

# Bcl-2 Orchestrates a Cross-talk between Endothelial and Tumor Cells that Promotes Tumor Growth

Tomoatsu Kaneko,<sup>1,6</sup> Zhaocheng Zhang,<sup>1</sup> Maria G. Mantellini,<sup>1</sup> Elisabeta Karl,<sup>1</sup> Benjamin Zeitlin,<sup>1</sup> Monique Verhaegen,<sup>2</sup> María S. Soengas,<sup>2,5</sup> Mark Lingen,<sup>7</sup> Robert M. Strieter,<sup>8</sup> Gabriel Nunez,<sup>3,5</sup> and Jacques E. Nör<sup>1,4,5</sup>

<sup>1</sup>Angiogenesis Research Laboratory, Department of Restorative Sciences, University of Michigan School of Dentistry; <sup>2</sup>Departments of Dermatology and <sup>3</sup>Pathology, University of Michigan School of Medicine; <sup>4</sup>Department of Biomedical Engineering, University of Michigan College of Engineering; <sup>5</sup>Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan; <sup>6</sup>Department of Restorative Sciences, Tokyo Medical and Dental University, Tokyo, Japan; <sup>7</sup>Departments of Pathology, Medicine, and Radiation and Cellular Oncology, University of Chicago School of Medicine, Chicago, Illinois; and <sup>8</sup>Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia

## Abstract

The current understanding of the interaction between the endothelium and cancer cells is fundamentally based on the concept that endothelial cells are responsive to differentiation and survival signals originating from the tumor cells. Whereas the effect of tumor cell-secreted factors on angiogenesis is well established, little is known about the effect of factors secreted by endothelial cells on tumor cell gene expression and tumor progression. Here, we show that *bcl-2* gene expression is significantly higher in the tumor-associated endothelial cells of patients with head and neck squamous cell carcinomas (HNSCC) as compared with endothelial cells from the normal oral mucosa. Bcl-2 induces vascular endothelial growth factor (VEGF) expression in neovascular endothelial cells through a signal transducer and activator of transcription 3 (STAT3)-mediated pathway. Endothelial cell-derived VEGF signals through VEGFR1 and induces expression of Bcl-2 and the proangiogenic chemokines CXCL1 and CXCL8 in HNSCC cells. Notably, inhibition of Bcl-2 expression in neovascular endothelial cells with RNA interference down-regulates expression of Bcl-2, CXCL8, and CXCL1 in HNSCC cells, and is sufficient to inhibit growth and decrease the microvessel density of xenografted HNSCC in immunodeficient mice. Together, these results show that Bcl-2 is the orchestrator of a cross-talk between neovascular endothelial cells and tumor cells, which has a direct effect on tumor growth. This work identifies a new function for Bcl-2 in cancer biology that is beyond its classic role in cell survival. [Cancer Res 2007;67(20):9685–93]

## Introduction

Angiogenesis plays a central role in the pathobiology of cancer (1). Selective elimination of tumor-associated endothelial cells is the goal of antiangiogenic therapeutic strategies that are being used today for the treatment of cancer (2, 3). Therapeutic blockade of vascular endothelial growth factor (VEGF) has shown promising results for the treatment of several malignancies including metastatic colorectal cancer and non-small-cell lung cancer (4, 5). Head and neck squamous cell carcinomas (HNSCC) are highly vascularized tumors. However, attempts to use antiangiogenic

therapeutic strategies in HNSCC have shown modest results. A phase II clinical trial with SU5416 (semaxanib), a synthetic small-molecule inhibitor of the tyrosine kinase domain of vascular endothelial growth factor receptor 2 (VEGFR2), in patients with recurrent or metastatic head and neck cancers showed one partial response and one minor response in 31 treated patients (6). The modest response of head and neck cancer patients to antiangiogenic therapies suggests the existence of potent interactions between endothelial cells and tumor cells that sustain tumor microvascular networks and contribute to resistance to therapy. The understanding of such interactions may have implications in the treatment of HNSCC and perhaps other malignancies that are resistant to current antiangiogenic therapies.

To acquire and sustain its microvascular network, tumor cells secrete an array of growth factors and cytokines that regulate the angiogenic switch, modulate endothelial cell survival, and promote tumor progression (1, 7–9). VEGF is considered a prototypic angiogenic factor that is expressed by most tumor cell types and enhances endothelial cell proliferation and migration (10, 11). VEGF was also shown to enhance endothelial cell survival by up-regulating Bcl-2 expression through a pathway mediated by VEGFR2 and phosphatidylinositol 3-kinase/Akt signaling (12, 13). Notably, VEGF expression levels have a strong correlation with survival of head and neck cancer patients (14, 15). In addition to VEGF, ELR (Glu-Leu-Arg) motif-positive CXC chemokines have been characterized as potent inducers of angiogenesis (16). CXCL1 [growth-related oncogene (GRO)- $\alpha$ ] and CXCL8 (interleukin-8) belong to this class of proangiogenic chemokines that contribute to cancer progression (16, 17). CXCL1 was initially identified in melanomas as an autocrine growth factor (18). CXCL8 was purified from conditioned medium of lipopolysaccharide-stimulated human monocytes (19). CXCL1 and CXCL8 share 43% homology at the amino acid level and present similar functions *in vivo* (20). VEGF, CXCL1, and CXCL8 have common characteristics of being secreted by tumor cells, binding to endothelial cell membrane receptors, and mediating signals that contribute to tumor angiogenesis and HNSCC growth.

Tumors of the head and neck are the sixth most common malignancy in the world (21). The 5-year relative survival rates of patients with oral cavity and pharynx cancer is about 59% in the United States and has shown modest improvement over the last 30 years (22). Notably, the incidence of head and neck cancer is still increasing in other countries (21). Better understanding of the molecular signals that define the pathobiology of HNSCC may lead to novel therapeutic interventions that might be beneficial for patients with these malignancies. Recently, a new pathway

**Requests for reprints:** Jacques E. Nör, Angiogenesis Research Laboratory, University of Michigan School of Dentistry, Room 2309, 1011 North University, Ann Arbor, MI 48109-1078. Phone: 734-936-9300; Fax: 734-936-1597; E-mail: jenor@umich.edu.

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involving contact-dependent signals that promote tumor angiogenesis has been described (23). This pathway is mediated by Jagged1 that is induced via the mitogen-activating protein kinase (MAPK) in HNSCC cells and activates Notch signaling in adjacent endothelial cells. This work led us to hypothesize that tumor cells and endothelial cells might engage in a two-way signaling cross-talk that is mutually supportive of both cell compartments. We have recently reported that Bcl-2 triggers an autocrine proangiogenic signaling cascade mediated by nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and CXC chemokine signaling through CXCR2 in endothelial cells (24). We have also shown that a small-molecule inhibitor of Bcl-2 (TW37) has an antiangiogenic effect at subapoptotic concentrations that is associated with the inhibition of Bcl-2-mediated CXCL1 and CXCL8 expression in endothelial cells (25). Here, we identified Bcl-2 as the regulator of a contact-independent cross-talk between tumor and endothelial cells, which is mediated by Bcl-2-induced VEGF secretion by endothelial cells, activation of VEGFR1 signaling in HNSCC cells, and up-regulation of Bcl-2 and CXC chemokine expression in HNSCC cells. The significance of this pathway to tumor biology is underscored by the demonstration that down-regulation of Bcl-2 expression in endothelial cells (RNA interference) is sufficient to slow down the progression of HNSCCs in immunodeficient mice.

## Materials and Methods

**Cells, neutralizing antibodies, proliferation assay, and ELISA.** The human dermal microvascular endothelial cells (HDMEC; Cambrex) stably transduced with Bcl-2 (HDMEC-Bcl-2) and empty vector controls HDMEC-LXSN were cultured in EGM2-MV (Cambrex). Human squamous cell carcinoma cell lines, UM-SCC-17B (gift from T. Carey, University of Michigan, Ann Arbor, MI) and OSCC3, were cultured in DMEM supplemented with 10% fetal bovine serum. When indicated, endothelial cells were cultured in the lower chamber and tumor cells (UM-SCC-17B or OSCC3) in the upper chamber of a noncontact coculture system for 24 to 48 h. Cells were separated by the 1- $\mu$ m pore membrane of the Transwell system (Becton Dickinson). For proliferation assays,  $8.5 \times 10^3$  UM-SCC-17B cells were seeded in the cell culture insert, and  $15 \times 10^3$  HDMEC-LXSN or HDMEC-Bcl-2 were plated in the bottom well of 24-well plates (Becton Dickinson). The cells were cultured with EGM2-MV medium for 24 to 48 h. UM-SCC-17B cells were harvested with trypsin-EDTA and counted with a hemocytometer. Cell viability was assessed on the basis of trypan blue exclusion. To inhibit VEGF or VEGFR1 signaling, cocultures were treated with 1  $\mu$ g/mL polyclonal antihuman VEGF (R&D Systems) or 2.1  $\mu$ g/mL polyclonal antihuman VEGFR1 (Flt-1) antibody (R&D Systems) for 24 h. VEGF expression was evaluated by ELISA (Quantikine; R&D Systems) according to the manufacturer's instructions.

**RNA interference.** Lentiviruses expressing a short hairpin RNA (shRNA) construct for silencing the *bcl-2* gene were generated in human embryonic kidney cells (293FT) as described (26). A scrambled oligonucleotide Bcl-2 sequence (shRNA-C) was used as control for these experiments. Supernatants were collected after 24 h and used to infect endothelial cells in DMEM containing 8  $\mu$ g/mL sequabrene (Sigma-Aldrich). Down-regulation of Bcl-2 expression was achieved by five consecutive rounds of infection lasting 4 h each, and the stability and specificity of the knockdown was confirmed by reverse transcription-PCR (RT-PCR).

**Severe combined immunodeficient mouse model of human angiogenesis.** Porous poly(L-lactic acid) scaffolds ( $6 \times 6 \times 1$  mm) with an average pore diameter of 180  $\mu$ m were fabricated as previously described (27). Just before implantation, scaffolds were seeded with  $0.9 \times 10^6$  endothelial cells (HDMEC-Bcl-2 or HDMEC-LXSN) and  $0.1 \times 10^6$  tumor cells (UM-SCC-17B or OSCC3) in a 1:1 Matrigel/EGM2-MV mix (27, 28). Five- to nine-week-old male severe combined immunodeficient (SCID) mice (CB.17.SCID, Taconic) were anesthetized with ketamine and xylazine, and two scaffolds were s.c. implanted bilaterally in each mouse. Twenty-eight

days after transplantation, mice were euthanized, and the scaffolds were retrieved, fixed overnight in 10% buffered formaldehyde at 4°C, and mounted on glass foiled pen slides (Leica) for laser capture microdissection (LCM). Immunohistochemical staining was done with factor VIII to identify blood vessels, as described (27, 28). The number of capillaries was counted in five random microscopic fields ( $0.646 \times 0.475$  mm) per scaffold, from at least five scaffolds per condition. The data are representative of three independent experiments. The care and treatment of experimental animals was in accordance with University of Michigan institutional guidelines.

**LCM.** Human head and neck tumor specimens were collected in accordance with Institutional Review Board approval from the University of Chicago. The head and neck tissue array was prepared from tumor biopsies and normal control samples (oral mucosa) and mounted on glass foiled pen slides for laser capture microdissection (LCM, Leica). Immunohistochemical staining was done with factor VIII to identify blood vessels, as described (27, 28). A three-step process was used for cell collection with a microdissection microscope (Leica AS LMD, Leica) with a pulsed 337-nm UV laser. Blood cells in factor VIII-positive capillaries were eliminated first. Then, factor VIII-positive endothelial cells were collected and the tumor cells were dissected. Approximately 1,500 endothelial cells and 10,000 cells from surrounding tissue were retrieved from each xenografted tumor, and 400 endothelial cells and 4,000 cells were collected from each human head and neck tumor biopsy. The RNA from at least five independent tumors was pooled for RT-PCR and real-time PCR analyses.

**RT-PCR analysis.** Total RNA from paraffin-embedded tissue sections was extracted using TRIzol reagent (Invitrogen) and purified with RNeasy Mini kits (Qiagen) according to the manufacturers' protocols. cDNA synthesis and PCR amplification were done in single tubes using simultaneously the primer set for the gene of interest and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set, with the use of SuperScript one-step reverse transcription-PCR (RT-PCR) and Platinum Taq kit (Invitrogen). PCR amplification was done using the following primers: Bcl-2, CTGCGAAGAACCTTGTGTGA (sense) and TGTCCTACCAACCAGAAGG (antisense); CXCL8, TAGCAAATTGAGGCCAAGG (sense) and AGCAGACTAGGGTTGCCAGA (antisense); CXCL1, CTCTCCGCTCCTCTCACAG (sense) and GGGACTTCACGTTACACT (antisense); Bcl-x<sub>L</sub>, GGCTGGGATACTTTTGTGGA (sense) and GGTGGGAGGGTAGAGTGGAT (antisense); VEGFR1, CAGAAGGGCTCTGTGGAAG (sense) and GCTCACACTGCTCATCCAAA (antisense); VEGFR2, AGCGATGGCCTCTTCTGTAA (sense) and ACACGACTCCATGTTGGTCA (antisense); E-cadherin, TGCCCAAAAATGAAAAGG (sense) and GGATGACACAGCGTGAGAGA (antisense); and GAPDH, CATGGCCTCCAAGGAGTAAG (sense) and AGGGGTCTACAGGCAACTG (antisense).

**Real-time PCR.** Total RNA was extracted and purified from LCM-retrieved samples as described above. First-strand complementary DNA (cDNA) synthesis was done with TaqMan reverse transcription reagents (Applied Biosystems). Probe and primer sets of TaqMan Gene Expression Assays (Bcl-2, Hs00608023\_m1; CXCL8, Hs00174103\_m1; CXCL1, Hs00605382\_gH; and 18S, Hs99999901\_s1) were obtained from Applied Biosystems. Total RNA at 0.02  $\mu$ g/30  $\mu$ L of reaction mixture was prepared by TaqMan Universal PCR Master Mix (Applied Biosystems). For standard RNA, we used TaqMan One-step RT-PCR Master Mix Reagents (Applied Biosystems). The reactions were done in a 96-well clear optical reaction plate using ABI7700 Sequence Detection System (Applied Biosystems), and the data were normalized by the data of 18S controls. All reactions were done in triplicate, with each run containing at least one negative and one positive control, and three independent experiments were done to verify reproducibility of results.

**Reporter gene assays.** HDMEC-LXSN or HDMEC-Bcl-2 ( $2 \times 10^5$ ) cultured in six-well plates were transiently transfected with signal transducers and activators of transcription 3 (STAT3; Panomics) or VEGF luciferase reporter (gift from Dr. Keller, University of Michigan, Ann Arbor, MI) constructs, using Lipofectin (Invitrogen) as recommended by the manufacturer. One day after transfection, cell lysates were prepared with Reporter Lysis buffer (Promega) and luciferase activity was detected by Dual luciferase reporter assay system (Promega) as described (24). Data were represented as firefly luciferase activity normalized by Renilla luciferase.

**STAT3 decoy transfection and ELISA.** The STAT3 decoy and mutant control decoy oligonucleotides (29) were kindly provided by Dr. Grandis (University of Pittsburgh, Pittsburgh, PA). The STAT3 decoy sequences were sense, 5'-CATTTCCCGTAAATC-3', and antisense, 3'-GTAAAGGGCATTAG-5'; the mutant control decoy sequences were sense, 5'-CATTTCCCTTAATC-3', and antisense, 3'-GTAAAGGGAATTTAG-5'. HDMEC-LXSN or HDMEC-Bcl-2 cells ( $5 \times 10^5$ ) were plated into 60-mm dishes and cultured overnight. The next day, 1 nmol/L STAT3 decoy or 1 nmol/L mutant control decoy oligonucleotide was transiently transfected using Lipofectin (Invitrogen). One day after transfection, cells were washed with sterile PBS and endothelial basal medium (Cambrex) was added to the dishes. After 24 h, supernatants were collected and VEGF expression levels were evaluated by ELISA (Quantikine, R&D Systems) as described (25, 28). Cells from individual plates were trypsinized, stained with trypan blue, and immediately counted in a hemocytometer. Each individual data point from the ELISA was normalized by cell number using the HDMEC-LXSN transfected with control oligonucleotides (scrambled sequence) as reference.

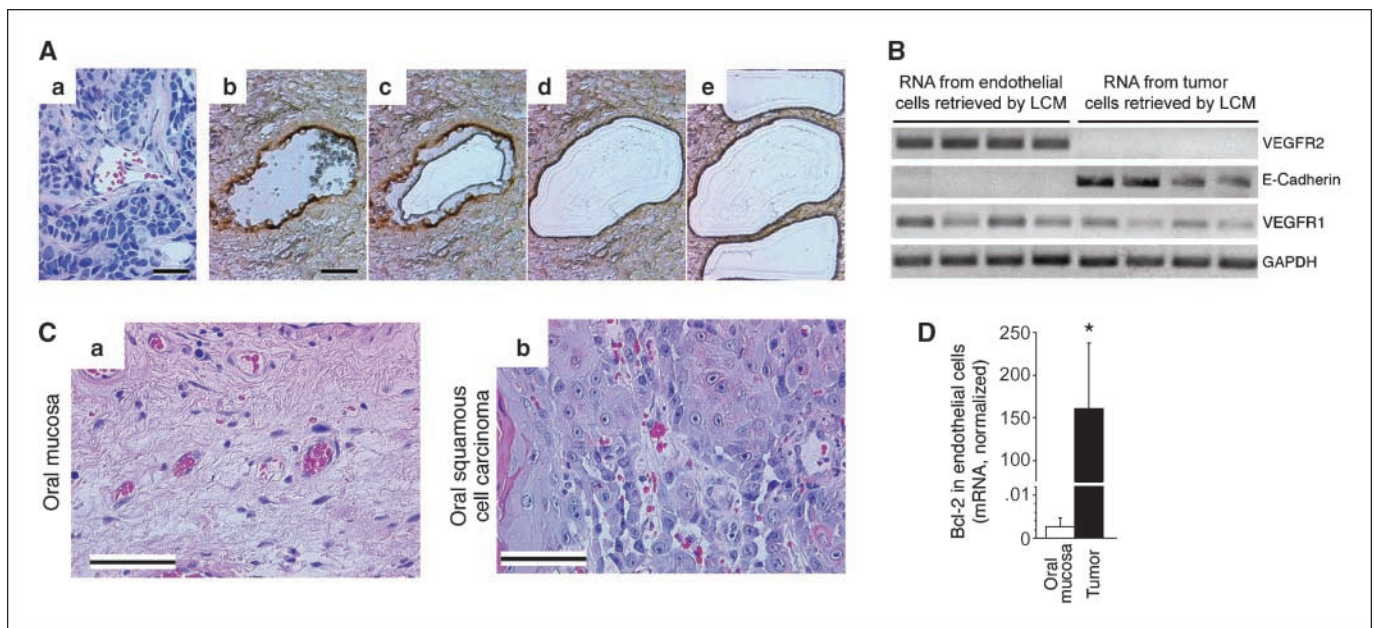
**Statistical analyses.** For statistical comparison of two experimental groups, we used Student's *t* tests, and for three or more groups, one-way ANOVA using Sigmastat 2.0 software (SPSS).

## Results

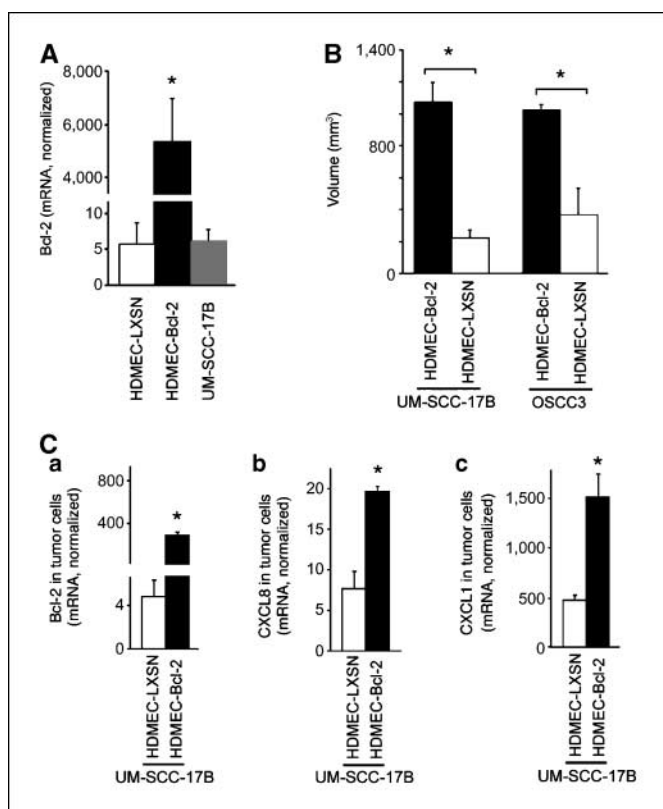
It is known that patients with head and neck cancer have elevated serum levels of VEGF (15) and that recombinant VEGF protein induces Bcl-2 expression in endothelial cells *in vitro* (13). However, the effect of the growth factor milieu secreted by HNSCC cells (which include proangiogenic and antiangiogenic proteins) on Bcl-2 expression in tumor-associated endothelial cells of human patients is not understood. Here, LCM from paraffin sections of tissue arrays that had been previously immunostained with factor VIII for identification of blood vessels (Fig. 1A) was done to retrieve either the endothelial cells lining the blood vessels (Fig. 1A, d) or

the tumor cells surrounding these blood vessels (Fig. 1A, e). RT-PCR using VEGFR2 as a marker for endothelial cells and E-cadherin as a marker for tumor cells of ectodermal origin (Fig. 1B) confirmed the origin and purity of the RNA samples used in the experiments described below. Notably, Bcl-2 mRNA expression levels were ~60,000-fold higher in endothelial cells retrieved from inside the tumor mass, as compared with endothelial cells retrieved from control normal mucosa from the same tissue arrays (Fig. 1C and D). We have also observed that both tumor-associated endothelial cells and HNSCC cells express VEGFR1 (Fig. 1B), which corroborates recent publications that have also shown VEGFR1 expression in tumor cells (30, 31).

To evaluate the effects of up-regulated Bcl-2 expression in endothelial cells on overall tumor progression, we used the SCID mouse model of human angiogenesis that allows us to engineer tumors with predefined populations of human endothelial cells and human tumor cells (27, 28). A retroviral vector encoding wild-type Bcl-2 was used to constitutively express *bcl-2* in primary HDMEC (Fig. 2A; ref. 13). A pool of G418-selected HDMEC-Bcl-2 or empty vector control endothelial cells (HDMEC-LXSN) was mixed with human squamous cell carcinoma (UM-SCC-17B) cells, seeded in biodegradable scaffolds, and s.c. implanted bilaterally in SCID mice to generate human tumors vascularized with human blood vessels, as described (27, 28). The tumors vascularized with HDMEC-Bcl-2 were consistently larger than the ones vascularized with HDMEC-LXSN (Fig. 2B). Coimplantation of another squamous cell carcinoma (OSCC3) with a different pool of G418-selected HDMEC-Bcl-2 showed that the responses observed above were not unique to one pool of HDMEC-Bcl-2 or to UM-SCC-17B xenografts (Fig. 2B). To evaluate the effect of modulated Bcl-2 expression in neovascular endothelial cells on tumor cell gene expression,



**Figure 1.** Quantitative analysis of Bcl-2 expression in endothelial cells lining head and neck tumor blood vessels or endothelial cells lining normal blood vessels from the oral mucosa. **A**, step-by-step characterization of the technique based on LCM used for retrieval of either endothelial cells or tumor cells from paraffin-embedded tissue sections. **a**, H&E staining. **Bar**, 500  $\mu$ m. **b**, factor VIII immunostaining of endothelial cells. **Bar**, 500  $\mu$ m. **c**, removal of blood cells. **d**, retrieval of factor VIII+ endothelial cells. **e**, retrieval of tumor cells adjacent to blood vessels. **B**, quality control check of RNA from endothelial cells or tumor cells using VEGFR2 (marker for endothelial cells) and E-cadherin (marker for cells of ectodermal origin). **C**, **a**, H&E staining of normal mucosa adjacent to a head and neck carcinoma. **b**, H&E staining of the head and neck carcinoma. **Bar**, 500  $\mu$ m. **D**, real-time PCR used to quantify Bcl-2 expression in endothelial cells retrieved either from the tumor mass or from normal mucosa. Specimens were obtained from a head and neck tissue array prepared from tumor biopsies and normal control samples (oral mucosa). Data presented from real-time PCR experiments reflect the expression level of Bcl-2 normalized by 18S. \*,  $P < 0.001$  (*t* test).



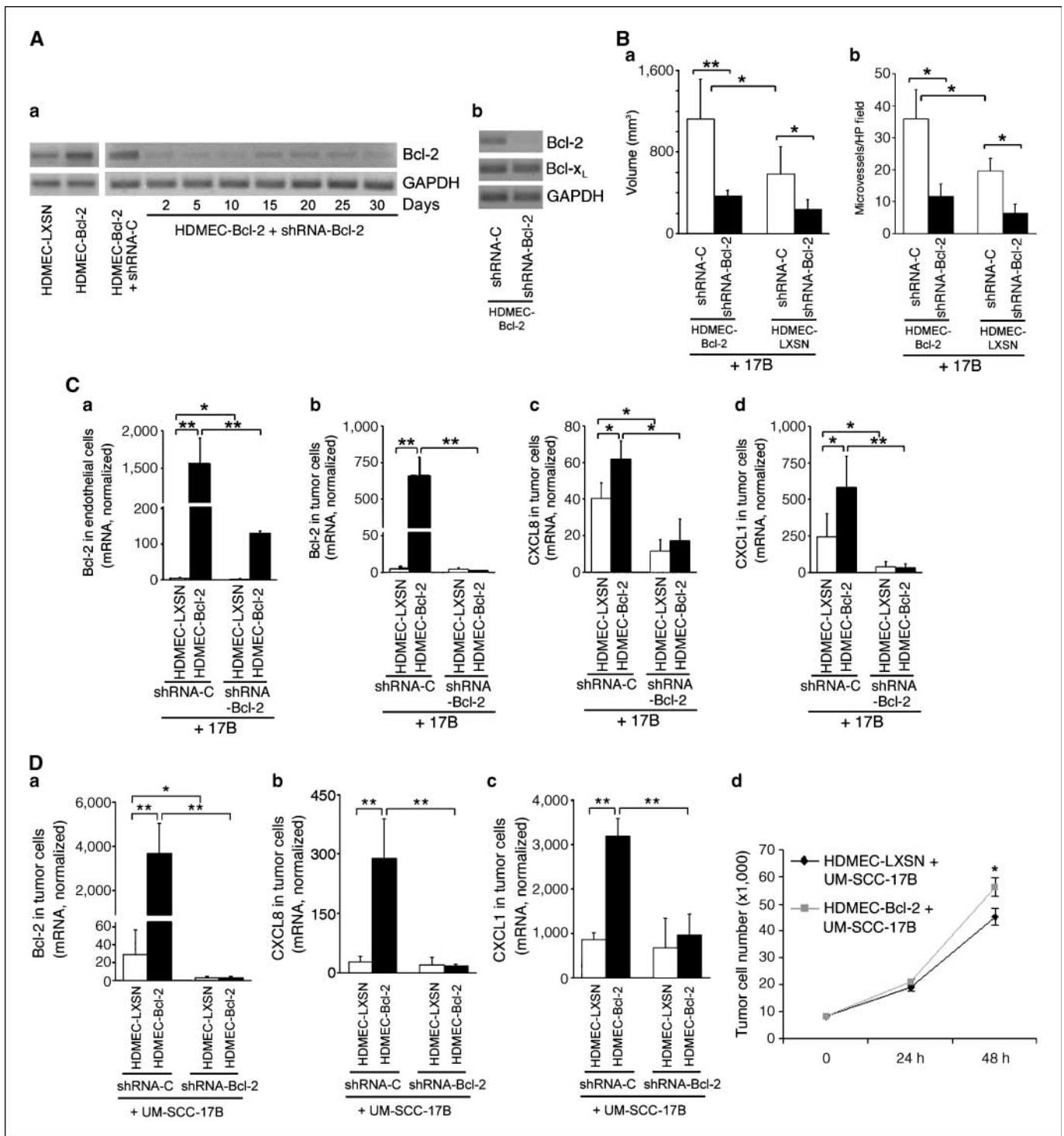
**Figure 2.** Constitutive expression of Bcl-2 in neovascular endothelial cells accelerates tumor progression and induces expression of Bcl-2, CXCL8, and CXCL1 expression in adjacent tumor cells. **A**, real-time PCR was used to evaluate baseline Bcl-2 mRNA expression levels in HDMEC-Bcl-2, HDMEC-LXSN, and UM-SCC-17B. **B**, volume of human tumors vascularized with human blood vessels were engineered in SCID mice by coimplanting human endothelial cells (HDMEC-LXSN or HDMEC-Bcl-2) and human carcinoma cells (UM-SCC-17B or OSCC3) in highly porous biodegradable scaffolds and endothelial cells in SCID mice ( $n = 8$  mice per experimental condition). Each mouse received one implant populated with HDMEC-Bcl-2 and tumor cells (UM-SCC-17B or OSCC3) and one implant populated with HDMEC-LXSN and tumor cells (UM-SCC-17B or OSCC3). **C**, real-time PCR to evaluate Bcl-2, CXCL8, and CXCL1 expression levels in tumor cells retrieved by LCM from the specimens analyzed in (B). Data presented from real-time PCR experiments reflect the expression level of the gene of interest normalized by 18S. \*,  $P < 0.001$  ( $t$  test).

real-time PCR assays were done using the RNA samples prepared from the tumor cell fraction of tumors that were either vascularized with HDMEC-Bcl-2 or control HDMEC-LXSN cells. We were specifically interested in evaluating the effect of modulated Bcl-2 expression levels in endothelial cells on the expression of Bcl-2 and CXC chemokines in tumor cells because of their known effects on tumor angiogenesis and tumor progression. The expression levels of Bcl-2 in tumor cells were ~80-fold higher in tumors vascularized with HDMEC-Bcl-2 cells than in tumors vascularized with HDMEC-LXSN ( $P < 0.001$ ; Fig. 2C, a). The purity of the source of each tumor RNA sample used here was confirmed by RT-PCR, as described above (Fig. 1B), to ensure that the samples were not contaminated with endothelial cell RNA. Notably, the expression levels of both CXCL8 (Fig. 2C, b) and CXCL1 (Fig. 2C, c) were significantly up-regulated in tumor cells from tumors vascularized with HDMEC-Bcl-2, as compared with tumors vascularized with control endothelial cells. Similar results were observed when OSCC-3 tumor cells were used in this model, confirming that this pathway is not unique to one carcinoma cell line (data not shown).

To eliminate the possibility that the effects observed above were artifacts induced by the methods used for constitutively expressing Bcl-2 in endothelial cells, we silenced Bcl-2 with RNA interference in these cells before implantation in the SCID mice. The short hairpin encoded in the lentiviral vector used here down-regulated Bcl-2 expression in endothelial cells for at least 30 days without selection (Fig. 3A, a). Specificity of Bcl-2 down-regulation was verified by the observation that Bcl-x<sub>L</sub> expression was not affected by our lentiviral vector (Fig. 3A, b). Down-regulation of Bcl-2 expression in the endothelial cells was sufficient to slow down tumor growth (Fig. 3B, a) and to decrease intratumoral microvascular density (Fig. 3B, b). Notably, down-regulation of Bcl-2 in neovascular endothelial cells (Fig. 3C, a) was directly correlated with down-regulation of Bcl-2, CXCL8, and CXCL1 in the tumor cells (Fig. 3C, b-d). Taken together, these findings support the hypothesis that endothelial cells play an active role in modulating tumor cell gene expression *in vivo* and that endothelial cell-initiated signaling events have a direct effect on tumor growth.

To understand mechanisms underlining these findings, we established a coculture system in which endothelial cells (bottom chamber) and tumor cells (upper chamber) were separated by a 1- $\mu$ m pore membrane. Real-time PCR assays showed that Bcl-2, CXCL8, and CXCL1 expression in UM-SCC-17B cells was significantly up-regulated when these cells were cocultured with HDMEC-Bcl-2, as compared with coculture with empty vector HDMEC-LXSN (Fig. 3D, a-c). Conversely, knockdown of Bcl-2 expression in endothelial cells with RNA interference prevented the up-regulation of Bcl-2, CXCL8, and CXCL1 expression in the tumor cells (Fig. 3D, a-c). Notably, UM-SCC-17B cells cocultured with HDMEC-Bcl-2 showed enhanced proliferation after 48 h, as compared with UM-SCC-17B cocultured with control endothelial cells (Fig. 3D, d). These *in vitro* data support our *in vivo* studies and suggest that endothelial cells secrete factor(s) that influence gene expression levels and proliferation of the tumor cells via a paracrine signaling pathway that is independent of direct cell-cell contact.

We next carried out experiments designed to identify signaling molecule(s) secreted by endothelial cells that might be responsible for the observed effect on tumor cell gene expression levels. It is known that VEGF induces Bcl-2 and CXC chemokine expression in neovascular endothelial cells (13, 24). Recent work has shown that VEGFR1 is expressed in various tumor cells including pancreatic and colorectal carcinomas (30, 31), and we had observed that tumor cells from HNSCC patients express VEGFR1 (Fig. 1B). The observation that both squamous cell carcinomas studied here (UM-SCC-17B and OSCC3) express VEGFR1 (Fig. 4A) led to the hypothesis that VEGF secreted by endothelial cells plays a role in the paracrine signaling pathway studied here. Indeed, Bcl-2 expression levels correlate directly with VEGF secretion by endothelial cells, as shown in experiments using either cells stably transduced and overexpressing Bcl-2 or cells that had Bcl-2 down-regulated by RNA interference (Fig. 4B). In addition, the cross-talk between HDMEC-Bcl-2 and tumor cells results in an increase in overall VEGF expression levels in the culture medium of cocultures, as compared with cocultures of the tumor cells and control endothelial cells (Fig. 4C). To begin to understand mechanisms underlying the correlation between Bcl-2 and VEGF expression levels, we evaluated the activity of STAT3, a known transcriptional regulator of VEGF (32). STAT3 transcriptional activity was significantly up-regulated in endothelial cells constitutively expressing Bcl-2 as compared with control endothelial cells (Fig. 4D, a). To



**Figure 3.** Analysis of the cross-talk between endothelial and tumor cells in an *in vitro* noncontact coculture system and *in vivo*. **A**, **a**, RT-PCR to determine stability of the knockdown of Bcl-2 expression levels mediated by a shRNA (shRNA-Bcl-2) in the endothelial cells used for the experiments included in this figure. A scrambled oligonucleotide sequence short hairpin (shRNA-C) was used as control for these experiments. **b**, RT-PCR to evaluate the specificity of the effect of the shRNA-Bcl-2 lentiviral vectors used here. **B**, volume (**a**) and microvessel density (**b**) of tumors generated by the implantation of human tumor cells (UM-SCC-17B) and human endothelial cells (HDMEC-LXSN or HDMEC-Bcl-2) in SCID mice ( $n = 6$  mice per experimental condition). The endothelial cells were transduced with either shRNA-Bcl-2 or control shRNA-C, as shown in (**A**). **C**, **a**, real-time PCR to evaluate Bcl-2 expression levels in endothelial cells retrieved by LCM from tumors engineered in SCID mice by the coimplantation of UM-SCC-17B cells and endothelial cells (HDMEC-LXSN or HDMEC-Bcl-2) that were transduced with either shRNA-Bcl-2 or control shRNA-C. **b** to **d**, real-time PCR to evaluate Bcl-2, CXCL8, and CXCL1 expression levels in tumor cells retrieved by LCM from the tumors described in (**B**). **D**, **a** to **c**, real-time PCR used to evaluate Bcl-2, CXCL8, and CXCL1 expression levels in tumor cells (UM-SCC-17B) cocultured with endothelial cells transduced with either shRNA-Bcl-2 or scrambled oligonucleotide sequence control shRNA-C. **d**, tumor cell proliferation as determined by the trypan blue exclusion method. Tumor cells (UM-SCC-17B) were cocultured with endothelial cells transduced with Bcl-2 (HDMEC-Bcl-2) or empty vector controls (HDMEC-LXSN). Data presented from real-time PCR experiments reflect the expression level of the gene of interest normalized by 18S. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  (one-way ANOVA).

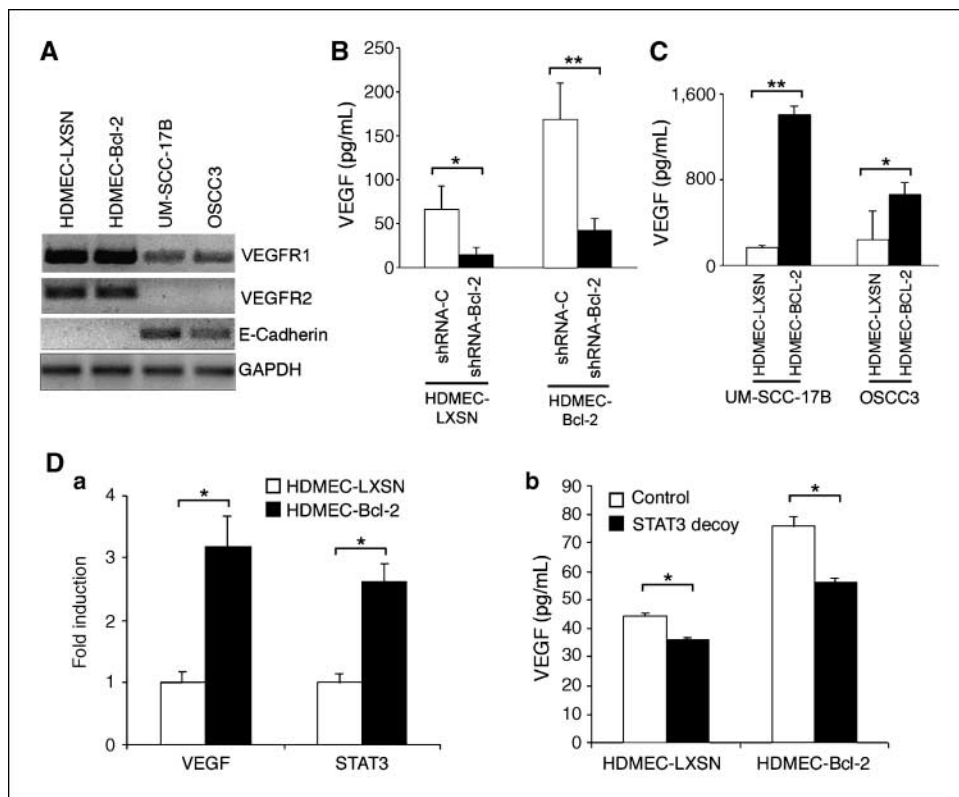
evaluate the role of STAT3 activity in Bcl-2-induced VEGF expression, we transfected the endothelial cells with a STAT3 decoy oligonucleotide (29). At the concentrations used here, the decoy STAT3 did not cause significant endothelial cell death. Notably, inhibition of STAT3 activity with the decoy oligonucleotide down-regulated VEGF expression in endothelial cells expressing Bcl-2 to a level comparable to the VEGF expression observed in HDMEC-LXSN transduced with control oligonucleotides (Fig. 4D, b). Taken together, these data show that Bcl-2-induced VEGF expression in endothelial cells is regulated, at least in part, by STAT3 activity.

To determine the involvement of VEGF in endothelial cell-induced Bcl-2 and CXC chemokine expression in tumor cells, anti-VEGF neutralizing antibody was added to the culture medium of endothelial cell/tumor cell cocultures. Blockade of VEGF signaling with a neutralizing antibody inhibited the ability of HDMEC-Bcl-2 to induce Bcl-2, CXCL8 and, CXCL1 expression in UM-SCC-17B cells (Fig. 5A, a-c). Similarly, blockade of VEGFR1 prevented up-regulation of Bcl-2 and CXC chemokine expression in the tumor cells cocultured with HDMEC-Bcl-2 (Fig. 5B, a-c). To exclude the possibility that the neutralizing antibodies blocked the VEGF signaling pathway in the endothelial cells (cocultured with tumor cells) and, therefore, the possibility that under these experimental conditions endothelial cells can secrete a different ligand that induces gene expression in the tumor cells, we repeated these experiments using conditioned medium. Treatment of the endothelial cell conditioned medium with antibodies against VEGF or VEGFR1 prevented HDMEC-Bcl-2-induced up-regulation of Bcl-2, CXCL8, and CXCL1 in tumor cells (Fig. 5C, a-c). These data show that the cross-talk initiated by endothelial cells that results in up-regulated Bcl-2, CXCL1, and CXCL8 expression in tumor cells is mediated by VEGF signaling through VEGFR1.

## Discussion

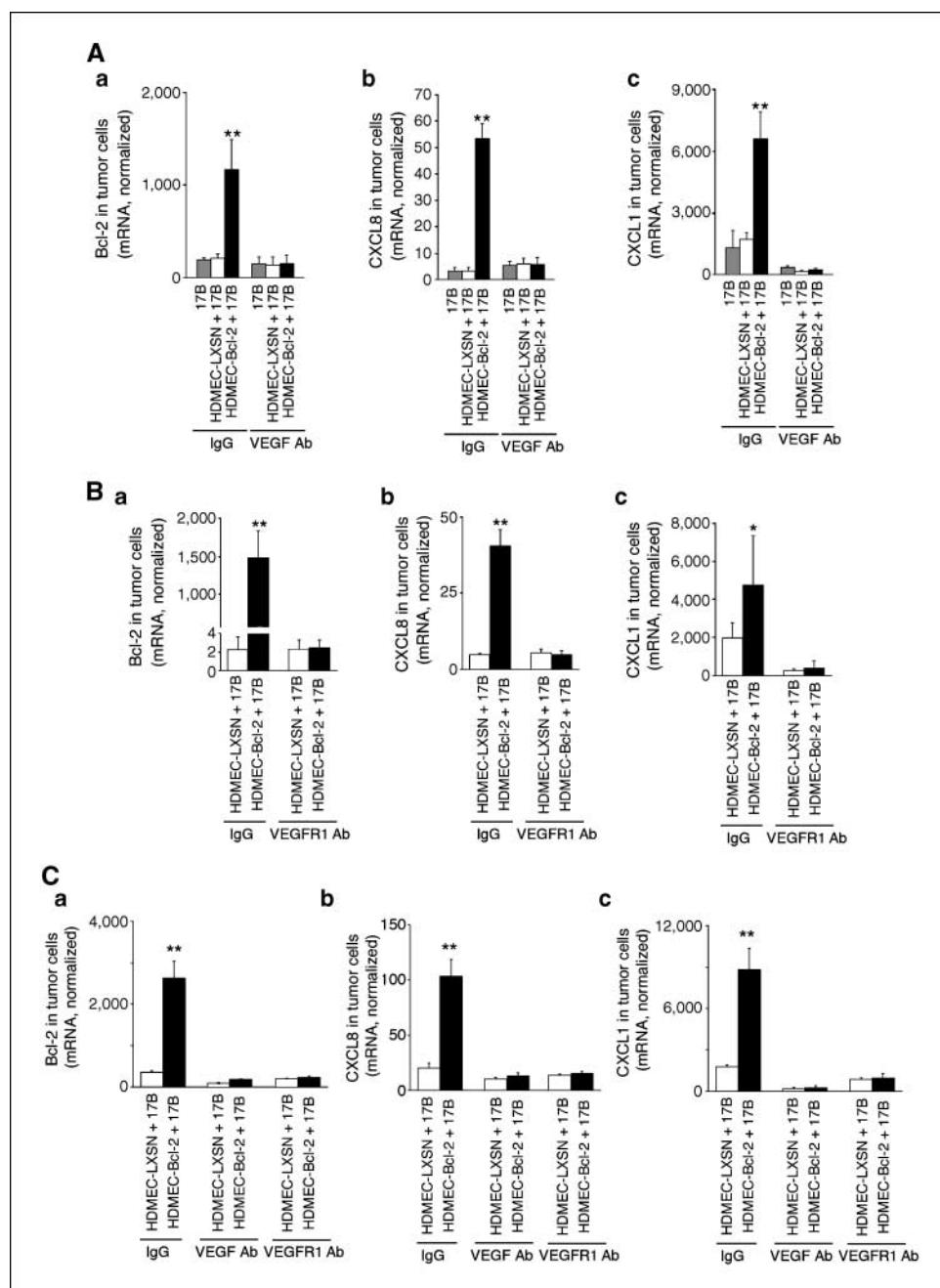
The present study identifies Bcl-2 as the orchestrator of a contact-independent cross-talk between HNSCC and endothelial cells. We observed that VEGF secreted by endothelial cells signals through VEGFR1 and modulates expression levels of Bcl-2, CXCL1, and CXCL8 in HNSCC cells. Although we could not find studies that show a direct effect of endothelial cells on tumor cell gene expression *in vivo*, several reports have shown the effects of stromal fibroblasts on tumor cell phenotype (33, 34). Soluble mediators released from irradiated fibroblasts enhance the invasive potential of pancreatic cancer cells through up-regulation of c-Met phosphorylation and MAPK activity (34). Another report has shown that tumor cells mediate p53 loss in stromal fibroblasts, making these cells highly proliferative in a mouse model of prostate cancer (35). Such studies suggest the existence of molecular exchanges between tumor cells and the stromal compartments that have an effect on tumor growth. The work presented here shows that besides stromal fibroblasts, endothelial cells also play an active role in modulating tumor cell gene expression and phenotype. Notably, this interaction has a significant effect on the pathobiology of the tumor because overexpression of Bcl-2 in the endothelial cells accelerates tumor growth, and selective blockade of Bcl-2 in the endothelial cells was sufficient to slow down tumor growth.

We have previously shown that Bcl-2 initiates an autocrine proangiogenic signaling pathway in endothelial cells that is mediated by NF- $\kappa$ B and results in up-regulated expression of angiogenic CXC chemokines (24). Here, we observed that VEGF signaling through VEGFR1 resulted in Bcl-2 up-regulation in HNSCC cells. Furthermore, we observed that Bcl-2 induces CXCL1 and CXCL8 up-regulation in HNSCC. These findings may provide a mechanism for the up-regulated expression of these chemokines



**Figure 4.** Bcl-2-induced VEGF expression in endothelial cells is mediated by STAT3. *A*, RT-PCR to evaluate baseline VEGFR1, VEGFR2, and E-cadherin expression levels in HDMEC-LXSN, HDMEC-Bcl-2, UM-SCC-17B, and OSCC-3. *B*, ELISA to evaluate VEGF expression levels in 24-h supernatant from endothelial cells transfected with either shRNA-Bcl-2 or control shRNA-C. *C*, ELISA to evaluate VEGF expression levels in 24-h supernatant from endothelial cells (HDMEC-Bcl-2 or HDMEC-LXSN) cocultured with tumor cells (UM-SCC-17B or OSCC3). *D*, *a*, luciferase assays for VEGF and STAT3 transcriptional activity in HDMEC-Bcl-2 or HDMEC-LXSN. Data for VEGF or STAT3 activity were normalized for transfection efficiency with a *Renilla* reporter gene. *b*, ELISA to evaluate VEGF expression levels in 24-h supernatant from endothelial cells transfected with either the STAT3 decoy or an inactive control oligonucleotide. Data presented in this figure were normalized by cell number. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  (one-way ANOVA).

**Figure 5.** Endothelial cell–derived VEGF signals through tumor cell VEGFR1 and regulates Bcl-2, CXCL1, and CXCL8 expression in tumor cells. **A**, real-time PCR was used to evaluate Bcl-2, CXCL8, and CXCL1 expression levels in tumor cells (UM-SCC-17B) cocultured with endothelial cells (HDMEC-Bcl-2 or HDMEC-LXSN) in the presence of polyclonal anti-human VEGF antibody or isotype-matched immunoglobulin control. *Asterisks*, statistically significant difference between UM-SCC-17B cocultured with HDMEC-Bcl-2 and UM-SCC-17B cocultured with HDMEC-LXSN. **B**, real-time PCR was used to evaluate Bcl-2, CXCL8, and CXCL1 expression levels in tumor cells (UM-SCC-17B) cocultured with endothelial cells (HDMEC-Bcl-2 or HDMEC-LXSN) in the presence of polyclonal anti-human VEGFR1 antibody or isotype-matched immunoglobulin control. *Asterisks*, statistically significant difference between UM-SCC-17B cocultured with HDMEC-Bcl-2 and UM-SCC-17B cocultured with HDMEC-LXSN. **C**, real-time PCR was used to evaluate Bcl-2, CXCL8, and CXCL1 expression levels in tumor cells (UM-SCC-17B) exposed to conditioned medium from endothelial cells (HDMEC-Bcl-2 or HDMEC-LXSN) in the presence of polyclonal anti-human VEGF antibody, polyclonal anti-human VEGFR1 antibody, or isotype-matched immunoglobulin control. *Asterisks*, statistically significant difference between UM-SCC-17B exposed to HDMEC-Bcl-2–conditioned medium and UM-SCC-17B exposed to HDMEC-LXSN–conditioned medium. Data presented reflect the expression level of the gene of interest normalized by 18S. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  (one-way ANOVA).



observed in several HNSCC cancers (36, 37). Notably, overexpression of Bcl-2 in prostate carcinoma cells and in a breast carcinoma cell line was shown to enhance VEGF expression and their angiogenic potential *in vivo* (38, 39). Our results support these findings and suggest that up-regulation of proangiogenic CXC chemokines is involved in the overall effect of Bcl-2 on tumor angiogenesis.

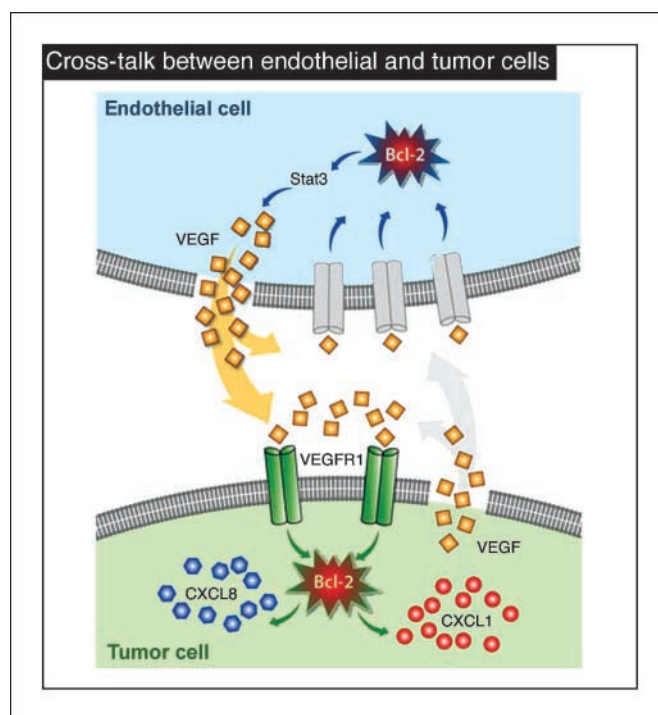
VEGF induces angiogenesis primarily through its interaction with two tyrosine kinase receptors expressed in vascular endothelial cells, VEGFR1 and VEGFR2 (40). Although VEGFR1 was originally reported as being exclusively expressed on endothelial cells, recent studies show that several tumor cells also express this receptor (30, 31). Here, we observed that HNSCC cells express VEGFR1, but not VEGFR2, and that VEGFR1 was the receptor used by endothelial cell–secreted VEGF to trigger the signaling pathway in HNSCC cells

that resulted in up-regulated Bcl-2 expression. These data show that VEGFR1 is a functional receptor in HNSCC cells that actively participates in a cross-talk between endothelial cells and tumor cells, which is regulated by Bcl-2 expression levels and mediated by VEGF. This is in contrast to VEGF signaling in endothelial cells, which seems to be primarily mediated by VEGFR2 (12).

The relatively low 5-year survival rates of patients with head and neck cancer have been frequently associated with acquired drug resistance. Notably, the expression levels of VEGF (14, 15) and Bcl-2 (41) have been directly associated with the clinical outcomes of patients with HNSCCs. The effect of Bcl-2 expression levels has been also associated with resistance to cancer treatment in other tumor models. Overexpression of Bcl-2 in prostate tumor cells is associated with the development of radiation-resistant tumors,

and its down-regulation with antisense oligodeoxynucleotides induced radiosensitization and inhibition of angiogenesis (42, 43). Furthermore, modulation of Bcl-2 expression might have been associated with the effect of inhibitors of VEGF receptors on resistance to therapy in other tumor models. Inhibition of VEGFR2 with SU5416 was shown to reverse the resistance of two refractory neoplasms (glioblastoma multiforme and melanoma) to radiotherapy (44). And combination of DC101 (a small-molecule inhibitor of VEGFR) and low-dose vinblastine was shown to mediate regression of large established xenografted neuroblastomas without signs of acquired drug resistance for 6 months (45). Kerbel (46) has elegantly discussed the fundamental mechanisms for the chemosensitizing activity of antiangiogenic drugs: normalization of the tumor vasculature, prevention of rapid tumor cell repopulation, and augmentation of the antivascular effects of chemotherapy. The results of the present study show that blockade of the VEGF signaling axis inhibits the cross-talk between endothelial and tumor cells that results in Bcl-2 up-regulation. It is known that Bcl-2 has a prosurvival effect for both endothelial cells and tumor cells and was shown to have a direct effect on resistance to treatment (13, 25, 43, 47, 48). Our data suggest that antiangiogenic therapies based on the blockade of VEGF signaling may function as a chemosensitization strategy for cancer by interfering with the endothelial cell and tumor cell Bcl-2-orchestrated cross-talk.

Our results collectively show that factors secreted by neovascular endothelial cells play an active role in determining tumor cell gene expression and tumor growth. Here, we showed an example of such interaction by characterizing a cross-talk that is modulated by Bcl-2 expression levels in neovascular endothelial cells (Fig. 6). This pathway may have functionally distinct, and perhaps synergistic, effects that contribute to tumor growth: (a) Bcl-2 enhances the survival of both endothelial cells and tumor cells; (b) VEGF enhances tumor angiogenesis and functions as a two-way signaling molecule between endothelial cells and tumor cells; and (c) CXCL1 and CXCL8 are proangiogenic and mitogenic for tumor cells. These observations challenge the existing paradigm that endothelial cells are passive responders of tumor cell-initiated stimuli. They show that interactions between endothelial cells and tumor cells are mutually relevant, and that factors secreted by endothelial cells have a direct effect on tumor cell gene expression and tumor growth.



**Figure 6.** Schematic drawing illustrating the proposed mechanism by which endothelial cell-derived VEGF initiates a paracrine signaling cascade that results in enhanced expression of Bcl-2, CXCL1, and CXCL8 in tumor cells in a signaling pathway mediated by VEGFR1. This cross-talk between endothelial cells and tumor cells is centrally regulated by Bcl-2. Interference with Bcl-2 expression in neovascular endothelial cells has a direct effect on tumor cell gene expression and tumor progression.

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## Bcl-2 Orchestrates a Cross-talk between Endothelial and Tumor Cells that Promotes Tumor Growth

Tomoatsu Kaneko, Zhaocheng Zhang, Maria G. Mantellini, et al.

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