Inhibition of Chemokine (CXC Motif) Ligand 12/Chemokine (CXC Motif) Receptor 4 Axis (CXCL12/CXCR4)-mediated Cell Migration by Targeting Mammalian Target of Rapamycin (mTOR) Pathway in Human Gastric Carcinoma Cells*

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Background: The cross-talk between CXCL12/CXCR4 axis and PI3K/mTOR pathway in migration of gastric carcinoma cells is unknown.

Results: p110β provided a conduit for CXCL12-stimulated signaling and targeting PI3K/mTOR blocked CXCL12-activated cell motility.

Conclusion: Targeting PI3K/mTOR pathway inhibited CXCL12-mediated cell migration.

Significance: Drugs targeting mTOR pathway may be used for the therapy of metastatic gastric cancer expressing high levels of CXCL12.

CXCL12/CXCR4 plays an important role in metastasis of gastric carcinoma. Rapamycin has been reported to inhibit migration of gastric cancer cells. However, the role of mTOR pathway in CXCL12/CXCR4-mediated cell migration and the potential of drugs targeting PI3K/mTOR pathway remains unelucidated. We found that CXCL12 activated PI3K/Akt/mTOR pathway in MKN-45 cells. Stimulating CHO-K1 cells expressing pEGFP-C1-Grp1-PH fusion protein with CXCL12 resulted in generation of phosphatidylinositol (3,4,5)-triphosphate, which provided direct evidence of activating PI3K by CXCL12. Down-regulation of p110β by siRNA but not p110α blocked phosphorylation of Akt and S6K1 induced by CXCL12. Consistently, p110β-specific inhibitor blocked the CXCL12-activated PI3K/Akt/mTOR pathway. Moreover, CXCR4 immunoprecipitated by anti-p110β antibody increased after CXCL12 stimulation and GTP protein inhibitor pertussis toxin abrogated CXCL12-induced activation of PI3K. Further studies demonstrated that inhibitors targeting the PI3K/mTOR pathway significantly blocked the chemotactic responses of MKN-45 cells triggered by CXCL12, which might be attributed primarily to inhibition of mTORC1 and related to prevention of F-actin reorganization as well as down-regulation of active RhoA, Rac1, and Cdc42. Furthermore, rapamycin inhibited the secretion of CXCL12 and the expression of CXCR4, which might form a positive feedback loop to further abolish upstream signaling leading to cell migration. Finally, we found cells expressing high levels of cxcl12 were sensitive to rapamycin in its activity inhibiting migration as well as proliferation. In summary, we found that the mTOR pathway played an important role in CXCL12/CXCR4-mediated cell migration and proposed that drugs targeting the mTOR pathway may be used for the therapy of metastatic gastric cancer expressing high levels of cxcl12.

Gastric carcinoma is a disease with a high death rate, making it the second most common cause of cancer death worldwide following lung cancer (1). Metastasis is a frequent cause of death in patients with advanced gastric carcinoma, and the prognosis of metastatic gastric cancer is poor with a six month survival rate of <15%. Recent research revealed that CXCL12 (chemokine (CXC motif) ligand 12) could be an independent prognostic factor in gastric cancer, with CXCL12-positive gastric cancer showing highly aggressive behavior, including metastasis (2).

CXCL12 is a 68-amino acid small cytokine that belongs to the CXC chemokine family, playing an important role in mediating tumor metastasis (3). CXCL12 is the only known ligand for chemokine (CXC motif) receptor 4 (CXCR4), which activates the receptor CXCR4 and attracts circulating CXCR4-expressing cells to peripheral tissues. The CXCL12/CXCR4 axis regulates a wide variety of downstream signal pathways related to chemotaxis, cell survival, and/or proliferation (4). PI3K is the major downstream transducer in CXCR4-mediated chemotaxis, which in turn regulates divergent signaling pathways (4). mTOR (mammalian target of rapamycin), a conserved serine/threonine kinase, sits in the center of the PI3K-mediated signaling pathways and regulates multiple processes, including...
mRNA translation, cell cycle progression, cell survival, and motility (5, 6). Although it has been reported that CXCL12 activates mTOR (7, 8), the precise mechanism underlying activation of the mTOR triggered by CXCL12 in gastric cancer cells remains elusive.

mTOR exists in two protein complexes, mTORC1 (mTOR, mLST8, and raptor) and mTORC2 (mTOR, mLST8, mSin1, and Rictor). mTORC1 integrates signals from growth factor or nutritional status and controls cap-dependent mRNA translation by phosphorylating its substrates 4E-BP1 and S6K1;4 mTORC2 phosphorylates AKT and SGK1 at a C-terminal site known as the hydrophobic motif and regulates the organization of the actin cytoskeleton, but the biological significance of these activities is not well understood (6, 9, 10). mTOR plays a critical role in the regulation of tumor cell motility and cancer metastasis (11). However, the underlying mechanism of mTOR regulating cell motility and mTOR inhibitors inhibiting tumor cell motility is understood poorly and controversial (12, 13).

In the present study, we studied the role of mTOR signaling pathway in cell migration mediated by CXCL12/CXCR4 axis in gastric cancer cells. We found that CXCL12 activated the CXCR4 receptor, which coupled G protein and interacted with p110β catalytic subunit and activated its activity and downstream signaling pathway, including phosphorylation of AKT and p70S6K1. Inhibitors targeting PI3K or/and mTOR inhibited cell migration induced by CXCL12, which is attributed primarily to inhibition of mTORC1 and related to decreased activity of RhoA, Cdc42 and Rac1 as well as prevention of F-actin reorganization. Interestingly, rapamycin decreased the secretion of CXCL12 and expression of its receptor CXCR4, which might form a positive feedback loop to further block cell migration. We also found that cells expressing high levels of CXCL12 are more sensitive to rapamycin in its ability in inhibiting cell migration induced by CXCL12, which is attributed to inhibition of mTORC1 and related to decreased activity of RhoA, Cdc42 and Rac1 as well as prevention of F-actin reorganization. Interestingly, rapamycin decreased the secretion of CXCL12 and expression of its receptor CXCR4, which might form a positive feedback loop to further block cell migration. We also found that cells expressing high levels of CXCL12 are more sensitive to rapamycin in its ability in inhibiting cell migration and proliferation. We proposed that inhibitors targeting the mTOR pathway may be used for therapy of metastatic gastric cancer expressing high levels of CXCL12.

MATERIALS AND METHODS

Cell Culture and Reagents—MKN-28, MKN-45, and SGC-7901 human gastric cancer cells were obtained from the Cancer Research Foundation of Japan and were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), l-glutamine (2 mM), penicillin (100 international units/ml), streptomycin (100 μg/ml), and HEPES (10 mM, pH 7.4). Cells were incubated in a humidified atmosphere of 95% air plus 5% CO₂ at 37 °C. AMD-3100, PI-103, TGX-221, rapamycin, PP-242, and pertussis toxin were obtained from Sigma-Aldrich, Cayman Chemical (Ann Arbor, MI), LC Laboratories (Woburn, MA), respectively. CXCL12 was purchased from R&D Systems (Minneapolis, MN).

Immunoblotting, Immunoprecipitation, and Immunofluorescence—Immunoblotting, immunoprecipitation, and immunofluorescence were conducted using standard procedures, using antibodies against S6K1, phosphorylated S6K1, 4EBP1, phosphorylated 4EBP1, Akt, phosphorylated Akt, and p110α (Cell Signaling Technology, Beverly, MA); p110β and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); and CXCR4 (Abcam, Cambridge, MA). Texas Red-X phallolidin was obtained from Invitrogen. Alexa Fluor 488 IgG was used as a fluorescent secondary antibody.

AKT Translocation Assay—CHO-K1 transfected with pEGFP-C1-Grp1-PH or pEGFP-C1 were seeded and further incubated in serum-free medium for 24 h. The cell was stained with CellMaskTM (Invitrogen) deep red plasma membrane stain (5 μg/ml) diluted in culture medium for 5 min at 37 °C. Then the stain solution was washed out and replaced with fresh medium. After addition of CXCL12 (300 ng/ml) for 2 min, the fluorescent images of the cell were captured with a confocal microscopy (FV1000-SIM; Olympus, Tokyo, Japan). The term co-localization refers to the coincidence of green and red fluorescence.

Real-time Quantitative PCR—Total RNA was extracted with TRIzol according to the manufacturer’s instructions and was transcribed using Prime ScriptTM RT reagent kit (TaKaRa, Dalian, China). The cDNA template was amplified by real-time PCR using the SYBR-Premix Ex Taq™ kit (TaKaRa). The primer sequences were as follows: 5′-CTTGGCAAAGCTAGTGAAG-3′ (forward) and 5′-AGAACCAGGAGGTGCTGA-3′ (reverse) for cxcl12; 5′-AGCTGTTGTGAAAGGTGGTCTATG-3′ (forward) and 5′-GCGCTCTGTTGGCCCCTTGAGGTG-3′ (reverse) for CXCR4; and 5′-GACCGGT-CAAGGCTGAAAC-3′ (forward) and 5′-GCTTCTCCATGTTGAAAAGGT-3′ (reverse) for GAPDH. Thermal cycling was programmed as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 15 s, and then 72 °C for 10 min. Gene expression was assessed by ΔC method, and mRNA levels of cxcl12 were normalized to those of the GAPDH internal standard.

Transfection of siRNA—The synthetic siRNAs targeting p110 catalytic subunit, Raptor, and Rictor were purchased from Shanghai GenePharma Co., Ltd with sequences as follows: 5′-GGUGGCCCAAGGAAGUUAATT-3′ (p110α); 5′-CGCAUUUGGUGGACCCAAAATT-3′ (p110β); 5′-CAGCGGAGAGCUCUAUCAAAT-3′ (Raptor siRNA1); 5′-GACACGGGAUGUUGAGCAATT-3′ (Raptor siRNA2); 5′-GCGAGCU-GAUGUAGAUAUATT-3′ (Rictor siRNA1); and 5′-GGGAA-UACAAUCUAAUUATT-3′ (Rictor siRNA2). Scrambled siRNA (5′-UUCUGACGUGUCAUGUGG-3′) was used as a negative control. The siRNA were transfected into MKN-45 cells using Oligofectamine™ reagent (Invitrogen) according to the instructions of the manufacturer.

Transwell Assay—Cell migration was evaluated using an 8-mm pore size Transwell system (Costar, Cambridge, MA). Briefly, cells were resuspended in serum-free RPMI 1640 medium at a density of 2 × 10⁵ cells/ml. The top chamber of the Transwell was loaded with 100 μl of cell suspension containing tested compounds, and the bottom chamber was loaded with 0.6 ml of RPMI 1640 medium containing CXCL12 (50 ng/ml) or 10% FBS. The total migrated cells to the lower chamber were fixed, stained with 0.1% crystal violet, and photographed after treatment. Crystal violet stained cells were dissolved with 10% acetic acid, and OD value was measured at 595 nm.
Inhibiting CXCR4-mediated Cell Migration by Targeting mTOR

Pull down Analysis of RhoA, Rac1, and Cdc42—Quantification of GTP-bound RhoA, Rac1, and Cdc42 was performed using the BK124, BK128, and BK127 G-LISA assay as instructed by the manufacturer (Cytoskeleton). Briefly, MNK-45 cells after treatment were suspended in lysis buffer. Cell extracts were incubated in separate wells of the G-LISA plate. The wells were probed with anti-RhoA, Rac1, or Cdc42 monoclonal antibodies and a secondary antibody. Finally, the plate was developed with a colorimetric substrate, and the absorbance was read at 490 nm with a multiwell plate reader.

Statistical Methods for Analysis of Rapamycin Activity and Gene Expression Data—Gene expression patterns for the NCI-60 cell lines assessed with HG-U133A Affymetrix chip. The probe sets were filtered to delete those with very little pattern (i.e. S.D. < 0.2 log2 units). The concentrations required to achieve 50% growth inhibition of the NCI-60 cells were obtained from the Developmental Therapeutics Program (http://www.dtp.nci.nih.gov/docs/dtp_search.html). Statistical analyses were performed by using the R package as described previously (14).

RESULTS

Activation of PI3K/AKT/mTOR Pathway by CXCL12—We first detected whether CXCL12 activates mTOR pathway in human gastric carcinoma MKN-45 cells. As shown in Fig. 1A, phosphorylated Akt at Thr-308 and Ser-473 as well as phosphorylated S6K1 increased significantly within 2 min after stimulation with CXCL12. As Akt may be phosphorylated by PDK1 at Thr-308 and by TORC2 at Ser-473 after PI3K activation, these results suggested that CXCL12 may stimulate PI3K and mTOR in a cascade.

To further verify that phosphorylation of Akt and S6K1 is resulted from activation of PI3K in gastric cancer cells, the redistribution of the Grp1-PH domain after stimulation of CXCL12 was determined in CHO-K1 cells expressing Grp1-PH fused to EGFP (15). The Grp1-PH domain is shown a specific binding affinity to PtdIns(3,4,5)P3 rather than phosphatidylinositol 4,5-bisphosphate in cells and aggregates around the cell membrane responding to the recruitment of PtdIns(3,4,5)P3 (15). As shown in Fig. 1B, the EGFP-Grp1-PH distributed in the cytoplasm in serum-deprived cells. However, CXCL12 stimulation resulted in the translocation of GFP-Grp1-PH to cellular membrane, which was indicated as the fluorescent foci of EGFP (green) co-localized with cell membrane stain (red), indicating that stimulation of cells with CXCL12 activates PI3K and results in production of PtdIns(3,4,5)P3 on the cellular membrane. On the other hand, the fluorescent foci of EGFP has distributed equally in the cytoplasm in cells transfected with EGFP despite CXCL12 stimulation.

Accordingly, pretreatment of CXCR4 antagonist AMD-3100, PI3K pan-inhibitor PI-103, or mTOR inhibitor rapamycin abrogated phosphorylation of Akt at Ser-473 and/or S6K1 at Thr-389 stimulated by CXCL12 (Fig. 1C). It appears that CXCL12 interacted with and activated CXCR4 receptor, which in turn activated PI3K and generated PtdIns(3,4,5)P3, and further activates the Akt/mTOR signaling cascade.

FIGURE 1. CXCL12 activated PI3K/Akt/mTOR signaling cascade. A, CXCL12 enhanced phosphorylation of Akt and p70S6K in MKN-45 cells. Serum-deprived MKN-45 cells were treated with CXCL12 (100 ng/ml) for the indicated times. Total Akt and p70S6K protein, as well as their phosphorylated forms were detected in whole cell lysates. GAPDH was employed as a loading control. B, CXCL12 increased PIP3 generation. CHO-K1 cells transfected with pEGFP-C1-Grp1-PH or pEGFP-C1 were incubated in serum-free media for 24 h. The cell was stained with CellMask Deep Red plasma membrane stains. Then, the stain solution was washed out and replaced with fresh medium. After addition of CXCL12 (300 ng/ml) for 2 min, the fluorescent images of the cell were captured with confocal microscopy. C, pretreatment of AMD3100 (100 ng/ml), PI-103 (100 nmol/liter), and rapamycin (10 nmol/liter) abrogated enhanced phosphorylation of Akt and/or p70S6K induced by CXCL12. Serum-deprived MKN-45 cells were treated with aforementioned inhibitors for 1 h and then stimulated with CXCL12 for 5 min. Phosphorylated Akt and p70S6K were detected in whole cell lysates. GAPDH was employed as a loading control. Data shown are representative of at least three independent experiments.
reduced in cells transfected with p110α-specific siRNA compared with those wild type cells. By contrast, p110β-specific siRNA blocked the CXCL12-stimulated Akt/mTOR signaling cascade. Consistently, phosphorylation of Akt and S6K1 induced by CXCL12 was abrogated by pretreatment of a p110β-specific inhibitor TGX-221 in MKN-45 cells (Fig. 2B), suggesting that p110β may be the main PI3K isofrom coupled with the CXCL12/CXCR4 axis in gastric cancer cells.

To further verify the obtained results, we analyzed the association of CXCR4 with p110β upon CXCL12 stimulation in MKN-45 cells with an immunoprecipitation assay. As shown in Fig. 2C, CXCR4 pulled down by an anti-p110β antibody significantly increased in MKN-45 cells after stimulation with CXCL12 for 20 s, indicating a significant increase in the association of CXCR4 with p110β. Thus, these results demonstrated that CXCR4 interacted with p110β and activated its downstream signaling. However, it is unknown whether CXCR4 interacts with p110β directly, which deserves to be further studied.

CXCR4 is a G protein-coupled receptor. We next investigated whether activation of p110β by the CXCL12/CXCR4 axis required G protein. As shown in Fig. 2D, Gi protein inhibitor pertussis toxin pretreatment abrogated phosphorylation of Akt at Ser-473 induced by CXCL12, indicating that G protein is required for activation of PI3K by CXCR4.

PI3K/mTOR Pathway Inhibitor Blocking Cell Migration Induced by CXCL12—Because CXCL12 promotes the motility of cells and we also validated that the CXCL12/CXCR4 axis activated the PI3K/mTOR signaling pathway in gastric cancer cells, we examined whether compounds targeting the PI3K/mTOR pathway would attenuate the motility of gastric cancer cells induced by CXCL12. To this end, a Transwell assay was utilized to detect migration of MKN-45 cell stimulated by CXCL12 or FBS in the presence of PI3K/mTOR dual inhibitor PI-103, mTORC1 inhibitor rapamycin or mTORC1/2 inhibitor PP-242. As shown in Fig. 3A, treatment of rapamycin at 10 nmol/liter for 24 h significantly inhibited CXCL12-stimulated migration of MKN-45 cells, with an inhibitory rate of ~40%. Similarly, PI-103 (10 nmol/liter) or PP-242 (100 nmol/liter) treatment for 8 h inhibited CXCL12-stimulated MKN-45 cells migration by ~45% (Fig. 3, B and C). These results indicated that compounds targeting the PI3K/mTOR pathway inhibited the motility of gastric cancer cells mediated by the CXCL12/CXCR4 axis.
To further distinguish the role of mTORC1 and mTORC2 in CXCL12-stimulated cell motility, we examined the migration of gastric cancer cells induced by CXCL12 after down-regulation of Raptor or Rictor by transiently transfection of respective siRNAs. As shown in the Fig. 3D, down-regulation of Raptor significantly inhibited CXCL12-stimulated migration of MKN-45 cells, whereas down-regulation of Rictor had little effect on this process. These data indicated that mTORC1 were more important in regulating the motility of gastric cancer MKN-45 cells.

Compounds Targeting PI3K/mTOR Pathway Prevented F-actin Reorganization and Down-regulated Active RhoA and RAC—Cell migration is a multistep cellular event, including cell polarization/protrusion (F-actin reorganization), adhesion, and de-adhesion, whereas the rapid reorganization of the actin cytoskeleton characterized as an early event of cell migration (17). Recently, it has been shown that rapamycin inhibition of insulin-like growth factor-induced cell motility is associated with its prevention of F-actin reorganization (18). We thus determined whether stimulation of CXCL12 resulted in F-actin reorganization and whether blocking PI3K/mTOR signaling prevented F-actin reorganization induced by CXCL12 in MKN-45 cells. As shown in Fig. 4A, F-actin distributed randomly across cells in serum-deprived MKN-45 cells with few lamellipodia formation, which was demonstrated by staining with Texas Red®-X phalloidin. CXCL12 stimulation resulted in the formation of lamellipodia and filopodia. Within the structure of lamellipodia, the condensation of F-actin at the leading edge indicated that stimulation of cells with CXCL12 results in F-actin reorganization. Pretreatment with PI-103 or PP-242 significantly abrogated F-actin reorganization at the leading edge.
induced by CXCL12. These results indicated that inhibition of cell motility by targeting the PI3K/mTOR pathway is correlated to disturbance of F-actin reorganization and lamellipodia formation.

The Rho family of small GTPase, in particular Rac1, Cdc42, and RhoA, consists of molecular switches that control the organization and dynamics of the actin cytoskeleton. It has been reported recently that rapamycin inhibits cytoskeleton reorganization and cell motility stimulated by insulin-like growth factor 1 via suppressing RhoA expression and activity (19). We thus detected the protein levels of RhoA, Rac1, and Cdc42 were analyzed by immunoblotting. GAPDH was employed as a loading control. C, down-regulation of the active form of RhoA, Rac1, and Cdc42. Cells were harvested for assays detecting the GTP-bound RhoA (upper panel), Rac1 (middle panel), and Cdc42 (lower panel) per the manufacturer’s instructions. Data shown are mean ± S.D. from three independent experiments. *, p < 0.05; **, p < 0.01, difference with CXCL12 group.

Compounds Targeting PI3K/mTOR Pathway Down-regulated Expression of CXCL12 and CXCR4—TORC1 plays a critical role in protein synthesis by phosphorylating S6 kinase and the translational inhibitor 4E-BP1. Phosphorylation of 4E-BP1 activates cap-dependent translation by dissociating with eIF-4E. Inhibition of TORC1 by rapamycin has been reported to down-regulate the expression of a number of proteins. We thus detected the effect of rapamycin on the expression of CXCR4 and CXCL12. As shown in Fig. 5A, CXCL12 enhanced the expression of CXCR4, whereas CXCR4 protein levels decreased significantly upon treatment with rapamycin for 6 h. The sim-
Inhibiting CXCR4-mediated Cell Migration by Targeting mTOR

It has been reported that CXCL12/CXCR4 axis is involved in the survival and proliferation of tumor cells (20). We next detected the anti-proliferative activities of rapamycin in these cells by sulforhodamine B assays. MKN-45 and MKN-28 were resistant to rapalogs. Rapamycin inhibited the proliferation of those cells by ~10% at the same concentration (Fig. 6D).

To investigate whether expression of cxcl12 is correlated with the anti-proliferative activity of rapamycin, we computed pairwise Pearson correlation coefficients (r) between rapamycin activity and genes expression patterns in NCI-60 cells. Analysis of 9,706 gene probes, including both proliferative and non-proliferative genes, discovered that expression of cxcl12 is correlated with the anti-proliferative activity of rapamycin.

**FIGURE 5.** Down-regulation of the expression of CXCL12 and CXCR4 by inhibiting mTOR pathway in MKN-45 cells. A, compounds targeting the PI3K/mTOR pathway down-regulated CXCR4 protein expression. MKN-45 cells were treated with PI-103, rapamycin, or PP-242 for 6 h, and CXCR4 protein was analyzed by immunoblotting. GAPDH was employed as a loading control. Data shown are representative of at least three independent experiments. B, PI3K pathway inhibitors inhibited the secretion of CXCL12. The supernatant was collected from the culture of MKN-45 cells after treatment with inhibitors for 24 h, and the CXCL12 level was assessed by human CXCL12 ELISA kit. The data (mean ± S.D. of three independent experiments) shown are fold change relative to the CXCL12 level in control group. *, p < 0.05; **, p < 0.01, difference with control group.

**FIGURE 6.** CXCL12 expression profiles and rapamycin anti-migratory or anti-proliferative activities in gastric cancer cells. A, mRNA levels of CXCL12 and CXCR4 in gastric cancer cells. Basal levels of CXCL12 or CXCR4 (normalized to GAPDH levels) were measured by real-time PCR. Data shown are fold-change relative to the CXCL12 or CXCR4 levels in SGC-7901 cells. B, activation status of the PI3K/AKT/mTOR pathway in gastric cancer cells were analyzed by immunoblotting. GAPDH was employed as a loading control. C, anti-proliferative activity was assessed by SRB assay after exposure to rapamycin for 72 h. D, effects of rapamycin on the cell migration was determined by the Transwell assay. Representative images (B and D) and quantitative data (A, C, and D; mean ± S.D. of three independent experiments) were shown. *, p < 0.05, difference with control (Ctrl) group.
significantly correlate with rapamycin activity \( (r = 0.395, p = 0.0011) \). Ten most positively correlated genes were listed in Table 1. Although the cell line samples in the present study are small, the results obtained provided a clue that mTOR inhibitor may be used for therapy of metastatic gastric cancer expressing high levels of **cxcl12**.

**DISCUSSION**

In the present study, we elucidated that the **CXCL12/CXCR4** axis activated **PI3K** and revealed that **p110β** catalytic isoform provided a conduit for **CXCL12**-activated **PI3K** signaling pathway in gastric cancer cells. Inhibitors targeting the **PI3K/mTOR** pathway blocked cell migration induced by **CXCL12**, which was attributed to decrease in the activity of Rac, Cdc42, and RhoA and inhibition of **F-actin reorganization**. Rapamycin also decreased the secretion of **CXCL12** and expression of its receptor **CXCR4**, which might form a positive feedback loop to further abolish upstream signaling leading to cell migration. We also found a positive correlation between **cxcl12** expression and rapamycin activity in inhibiting cells migration and proliferation. Taken together, we proposed that inhibitors targeting the mTOR pathway may be used for therapy of metastatic gastric cancers expressing high levels of **cxcl12**.

The chemokine **CXCL12** binds primarily to the **CXCR4** receptor and constitutes a biological axis, which is related to tumor progression, angiogenesis, metastasis, and survival (3). Blocking **CXCL12/CXCR4** interaction or inhibiting downstream intracellular signaling pathway may be useful for cancer therapy (4). We found that **CXCL12** stimulation activated **PI3K/Akt/mTOR** signaling cascade in gastric cancer **MKN-45** cells, which is consistent with the previous report, where phosphorylated Akt was utilized as an indirect marker for the activation of **PI3K** (7, 22–25). By employing CHO-K1 cells stably expressing pEgfp-C1-Grp1-PH fusion protein, we demonstrated that **CXCL12** stimulation resulted in generation of **PtdIns(3,4,5)P3** and the translocation of protein contained **PH** domain to cellular membrane, providing direct evidence that **CXCL12** activated **PI3K**. We further revealed that **p110β** was the main **PI3K** isofrom coupled with the **CXCL12/CXCR4** axis in gastric cancer cells, which was further supported by the fact that **CXCL12** enhanced the association between **CXCR4** and **p110β**. It has been reported that **CXCL12** activate the activity of **PI3K** in **G1** protein inhibitor pertussis toxin-treated cells, indicating both **Gαi** and **Gα13** proteins can mediate **PI3K** activation or this activation is **G-protein-independent** (8). However, **G1** protein was reported to be required for the activation of **PI3K** by **CXCL12** in T lymphocytes (26). In aforementioned studies, only **p110y** was detected to be associated with **CXCL12** after **CXCL12** stimulation. Recently, it has been reported **p110β** isoform signals downstream of **G** protein-coupled receptors, including **CXCR4**, and is functionally redundant with **p110y** (27). We found that pertussis toxin pretreatment abrogated phosphorylation of Akt at Ser-473 induced by **CXCL12**, indicating that **G1** protein is required for activation of **PI3K** by **CXCR4**. Although how CXCR4 interacts with **p110β** deserves to be further studied, our data shed new light on the understanding of the precise signaling underlying the activation of mTOR by CXCL12 in gastric cancer cells.

It has been reported that expression of **CXCR4** and **CXCL12** was associated with lymph node and liver metastasis in intestinal-type gastric cancer (28). The **CXCL12/CXCR4** axis and its downstream signaling could be therapeutic targets for preventing metastasis of gastric cancer. Moreover, it has been reported that both mTORC1 and mTORC2 play critical roles in the regulation of tumor cell motility and cancer metastasis (12, 19). We found that the **CXCL12/CXCR4** axis was able to activate the **PI3K/mTOR** pathway and stimulate cell migration in human **MKN-45** gastric cancer cells. On the other hand, inhibition of **PI3K/mTOR** pathway by **PI3K/mTOR** dual inhibitor **PI-103**, **TORC1** inhibitor rapamycin, or **TORC1/2** inhibitor **PP-242** significantly blocked the chemotactic responses of **MKN-45** cells triggered by **CXCL12**, which is related to prevention of **F-actin reorganization** and down-regulation of active **RhoA**, **Rac1**, and **Cdc42**. Interestingly, inhibition of cell migration by compounds targeting the **PI3K/mTOR** pathway was primarily attributed to their action against **mTORC1**, as down-regulation of Raptor but not Rictor impeded migration of **MKN-45** cells.

Furthermore, rapamycin treatment simultaneously decreased the secretion of chemokine **CXCL12** and the expression of its receptor **CXCR4** in **MKN** cells (Fig. 5). It has been reported that **CXCR4** is a transcriptional target of **HIF-1α** (25), and Song et al. reported that **PDGF-BB** induces **CXCL12** expression through **HIF-1α** activation (29). Inhibition of mTOR by rapamycin results in down-regulation of **HIF-1α** via block of cap-dependent translation. Therefore, decreased **CXCR4** and **CXCL12** expression by rapamycin could either due to global inhibition of protein translation or decreased transcription mediated by **HIF-1α**, which deserves further exploration. Autocrine of

### TABLE 1

| Rank | Gene name     | Gene description                          | r   | p value   |
|------|---------------|-------------------------------------------|-----|-----------|
| 1    | CDKN1B        | Cyclin-dependent kinase inhibitor 1B (p27, Kip1) | 0.473 | 0.0000   |
| 2    | ATP8A2        | ATPase, aminophospholipid transporter-like, class I, type 8A, member 2 | 0.436 | 0.0005   |
| 3    | CYFIP2        | Cytoplasmic FMR1-interacting protein 2    | 0.408 | 0.0010   |
| 4    | SOX1          | SRY (sex determining region Y)-box 1     | 0.402 | 0.0011   |
| 5    | Csof13        | Chromosome 5 open reading frame 13        | 0.399 | 0.0007   |
| 6    | CXCL12        | Chemokine (CXC motif) ligand 12 (stromal cell-derived factor 1) | 0.395 | 0.0011   |
| 7    | PIP5K2A       | Phosphatidylinositol 4-phosphate 5-kinase type II α | 0.384 | 0.0009   |
| 8    | LOC283567     | Hypothetical protein LOC283567            | 0.382 | 0.0008   |
| 9    | MGC16169      | Hypothetical protein MGC16169             | 0.382 | 0.0010   |
| 10   | NEK11         | NIMA (never in mitosis gene a)-related kinase 11 | 0.3792 | 0.0031   |

**Inhibiting CXCR4-mediated Cell Migration by Targeting mTOR**
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CXCL12 from tumor cells is able to activate CXCR4 receptor, which will promote cell motility as well as the viability of tumor cells at distant metastasis (2). Taken together, rapamycin not only inhibited CXCL12/CXCR downstream signaling but also down-regulated the expression of the chemokine and its receptor, which might form a positive feedback loop to block cell migration. Targeting the PI3K/mTOR pathway may be a new strategy for preventing gastric tumor metastasis mediated by the CXCL12/CXCR4 axis, even though additional preclinical and clinical studies are required to validate this proposal.

Recent study revealed that CXCL12 could be an independent prognostic factor in gastric cancer, with CXCL12-positive gastric cancer showing more aggressive behavior, including metastasis (2). We found that rapamycin-inhibited CXCL12 stimulation activated the PI3K/mTOR pathway and displayed selectivity to CXCL12-stimulated cell migration. These results were consistent with the observation that cells expressing high levels of CXCL12 were more sensitive to rapamycin-induced anti-metastatic activity. Meanwhile, we also found the positive correlation of cxcl12 expression and rapamycin anti-proliferative activity in gastric cancer cells. This point was also confirmed by studying the correlation between cxcl12 gene expression profiles and rapamycin activity in NCI-60 cell lines. Sporadic studies indicated that CXCL12 mediated the proliferation of cancer cells, which may be related to activation of Akt, Erk, Akt-1/FOXO-3a, Stat-3, and etc. (7, 20, 21, 30, 31). Although additional study is warranted to elucidate the correlation between rapamycin anti-proliferative activity and cxcl12 expression, this finding further supports that mTOR inhibitor may be useful for gastric cancer therapy.

In conclusion, we have shown that mTOR pathway play an important role in cell migration mediated by the CXCL12/CXCR4 axis and inhibitors targeting mTOR pathway could inhibit CXCL12-stimulated cell migration. On the other hand, cells that express high levels of cxcl12 were more sensitive to rapamycin in its activity in inhibiting both cell migration and proliferation. We proposed that drugs targeting the mTOR pathway may be used for the therapy of metastatic gastric cancer expressing high levels of cxcl12.

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