Diffuse-type gastric cancer cells switch their driver pathways from FGFR2 signaling to SDF1/CXCR4 axis in hypoxic tumor microenvironments

Haruhito Kinoshita1, Masakazu Yashiro1,2,*, Tatsunari Fukuoka1, Tsuyoshi Hasegawa1, Tamami Morisaki1, Hiroaki Kasashima1, Go Masuda1, Satoru Noda1 and Kosei Hirakawa1

1Department of Surgical Oncology and 2Oncology Institute of Geriatrics and Medical Science, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

*To whom correspondence should be addressed. Tel: +81 6 6645 3838; Fax: +81 6 6646 6450; Email: m9312510@med.osaka-cu.ac.jp

Abstract

Cancer-associated fibroblasts (CAFs) have been considered to play an important role for tumor progression of cancer. Solid tumors contain heterogeneous distribution of oxygen in their microenvironments. This study investigated the growth signaling of gastric cancer (GC) cells in focus on the interaction with CAFs and GC cells under normoxia and hypoxia. Four diffuse-type GC cell lines, two intestinal-type GC cell lines and three CAF cell lines were used. Cells were examined for expression of C-X-C chemokine receptor 4 (CXCR4), fibroblast growth factor receptor 2 (FGFR2) and stromal-derived factor 1 (SDF1) by RT-PCR, western blot, ELISA and immunohistochemical staining of xenografted tumors. GC cell proliferation was examined under hypoxia in the presence or absence of CAFs, a FGFR2 inhibitor, a CXCR4 inhibitor and HIF1α siRNA.

Proliferation of diffuse-type GC cells, but not intestinal-type GC cells, was significantly increased by CAFs. CXCR4 expression by diffuse-type GC cells was significantly increased in hypoxia, while FGFR2 expression was decreased. CXCR4 expression was correlated with hypoxic microenvironment of xenografted tumor, but FGFR2 expression was not. FGFR2 inhibition significantly decreased the growth-stimulating activity of CAFs for diffuse-type GC cells in normoxia. In contrast, CXCR4 inhibition significantly decreased the growth-stimulating activity of CAFs in hypoxia. SDF1 production by CAFs was increased in hypoxia, while cancer cells did not produce SDF1. HIF1α siRNA significantly decreased both CXCR4 expression by diffuse-type GC cells and SDF1 production by CAFs. These findings suggest that diffuse-type GC cells might switch their driver pathways from FGFR2 signaling to SDF1/CXCR4 axis through HIF1 in hypoxic tumor microenvironments.

Introduction

Tumor progression has been recognized as the result of an evolving crosstalk between the cancer cells and its surrounding environment (1). Solid cancers, including gastric cancer (GC), have a heterogeneously hypoxic environment, which is considered to be associated with an aggressive tumor phenotype (2–4). Current studies on tumor microenvironments report that cancer-associated fibroblasts (CAFs) might play an important role for the progression of solid carcinomas (5–7). Analysis of the interaction between cancer cells and stromal fibroblasts in a hypoxic microenvironment is important for understanding the mechanisms responsible for the solid tumor progression.

We previously reported that fibroblast growth factor 7 (FGF7) produced by CAFs is associated with the proliferation of diffuse-type of GC cells (8,9), suggesting that FGF7 and fibroblast growth factor receptor 2 (FGFR2) signaling plays an key role for the proliferation of GC cells under normoxic conditions (5,8,10). Recently, we reported that hypoxic conditions resulted in increasing of transforming growth factor β (TGFβ) signaling, and affected on...
the ability of diffuse-type GC cells to invade and adhere to peritoneum (11–13). Hypoxic conditions might effect not only the signaling for invasion but also the growth signaling of cancer cells. However, the signaling responsible for the proliferation of GC cells in hypoxic conditions remains unclear.

The aim of this study was to clarify the effect of hypoxic conditions on the growth signaling of GC cells, especially on the growth interaction between the CAFs and diffuse type of GC cells. In this study, we have demonstrated for the first time that diffuse type of GC cells switch the growth signaling between CAFs and cancer cells from FGF7/FGFR2 signaling to stromal-derived-factor 1 (SDF1)/CXCR chemokine receptor 4 (CXCR4) signaling in hypoxic conditions.

Materials and methods

Cell culture and cell lines

Six GC cell lines, OCUM-2MD3, OCUM-12, KATO-III, NUGC4, MNK-7 and MNK-74, were used. OCUM-2MD3, OCUM-12, KATO-III and NUGC4 were derived from diffuse-type GC, and MNK-7 and MNK-74 were derived from intestinal-type GC. Three CAFs cell lines, CaF45, CaF53 and CaF58, were established from surgical GC tissue of each patients with gastric carcinoma, as previously reported [14,15]. OCUM-2MD3 [16], OCUM-12 [11], CaF45, CaF53 and CaF58 were established at our department. OCUM-2MD3 and OCUM-12 were authenticated by STR profiling. KATO-III, NUGC4, MNK-7 and MNK-74, were purchased from JCRB Cell Bank (Osaka, Japan), where the cell lines were authenticated by STR profiling before distribution. Cells were cultured at 37°C 21% O2 and 5% CO2 balanced with N2 gas. The culture medium consisted of Dulbecco's modified Eagle medium (DMEM; Nikken Seed Co., Tokyo, Japan) with 5% CO2, 100 IU/ml penicillin (ICN Biomedicals, Costa Mesa, CA), 100 mM sodium pyruvate (Cambrex, Germany) according to the manufacturer's recommendation. Diffuse-type GC cells and CAFs were prepared at 50-60% confluence in six-well dishes. The transfection mixture was prepared by adding 250 μl of Opti-MEM including 5 μl of lipofectamine RNAiMAX Reagent (Life technologies, Carlsbad, CA) for knockdown experiments. Diffuse-type GC cells and CAFs were prepared at 50-60% confluence in six-well dishes. The transfection mixture was prepared by adding 250 μl of Opti-MEM including 5 μl of lipofectamine RNAiMAX Reagent (Life technologies) to 250 μl of Opti-MEM including 60 pmol of siRNA and incubating for 5 min at room temperature. Finally, the above transfection mixture was added to six-well dish containing 2 ml of DMEM with 10% fetal bovine serum (final siRNA concentration was 30 nM). Twenty-four hours after transfection under normoxia.

Preparation of serum-free conditioned medium from CAFs

Serum-free conditioned medium (SF-CM) from CAFs was prepared as follows. CAFs (5.0×10^4) were seeded into 100mm plastic dishes with 10 ml of DMEM containing 10% fetal bovine serum, and incubated at 37°C for 3 days. To obtain the SF-CM, CAFs were washed twice with Dulbecco’s phosphate-buffered saline (PBS) and then incubated for 3 days with 3 ml of DMEM. The number of CAFs in each dish was ~2×10^5 cells at the collection of SF-CM. The SF-CM was collected and stored at −20°C until use. The fibroblasts were used before the 8th passage in culture.

Proliferation assay of GC cells cocultured with CAFs

The effect of co-culture with diffuse-type GC cells and CaF53 on growth was examined by using the double chamber method. GC cells (1×10^4) were seeded under the membrane, and CaF53 (1×10^4) were seeded in the upper chamber with a pore size of 3 μm (Millipore, Billerica, MA) in the same wells, furthermore, added to 5 μM CXCR4 antagonist, AMD3100 (Sigma–Aldrich, St. Louis, MO) and FGFR2 antagonist, KG3057 (KIRIN Brewery Co., Gunma, Japan) [10]. There were incubated under normoxia or hypoxia for 72 h. The number of cancer cells was counted by using cell counter (TC10 Automated cell counter, BIO-RAD).

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time PCR was performed to examine hypoxia-inducible factor-1α (HIF1α), CXCR4, CXCR2, FGFR2, TGFβ1, TGFβ2 and epidermal growth factor (EGFR) mRNA expression. GCCs were incubated under normoxic conditions or under hypoxic conditions for 48 h. After incubation, the total cellular RNA was extracted using RNeasy Mini (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After the removal of genomic DNA by DNase, cDNAs were prepared from 1 μg RNA with Maloney mouse leukemia virus reverse transcriptase (Invitrogen Carlsbad, CA) using random primers (Invitrogen). To determine fold changes in each gene, real-time RT-PCR was performed on the ABI Prism 7000 (Applied Biosystems, Foster City, CA) using commercially available gene expression assays for HIF1α (Hs01565584), CXCR4 (Hs00237052), CXCR2 (Hs00174304), FGFR2 (Hs00253682), TGFβ1 (Hs00610319), TGFβ2 (Hs00595661) and EGFR (Hs0107691). PCR was performed at 95°C for 15 s and 60°C for 60 s for 40 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize mRNA levels for differences in sample concentration and loading.

Western blot analysis

D-GCCs were incubated under normoxia or hypoxia for 48 h. The cells were lysed, and 20 μg of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the protein bands were transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was placed in a TBS-T solution (10 mM TBS and 0.05% Tween 20) containing the primary Anti-CXCR4 antibody (ab2074; 1:1000, Abcam, Cambridge, MA), Anti-HIF-1α antibody (GTX127309; 1:1000; GeneTex, LA) or β-actin (1:1000, Cell Signaling) and allowed to react at 4°C overnight. Then, a peroxidase-labeled secondary antibody (GE healthcare, Buckinghamshire, UK) was added. The bands were detected using an enhanced chemiluminescence system (Wako, Osaka, Japan).

Small interfering RNA

Silencer predesigned siRNA targeting HIF1α#1 (ID #56539), HIF1α#2 (ID #56541), CXCR4#1 (ID #515412), CXCR4#2 (ID #515413) and Silencer™ negative control siRNA (ID #4390843) were purchased from Ambion (Life technologies, Carlsbad, CA) for knockdown experiments. Diffuse-type GC cells and CAFs were prepared at 50-60% confluence in six-well dishes. The transfection mixture was prepared by adding 250 μl of Opti-MEM including 5 μl of lipofectamine RNAiMAX Reagent (Life technologies) to 250 μl of Opti-MEM including 60 pmol of siRNA and incubating for 5 min at room temperature. Finally, the above transfection mixture was added to six-well dish containing 2 ml of DMEM with 10% fetal bovine serum (final siRNA concentration was 30 nM). Twenty-four hours after transfection under normoxia.

Enzyme-linked immunosorbent assay

The production of stromal-derived-factor 1 (SDF1) in SF-CM from CAFs and cancer cells was determined using a ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendation.

Effect of various factors on the proliferation of GC cells under hypoxic condition

Diffuse-type GC cell lines were cultured in 96-well plates at a concentration of 5000 cells per well with culture medium as the control, recombinant human FGF7 30 ng/ml (R&D Systems), recombinant human SDF-1 100 ng/ml (R&D Systems) and 50% CM from CaF53, furthermore, added to 1 μM CXCR4 antagonist AMD3100, negative siRNA, HIF1α siRNA and CXCR4 siRNA. After incubation for 72 h under hypoxic condition, the proliferation of diffuse-type GC cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT; Sigma) assay was measured as absorbance at 570 nm using a microtiter plate reader (PM2004, Wako).

Animal models

 Xenografts were established by injecting 10°C OCUM-2MD3 cells into the flanks of female athymic BALB/c nude mice (4-week-old female mice; Clea
Oxidative conditions affect the expression level of growth factor receptors on GCs

Since oxidative conditions might affect on the signaling pathway, the expression level of growth factor receptors of six GC cell lines were examined. The expression of CXCR4 mRNA was significantly increased in all four diffuse-type GC cell lines, but not the intestinal-type GC cell lines, in hypoxic compared with normoxic conditions. In contrast, the expression of CXCR2 and FGFR2 mRNA was significantly decreased in hypoxic conditions in all six GC cell lines. The expression of TGFβR1, TGFβR2 and EGFR mRNA was significantly increased in OCUM-2MD3 and OCUM-12 cell lines when in hypoxia compared with normoxia (Figure 2A). The expression of CXCR4 proteins was significantly increased in all four diffuse-type GC cell lines in hypoxic to normoxic conditions (Figure 2B). Diffuse-type GC cells may switch from FGF7/FGFR2 to the SDF1/CXCR4 axis to promote proliferation in hypoxia.

**HIF1α siRNA regulates the expression of various growth factor receptors when in hypoxia**

Hypoxia-inducible factor 1 (HIF1) activates transcription of genes in hypoxic cells. To determine whether HIF1 mediates cell-growth signaling to hypoxia, we have compared the expression of various growth factor receptors in cancer cells cultured with HIF1α siRNA under hypoxic conditions. HIF1α mRNA expression of diffuse-type GC cells in hypoxia was significantly increased, 16.24-fold (P < 0.01) for OCUM-2MD3 cells, 9.01-fold (P < 0.01) for OCUM-12 cells, 1.51-fold (P < 0.01) for KATO-III cells and 1.75-fold (P < 0.01) for NUGC4 cells. In contrast, HIF1α mRNA expression of intestinal-type GC cells was decreased, 0.32-fold for MKN-7 cells and 0.82-fold for MKN-7 cells in hypoxia (Figure 3A). HIF1α protein level is increased under hypoxia in diffuse-type GC cells, OCUM-2MD3, OCUM-12, KATOIII and NUGC4, in comparison with that in normoxia (Figure 3B). HIF1α siRNA successfully decreased the HIF1α mRNA expression in all four diffuse-type GC cell lines under hypoxic conditions, in comparison to cells treated with negative control siRNA (Figure 3C). The expression of CXCR4 mRNA in hypoxia was significantly decreased in all four diffuse-type GC cell lines by HIF1α siRNA, compared with those treated with the negative control siRNA. In contrast, HIF1α siRNA significantly increased the expression of FGFR2 in OCUM-2MD3 and NUGC4 cells. HIF1α siRNA significantly decreased TGFβR1 and TGFβR2 expression in all four diffuse-type GC cell lines and increased the expression of EGFR in OCUM-2MD3 cells and KATO-III cells (Figure 3D). The expression of CXCR4 proteins under hypoxia decreased in all four diffuse-type GC cell lines by HIF1α siRNA treatment, compared with negative control siRNA (Figure 3E). HIF1 regulates the expression of cell-growth signaling in hypoxic GC cells.

**The effect of hypoxia on SDF1 production by CAFs**

Oxidative conditions might affect on not only the expression of receptors of GC cells but also growth factor production from CAF. SDF1 production by CAFs were examined under hypoxia compared with normoxia in the presence and absence of HIF1α siRNA. HIF1α siRNA successfully down-regulated the HIF1α mRNA expression in all three CAF cell lines when in hypoxia, in comparison with negative control siRNA (Figure 4A). SDF1 production by CaF45, CaF53 and CaF58 significantly increased in hypoxia (106, 245 and 359 pg/ml, respectively), in comparison to their production in normoxia (44, 139 and 224 pg/ml, respectively). In hypoxia, HIF1α siRNA significantly decreased SDF1 production by CaF45, CaF53 and CaF58 cells (76, 150 and 245 pg/ml).
235 pg/ml, respectively), in comparison to the cells treated with the negative control siRNA (113, 235 and 351 pg/ml, respectively) (Figure 4B). Hypoxia upregulated the production of SDF1 by CAFs via HIF1 signaling. On the other hand, SDF1 production from diffuse type GC cell lines, OCUM-2MD3, OCUM-12, KATO-III and NUGC4, was not detected (<16 pg/ml).

The significance of SDF1/CXCR4 signaling on the proliferation of diffuse-type GC cells in hypoxia

To examine whether CXCR4 is driver signaling on the proliferation of diffuse-type GC cells in hypoxia, we compared the inhibitory-effect of FGFR2 inhibitor and CXCR4 inhibitor in diffuse-type GC cells in hypoxia. SDF1 and conditioned medium (CM) produced by CaF53 cells significantly stimulated the proliferation of diffuse-type GC cells in hypoxia. SDF1 and conditioned medium (CM) produced by CaF53 cells significantly stimulated the proliferation of diffuse-type GC cells in hypoxia. HIF1α siRNA and CXCR4 siRNA significantly decreased the proliferation of diffuse-type GC cells stimulated by the CM produced by CaF53 cells in hypoxia (Figure 5). Diffuse-type GC cells may switch from FGFR7/FGFR2 to the SDF1/CXCR4 axis to promote proliferation in a hypoxic microenvironment.

The expression of CXCR4, FGFR2/KsamII and CA-9 on tumors in vivo

The expression of CA9 and CXCR4 was mainly observed in the cell membrane of tumor cells. FGFR2 was expressed at the membrane and in the cytoplasm of cancer cells. The tumor lesion with CXCR4 expression was associated with that with CA9 expression. CXCR4 expression was correlated with hypoxic microenvironment. The size of xenografted tumors by OCUM-2MD3 cells was smaller after Ki23057 than those of the untreated control. In tumor with Ki23057 treatment, the lesion of both CXCR4 expression and CA9 expression was frequently found. In contrast, FGFR2/KsamII expression was substantially reduced in tumor with Ki23057 treatment, in compared with that in control tumor (Figure 6). CXCR4 expression was correlated with hypoxic microenvironment of xenografted tumor in vivo.

Discussion

We examined the effect of a hypoxic environment on the proliferation of GC cells, focusing on the interaction between cancer cells and stromal fibroblasts. CAFs significantly increased the proliferation of diffuse-type GC cells in both normoxia and hypoxia, but did not increase proliferation of intestinal-type GC cells. Taken together, we examined the effect of CAFs and normal fibroblasts (NFs) on the proliferation of diffuse-type GC cells by using the double chamber method. CAFs increased the proliferation of diffuse-type GC cells, OCUM-2MD3 and OCUM-12, but NF did not increase the proliferation of diffuse-type GC cells (Supplementary Figure 1, available at Carcinogenesis Online). This proliferation-stimulating activity of CAFs for diffuse-type GC cells was greater in hypoxic conditions compared with...
normoxic conditions. These findings suggest that the oxidative conditions may effect the growth-stimulating interaction between CAFs and diffuse-type GC cells. We previously reported that gastric fibroblasts stimulate the proliferation of diffuse-type GC cells through FGF7/FGFR2 signaling in normoxia (8,10). In this study, we clarified the growth stimulating signalings of CAFs for diffuse-type GC cells in hypoxic conditions and compared the results with those in normoxic conditions.

Interestingly, the expression level of FGFR2 in diffuse-type GC cells was significantly decreased under hypoxic conditions, whereas the expression of CXCR4, TGFβR and EGFR was significantly increased. Conversely, the expression of these growth factor receptors decreased under hypoxic conditions in the two intestinal-type GC cell lines. HIF1α mRNA expression of all 4 diffuse-type GC cells was significantly increased in hypoxia whereas HIF1α mRNA of intestinal-type GC cells was decreased. These differential responses in hypoxia might be associated with the histologic formation of GC.

We previously reported that a FGFR2 phosphorylation inhibitor decreased the proliferation of diffuse-type GC cells in normoxia and prolonged the survival of mice with diffuse-type GC, however, all mice died later due to peritoneal growth of GC.
cells despite of continuous administration of the inhibitor (10). FGFR2 inhibitor reduced tumor cells with FGFR2 expression, which might result in the increase of cancer cells with CXCR4 expression in xenografted tumor treated by Ki23057. This indicates that diffuse-type GC cells in hypoxic microenvironments may have switched the growth-signaling pattern that bypasses FGFR2, thereby enabling resistance against the FGFR2 monotherapy. A tyrosine kinase inhibitor that prevented CXCR4 phosphorylation significantly decreased the proliferation-inducing effects of CAFs in hypoxia. Pre-clinical investigations have demonstrated that a CXCR4 inhibitor decreased the proliferation of GC in vitro and in vivo (21,25,26). GC frequently shows tumor heterogeneity, suggesting that there might exist many kinds of driver gene and passenger gene in a single tumor. These findings indicated that FGFR2 is a driver gene and CXCR4 is a passenger gene under normoxia, meanwhile CXCR4 is a driver gene and FGFR2 is a passenger gene under hypoxia. A combination therapy consisting of a FGFR2 inhibitor and a CXCR4 inhibitor may provide a significant clinical benefit for diffuse-type GC patients as it will target GC cells in both hypoxic and normoxic conditions. Effective cancer therapy should consist of components targeting both normoxic and hypoxic tumor tissues.

Diffuse-type GC frequently metastasizes to the peritoneum (10,27,28). Cancer cells leaving the primary tumor might be exposed to low oxygen levels in the abdominal cavity because no feeding vessel is found around these free cancer cells. In fact the abdominal cavity filled with ascites was severely hypoxic (<10 mmHg) at the terminal stage after orthotopic implantation.
of human cancer cell line (29). Our data indicated that hypoxia in the peritoneal cavity might affect the phenotype of diffuse-type cancer cells by changing their driver pathways from FGFR2 signaling to CXCR4 signaling. Yasumoto K. et al also reported that the CXCL12/CXCR4 axis is involved in the development of peritoneal carcinomatosis from diffuse-type GC. The upregulation of CXCR4 in GC cells in hypoxia might be one of the mechanisms responsible for high metastatic potential of diffuse-type GC to the peritoneum.

HIF1α mRNA expression of diffuse-type GC cells, but not intestinal-type GC cells, was significantly increased in hypoxia. Oxygen sensors, such as the prolyl hydroxylase domain family and the factor inhibiting HIF, play a pivotal role in regulating HIF stability (30,31). The activity of these oxygen sensors might be different between diffuse-type GC cells and intestinal-type GC cells. HIF1α knockdown by HIF1α siRNA significantly prevented the up-regulation of CXCR4 by diffuse-type GC cells in hypoxic conditions and also significantly decreased the hypoxia-induced production of SDF1 by CAFs, resulting that HIF1α siRNA decreased the proliferation-inducing activity of CAFs to diffuse-type GC cells under hypoxia. Several studies have also reported that hypoxic conditions up-regulate the expression level of SDF1 and CXCR4 through activation of HIF1α (25,32,33). These findings indicate that hypoxia increased the proliferation-inducing stimulus provided by CAFs to diffuse-type GC cells via the CXCR4/SDF1 axis by inducing HIF1α signaling. These findings suggested that the key proliferation-inducing signaling between CAFs and cancer cells in hypoxia might be different from those in normoxia. GC has both normoxic and hypoxic lesions in the tumor microenvironment (3), and diffuse-type GC cells may switch their signaling pathways for proliferation in order to adapt to the different oxidative conditions.

TGFβR and EGFR were up-regulated by diffuse-type GC cells in hypoxic conditions, as previously reported (14,34). However, TGFβ/TGFβR signaling did not increase the proliferation of GCs in hypoxia (data not shown). Also, EGF and TGFα were not detected in the conditioned medium of CAFs (data not shown). These findings indicate that TGFβR and EGFR signaling are not associated with the proliferation stimulus provided by CAFs to GCs in hypoxic conditions.

In conclusion, diffuse-type GC cells might switch their signaling pathways in hypoxic conditions from FGFR7/FGFR2 to SDF1/CXCR4, which may enable proliferation in hypoxic tumor microenvironments. This study provides new insight into the growth mechanisms responsible for the progression of GC cells in hypoxic microenvironments and the development of effective cancer therapeutics targeting pathway.
Figure 4. The effect of hypoxia on SDF1 production by CAFs. (A) HIF1α mRNA expression. In hypoxic conditions, HIF1α siRNA, siHIF1α #1 and siHIF1α #2, downregulated HIF1α expression in all three CAFs. (B) Results from an ELISA showed that hypoxic conditions significantly increased the production of SDF1 by all of CAFs. In addition, knockdown of HIF1α significantly decreased SDF1 protein levels produced by CAFs. *P < 0.05; **P < 0.01.

Figure 5. The effect of SDF1/CXCR4 signaling on the proliferation of diffuse-type gastric carcinoma cells under hypoxia. The proliferation of diffuse-type GC cells under hypoxia was significantly increased in the presence of SDF1 and conditioned medium from CAFs. HIF1α siRNA and CXCR4 siRNA, significantly decreased the growth-stimulating activity of CAFs. The results are shown as the means of six samples and the bars correspond to the standard deviations. *P < 0.05; **P < 0.01.
Figure 6. Immunohistochemical determination of CXCR4, FGFR2/KsamII and CA-9 in xenografted tumors. Representative staining of CXCR4, FGFR2 and CA-9 in control tumor and Ki23057 treated tumor. A distinctive pattern of membranous staining of CXCR4 and CA-9 was observed at the cell-cell boundaries. FGFR2/KsamII antibody stained in the cytoplasm of tumor cells. CXCR4 expression was recognized at the tumor lesions with CA-9 expression. In contrast, FGFR2/KsamII expression was weak at the lesions with CA-9 expression. Ki23057 decreased immunohistochemical staining of FGFR2 and increased CXCR4 expression in OCUM-2MD3 tumor xenografts compared to the control.

Supplementary material
Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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