Wnt5a-Ror2 signaling in mesenchymal stem cells promotes proliferation of gastric cancer cells by activating CXCL16–CXCR6 axis

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Wnt5a-Ror2 signaling has been shown to play important roles in promoting aggressiveness of various cancer cells in a cell-autonomous manner. However, little is known about its function in cancer-associated stromal cells, including mesenchymal stem cells (MSCs). Thus, we examined the role of Wnt5a-Ror2 signaling in bone marrow-derived MSCs in regulating proliferation of undifferentiated gastric cancer cells. Coculture of a gastric cancer cell line, MKN45, with MSCs either directly or indirectly promotes proliferation of MKN45 cells, and suppressed expression of Ror2 in MSCs prior to coculture inhibits enhanced proliferation of MKN45 cells. In addition, conditioned media from MSCs, treated with control siRNA, but not siRNAs against Ror2, can enhance proliferation of MKN45 cells. Interestingly, it was found that expression of CXCL16 in MSCs is augmented by Wnt5a-Ror2 signaling, and that recombinant chemokine (C-X-C motif) ligand (CXCL)16 protein can enhance proliferation of MKN45 cells in the absence of MSCs. In fact, suppressed expression of CXCL16 in MSCs or an addition of a neutralizing antibody against CXCL16 fails to promote proliferation of MKN45 cells in either direct or indirect coculture with MSCs. Importantly, we show that MKN45 cells express chemokine (C-X-C motif) receptor (CXCR)6, a receptor for CXCL16, and that suppressed expression of CXCR6 in MKN45 cells results in a failure of its enhanced proliferation in either direct or indirect coculture with MSCs. These findings indicate that Wnt5a-Ror2 signaling enhances expression of CXCL16 in MSCs and, as a result, enhanced secretion of CXCL16 from MSCs might act on CXCR6 expressed on MKN45, leading to the promotion of its proliferation.
Materials and Methods

Cell culture. MKN45-Luc cells, which express luciferase stably, were obtained from JCRB cell bank (Osaka, Japan) and maintained in RPMI-1640 medium (Nacalai Tesque, Tokyo, Japan) containing 10% FBS. Mesenchymal stem cells, primary human MSCs derived from bone marrow, were purchased from Lonza (Basel, Switzerland). The cells were maintained in MSCGM (Lonza) and used by passage 5. These cells were incubated at 37°C, with 5% CO₂ at 95% humidity. In some experiments, MKN45-Luc cells were treated with soluble recombinant human CXCL16 (PeproTech, Oak Park, CA, USA) at a final concentration of 1.0 ng/mL.

Coculture. For monolayer culture, MKN45-Luc cells were plated in 12-well plates at a density of 1 × 10⁴ cells per well with 2 mL MSCGM. For direct coculture, MSCs and MKN45-Luc cells were plated in the same well of 12-well plates at a density of 1 × 10⁴ cells per well for each cell type with 2 mL MSCGM. For indirect coculture, Transwells with 0.4-µm pore membrane in 12-well plates (Costar, Cambridge, MA, USA) were used to allow both types of cells to share media without making any direct contact. Unless otherwise indicated, MSCs (1 × 10³ cells) were seeded in the upper chamber with 500 µL MSCGM, and MKN45-Luc cells (1 × 10³ cells) were seeded in the lower chamber with 1500 µL MSCGM. To neutralize CXCL16, anti-human CXCL16 antibody (R&D Systems, Minneapolis, MN, USA) or control IgG (R&D Systems) was added to the media at a concentration of 0.25 µg/mL.

Conditioned media. Mesenchymal stem cells untreated or treated with the respective siRNAs were plated at 1 × 10⁴ cells/mL in MSCGM and cultured for 6 days. The cell supernatants were collected as the conditioned media. To culture MKN45-Luc cells with the conditioned media, cells were plated at 1 × 10⁴ cells/mL in the well of 12-well plates with 25% (v/v) of conditioned medium and 75% (v/v) of MSCGM.

Luciferase assay. Cells were lysed in Glo Lysis buffer (Promega, Madison, WI, USA). Aliquots of cell lysates were mixed with ONE Glo Luciferase Assay buffer (Promega), and the luciferase activities were measured by using the GloMax 96 microplate luminometer (Promega).

Enzyme-linked immunosorbent assay. The culture supernatants from MSCs treated with si-Ror2 or si-CXCL16 siRNAs were collected. The CXCL16 concentration in the culture supernatants was determined using Quantikine ELISA kit (R&D Systems), according to the manufacturer’s instructions.

Flow cytometric analysis. MKN45-Luc cells treated with si-CXCR6 for 5 days were collected and fixed with 10% (v/v) formalin in PBS. Then cells were washed with PBS and incubated with mouse anti-human CXCR6 antibody (R&D Systems) at a final concentration of 25 µg/mL, followed by incubation with Alexa488-conjugated anti-mouse IgG (Life Technologies, Carlsbad, CA, USA). Flow cytometric analysis was performed using the BD FACSVerse (Becton Dickinson, Franklin Lakes, NJ, USA).

Human chemokine array. Conditioned media obtained from Ror2-silenced MSCs were analyzed using the Proteome Profiler Human Chemokine Array Kit (ARY017; R&D Systems), according to the manufacturer’s instructions. Briefly, array membranes, spotted with capture antibodies to specific target proteins, were incubated overnight at 4°C with the conditioned media pretreated with a cocktail of biotinylated detection antibodies. Then, membranes were washed and incubated with streptavidin–HRP for 30 min at room temperature. After washing the membranes, captured proteins on the membranes were visualized using chemiluminescent detection reagents. Supplementary materials and methods are described in Data S1.

Results

Promoted proliferation of MKN45 cells by direct and indirect coculture with MSCs. We first examined a possible role of human bone marrow–derived MSCs on the proliferation of an undifferentiated gastric cancer cell line, MKN45 cells which express Wnt5a at marginal levels, if any (16). To this end, we used MKN45-Luc cells, which express luciferase stably, to monitor proliferation of MKN45(-Luc) cells. When MKN45-Luc cells were cultured singly for 12 h at different densities, both total viable cell numbers and luciferase activities increased in proportion to increased cell densities (Fig. S1), indicating that measurement of luciferase activities can be a reliable readout of proliferation of MKN45-Luc cells.

When MKN45-Luc cells were either cultured singly (MSCS−) or cocultured directly with MSCs (MSCS+), proliferation of MKN45-Luc cells was promoted remarkably at day 9 after coculture with MSCs (Fig. 1a). We next examined whether the effect of coculture with MSCs on promoted proliferation of MKN45-Luc cells would be mediated by direct cell–cell interaction or by a soluble mediator(s) produced by MSCs. Thus, we carried out indirect cocultures of MKN45-Luc cells and MSCs by using Transwells that prohibit any direct contact between cells in the lower and upper chambers. As shown in Figure 1(b,c), indirect coculture of MKN45-Luc cells with MSCs promoted proliferation of MKN45-Luc significantly at day 9 after coculture with MSCs, indicating that a soluble mediator(s) produced by MSCs might play a role in promoting proliferation of MKN45 cells.

Role of Wnt5a-Ror2 signaling in MSCs in promoting proliferation of MKN45 cells. It was found that expression levels of both Ror2 and Wnt5a were relatively high in MSCs, but were marginal if any in MKN45-Luc cells (Fig. S2). Thus, we then examined a possible role of Wnt5a-Ror2 signaling in MSCs in promoting proliferation of MKN45-Luc cells. Suppressed expression of Ror2 or Wnt5a in MSCs resulted in the inhibition of promoted proliferation of MKN45-Luc cells by direct or indirect coculture with MSCs (Figs 2, S3). Furthermore, proliferation and viability of MSCs were unaffected by suppressed expression of Ror2 in the cells as assessed by WST assay (Fig. S4a). These results suggest that Wnt5a-Ror2 signaling in MSCs might play a role in promoting proliferation of MKN45 cells.

Induced expression of CXCL16 by Wnt5a-Ror2 signaling in MSCs. To assess further the findings that MSCs produce a soluble mediator(s) that promotes proliferation of MKN45 cells which we first tested the effect of conditioned media from MSCs on proliferation of MKN45-Luc cells. As expected, conditioned media from MSCs promoted proliferation of MKN45-Luc cells (Fig. 3a). Interestingly, suppressed expression of Ror2 in MSCs inhibited the effect of MSC-conditioned media to
promote proliferation of MKN45-Luc cells (Fig. 3b), indicating that Wnt5a-Ror2 signaling in MSCs might play a role in producing a soluble mediator(s) that promotes proliferation of MKN45 cells.

To identify a candidate soluble mediator(s) produced by MSCs through Wnt5a-Ror2 signaling, we carried out a human chemokine array analysis using conditioned media from either MSCs treated with control (ctrl) siRNA or siRNAs against Ror2. As a result, among several candidate chemokines identified, CXCL16 was the most prominent one whose expression was inhibited significantly and reproducibly by suppressed expression of Ror2 (Fig. 3c). In fact, expression of CXCL16 was inhibited by suppressed expression of Ror2 in MSCs as assessed by quantitative RT-PCR (Fig. 3d). As CXCL16 can...
exist as a transmembrane protein and a soluble protein by cleavage with the ADAM family of proteases, we measured amounts of soluble CXCL16 secreted from MSCs following treatment with either ctrl siRNA or siRNAs against Ror2 by ELISA. As shown in Figure 3(e), secretion of soluble CXCL16 from MSCs was inhibited significantly by suppressed expression of Ror2. These results indicate that Wnt5a-Ror2 signaling in MSCs plays an important role in promoting expression and secretion of CXCL16.

Critical role of soluble CXCL16 in promoting proliferation of MKN45 cells. We next examined the effect of soluble recombinant CXCL16 (rCXCL16) on proliferation of MKN45 cells. Importantly, proliferation of MKN45-Luc cells was promoted by the addition of rCXCL16 after day 7 (Fig. 4a). To further assess the role of soluble CXCL16 secreted from MSCs in the promotion of MKN45 cell
proliferation, MKN45-Luc cells were cocultured directly or indirectly with MSCs pretreated with either ctrl siRNA or siRNA against CXCL16. As shown in Figure 4(b,c), expression of CXCL16 mRNA and secretion of soluble CXCL16 were inhibited drastically by pretreatment of MSCs with CXCL16 siRNA. After 6 days in culture, mRNA levels of CXCL16 were measured by quantitative RT-PCR analyses (b). Conditioned media from the siRNA-treated MSCs were collected to measure relative amounts of CXCL16 protein by ELISA (c). (d,e) MKN45-Luc cells were cultured singly or cocultured with siRNA-treated MSCs either directly (d) or indirectly (e). Luciferase activities were measured at the indicated time points. Data are expressed as mean ± SD (n = 3). **P < 0.001, t-test. (f,g) Effect of neutralizing antibody against CXCL16 on proliferation of MKN45-Luc cells cocultured with MSCs. MKN45-Luc cells were cocultured with MSCs either directly (f) or indirectly (g) in the presence of anti-CXCL16 neutralizing antibody or control IgG. Luciferase activities were measured at the indicated time points. Data are expressed as mean ± SD (n = 3). **P < 0.001, t-test.

Fig. 4. Expression of CXCL16 in mesenchymal stem cells (MSCs) is required for the ability of MSCs to promote proliferation of MKN45-Luc cells in coculture. (a) Recombinant CXCL16 (rCXCL16) promotes proliferation of MKN45-Luc cells. MKN45-Luc cells were cultured in the absence (vehicle) or presence of 1 ng/mL rCXCL16, and luciferase activities were measured at the indicated time points. Data are expressed as mean ± SD (n = 3). **P < 0.001, t-test. (b,c) Suppressed expression of CXCL16 in MSCs. MSCs were transfected with either ctrl or CXCL16 siRNA. After 6 days in culture, mRNA levels of CXCL16 were measured by quantitative RT-PCR analyses (b). Conditioned media from the siRNA-treated MSCs were collected to measure relative amounts of CXCL16 protein by ELISA (c). (d,e) MKN45-Luc cells were cultured singly or cocultured with siRNA-treated MSCs either directly (d) or indirectly (e). Luciferase activities were measured at the indicated time points. Data are expressed as mean ± SD (n = 3). **P < 0.001, t-test. (f,g) Effect of neutralizing antibody against CXCL16 on proliferation of MKN45-Luc cells cocultured with MSCs. MKN45-Luc cells were cocultured with MSCs either directly (f) or indirectly (g) in the presence of anti-CXCL16 neutralizing antibody or control IgG. Luciferase activities were measured at the indicated time points. Data are expressed as mean ± SD (n = 3). **P < 0.001, t-test.
cocultured directly or indirectly with MSCs. As expected, proliferation of MKN45-Luc cells by direct or indirect coculture with MSCs was inhibited significantly by an addition of the neutralizing anti-CXCL16 antibody (Fig. 4f,g), confirming the critical role of soluble CXCL16 in promoting proliferation of MKN45.

Critical role of CXCL16–CXCR6 signaling in promoted proliferation of MKN45 cells. It has been shown that CXCR6 acts as a receptor for soluble and transmembranous forms of CXCL16.\(^{18,19,22}\) Therefore, it can be envisaged that CXCR6 might be expressed on MKN45 cells to mediate CXCL16-induced proliferation signaling. In fact, quantitative RT-PCR analysis revealed remarkably high levels of CXCR6 mRNA in MKN45-Luc cells compared with those in MSCs (Fig. S5). Treatment of MKN45-Luc cells with siRNA against CXCR6 inhibited expression of CXCR6 (Fig. 5a). Flow cytometric analysis also showed expression of cell surface CXCR6 proteins on MKN45-Luc cells and its inhibition by the siRNA treatment (Fig. 5b). It was found that suppressed expression of CXCR6 in MKN45-Luc cells by itself had marginal effect, if any, on proliferation of MKN45-Luc cells (monoculture) (Fig. S6). However, suppressed expression of CXCR6 in MKN45-Luc cells significantly inhibited the promotion of MKN45-Luc cell proliferation by direct or indirect coculture with MSCs (Figs 5c,d,S6), indicating that CXCR6 expressed on MKN45 cells plays a critical role in the promotion of MKN45 cell proliferation by CXCL16 secreted from MSCs.

Discussion
Growing evidence indicates the important roles of Wnt5a-Ror2 signaling in the progression of various types of cancer cells.\(^{4–8}\) Here we show that Wnt5a-Ror2 signaling plays an important role in bone marrow-derived MSCs to produce soluble CXCL16, which in turn, acting on CXCR6, a receptor for CXCL16 expressed on MKN45 undifferentiated gastric cancer cells, to mediate the promotion of its proliferation. To our knowledge, this is for the first time the role of Wnt5a-Ror2 signaling has been revealed by an external cue, in this case MSCs, to promote cancer cell progression.

In this study we used undifferentiated MKN45 gastric cancer cells to exclude the possibility of cancer cells proliferating in a cell-autonomous manner through Wnt5a-Ror2 signaling. MKN45 cells express both Wnt5a and Ror2 marginally, if at all, whereas MSCs express them at relatively high levels (Fig. S2). Although the extent of the luciferase activities detected was varied under different experimental settings, proliferation of MKN45-Luc cells was promoted remarkably at day 9 after direct or indirect coculture with MSCs through Wnt5a-Ror2 signaling (Figs 1,2,S3). We further showed that CXCL16, expressed in and secreted from MSCs by
Wnt5a-Ror2 signaling, acting on CXCR6 expressed on MKN45-Luc cells, thereby promoting proliferation of MKN45 cells (Figs 3–5,S4b). In this respect, it should be noted that the CXCL16–CXCR6 axis has been shown to play an important role in promoting progression of prostate cancer cells by activating the AKT–mTOR pathways.\(^{23–26}\)

As shown, the promoted effects of coculture with MSCs, and the addition of conditioned media from MSCs and rCXCL16 on proliferation of MKN45 cells can be detected only after day 7 or day 9 in culture (Figs 1–5,S3). It is conceivable that the CXCL16–CXCR6 axis might activate further signaling to promote the eventual proliferation of MKN45 cells. Further study will be required to clarify this issue. It has also been reported that in vitro culture of MSCs alone or with cancer-dermed medium can induce its conversion into CAFs.\(^{1,25,27}\)

Although expression levels of Ror2 and Wnt5a increased and decreased, respectively, during in vitro culture of MSCs alone (Fig. S2c), suppressed expression of either Ror2 or Wnt5a failed to affect expression patterns and levels of CAF marker genes, including α-SMA, SDF-1, and FAP (data not shown), suggesting that Wnt5a-Ror2 signaling in MSCs might not affect its conversion into CAFs and vice versa.

Collectively, our present findings reveal that soluble CXCL16, produced by MSCs through Wnt5a-Ror2 signaling, can promote proliferation of MKN45 cells expressing CXCR6. Thus, it can be assumed that CXCL16, produced by MSCs, might also contribute to the promoted proliferation of other cancer cells that express CXCR6. Therefore, Wnt5a-Ror2 signaling in MSCs and/or the CXCL16–CXCR6 axis between MSCs and cancer cells might be effective therapeutic targets to prevent cancer progression. Future study with in vivo xenograft analyses will be required to test this possibility.

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Disclosure Statement

The authors have no conflict of interest.
Fig. S2. Expression levels of Ror2 and Wnt5a in MKN45-Luc cells and mesenchymal stem cells.

Fig. S3. Expression of Wnt5a in mesenchymal stem cells is required for the ability of mesenchymal stem cells to promote proliferation of MKN45-Luc cells in culture.

Fig. S4. Effect of suppressed expression of Ror2 or Wnt5a on viability of mesenchymal stem cells and expression of CXCL16 in mesenchymal stem cells, respectively.

Fig. S5. Expression levels of CXCR6 in MKN45-Luc cells and mesenchymal stem cells.

Fig. S6. Effects of suppressed expression of CXCR6 on proliferation of MKN45-Luc cells cultured singly or cocultured with mesenchymal stem cells.

Data S1. Supplementary materials and methods.