New Insight into the SDF-1/CXCR4 Axis in a Breast Carcinoma Model: Hypoxia-Induced Endothelial SDF-1 and Tumor Cell CXCR4 Are Required for Tumor Cell Intravasation

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Abstract

The SDF-1/CXCR4 axis has been implicated in breast cancer metastasis. In contrast to its well-established role in organ-specific homing and colonization of tumor cells, the involvement in intravasation, especially in a hypoxic environment, is still poorly understood. Initially, we detected both, the chemokine SDF-1 and its receptor CXCR4 in microvessels in invasive ductal cancer samples. To elucidate the role of the SDF-1/CXCR4 axis in vascular endothelium for tumor intravasation, we evaluated the effects of CXCR4 activation in human umbilical vein and dermal microvascular endothelial cells (HUVEC and HDMEC) and in cultured mammary carcinoma cells (MDA MB231, and MCF7). We observed an upregulation of SDF-1 and CXCR4 in HUVECs in hypoxia, which led to proliferation, migration, and tube formation. Hypoxia induced adhesion of tumor cells to endothelial cells and stimulated transendothelial migration. The effects of hypoxia were dependent on the activity of the transcription factor hypoxia-inducible factor. Adhesion to and migration through a HUVEC monolayer were significantly reduced by lentiviral inhibition of CXCR4 in breast carcinoma cells or treatment of endothelial cells with an anti-SDF-1 neutralizing antibody. These data show that the interaction of SDF-1 secreted by ECs with tumor cell CXCR4 is sufficient to stimulate transendothelial migration of the tumor cells. Our results suggest that the SDF-1/CXCR4 axis is important in angiogenesis and tumor cell intravasation. Because both proteins were readily identifiable in a significant fraction of human breast cancer samples by immunohistochemistry, CXCR4 may constitute a molecular target for therapy when both, SDF-1, and CXCR4 are expressed.

Introduction

In the United States, breast cancer is the most frequent type of cancer with nearly 210,000 new cases diagnosed and almost 40,000 deaths in 2010 (1). Metastasis, that is transport of tumor cells to and growth at secondary sites, is a major cause for morbidity and mortality (2, 3). The metastatic process involves multiple steps including tumor cell mobilization, angiogenesis, intravasation, survival in the circulation, extravasation, and proliferation in a new tissue environment. Despite increasing knowledge, all these steps are incompletely understood. For example, at the primary site, the communication of the tumor cells with their cellular microenvironment which is composed of fibroblasts, neutrophils, mast cells, and endothelial cells, not only promotes tumor growth, but also facilitates emigration from the original location (4, 5). These studies strikingly suggest a functionally important interaction between host-stroma and tumor. However, the mechanisms of intravasation are mostly elusive.

In a hypoxic microenvironment, the function of endothelial cells is modified toward an angiogenic phenotype. This response to hypoxia is indeed physiological, for example blood vessel development in the mammalian embryo is also stimulated by hypoxia (6). In tumor biology, this pathway is hijacked by the tumor cells. When a tumor grows beyond a diameter of 2 mm, the tumor cells commonly turn hypoxic. As a response, the transcription factor complex hypoxia-inducible factor (HIF) is activated. HIF stimulates secretion of growth factors including VEGF, which acts on endothelial cells to initiate angiogenesis. The developing capillary network in the tumor not only delivers nutrients and oxygen, but also provides the escape route by which detached cancer cells can enter into the circulation.

Stromal cell-derived factor-1 (SDF-1) is a highly conserved chemokine that acts through its cognate receptor CXCR4. Chemokines are low molecular weight signaling
peptides frequently involved in leucocyte mobilization and homing. Specifically, the SDF-1/CXCR4 axis plays a critical role in B-cell development (7), angiogenesis (8), HIV infection (9), as well as stem cell mobilization and homing (10). Both SDF-1 and CXCR4 have been reported to be hypoxia-inducible (11, 12). Several lines of evidence suggest that SDF-1 and CXCR4 are involved in tumor progression and the development of distant metastases: SDF-1 secreted by tumor-associated fibroblasts has been reported to be proangiogenic (5). In a landmark study it was showed that expression of CXCR4 facilitates orthotopic tumor growth and metastatic potential in SCID mice although, notably, tumor cells were injected i., which indicates that the experiments reflect late steps of metastasis (13). In another report CXCR4 was identified in a human breast cancer cell line as 1 of 5 proteins which gives rise to bone metastases after intraventricular injection in mice (14). Correlation of CXCR4 expression to HER2, a well-established prognostic factor in mammary carcinoma was also observed in human breast tumors (15).

However, neither SDF-1 nor CXCR4 has been identified as a prognostic marker in large microarray DNA screens published by several independent groups (16, 17). A low molecular weight CXCR4 antagonist did not inhibit growth in a murine tumor model (18). In one study it was found that expression of SDF-1 reduced the metastatic potential of breast carcinoma cell lines (19) and, furthermore, an immunohistochemical analysis of SDF-1 expression in human breast cancer patients came to the result that SDF-1 expression was a marker of longer disease-free and overall survival (20). Collectively, these reports show that the role of SDF-1 and CXCR4 in human tumors is still controversial, and in particular, that the effect of SDF-1/CXCR4 activation in tumor-host interaction at the primary site is elusive.

It is still an open question whether tumor cells enter into the vasculature passively or whether they migrate through blood vessels actively. Evidence for active metastasis is provided by the observation that the tumor microenvironment can induce migration-related pathways (21, 22) or collaboration of stromal and tumor cells in metastasis (4, 23). However, currently there is no study available in which the whole sequence of events is followed in a single coculture model. Therefore, it was the aim of our study to provide mechanistic insight into the role of the SDF-1/CXCR4 axis in the early steps of metastasis with particular emphasis on the effects of hypoxia in a coculture system of breast cancer and endothelial cells. We observed that endothelial SDF-1 stimulated active adhesion of CXCR4 expressing breast carcinoma cells to endothelial cells and transendothelial migration. Importantly, we confirmed endothelial SDF-1 and tumor cell CXCR4 expression in a significant fraction of samples from mammary tumors which pinpoints the relevance of our data in a clinical setting.

Materials and Methods

Immunohistochemistry and immunofluorescence

Staining of SDF-1 and CXCR4 was carried out in 140 samples of primary breast carcinomas chosen from a collection of our Institute of Pathology. The patients presented between 1989 and 1993 were operated with curative intent at the Department of Obstetrics and Gynaecology, University Hospital Essen. The study was carried out according to the Declaration of Helsinki and approved by the local Ethics Committee. Immunohistochemistry was conducted as described previously (24). Following individually optimized heat-based antigen retrieval for each antibody, SDF-1 was stained with a monoclonal anti- CXCL12/SDF1 [clone 79018 (R&RD Systems, Inc.) dilution 1:1000, antigen retrieval as for proangiogenic (5)]. In a landmark study it was showed that expression of CXCR4 facilitates orthotopic tumor growth and metastatic potential in SCID mice although, notably, tumor cells were injected i., which indicates that the experiments reflect late steps of metastasis (13). In another report CXCR4 was identified in a human breast cancer cell line as 1 of 5 proteins which gives rise to bone metastases after intraventricular injection in mice (14). Correlation of CXCR4 expression to HER2, a well-established prognostic factor in mammary carcinoma was also observed in human breast tumors (15).

Cell culture

Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC), both from PromoCell were grown in endothelial cell growth medium (ECGM; PromoCell) containing the recommended additives. ECs were used for experiments from passage 3 to 6. HEK-293T, MDA-MB 231, and MCF7 cells were obtained from ATCC. MDA-MB 231 were maintained in DMEM (Invitrogen), MCF-7 cells were grown in RPMI1640 (Invitrogen), supplemented with 10% fetal calf serum, penicillin, and streptomycin. Hypoxic culture conditions were achieved in a hypoxic chamber (Toepffer Lab Systems) by inflation of N2 and CO2 into the air-filled chamber until 1% O2 and 5% CO2 were reached.

Chemokines, antibodies, and reagents

Recombinant CXCL12/SDF-1α, CXCL12/SDF-1 monoclonal antibody (MAb) (Clone 79018, Mouse IgG1), CXCR4 (Fusin) MAb (Clones 12G5 and 44716), and affinity-purified HIF-2α antibody were purchased from R&D Systems. CXCR4 polyclonal antibodies were from Abcam. HIF-1α MAb was from BD Transduction Laboratories. Mouse Ig G2a (Clone G155-178) was purchased from BD Biosciences. Human α-tubulin MAb was from Santa Cruz Biotechnology. Human actin antibody was from Sigma. Dimethylxalylglycine (DMOG) was purchased from Alexis Biochemicals.

Bacterial strains, plasmids, lentiviral transduction, and siRNA transfection

Escherichia coli XL1-blue served as the general host for plasmid preparation. E. coli SfiI3 (Invitrogen) were used for production of plKO.1 (8453, Addgene), plKO.1-shRNA-HIF-1α-1 (Mission TRC shRNA TRCN000003810; Sigma), plKO.1-shRNA-HIF-1α-2 (Mission TRC shRNA

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were regarded as a tube.

Twenty-four hours later, photographs were taken with a Leica DFC 290 camera (Leica Microsystems). A total of 2 × 10⁵ MCF7 cells were transacted, the final concentration of the siRNA was 25 nmol/L. The cells were incubated with the transfection solution for 72 hours. Gene silencing was monitored by Western blotting.

**Western blot**

Whole-cell lysates from HUVECs, MDA-MB 231, and MCF-7 were prepared and Western blotting was carried out as reported (25).

**Fluorescent labeling and flow cytometry**

The fluorescent labeling of HUVECs, MDA-MB 231, and MCF7 cells with calcein-AM (Invitrogen) was conducted according to the manufacturer’s protocol. Fluorescence was detected using a Zeiss Axiosvert 200 M confocal microscope (Carl Zeiss). For FACS analysis, HUVECs or MDA-MB 231 cells were detached with 2 mmol/L EDTA in PBS. For CXCR4 determination and membrane SDF-1 expression, the untrained cells were fixed by incubation with 1% paraformaldehyde (PFA) for 5 minutes. The cells were incubated with 20 μL PE-conjugated human CXCR4 MAB (Clone 12G5) for 30 minutes at 4°C. For intracellular SDF-1 detection, the cells were permeabilized with Cytotox/ Cytoperm (BD Biosciences) and incubated with 25 μg/mL CXCL12/SDF-1 MAB (Clone 79018) for 30 minutes at 4°C. SDF-1 was detected with a PE-conjugated goat anti-mouse antibody (BD Pharmingen). Cells were analyzed with a Beckman Coulter FACSCalibur. A total of 10,000 events were assessed. Data were analyzed by CELLQuest software (BD Biosciences).

**Tube formation assay**

HUVECs (2 × 10⁵/well) were transduced with pLKO.1-shRNA-HIF-1α, pLKO.1-shRNA-HIF-2α or pLKO.1-shRNA-CXCR4 and seeded into Matrigel-coated wells of a 96-well plate. Twenty-four hours later, photographs were taken with a Leica DFC 290 camera (Leica Microsystems). Only perfectly continuous tubes between 2 branching points were regarded as a tube.

**Proliferation assay**

To assess HUVEC proliferation, 1 × 10⁴ HUVECs were seeded into 24-well plates in the absence or presence of recombinant human SDF-1 (1–100 ng/mL). CXCR4 neutralizing antibodies (10 μg/mL) or control mouse IgG (BD Biosciences) were added 12 hours before treatment with SDF-1. The viable cells were counted at day 3 and 6. When the effects of endogenous SDF-1 were to be blocked, 1 × 10⁴ HUVECs were seeded into a 24-well plate and cultured in serum-free ECGM in the absence or presence of an SDF-1 neutralizing antibody for 3 days. For proliferation assays after HIF-1α, HIF-2α, or CXCR4 knockdown, 8 × 10⁴ HUVECs was transduced with the appropriate vectors and cultured for 3 days. To quantify tumor cell proliferation, 1 × 10⁵ MDA-MB 231 were plated and grown for 4 days.

**Tumor cell-to-endothelium adhesion assay**

A total of 5 × 10⁴ HUVECs transduced with pLKO.1-shRNA-CXCR4, or pLKO.1 were exposed to normoxia or hypoxia for 12 hours after the monolayer had formed. A total of 1 × 10⁵ calcine-labeled MDA-MB 231 cells were added, adhesion was allowed to occur for 2 hours at 37°C. Non-adherent cells were washed off, adherent MDA-MB 231 cells were quantified.

**Adhesion of tumor cells to endothelial tubes**

A total of 1 × 10⁴ calcine-labeled MDA-MB 231 or MCF7 cells were cocultured with 2 × 10⁴ HUVEC or HDMEC on a Matrigel-coated 96-well plate. Ten μg/mL CXCR4 neutralizing antibody or 6 to 12 μg/mL SDF-1 antibody (both from R&D Systems) was added to block CXCR4 or SDF-1, respectively. After 24 hours, the adherent tumor cells were quantified.

**Cell migration assays**

All cell migration assays were conducted in a modified Boyden chamber, that is, with 24-well plates in which transwell inserts (polycarbonate membrane insert with 6.5-mm diameter and 8.0 μm pores; Corning) were added. To assess endothelial cell migration, 2 to 5 × 10⁴ HUVECs were seeded into the upper compartment and incubated for 24 hours. The nonmigrated cells on the top side of the surface were removed. The migrated cells on the bottom side of the membrane were counted in 12 standardized fields. To test endothelial effects on tumor cell migration, 1 × 10⁵ HUVECs or HDMECs were seeded in the lower compartment of the chamber. When the cells had formed a monolayer, they were treated with SDF-1 neutralizing antibodies (R&D Systems) before calcine-labeled MDA-MB 231 cells were seeded into the upper compartment. Exposure to hypoxia was for 24 hours. Migrated cells in the lower compartment were counted and photographs were taken. For transendothelial migration assays, HUVECs were cultured in the upper compartment which was precoated with 0.1% gelatine. Leaksiness of the endothelial monolayer was tested with FITC dextran before addition of tumor cells. A total of 2 × 10⁴ calcine-labeled MDA-MB 231 cells were seeded into the upper compartment and incubated for 24 hours. The migrated cells were quantified. Because only the tumor cells were labeled, this assay allows discrimination of tumor cells from migrated endothelial cells.

**Statistical analysis**

Data are expressed as the mean ± SD. Studies involving more than 2 groups were analyzed by 1-way ANOVA.
coupled to post hoc comparison tests for multiple pairwise comparisons. All other experiments were analyzed with an unpaired 2-tailed Student $t$ test. In all figures ß indicates $P < 0.05$, whereas ßß indicates $P < 0.001$.

Results

**SDF-1 and CXCR4 are expressed in microvessels in human breast carcinoma tissues**

SDF-1 and CXCR4 are expressed in a number of tissues including bone marrow, lung, and lymph nodes. Because these factors were implicated in breast cancer progression and metastasis, we regarded it as a prerequisite that they are indeed expressed in breast cancer samples. Immunohistochemistry showed that SDF-1 was expressed in the tumor cells in approximately 50% of 140 samples chosen from a collection of mammary tumor samples. We carried out double immunofluorescence by staining SDF-1 and vWF in 10 of these samples. A significant fraction of the cells stained positive for SDF-1 but negative for vWF (Fig. 1A, case 1). These cells are most likely tumor cells. Interestingly, we detected SDF-1 also in the endothelium of microvessels. In 3 of 10 samples, SDF-1 was predominantly expressed in endothelial cells (Fig. 1A, case 2). Within the 140 samples, CXCR4 was expressed in 13 cases in the tumor cells (Fig. 1B, top). Interestingly, 23 samples showed necrotic areas, in 7 of these cases CXCR4 was apparently induced in the proximity of the necroses (Fig. 1B, center). Necroses are likely to be hypoxic, which is in line with the idea that CXCR4 is hypoxia-inducible. From the 140 samples, a subgroup of 24 samples showed CXCR4 expression in blood vessels (Fig. 1B, bottom). Which molecular events or pathways govern CXCR4 expression in tumor cells as opposed to endothelial cells is unknown. There was no apparent correlation between CXCR4 and SDF-1 expression. In 13 of 130 cases, tumor cells stained positive for CXCR7. Again, expression of CXCR7 did not correlate to SDF-1 or CXCR4 expression. Tumor cells stained positive for CXCR4 and CXCR7 simultaneously in only 2 cases.

**Autocrine SDF-1/CXCR4 signaling in hypoxia is required for ECs angiogenesis**

In initial experiments, we observed that hypoxia induced proliferation, tube formation, and migration in HUVECs (data not shown). To analyze the regulation of CXCR4 under hypoxic conditions, we assessed the expression of CXCR4 by FACS (Supplementary Fig. S1A). As expected,
CXCR4 increased markedly under hypoxia as compared with normoxia. Hypoxic induction was confirmed by Western blotting (Supplementary Fig. S1B). As shown in Supplementary Fig. S1C, an intracellular SDF-1 upregulation by hypoxia was detected by FACS, which probably reflects SDF-1 being processed before secretion because storage of chemokines is uncommon. Importantly, secretion was also elevated as shown by ELISA in samples of the conditioned media (Supplementary Fig. S1D). In both, HUVEC and HDMEC, CXCR7 was neither detectable by Western blotting nor by immunofluorescence in our hands. Next, we analyzed whether SDF-1 induced proliferation and angiogenesis. To do so, HUVECs were cultured in the absence or presence of increasing concentrations of SDF-1 (1–100 ng/mL), CXCR4 neutralizing antibody (10 μg/mL), or IgG control antibody. SDF-1 stimulated HUVEC proliferation (Supplementary Fig. S2A) and tube formation (Supplementary Fig. S2B) in a dose-dependent manner. Anti-CXCR4 neutralizing antibodies inhibited proliferation and tube formation significantly.

To assess the importance of CXCR4 in the endothelial cells, HUVECs were transduced with pLKO.1-shRNA-CXCR4. Expression of CXCR4 was significantly reduced as compared with empty vector (Fig. 2A). Strikingly, reduction of CXCR4 retarded HUVEC proliferation (Fig. 2A). We next tested whether CXCR4 also plays a role in migration. As shown in Fig. 2B, HUVECs expressing shCXCR4 exhibited attenuated migration under normoxia and hypoxia as compared with controls. To show the involvement of CXCR4 in capillary morphogenesis, we analyzed tube formation in HUVEC. After CXCR4 knockdown the tube numbers were greatly reduced (Fig. 2C and D). We observed that serum-deprived HUVECs died at day 3 in normoxia. However, many of the HUVECs survived when kept in hypoxia. An SDF-1 neutralizing antibody partly abrogated this effect in a dose-dependent manner (Fig. 2E and F). Furthermore, we carried out tube formation assays with the SDF-1 neutralizing antibody. We observed that blocking of the SDF-1 signal reduced tube numbers under normoxia and under hypoxia (Fig. 2G and H).

**HIF-1α and HIF-2α are essential for angiogenesis**

In general, hypoxia significantly stimulates angiogenesis. As in HUVEC, both HIF-1α subunits were detectable and induced by hypoxia in HDMEC. We also confirmed that CXCR4 is hypoxia-inducible in HDMEC (Supplementary Fig. S4A). The relative contribution of HIF-1α as opposed to HIF-2α to the transcriptional response of endothelial cells in hypoxia is controversial (26, 27). To elucidate the role of HIF-1α or HIF-2α in endothelial CXCR4 expression and angiogenesis, HUVECs were transduced with pLKO.1-shRNA-HIF-1α or pLKO.1-shRNA-HIF-2α alone or in combination. We then induced HIF activity chemically using the prolyl hydroxylase inhibitor DMOG. Analyzing CXCR4 expression by Western blotting, we showed that both HIF-1α and HIF-2α regulated CXCR4 expression in HUVECs (Fig. 3A). Apparently, both HIF-α subunits affected CXCR4 expression, although the precise contribution of each subunit is difficult to quantify because knockdown of 1 subunit affected also the other. HIF-1α and HIF-2α knockdown reduced proliferation (Fig. 3B). HIF-2α appeared to have a more pronounced effect. Apparently, there was some degree of synergy between the 2 factors because double knockdown of HIF-1α and HIF-2α had the most striking effect. Similarly, migration of HUVECs after HIF-1α or HIF-2α knockdown was reduced markedly, particularly after HIF-1α/2α double knockdown (Fig. 3C). In addition, transduction of HIF-1α shRNA or HIF-2α shRNA abrogated hypoxic induction of tube formation (Fig. 3D and E). Again, the most significant suppression was achieved by combined HIF-1α/2α knockdown.

**Hypoxia induces CXCR4 expression in MDA-MB 231 and MCF7 and stimulates transendothelial migration toward an SDF-1 gradient**

Hypoxia was shown previously to enhance CXCR4 expression in cultured tumor cells. Although CXCR4 expression was readily detectable, we were unable to show expression of CXCR7 in normoxia or hypoxia by Western blotting or cell surface expression by immunofluorescence or FACS. To test regulation of CXCR4 by hypoxia, we exposed 2 breast carcinoma cell lines, MDA-MB 231 (aggressive type) and MCF7 (moderately aggressive) to hypoxia or DMOG treatment. In the case of MDA-MB 231, 24 hours of hypoxia or DMOG led to a comparable increase in CXCR4 expression (Fig. 4A and B). However, 4 hours in hypoxia were insufficient to increase CXCR4 levels (Fig. 4B). Similarly, CXCR4 expression of MCF7 showed a marked increase after treatment with DMOG or hypoxia (Supplementary Fig. S3). Knockdown of HIF-1α or HIF-2α led to decreased CXCR4 expression in MDA-MB 231 (Fig. 4C) and MCF7 (Supplementary Fig. S3). Most efficient attenuation of CXCR4 expression was observed by combined knockdown of HIF-1α/2α (Fig. 4C). Next, we observed that hypoxia facilitated transendothelial migration of MDA-MB 231 cells, in particular when an SDF-1 gradient was present (Fig. 4D).

**Collaboration of endothelial and breast carcinoma cells mediates active adhesion and migration through the SDF-1/CXCR4 axis**

To analyze whether hypoxia, CXCR4 and SDF-1 affect the interaction of tumor cells with endothelial cells, a tumor-to-endothelium adhesion assay was developed. Hypoxia promoted the adhesion of MDA-MB 231 cells to HUVECs. This interaction was disrupted by selectively blocking CXCR4 in MDA-MB 231 cells, not by blocking CXCR4 in HUVECs (Fig. 5A and B). This result indicated that CXCR4 expression by tumor cells is important for the adhesive function. Next, calcein-labeled MDA-MB 231 were cocultured with HUVECs in Matrigel for 24 hours. We observed that MDA-MB 231 were closely associated with the tubes formed by HUVECs. Again, blocking CXCR4 in MDA-MB 231 cells, but not in HUVECs inhibited this association (Fig. 5C and D). We carried out...
transendothelial migration assays to investigate whether CXCR4 influences transendothelial migration. We observed that CXCR4 knockdown in MDA-MB 231 cells, but not in HUVECs reduced transendothelial migration under normoxia and hypoxia (Fig. 5E and F). CXCR4 knockdown using siRNA in MCF7 significantly reduced migration (Fig. 5G and H). Because CXCR4 in HUVECs did not regulate these processes we asked whether HUVEC-derived SDF-1 regulates these steps. The presence of an SDF-1 neutralizing antibody attenuated the association of MDA-MB 231 as well as MCF7 with HUVECs remarkably (Fig. 6A–D). Importantly, adhesion of MDA-MB 231 to HDMEC tubes was also inhibited by addition of an SDF-1 antibody (Supplementary Fig. S4B). When we blocked the activity of the SDF-1 secreted by HUVECs or HDMECs with an SDF-1 neutralizing antibody for 12 hours, migration of MDA-MB 231 or MCF7 was substantially reduced in hypoxia (Fig. 6E and F, Supplementary Fig. S4C). Taken together, these findings strongly indicate that SDF-1 expressed in HUVECs or HDMECs promotes tumor-to-endothelium adhesion and transendothelial migration of tumor cells.
Discussion

In this study, we show that the chemokine SDF-1 and its cognate receptor CXCR4 are expressed in breast cancer in vivo and analyze the effects of manipulating these proteins in a coculture model of mammary carcinoma and vascular endothelial cells. The evidence presented suggests that activation of the SDF-1/CXCR4 axis in a growing tumor contributes to angiogenesis and the initial steps of metastasis. CXCR4 activation had surprisingly similar effects on tumor and endothelial cells: it induced proliferation and migration. In addition, SDF-1 binding to CXCR4 led to specific effects on endothelial and tumor cells: in endothelial cells, CXCR4 stimulated tube formation which may point to proangiogenic activity in vivo, whereas in the tumor cells activation of this receptor resulted in adhesion to endothelial cells and transendothelial migration.

In addition to their hematopoietic activities, SDF-1 and CXCR4 have been implicated in the regulation of angiogenesis in a number of (patho)physiological situations: the retina, the gastrointestinal tract, and the myocardium after infarction have been shown to rely on SDF-1 and CXCR4 for angiogenesis (28–30). Tumors also depend on angiogenesis because diffusion of oxygen and nutrients is insufficient to sustain tumor growth beyond a size of approximately 2 mm. Therefore, the antiangiogenic antibody bevacizumab (Avastin) has been developed and is used to antagonize the proangiogenic molecule VEGF. With respect to breast cancer, however, the use of this antibody is discussed controversially (31). It is important to note that our data do not simply suggest that the SDF-1/CXCR4 axis is proangiogenic. This is only one component which we have observed in vitro, as we have clearly shown that SDF-1 stimulates proliferation, migration, and tube formation of endothelial cells. In addition, and importantly, CXCR4 signaling also fostered growth of cultured tumor cells (data not shown) and the interaction between tumor and endothelial cells, which resulted in transendothelial migration. Transposed to the in vivo situation, these processes may result not only in angiogenesis but also in intravasation and metastasis.

The in vivo sequence of events which we propose and which we have followed meticulously in vitro is as follows: rapid proliferation of tumor cells leads to areas of hypoxia within the primary tumor. Hypoxia then activates the ubiquitous transcription factor HIF, which results in activation of an array of HIF target genes including SDF-1 and CXCR4 which contribute to tumor cell migration and adhesion to endothelial cells. Eventually, activation of the SDF-1/CXCR4 axis results in transendothelial migration. Within this scenario, angiogenesis provides the escape route

Figure 3. HIF-1α and HIF-2α contribute to the effects of hypoxia on HUVECs. Endothelial cells were transduced with pLKO.1-shRNA-HIF-1α or pLKO.1-shRNA-HIF-2α. A, CXCR4 expression in HUVECs after HIF-1α or HIF-2α knockdown. HUVECs were treated with 1 mmol/L DMOG for 4 hours. Data represent at least 3 independent experiments. B, hypoxia survival assay: HUVEC were cultured in serum-free ECGM under hypoxia for 3 days. Representative photographs from 2 independent experiments are shown. C, HUVEC migration assay after HIF-1α or HIF-2α knockdown. Columns give average of 3 independent experiments. D, tube formation assay: HUVECs were exposed to hypoxia for 24 hours. Representative data from 3 independent experiments are shown. E, quantification of tube numbers shows means of 3 independent experiments.
for tumor cells. The secretion of SDF-1 by endothelial cells (potentially also by tumor and stroma cells) and CXCR4 expression by the tumor cells results in entering of the tumor cells into the blood vessels. In principle, CXCR4 expression of tumor cells also favors extravasation in tissues where SDF-1 is expressed. Indeed, this effect has been reported previously (14, 32) and is regarded as a reason for the organ-specific pattern of metastasis seen in many cases of breast cancer. Because our experiments focused on endothelial SDF-1 secretion and also showed effects on tumor cell migration, our data suggest that in particular in hypoxic regions of the tumor an SDF-1 gradient develops, which attracts tumor cells to the blood vessels and results in tumor cell intravasation at the site of primary tumor growth.

Hypoxia is an important feature in many solid tumors. Effects of hypoxia are associated with several aspects of tumor biology including angiogenesis, metastasis as well as resistance to radiation and chemotherapy (33–35). In a hypoxic environment, angiogenesis is induced by a number of genes including vascular endothelial growth factor-A (VEGF-A), VEGFR1, Angiopoietin-2 (Ang-2), and others (36, 37). Hypoxia also stimulates expression of CXCR4 in different cell types. We observed that both SDF-1 and CXCR4 in HUVECs are hypoxia-inducible. The key regulators of the cellular responses to low oxygen concentrations are two distinct α-subunits termed HIF-1α and HIF-2α. The individual contributions of HIF-1α and HIF-2α to angiogenesis are under debate. We observed that HUVECs expressed both HIF-1α and HIF-2α when cultured under hypoxic conditions. In our hands, both HIF-1 and HIF-2 contributed to migration and tube formation in response to hypoxia because inhibition of either HIF-1α or HIF-2α provoked partial loss of induction of CXCR4 in response to hypoxia. Previously a reciprocal interaction between the two α-subunits has been reported in tumor cells of renal origin (38). We could not observe this effect in our cells but frequently noticed that inhibition of 1 α-subunit led to a parallel decrease of the other. The molecular basis of this discrepancy is unclear. Because the effects of HIF-1α and HIF-2α knockdown were additive, we believe that both subunits contribute to CXCR4 induction by hypoxia.

Importantly, we have showed by immunohistochemistry that SDF-1 and CXCR4 are expressed in a significant fraction of mammary tumors. We observed that SDF-1 is expressed in approximately 50% of our samples. Within these samples SDF-1 was mainly expressed by the tumor cells but frequently also in endothelial cells as showed by colocalization with vWF. SDF-1 has also been detected in endothelial cells of human breast cancer samples in a previous study (5, 20). Interestingly, none of our tumor cell lines (MDA MB231 and MCF-7) expressed SDF-1. However, CXCR4 expression in breast cancer samples was more difficult to assess than anticipated. Several commercial antibodies led to nuclear staining of almost all tumor cells,
which we regarded as unreliable. Using another antibody which has been characterized extensively very recently (39), we observed that nearly 10% of the tumors we analyzed were positive for CXCR4. From our data it is indeed conceivable that these patients would benefit from antagonizing CXCR4 signaling. Of note, very recently an in vitro study was published which implies that the effects of CXCR4 and CXCR7 inhibition may act synergistically (40). Importantly, CXCR4 and CXCR7 positive tumors can be identified on a routine basis by immunohistochemistry. For some tumor entities it was reported that the chemokine receptor termed CXCR7 can also bind SDF-1 and thus modulate CXCR4 signaling. Because expression of both receptors was only detectable in 2 out of approximately 130 cases in our cohort, functional interaction is of course possible but seems to be a rare event. However, CXCR7 could also transmit proliferative or angiogenic signals. Overall, our data suggest that inhibition of CXCR4 may reduce growth and metastasis in breast carcinoma. Interestingly, a low molecular weight compound termed AMD3100, which antagonizes CXCR4 function is already in clinical use to mobilize hematopoietic stem cells and has been reported to have a tolerable side effect profile (41).

Collectively, we discovered a crosstalk mechanism between breast carcinoma and endothelial cells. Hypoxia-dependent induction of the SDF-1/CXCR4 pathway links metabolism to tumor angiogenesis and active transendothelial migration. Inhibition of the activated SDF-1/CXCR4 signaling pathway in a primary tumor environment may therefore attenuate angiogenesis and carcinoma intravasation. Our data are primarily applicable to breast cancer because we have analyzed cultured breast carcinoma cell lines.
lines and confirmed SDF-1 and CXCR4 expression in samples of mammary carcinoma. Because importance of CXCR4 and SDF-1 has already been proposed for tumors of other origin, our data may also be relevant for other tumor types.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: F. Jin, F. Otterbach, E. Metzen
Development of methodology: F. Jin, U. Brockmeier
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Jin, F. Otterbach
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Jin, F. Otterbach
Writing, review, and/or revision of the manuscript: F. Jin, U. Brockmeier, F. Otterbach, E. Metzen

References
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