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The Role of CXCR2/CXCR2 Ligand Biological Axis in Renal Cell Carcinoma

Javier Mestas,* Marie D. Burdick,* Karen Reckamp,* Allan Pantuck, † Robert A. Figlin,* and Robert M. Strieter2*§§

Renal cell carcinoma (RCC) accounts for 3% of new cancer incidence and mortality in the United States. Studies in RCC have predominantly focused on VEGF in promoting tumor-associated angiogenesis. However, other angiogenic factors may contribute to the overall angiogenic milieu of RCC. We hypothesized that the CXCR2/CXCR2 ligand biological axis represents a mechanism by which RCC cells promote angiogenesis and facilitate tumor growth and metastasis. Therefore, we first examined tumor biopsies and plasma of patients with metastatic RCC for levels of CXCR2 ligands, and RCC tumor biopsies for the expression of CXCR2. The proangiogenic CXCR2 ligands CXCL1, CXCL3, CXCL5, and CXCL8, as well as VEGF were elevated in the plasma of these patients and found to be expressed within the tumors. CXCR2 was found to be expressed on endothelial cells within the tumors. To assess the role of ELR+ CXC chemokines in RCC, we next used a model of syngeneic RCC (i.e., RENCA) in BALB/c mice. CXCR2 ligand and VEGF expression temporally increased in direct correlation with RENCA growth in CXCR2+/− mice. However, there was a marked reduction of RENCA tumor growth in CXCR2−/− mice, which correlated with decreased angiogenesis and increased tumor necrosis. Furthermore, in the absence of CXCR2, orthotopic RENCA tumors demonstrated a reduced potential to metastasize to the lungs of CXCR2−/− mice. These data support the notion that CXCR2/CXCR2 ligand biology is an important component of RCC tumor-associated angiogenesis and tumorigenesis. The Journal of Immunology, 2005, 175: 5351–5357.
and CXCR2<sup>+/−</sup> BALB/c mice determined that CXCR2 contributes to tumor-associated angiogenesis and tumor growth. CXCR2 ligands and VEGF expression temporally increased in direct correlation with RENCA growth in CXCR2<sup>+/−</sup> mice. However, RENCA tumor growth was markedly reduced in CXCR2<sup>−/−</sup> mice, which correlated with decreased angiogenesis and increased tumor necrosis. In the absence of CXCR2, RENCA tumors demonstrated a reduced potential to metastasize to the lungs of CXCR2<sup>−/−</sup> mice. Taken together, these results underline the importance of CXCR2/ CXCR2 ligand biology with respect to tumor-associated angiogenesis and provide an alternative avenue of investigation in the development of novel approaches to combat RCC tumorigenesis.

Materials and Methods

Reagents

Polyclonal rabbit anti-human CXCL3 and CXCL8 sera was produced by immunization of rabbits with CXCL3 or CXCL8 (R&D Systems) in multiple intradermal sites with CFA. This Ab has been previously well characterized for its ability to detect CXCL3 and CXCL8 with high sensitivity (13). Both antisera were found to be specific for CXCL3 or CXCL8. Furthermore, in a sandwich ELISA, this Ab is specific for CXCL3 or CXCL8 without cross-reactivity to a panel of 12 other recombinant human cytokines or the murine chemokines KC and MIP-2.

Cytokine protein quantification

The quantity of mouse KC and MIP-2, human CXCL1, CXCL3, CXCL5, CXCL8, and human and mouse VEGF present in human plasma, mouse plasma, and tissue homogenates was determined by species-specific ELISA, using a modification as previously described (19–24). Briefly, flat-bottom 96-well microtiter plates were coated with specific polyclonal anti-human CXCL1, CXCL3, CXCL5, CXCL8, or VEGF or anti-mouse KC, MIP-2, or VEGF Ab (R&D Systems) (1 μg/ml in 0.6 M NaCl, 0.26 M H<sub>2</sub>BO<sub>3</sub>, and 0.08 N NaOH; pH 9.6) for 24 h at 4°C, and then washed with PBS (pH 7.5) plus 0.05% Tween 20 (wash buffer). Plates were blocked with 2% BSA in PBS for 1 h at 37°C and then washed three times with wash buffer. Sample or standard was added, and the plates were incubated at 37°C for 1 h. Plates were washed three times. Biotinylated monoclonal anti-human CXCL1, CXCL3, CXCL5, CXCL8, or VEGF or anti-mouse KC, MIP-2, or VEGF Ab (R&D Systems) (3.5 μg/ml in PBS (pH 7.5), 0.05% Tween 20, and 2% BSA) was added, and plates were incubated at 37°C for 45 min. Plates were washed three times, streptavidin-peroxidase conjugate was added, and the plates were incubated for 30 min at 37°C. Plates were washed again, and 3,3’5,5’-tetramethylbenzidine chromogenic substrate was added. Plates were incubated at room temperature to the desired extinction, and the reactions were terminated by the addition of 1 M H<sub>2</sub>PO<sub>4</sub>. Plates were read at 450 nm in an automated microtiter plate reader, and the amount of human CXCL1, CXCL3, CXCL5, CXCL8, and VEGF or mouse KC, MIP-2, and VEGF present was determined by interpolation of a standard curve generated by known amounts of recombinant human CXCL1, CXCL3, CXCL5, CXCL8, and VEGF or mouse KC, MIP-2, and VEGF (R&D Systems), respectively. The sensitivity for human CXCL1, CXCL3, CXCL5, CXCL8, and VEGF and mouse KC, MIP-2, and VEGF ELISA was ~1 pg/ml, and this assay failed to cross-react with a panel of other known cytokines and chemokines. Mouse MIP-2, and VEGF expression in the orthotopic tumor was also measured using a three-head multiplex system (KC (Upstate Biotechnology) and MIP-2 and VEGF (R&D Systems)), as previously described (25). Briefly, single cell suspension preparations were made from either kidney of mice from the primary tumor or kidneys from naive mice. The latter were labeled with carboxyfluorescein succinimidyl ester. Samples were incubated for 2 h at room temperature with a mixture of anti-KC, anti-MIP-2, and anti-VEGF Abs and were added to each well and the plate was incubated for 1.5 h. Streptavidin-PE was added to the wells and the plate was incubated for 30 min. Next, 0.2% paraformaldehyde was added to the wells and the plate was read on a LumineX 100 IS instrument. The concentration of cytokine was determined by interpolation of a standard curve, using a mixture of known amounts of recombinant KC, MIP-2, and VEGF. The levels of cytokines measured from tissue specimens were further normalized to total protein.

Heterotopic and orthotopic RENCA model

BALB/c CXCR2<sup>+/−</sup> and CXCR2<sup>−/−</sup> mice 6–to-8 wk-old (The Jackson Laboratory) were injected with RENCA, provided as a gift from Dr. A. Belldegrun (David Geffen School of Medicine at UCLA, Los Angeles, CA), s.c. (heterotopic; 10<sup>6</sup> cells per 100 μl) into one flank or with RENCA cells expressing GFP subcapsular (orthotopic; 10<sup>6</sup> cells per 20 μl) into the left kidney using a modification, as previously described (12, 20, 26, 27). The animals were maintained under specific pathogen-free conditions and sacrificed in groups of six each at specified time points. Heterotopically placed RENCA tumors were dissected from the mice and measured with a Thorel corp (Biomedical Research Institute). Tumor volume was calculated using the formula: volume = (d<sub>1</sub> × d<sub>2</sub> × d<sub>3</sub>) / 2, where d<sub>1</sub> represents the mean orthogonal diameter measurements. Tumor size of orthotopically placed kidney tumors was evaluated by FACS analysis, as determined by the presence of GFP<sup>+</sup> cells. Tumor and tissue specimens were then processed for: 1) histo-logic analysis and immunohistochemistry by fixing with 4% paraformaldehyde and embedding in paraffin for sectioning; 2) FACS analysis of GFP<sup>+</sup> cells by digesting tissue and generating single cell suspensions; or 3) analysis of cytokine levels by ELISA through the homogenization and sonication of tissue, as previously described (19–24).

FACS analysis of primary tumors and metastatic lesions

Single cell suspension preparations of the primary tumors and lungs were made using a method previously described (19, 24). Briefly, primary tu-mors or entire left kidney and lungs were harvested at 4 wk. Tissue was minced with scissors to a fine slurry in 15 ml of digestion buffer (RPMI 1640, 5% FCS, 1 mg/ml collagenase (Boehringer Mannheim), and 30 μg/ml DNAse (Sigma-Aldrich). Tissue slurry was vortexed for 45 min at 37°C. Undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total cell suspension was pelleted and resuspended in FACS analysis buffer. Cell counts and viability were determined using trypan blue exclusion on a hemocytometer. Single cell suspensions from tumors were stained with a primary biotinylated pan-endothelial cell Ab (MECA-32; BD Pharmingen) or isotype controls. The primary Abs were detected with streptavidin-FITC. Cell suspensions from tumors and lung tissue were analyzed for GFP expression without staining. Cells were analyzed on a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences), as previously described (19, 24).

Proliferation of RENCA cells

Proliferation of RENCA cells was assessed using 96-well culture plates seeded with either 5000 RENCA cells/well, which were starved overnight in growth medium containing 1% FCS. The following morning, medium containing 10% serum was added to the cultures along with 30 ng/ml KC, MIP2, anti-CXCR2 Ab (1/3000) or goat serum as a control, and the cells were allowed to grow for 72 h. At this time, 1 μCi/well [<sup>3</sup>H]thymidine was added, and the cultures were incubated for a further 18 h. Finally, the cells were harvested using a cell harvester, and [<sup>3</sup>H]thymidine incorporation was quantitated by scintillation counting. Mitomycin C-treated cells were used as negative control for proliferation.

RENA cell chemotaxis

RENA cells were harvested by trypsinization, counted and resuspended in RPMI 1640 containing 5% FCS at a concentration of 10<sup>6</sup/ml. Neuro-probe filters (5 μm diameter) and 12-well chemotaxis chambers were used in this assay. KC, MIP-2, or CXCL12 (30 ng/ml; PeproTech) was added to the lower wells and 10<sup>6</sup> cells were added to each of the upper wells. The chambers were then incubated for 6 h at 37°C. After fixing in methanol and staining in 2% toluidine blue, the number of cells that migrated to the underside of the filters was calculated by counting the total number of cells in 15 separate fields of view under x20 magnification.

Immunohistochemistry of CXCR2, CXCR2 ligands, and Factor VIII-related Ag

Histo Array slides for human kidney cancer (IMH-313 CL; IMGENEX) were processed for immunohistochemical staining of CXCR2 and Factor VIII-related Ag, as well as CXCL3, CXCL5, CXCL8, and VEGF using the Vectastain ABC system (Vector Laboratories), according to the manufacturer’s instructions. Briefly, tissue sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. Non specific binding sites were blocked with powerblock (BioGenex Laboratories), washed, and overlaid with control, anti-human CXCR2 (R&D Systems), anti-human Factor VIII-related Ag (Sigma-Aldrich), anti-human CXCL3, anti-human...
CXCL5 (PeproTech), anti-human CXCL8 or anti-human VEGF (PeproTech). Slides were then rinsed and overlaid with secondary biotinylated Ab and incubated for 30 min. After washing twice with PBS, slides were overlaid with Vectastain ABC systems peroxidase-conjugated streptavidin and incubated for 30 min. The 3,3’-diaminobenzidine tetrahydrochloride reagent was used for chromogenic localization of CXCR2 or Factor VIII-related Ag. After optimal color development, sections were counterstained with Mayer’s hematoxylin and coverslipped with mounting solution.

Morphometric analysis of tumor necrosis

Morphometric analysis was performed on 10 different fields of H&E stained sections from each of the heterotopic tumors. Necrosis was defined as acellular areas (18, 28). Necrosis was not distinguished from apoptosis, as a lack of cells precludes the detection of apoptosis. An Olympus BH-2 microscope coupled to a Sony 3CCD camera was used to capture images that were then analyzed to obtain the average percentage of necrotic area per high power field (magnification, ×200) and this value was then multiplied by the tumor area, yielding the average necrotic area for each tumor.

Statistical analysis

The animal studies involved six mice for each treatment group. Data were analyzed on an Apple Powerbook computer using the Statview 5.0 statistical package (Abacus Concepts). Comparisons were evaluated by the unpaired t test. Data were considered statistically significant for values of p < 0.05.

Results

Patients with mRCC exhibit elevated levels of proangiogenic CXCR2 ligands

Because multiple angiogenic factors may contribute to angiogenesis associated with RCC, we first assessed the levels of CXCR2 ligands, as compared with VEGF, in 21 patients with known mRCC and 11 normal healthy donors. We examined the plasma levels of CXCL1, CXCL3, CXCL5, and CXCL8, as well as VEGF in 21 patients with mRCC. The plasma levels of all the CXCR2 ligands and VEGF were significantly elevated in patients with mRCC compared with the healthy control subjects (Fig. 1A). Immunohistochemical analysis of RCC tumor sections revealed the expression of CXCL3, CXCL5, CXCL8, and VEGF (Fig. 1B).

Given the elevated levels of CXCR2 ligands, we next assessed RCC tumor specimens for the expression of CXCR2, which is the common receptor for CXCL1, CXCL3, CXCL5, and CXCL8. Immunohistochemistry was performed on Histo Array slides (IMGENEX) containing 30 samples of human clear cell type mRCC. CXCR2 expression was found on vascular endothelial cells, which were positive for Factor VIII-related Ag, suggesting a role for CXCR2/CXCR2 ligands in promoting tumor-associated angiogenesis (Fig. 1C).

CXCR2 ligands correlate with tumor growth in a preclinical heterotopic murine model of RCC (RENCA)

To determine the significance of elevated ELR+ CXC chemokines in renal tumors and further investigate the role of CXCR2 in tumor growth and progression, we used a syngeneic murine model of RCC (i.e., RENCA). Heterotopic RENCA tumors were generated s.c. in the flank of BALB/c mice. Tumor size was measured every week over a 4-wk period, as were the levels of KC, MIP-2, and VEGF from the tumor specimens and plasma. Tumor size increased over the 4-wk period (Fig. 2A). Because KC and MIP-2 bind to CXCR2 in a similar stoichiometric relation, we arithmetically summed the two CXCR2 ligands and VEGF levels within the tumor directly correlated with tumor growth. As expected, plasma levels of CXCR2 ligands and VEGF were detected, but at much lower values. The plasma levels paralleled the expression of these cytokines during tumor growth (Fig. 2B).

RENCA tumor growth is attenuated in CXCR2−/− mice

We next established heterotopic RENCA tumors in CXCR2−/− BALB/c mice to determine the effects of ELR+ CXC chemokines on tumor growth and tumor-associated angiogenesis. Tumor size was measured over a 4-wk period and was significantly reduced in CXCR2−/− mice compared with CXCR2+/+ mice (Fig. 3). Morphometric analysis of H&E stained tumor sections demonstrated an increase in the mean necrotic area of tumors in CXCR2−/− mice, as compared with CXCR2+/+ mice (Fig. 4A). In addition, tumor-associated angiogenesis, as determined by the number of endothelial cells measured by FACS analysis of the pan-endothelial marker MECA-32 in single cell suspensions of tumors, was markedly reduced in tumors grown in CXCR2−/− mice, as compared with CXCR2+/+ mice (Fig. 4B).
We next used an orthotopic murine model of RENCA tumor growth to confirm the results of the heterotopic model, and to investigate the effects of CXCR2 depletion on the metastatic potential of primary orthotopic RENCA tumors. We established orthotopic RENCA tumors by injecting GFP$^+$ RENCA cells into the subcapsular space of the left kidney of CXCR2$^{+/+}$ and CXCR2$^{-/-}$ BALB/c mice. Tumors were allowed to grow for 4 wk, at which time the animals were sacrificed and tumor size was measured using FACS analysis to determine the number of GFP$^+$ cells in single cell suspension preparations of the entire left kidney containing the RENCA tumor. Tumor size was markedly reduced in CXCR2$^{-/-}$ mice compared with CXCR2$^{+/+}$ mice (Fig. 5A).

The levels of KC, MIP-2, and VEGF within the kidney containing the RENCA tumor, as well as those in naive kidneys, were also measured. There was not a significant difference in the kidney tumor levels of KC, MIP-2, and VEGF between CXCR2$^{+/+}$ and CXCR2$^{-/-}$ mice. However, animals with tumors within the left kidney had significantly elevated levels of CXCR2 ligands compared with those that did not receive (naive) RENCA cells (Fig. 5B).

To exclude a direct effect of CXCR2 ligands on RENCA cell proliferation, RENCA cells were stimulated with KC or MIP-2 and cell proliferation was measured by [3H]thymidine incorporation. (data not shown). KC and MIP-2 did not cause RENCA cell proliferation. The ability of RENCA cells to migrate in response to CXCR2 ligands was also tested. KC and MIP-2 had no effect on RENCA cell motaxis (data not shown).

Loss of CXCR2 decreases the metastatic potential of primary RENCA tumors

One of the hallmarks of RCC cells is their propensity to metastasize to other organs, specifically the lungs (2). Given the marked reduction of vessel density in primary RENCA tumors in CXCR2$^{-/-}$ mice, we were interested in determining the effects of CXCR2 depletion on the metastatic potential of primary RENCA tumors. Orthotopic GFP$^+$ RENCA tumors were allowed to grow in the left kidney for 4 wk, at which time the animals were sacrificed and metastatic lesions to the lungs were measured using FACS analysis of single cell suspension preparations of the lungs to determine the number of GFP$^+$ cells. CXCR2$^{-/-}$ mice exhibited a 5-fold decrease in the amount of GFP$^+$ RENCA cells that had metastasized to the lungs, as compared with cells in CXCR2$^{+/+}$ mice (Fig. 6). In addition, histological analysis of lung tissue demonstrated that the metastatic lesions in CXCR2$^{-/-}$ mice were markedly smaller than those found in CXCR2$^{+/+}$ animals (data not shown).

Discussion

The regulation of angiogenesis is dependent on the expression of opposing angiogenic and angiostatic factors, the balance of which controls the net angiogenic process. Several members of the CXC chemokine family are potent promoters of angiogenesis (12, 29–31), whereas others inhibit the angiogenic process (27, 32–34). This disparity in angiogenic activity among CXC chemokine family members is attributed to a three amino acid structural domain at the N terminus, Glu-Leu-Arg (ELR), which is present in angiogenic (i.e., CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7,
and CXCL8) (11, 12, 35), but not angiostatic (i.e., CXCL4, CXCL9, CXCL10, and CXCL11) CXC chemokines (32).

The ELR CXC chemokines play an important role in tumor growth and progression in a number of tumor model systems. In particular, CXCL8 is significantly elevated in NSCLC and augments tumor-associated angiogenesis in NSCLC (13, 14). CXCL8 is also found elevated in glioblastomas, compared with normal brain tissue, along with increased vascular density (15). Furthermore, CXCL1 is elevated in human malignant melanoma lesions and promotes the progression of these tumors (11).

In the present study, we found CXCL1, CXCL3, CXCL5, and CXCL8 elevated in the plasma of patients with mRCC, as compared with normal healthy volunteers. The low CXCR2 ligand levels detected in plasma were not surprising due to the nature of these molecules. The ELR CXC chemokines are small, secreted proteins with heparin-binding motifs, and therefore can bind to proteoglycans found within the extracellular matrix. These proteins can also bind to Duffy Ag receptor for chemokines (DARC), a promiscuous chemokine receptor that binds all ELR CXC chemokines in the absence of any detectable signal transduction (28), therefore any detection of these molecules in plasma represents only a fraction of the true produced levels. CXCL3, CXCL5, CXCL8, and VEGF were also detected within human RCC tumors. Immunohistochemical analysis revealed a diffuse staining pattern for these cytokines, typical of heparin-binding proteins that can bind to the extracellular matrix. Moreover, when orthotopically placed RENCA tumors were examined for KC, MIP-2, and VEGF, the levels of the CXCR2 ligands were found elevated in kidneys containing RENCA tumors as opposed to naive kidneys, suggesting that elevated CXCR2 ligand levels observed in human plasma and the RENCA tumors were expressed within the tumor. Interestingly, CXCR2 expression was found on vascular endothelial cells in tumor biopsies from patients with mRCC. Together, these findings support a role for CXCR2/CXCR2 ligands in the promotion of angiogenesis associated with RCC.

CXCR2 is expressed by vascular endothelial cells and is the primary functional chemokine receptor in mediating endothelial cell chemotaxis in response to CXCR2 ligands (17, 36). Therefore, it is not surprising that CXCR2 is responsible for the induction of angiogenesis mediated by ELR CXC chemokines in vivo (17). Our previous findings show that Lewis lung cancer tumor growth is inhibited in CXCR2 mice (18). In this study, we demonstrate similar results in a heterotopic model of RCC. The heterotopic RENCA model revealed that tumor growth directly correlated with increased CXCR2 ligand expression. We also found that tumor necrosis was increased and tumor-associated angiogenesis was decreased in CXCR2 mice, as compared with CXCR2 mice. These results are consistent with the concept that in the absence of CXCR2 on host endothelial cells within the tumor, tumor-associated angiogenesis is markedly reduced and the tumor tissue becomes necrotic. Similar results were obtained in a recent study in which DARC was overexpressed in NSCLC cells (28). Overexpression of DARC resulted in decreased tumor-associated angiogenesis and increased tumor necrosis, suggesting that sequestration of CXCR2 ligands by DARC and the subsequent loss of CXCR2 signaling in endothelial cells contributed to these effects. Additional support is provided by the observation that stimulation of RENCA cells by KC and MIP-2 did not cause tumor cell proliferation or chemotaxis, indicating that CXCR2 on host endothelial

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** Absence of CXCR2 is associated with reduced orthotopic tumor growth. A, FACS analysis of GFP expressing RENCA cells in the left kidney of six CXCR2 and eight CXCR2 BALB/c mice at 4 wk. B, Levels of ELR CXC chemokines in the orthotopic tumors. Protein levels were measured by ELISA from the left kidneys of naïve CXCR2 and CXCR2 mice or mice implanted with RENCA tumors. *, p < 0.05.

![Figure 6](http://www.jimmunol.org/)

**Figure 6.** Absence of CXCR2 is associated with reduction of orthotopic RENCA tumor metastasis to the lungs. FACS analysis of GFP RENCA cells from single cell suspensions of lungs from five CXCR2 and seven CXCR2 BALB/c mice bearing orthotopic (left kidney) GFP RENCA tumors. *, p < 0.05.
cells, and not RENCA cells, mediates the effect of CXCR2 ligands on tumor growth.

In the heterotopic RENCA model, tumor cells were implanted s.c. onto the flank of the mouse. Although RENCA tumors grow well in this environment, it has been shown that spontaneous metastasis can more optimally occur if placed in the orthotopic subcapsular region of the kidney (37). Therefore, we used the orthotopic RENCA model to determine the effects of CXCR2 depletion related to metastasis of the primary tumor. Similar to the heterotopic model, RENCA tumors implanted into the subcapsular region of the kidney grew over a 4-wk period, however those implanted into CXCR2/−/− mice were significantly reduced in size. Because the lung is a preferred metastatic site for RCC, we found that RENCA tumor metastatic lesions were found to be a greater extent in the lungs of CXCR2/−/−, as compared with CXCR2+/+ mice (2). These findings suggest that the loss of CXCR2 on host responding cells (i.e., endothelial cells) has a profound effect on tumor growth, tumor necrosis, tumor-associated angiogenesis, and tumor metastatic potential.

The metastatic potential of primary tumors is highly associated with tumor growth and with the degree of tumor-associated angiogenesis. Solid tumors cannot grow more than several cubic millimeters in the absence of a vascular network. The lack of angiogenesis creates areas of hypoxia, which mediate the expression of proangiogenic factors, such as VEGF. Hypoxia also mediates the expression of CXCR4, which has been shown to mediate metastasis of NSCLC cells in an angiogenesis-independent manner (20). Hypoxia-mediated expression of CXCR2 has also been shown in aortic endothelial cells (38) and microvascular endothelial cells (our unpublished observations). Our results suggest that the role of CXCR2 in tumor metastasis is related to its expression on endothelial cells. Tumor growth is promoted via CXCR2-mediated angiogenesis, which provides tumors cells access to the vasculature. However, we cannot exclude other potential mechanisms involved in metastases of RENCA tumors that were attenuated in CXCR2/−/− mice.

Although the absence of CXCR2 impaired RENCA tumorigenesis and metastases, tumor growth was not completely reduced, which is consistent with the presence of other factors, such as VEGF. Many clinical trials have focused on targeting of VEGF for which multiple angiogenic mediators are targeted is currently un-antiangiogenic therapy; however targeting VEGF alone has not been effective in human non-small cell lung cancer in XCD mice. J. Clin. Invest. 107: 2792–2800. 1996. Dimerization of ENA-binding, and transcription regulations of hypoxia-inducible factor 1. J. Biol. Chem. 271: 17771–17778. 1996. Cellular and developmental control of O2 homeostasis by hypoxia-inducible fac-


