1/3 expressed on MKN45 cells, thereby activated CCL5-CCR1/3 signaling can promote migration of MKN45 cells (Lower panel).