Endothelial Cell-Secreted EGF Induces Epithelial to Mesenchymal Transition and Endows Head and Neck Cancer Cells with Stem-like Phenotype

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Abstract

Emerging evidence suggests that endothelial cell-secreted factors contribute to the pathobiology of squamous cell carcinoma (SCC) by enhancing invasive migration and resistance to anoikis. Here, we report that SCC cells within the perivascular niche have undergone epithelial to mesenchymal transition (EMT) in a primary human SCC of a patient that developed distant metastases. Endothelial cell–secreted EGF induced EMT of human SCC cells in vitro and also induced acquisition of a stem-like phenotype. In vivo, tumor xenografts vascularized with EGF-silenced endothelial cells exhibited a smaller fraction of cancer stem-like cells (ALDH+CD44+) and were less invasive than tumors vascularized with control endothelial cells. Collectively, these results demonstrated that endothelial cell-EGF induces EMT and acquisition of stem-like properties by head and neck tumor cells. On this basis, we suggest that vascular endothelial cells contribute to tumor dissemination by secreting factors that endow carcinoma cells with enhanced motility and stemness.

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Introduction

Epithelial to mesenchymal transition (EMT) enables the migration of epithelial cells into connective tissues, a critical process for embryonic development (1). In cancer, it has been shown that epithelial tumor cells exploit EMT to detach from the primary tumor mass and disseminate into the surrounding stroma (2). It has also been shown that epithelial cells can be endowed with stem-like properties through EMT (3). Such findings demonstrate that EMT plays a key role in the progression of epithelial tumors (4, 5). However, the cellular source and the nature of the signals that turn on and off the EMT cascade in cancer remain largely unknown. Such knowledge is critical for the understanding of the pathobiology of local invasion and metastasis of epithelial tumors, and may unveil novel therapeutic targets for this type of malignancy.

Loss of E-cadherin, a hallmark of EMT, is an important step in the progression of papilloma to invasive carcinoma (6). It has been recently shown that EMT is modulated by transcription factors that regulate E-cadherin, for example, Snail (3, 7), Twist (8), and ZEB1 (9). For example, Snail controls EMT by repressing E-cadherin expression in development and cancer (10). Twist is required for EMT and breast cancer metastasis (8). In general, expression of these transcription factors is correlated with increased invasiveness-metastasis and poorer clinical prognosis (11).

EGF is a well-known mitogen that plays important roles in cell proliferation survival in physiologic settings and in cancer (12–14). Continuous EGF treatment has been shown to down-regulate E-cadherin expression and results in loss of cell–cell adherence junctions (15). EGF treatment enhances tumor progression and induces EMT in breast cancer cells and cervical cancer cells (13, 14). Notably, a recent study showed that the overexpression of EGF receptor (EGFR) results in the enrichment of a subset of esophageal cells that is capable of undergoing EMT in response to TGF-β through ZEB transcription factor (16). However, the cellular source of EGF within the tumor microenvironment remains unclear.

Mounting evidence demonstrates that epithelial tumors contain a small subpopulation of cells with stem-like and/or progenitor characteristics (17–19). These cells are highly tumorigenic, exhibit self-renewal, and are capable of differentiating into complex new tumors (20). The origin of these cancer stem cells remains unclear. However, exciting new evidence suggests that EMT allows for the generation of cells with stem cell properties in tumor models (3, 21, 22).

We have showed that EGF secreted by endothelial cells induces motility and protects human squamous cell carcinoma (SCC) against anoikis (23). In addition, we have shown that endothelial cell–secreted factors enhance the survival, self-renewal, and tumorigenicity of cancer stem cells (24). Here,
we hypothesized that EGF secreted by endothelial cells enables tumor cell motility by inducing EMT and by endowing SCC cells with stemness. This work showed that endothelial cell-secreted EGF induces Snail through the PI3k-Akt pathway and induces EMT of SCC cells, as shown by downregulation of epithelial markers (E-cadherin, desmoplakin), upregulation of mesenchymal markers (vimentin, N-cadherin), induction of cell motility and acquisition of stem-like properties [expression of aldehyde dehydrogenase (ALDH) and CD44], and growth as nonadherent or ospheres. Notably, specification of cell motility and acquisition of stem-like properties of epithelial markers (E-cadherin, desmoplakin), upregulation of mesenchymal markers (vimentin, N-cadherin), induction of cell motility and acquisition of stem-like properties [expression of aldehyde dehydrogenase (ALDH) and CD44], and growth as nonadherent os spheres. Notably, specific silencing of EGF in endothelial cells slowed down tumor growth and decreased the fraction of stem-like cells in xenograft models. Collectively, these data demonstrate that signals secreted by tumor-associated endothelial cells enhance the aggressive behavior (motility and stemness) of epithelial cancer cells.

Materials and Methods

Cell culture

University of Michigan squamous cell carcinoma (UM-SCC) cells, UM-SCC-1, UM-SCC-11A, UM-SCC-11B, UM-SCC-14A, UM-SCC-14B, UM-SCC-17A, UM-SCC-17B, UM-SCC-22A, UM-SCC-22B, UM-SCC-74A, and UM-SCC-74B (Tissue Biorepository, University of Michigan Head and Neck SPOR E) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen), supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell lines were genotyped and authenticated before use in the experiments included in this manuscript. Pooled primary human dermal microvascular endothelial cells (HDMEC; Lonza) were cultured in epithelial growth medium-2 for microvascular cells (EGM2-MV; Lonza). Cancer cells were serum starved overnight, and treated with 0 to 50 ng/mL rhEGF, anti-human EGF-neutralizing antibody (Cat. MAB236; R&D Systems) for indicated time points. Alternatively, HDMECs were cultured in serum-free medium (EBM2, Lonza) for 24 hours, and the supernatants were collected as endothelial cell conditioned medium (ECCM). Notably, EGF levels in culture supernatants were normalized to cell number for all experiments included here. SCCs were starved overnight and incubated with ECCM for 0 to 24 hours. In selected experiments, cells were preincubated with 0 to 20 μmol/L Static V (Stat3 inhibitor; Calbiochem), 0 to 20 μmol/L U0126 (MEK1/2 inhibitor; Cell Signaling Technology), or 0 to 20 μmol/L Ly294002 (phosphoinositide 3-kinase; PI3K inhibitor; Cell Signaling Technology) for 1 hour, and then treated with 0 to 50 ng/mL EGF for 0 to 4 hours.

Western blot analyses

SCC cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mmol/L Tris-HCL, PH 7.4, 10% glycerol, 200 mmol/L NaCl, and 2 mmol/L MgCl2) containing protease inhibitors. Protein lysates were loaded onto 8% to 15% SDS-PAGE. Membranes were blocked with 5% nonfat milk in 1 × TBS containing 0.3% Tween-20, then incubated with the following primary antibodies overnight at 4 °C: rabbit anti-human phospho EGFR (Tyr 845, SC-23420-R), EGFR (SC-03), rabbit anti-human E-cadherin (SC-7870), rabbit anti-human Twist (SC-15393), rabbit anti-human desmplakin (SC-33555), rabbit anti-human pan-cytokeratin (SC-81714), rabbit anti-human β-actin conjugated with horseradish peroxidase (HRP; SC-47778 HRP; Santa Cruz Biotechnology); mouse anti-human phosphor STAT3 (Tyr 705, Cat. 9138), rabbit anti-human STAT3 (Cat. 9132), mouse anti-human ERK1/2 (Cat. 4696), rabbit anti-human phosphor-ERK1/2 (Thr 202/Tyr 204, Cat. 4376), rabbit anti-human phosphor-AKT (Ser 473, Cat. 9271), rabbit anti-human AKT (Cat. 9272), mouse anti-human Snail (Cat. 3895), rabbit anti-human N-cadherin (Cat. 4061), mouse anti-human vimentin (Cat. 2309), mouse anti-human CD44 (Cat. 3570; Cell Signaling Technology), mouse anti-human ALDH (Cat. 611195; BD Transduction Laboratories), mouse anti-GAPDH (MAB374; Chemicon), rabbit anti-human Bmi-1 (Cat. SAB4200034; Sigma). Affinity-purified second antibodies conjugated with HRP (Jackson Laboratories) were used, and immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to X-ray film.

Stem cell staining and flow-cytometric analysis

Starved SCC cells were treated with 0 to 50 ng/mL EGF, ECCM, or unconditioned medium for 24 hours. Stemness was assessed by ALDH activity and CD44 expression, as we showed (24). Briefly, cells were collected and stained with Aldefluor (Aldagen Inc) using diethylaminobenzaldehyde (specific inhibitor of ALDH activity) as negative control. Cells were also immunostained with mouse anti-human CD44 conjugated with APC (Cat. 559942; BD Biosciences) using IgG-APC as isotype control. Percentage of ALDH- and CD44-positive cells was determined by flow cytometry. 7-Aminoactinomycin D (eBioscience) staining was used for elimination of dead cells.

Orosphere assay

UM-SCC-1, UM-SCC-22A, or UM-SCC-22B cells (1,000–3,000) were cultured in ultralow attachment plates (Corning Inc) with serum-free medium containing 0 to 50 ng/mL EGF, ECCM, or unconditioned medium for 7 to 15 days, as we showed (25). Orospheres, defined as nonadherent spheres of ≤25 cells, were photographed and counted under light microscopy.

Immunohistochemistry

Of note, 4 μm-thick sections were deparaffinized and rehydrated. Antigen retrieval was performed, and sections were incubated with primary antibodies for CD44 (clone EPR1013Y; Abcam, 1:100), ALDH (clone 44; BD Transduction Laboratories, 1:50), vimentin (clone 3B4; Dako, 1:100), E-cadherin (clone 24E10; Cell Signaling Technology, 1:400), Factor VIII related antigen/Von Willebrand Factor Ab-1 (RB-281-A; Thermo Scientific, 1:100), and phospho-EGFR (Tyr 1068, Cat. 2236, Cell Signaling Technology, 1:100) overnight at 4 °C. The EnVision+ system (Dako) and 3,3′-diaminobenzidine (Dako) were utilized for visualization. Isotype-matched nonspecific immunoglobulin G (IgG) was used as negative control.

ELISA

HDMEC and tumor cells were cultured with serum-free medium (EBM and DMEM, respectively) for 24 hours. The
Figure 1. EMT in human HNSCC. A, photomicrographs of serial histologic sections of a primary human HNSCC containing tumor cells inside the lumen of a blood vessel. This tumor was surgically excised from a Caucasian male, 72 years old, smoker, with a HNSCC in the floor of the mouth extending into base of the tongue, classified as stage T3N2M0 at time of diagnosis of the primary tumor. This patient was diagnosed with distant metastases 1 year after surgical removal of the primary tumor depicted here. E-Cadherin, vimentin, ALDH1, and CD44 expression was examined by immunohistochemistry (brown). Low magnification (×100, top row) and high magnification (×200, bottom row) of the same area of the HNSCC (box) depict in detail the tumor cell cluster within a blood vessel. B, photomicrographs (×100) of two representative pairs of HNSCC cell lines with distinctly different morphologies. The UM-SCC-22 pair exhibits epithelial, cobblestone-like morphology, whereas the UM-SCC-74 pair exhibits a more elongated, mesenchymal-like shape. C, Western blot for analysis of constitutive expression of epithelial markers (E-cadherin, desmoplakin), mesenchymal markers (N-cadherin, vimentin), EMT-related transcription factors (Snail, Twist), and self-renewal marker (Bmi-1) in a series of established HNSCC cell lines. The origin of the human HNSCC cell lines is as follows: UM-SCC-11A pretreatment biopsy; UM-SCC-11B postchemotherapy biopsy from same patient as UM-SCC-11A; UM-SCC-14A wide local excision after excisional biopsy; UM-SCC-14B recurrence after surgery and radiation; UM-SCC-17A primary tumor from the endolarynx; UM-SCC-17B from tumor extending outside the thyroid cartilage; UM-SCC-22A from primary site; UM-SCC-22B from lymph node metastasis; UM-SCC-74A surgical resection after chemotherapy and radiation; UM-SCC-74B second surgery for persistent cancer. D and E, Western blot analyses for EGFR, vimentin, Snail, Twist, E-cadherin, desmoplakin and N-cadherin in tumor cells (UM-SCC-74B) stably transduced with EGFR (D) or tumor cells (UM-SCC-14A) stably transduced with shRNA-EGFR (E).
supernatant was collected and ELISA for EGF (Cat. DEG00; R&D Systems), TGF-α (Cat. DTG00; R&D Systems), and Amphiregulin (Cat. DAR00; R&D Systems) was performed.

**EGF, EGFR silencing, and EGFR overexpression**

HEK293T cells were transiently cotransfected with the lentiviral packaging vectors psPAX2, pMD2G, and shRNA-EGF (3 clones: TAGCTGATAGGAGAGAATG, TTCAATCACA-GAVTGCTTG, AACATCTTCACAGTACTTC, for endothelial cell infection), shRNA-EGFR (2 clones: TTTCCAAATTCC-CAAGGAC, TTCCGTTACACACTTTGCG, for UM-SCC-14A infection), or scramble sequence control shRNA-C (Vector Core, University of Michigan, Ann Arbor, MI) by the calcium phosphate method. Alternatively, we cotransfected HEK293T cells with retrovirus packaging vector pVpack-GP, pVpack-VSV-G, and pBABE-EGFR (for UM-SCC-74B EGFR overexpression) or control vector pBABE-puro (Addgene) by the calcium phosphate method. Recipient cells were infected

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**Figure 2.** Endothelial cell-secreted EGF induces phenotypic changes consistent with EMT in HNSCC cells. A, photomicrographs of HNSCC cells cultured in presence or absence of EGF for 24 hours. B, ELISA for EGF in the supernatant of a panel of UM-SCC cell lines and in pooled primary HDMECs. C, ELISA for EGFR ligands (EGF, TGF-α, AREG) in the supernatant of HDMEC. D and E, UM-SCC-22B cells were starved overnight and treated with 0 to 50 ng/mL EGF for 25 minutes (D) or 4 hours (E). Western blot analyses for P-EGFR, EGFR, P-AKT, AKT, P-ERK, ERK (D), desmoplakin, vimentin, Snail, and Twist (E). F–H, UM-SCC-22B cells were starved overnight and treated with 50 ng/mL EGF (F and G) or ECCM (G and H) for indicated time points. H, UM-SCC-22B cells were starved overnight and then treated with ECCM with or without 10 μg/mL EGF neutralizing antibody for 24 hours. Western blot analyses were performed for desmoplakin, vimentin, Bmi-1, Snail, and Twist. *P < 0.05.
with supernatants containing lentivirus or retrovirus and selected with 1 μg/mL of puromycin (Sigma-Aldrich) for at least 1 week. Here, and throughout this manuscript, EGF expression was evaluated by ELISA, and EGFR expression was detected by Western blot analysis.

SCID mouse model of human tumor angiogenesis

Xenograft human tumors vascularized with human blood vessels were generated under an University Committee on Use and Care of Animals-approved protocol. Briefly, highly porous poly-L(lactic) acid (Boehringer Ingelheim) scaffolds were seeded with 9 × 10^5 HDMECs stably transduced with shRNA-EGF (or control shRNA-C) and 1 × 10^5 UM-SCC-22B cells. Immunodeficient mice (CB.17.SCID; Taconic) were anesthetized with ketamine and xylazine, and scaffolds were implanted in the subcutaneous space of the dorsal region (10 mice per experimental condition), as we showed (24). When tumors reached 0.5 cm^3, mice were euthanized and tumors were removed, single cell suspensions were prepared and analyzed by flow cytometry, as described above.

**Statistical analysis**

A t-test or one-way ANOVA followed by appropriate post hoc tests was performed with SigmaStat 2.0 software (SPSS). Statistical significance was determined at \( P < 0.05 \).

**Results**

**Endothelial cell-secreted EGF induces EMT of squamous cell carcinoma cells**

A hallmark of cells undergoing EMT is the loss of epithelial markers (e.g. E-cadherin, desmoplakin) and concomitant acquisition of mesenchymal markers (e.g. vimentin, N-cadherin; ref. 26). We analyzed the primary head and neck squamous cell carcinoma (HNSCC) of a patient that presented with distant metastases 1 year after surgical removal of the primary tumor (Fig. 1A). We observed a group of tumor cells approaching a blood vessel and a cluster of tumor cells in the lumen of this blood vessel (Fig. 1A). Interestingly, the tumor cells approaching the blood vessel exhibited features of EMT, as characterized by low E-cadherin and high vimentin expression. In contrast, the tumor cells within the lumen have reversed back to an epithelial phenotype characterized by high E-cadherin and low vimentin,
suggestive of mesenchymal to epithelial transition (MET; Fig. 1A). A link between EMT and acquisition of stemness has been proposed (3). Here, we observed that many tumor cells in close proximity to the blood vessel express the stem cell markers ALDH and CD44, and that this expression is visibly enhanced in the tumor cells within the lumen of the blood vessel (Fig. 1A). These data underline the plasticity of invasive epithelial tumor cells and suggest that once the epithelial cells have invaded through the connective tissue, and EMT is no longer beneficial, they quickly revert back to their original epithelial phenotype.

Figure 4. Endothelial cell-secreted EGF induces Snail expression via PI3K/Akt signaling. A–C, UM-SCC-22B cells were starved overnight and treated with 50 ng/mL EGF (A) or ECCM (B and C) for indicated time points. C, UM-SCC-22B cells were starved overnight and then treated with ECCM with or without 10 μg/mL EGF-neutralizing antibody for 30 minutes. Western blot analyses were performed for P-Stat3, Stat3, P-Akt, Akt, P-ERK, and ERK. D–G, UM-SCC-22B cells were starved overnight, preincubated with 20 μmol/L Stat3 inhibitor (Stattic V), 20 μmol/L PI3K/Akt inhibitor (Ly294002), 20 μmol/L MEK1/2 inhibitor (UO126) for 1 hour, and then exposed to 50 ng/mL EGF (F) or ECCM (G) for 4 hours. Western blot analyses were performed for P-Stat3, Stat3, P-Akt, Akt, P-ERK, and ERK (D and E) or Snail (F and G). H and I, Western blot analyses for desmoplakin, Snail, Twist, and vimentin. UM-SCC-22B cells were starved overnight, preincubated with 20 μmol/L LY294002 for 1 hour, then treated with 50 ng/mL EGF (H) or ECCM for 4 hours (I).
To begin to understand the molecular mechanisms underlying this phenomenon, we screened a panel of HNSCC cell lines for expression of markers and regulators of EMT and stemness (Fig. 1B and C). This screen revealed a wide diversity of gene expression patterns. Noteworthy is the observation that one pair of cells lines, UM-SCC-74A (surgical resection after chemotherapy and radiation) and UM-SCC-74B (second surgical for persistent cancer) derived from the same patient (27), exhibits a gene expression profile consistent with constitutive EMT, as shown by mesenchymal-like morphology, low E-cadherin, desmoplakin, and high N-cadherin, vimentin, and Twist (Fig. 1B and C). In contrast, UM-SCC-22A (primary tumor of the hypopharynx) and UM-SCC-22B (neck metastasis) from the same patient (27) showed a gene expression profile that is more consistent with epithelial cells (i.e., high E-cadherin, desmoplakin, and low N-cadherin, vimentin, and Twist). To determine whether EMT markers are correlated with EGFR expression in head and neck cancer cell lines, we performed EGFR overexpression in UM-SCC-74B cells (Fig. 1D) and EGFR silencing with shRNA-EGFR in UM-SCC-14A (Fig. 1E). Overexpression of EGFR in UM-SCC-74B cells by stable transduction with lentivirus þ selection correlated with increased expression of the epithelial marker E-cadherin, reduced expression of the mesenchymal markers N-cadherin and vimentin, and reduced expression of the EMT transcriptional factors snail and twist (Fig. 1D). On the other hand, knockdown of EGFR by short hairpin (shRNA) in UM-SCC-14A cells correlated with downregulation of E-cadherin and desmoplakin, and upregulation of vimentin, snail, and twist (Fig. 1E).
their typical cobblestone appearance and assuming a more elongated, fibroblast-like morphology (Fig. 2A). Surprisingly, the expression levels of EGF in HNSCC cells are significantly lower than in microvascular endothelial cells (Fig. 2B), suggesting that the endothelial cells constitute a major source of EGF in the tumor microenvironment. To verify the relative expression of EGF in comparison with other EGFR ligands, we performed ELISA tests that demonstrated that the expression of EGF is 25-fold higher than that of TGF-α and 62.5-fold higher than that of amphiregulin AREG (4 pg/mL) in endothelial cells (Fig. 2C).

To explore a role of endothelial cells on the induction of EMT in head and neck cancer, we exposed HNSCC cells to EGF or to ECCM. First, we checked dose response of cancer cells upon EGF stimulation (Fig. 2D and E) and time dependent upon EGF and ECCM treatment (Fig. 2F and G) and observed that not only P-EGFR, P-Akt, and P-ERK related to proliferation and survival were upregulated (Fig. 2D), but also gene expression changes were consistent with EMT (Fig. 2E–G). Both EGF and ECCM decreased expression of an epithelial marker (desmoplakin), increased the expression of a mesenchymal marker (vimentin), and activated expression of EMT transcriptional factors (Snail and Twist) in UM-SCC-22B cells (Fig. 2E–G). To evaluate the role of EGF in the responses induced by endothelial cell-secreted factors, we used a neutralizing anti-EGF antibody and observed that EGF inhibition restored baseline levels of desmoplakin, vimentin, and Twist, and partially inhibited upregulation of Snail (Fig. 2H). As expected, both EGF and ECCM treatment mediated downregulation of total EGFR expression, which was rescued with the use of anti-EGF antibody (Fig. 2H). These studies were repeated with a second cell line (UM-SCC-1; Supplementary Fig. S1E). To further understand the effect of PI3K/Akt signaling on EMT, we performed Western blot analyses that demonstrated that LY294002 inhibits rhEGF-induced or ECCM-induced upregulation of snail, twist, and vimentin and downregulation of desmoplakin in tumor cells (Fig. 4H and I). These data suggest that PI3K/Akt signaling plays an important role in the EMT mediated by EGF.

**Endothelial cell-secreted EGF endows epithelial tumor cells with stem-like characteristics**

To evaluate the potential mechanism of action of endothelial cell-secreted EGF in tumor cells, we seeded SCC cells in ultra-low attachment plates and cultured them in serum-free medium with or without EGF, or in ECCM, for 7 to 14 days (Fig. 5). EGF supplementation or treatment with endothelial cell-secreted factors induced a significant increase in the number of orospheres when compared with controls (Fig. 5A–C). Notably, recombinant human EGF or the full milieu of growth factors secreted by endothelial cells induced expression of Bmi-1 (Fig. 5D and E), an important regulator of self-renewal and stemness (29, 30). The Bmi-1 activation in response to the endothelial cell growth factor milieu was strictly dependent on EGF (Fig. 5E).

Next, we explored a possible role of endothelial cell-secreted EGF on the fraction of cancer stem-like cells in HNSCC, as determined by the activity of ALDH and expression of CD44. EGF increased significantly the fraction of ALDH<sup>+</sup>/CD44<sup>+</sup> cells (Fig. 6A and Supplementary Fig. S5). Notably, the increase in the fraction of ALDH<sup>+</sup>/CD44<sup>+</sup> cells mediated by endothelial cells was blocked by EGF-neutralizing antibody (Fig. 6B and D) and PI3K inhibitor LY294002 (Fig. 6C and D). Collectively, these data suggest that endothelial cell-secreted factors enhanced the fraction of ALDH<sup>+</sup>/CD44<sup>+</sup> cells in an EGF-dependent manner via the PI3K/Akt signaling pathway.

**Endothelial cell-secreted EGF enhances tumor growth and cancer stem cell fraction**

To evaluate the effect of endothelial cell-secreted EGF on the pathobiology of HNSCC, we engineered xenograft tumors vascularized with endothelial cells stably transduced with EGF phosphorylation was sustained for 24 hours, phosphorylation of Akt and ERK was more transient (Fig. 4B). The use of a neutralizing anti-EGF antibody abolished endothelial cell-induced phosphorylation of Akt and STAT3, while partially inhibiting phosphorylation of ERK (Fig. 4C). Inhibition of ERK with U0126 potentiated EGF-induced phosphorylation of STAT3 and Akt (Fig. 4D and E). Notably, inhibition of PI3K/Akt signaling with LY294002 prevented EGF- and endothelial cell-induced Snail expression (Fig. 4F and G). These results were confirmed in a second cell line (UM-SCC-1; Supplementary Fig. S4). To further understand the effect of PI3K/Akt signaling on EMT, we performed Western blot analyses that demonstrated that LY294002 inhibits rhEGF-induced or ECCM-induced upregulation of snail, twist, and vimentin and downregulation of desmoplakin in tumor cells (Fig. 4H and I). These data suggest that PI3K/Akt signaling plays an important role in the EMT mediated by EGF.

**Endothelial cell-secreted EGF induces EMT through PI3K/Akt signaling**

To evaluate the effect of endothelial cell-secreted EGF on the activity of signaling pathways that play a key role in HNSCC survival and motility (23), we evaluated signaling through STAT3, Akt, and extracellular signal-regulated kinase (ERK) in UM-SCC-22B (Fig. 4A–C) and UM-SCC-1 (Supplementary Fig. S3). We observed that EGF and endothelial cell-secreted factors potently induce activation of STAT3, Akt, and ERK within 15 minutes (Fig. 4A and B). Although STAT3 phosphorylation was sustained for 24 hours, phosphorylation of Akt and ERK was more transient (Fig. 4B). The use of a neutralizing anti-EGF antibody abolished endothelial cell-induced phosphorylation of Akt and STAT3, while partially inhibiting phosphorylation of ERK (Fig. 4C). Inhibition of ERK with U0126 potentiated EGF-induced phosphorylation of STAT3 and Akt (Fig. 4D and E). Notably, inhibition of PI3K/Akt signaling with LY294002 prevented EGF- and endothelial cell-induced Snail expression (Fig. 4F and G). These results were confirmed in a second cell line (UM-SCC-1; Supplementary Fig. S4). To further understand the effect of PI3K/Akt signaling on EMT, we performed Western blot analyses that demonstrated that LY294002 inhibits rhEGF-induced or ECCM-induced upregulation of snail, twist, and vimentin and downregulation of desmoplakin in tumor cells (Fig. 4H and I). These data suggest that PI3K/Akt signaling plays an important role in the EMT mediated by EGF.

**Endothelial cell-secreted EGF enhances tumor growth and cancer stem cell fraction**

To evaluate the effect of endothelial cell-secreted EGF on the pathobiology of HNSCC, we engineered xenograft tumors vascularized with endothelial cells stably transduced with...
shRNA-EGF or vascularized with endothelial cells transduced with scramble sequence control lentiviral vectors. ELISA demonstrated that one of the shRNA sequences (clone 2) produced significant and stable silencing of EGF expression in endothelial cells (Fig. 7A). EGF silencing in the endothelial cells was sufficient to slow down tumor growth over a period of 50 days (Fig. 7B and C). Mice were euthanized 50 days after cell transplantation, tumors were retrieved, and single cell suspensions were prepared for ALDH analyses (Fig. 7D and E). Tumors vascularized with EGF-silenced endothelial cells showed a significant reduction in the proportion of ALDH+ cells, as determined by flow cytometry (Fig. 7D and E) and immuno-histochemistry staining (Fig. 7F). Notably, the tumors vascularized with EGF-silenced endothelial cells were more differentiated and less invasive, as compared with tumors vascularized with control endothelial cells (Fig. 7F). Interestingly, we observed strong phosphorylation of EGFR in tumor cells near blood vessels in xenograft tumors vascularized with control endothelial cells (Supplementary Fig. S6A). Nonetheless, there was no difference in microvascular density when xenograft tumors were vascularized with control or EGF-silenced endothelial cells (Supplementary Fig. S6B and S6C). Collectively, these data demonstrate a direct correlation between the level of EGF secretion by endothelial cells and the fraction of stem-like cells in an animal model of HNSCC.

Discussion

The cross-talk between endothelial cells and tumor cells has been characterized as one of the key cell-cell interactions within the tumor microenvironment. For many years, the paradigm explaining this cross-talk has been centered on a dominant role for tumor cell-initiated signals (e.g., VEGF) that induce the recruitment and enhance the survival of blood vessels required for the influx of oxygen and nutrients necessary for the high metabolic demands of tumor cells. However, recent studies demonstrated that this cross-talk is a "two-way street," where endothelial cell-initiated events have a profound impact on the behavior of tumor cells. Indeed, endothelial cell-initiated signaling enhances tumor cell proliferation, migration, and anoikis resistance (23, 31, 32). And, more recently we showed that endothelial cell-initiated signaling promotes the survival and enhances the tumorigenic potential of cancer stem cells (24). Here, we demonstrated that endothelial cell-secreted EGF induces EMT and acquisition of stemness by epithelial tumor cells. Collectively, these studies showed that vascular endothelial cells play an active role in the pathobiology of cancer that is not limited to "blood vessel making." Rather, endothelial cells secrete factors that modulate the aggressiveness of epithelial tumor cells.

The rationale for this study is based on the following observations: (i) endothelial cells secrete significantly more EGF than SCC cells; (ii) EGF secreted by endothelial cells induces EMT of SCC cells; and (iii) human epithelial tumor cells approaching a blood vessel undergo EMT (low E-cadherin, high vimentin). Surprisingly, once the tumor cells have entered the blood vessel, they revert back to an epithelial phenotype (high E-cadherin, low vimentin). This finding might be explained by MET and underscores the plasticity of these tumor cells. Transitions between epithelial and mesenchymal states are involved in the acquisition of malignant and stem cell traits (33). Indeed, cell plasticity may allow the conversion of normal and neoplastic nonstem cells into a stem-like cell (34). MET often converts the disseminated mesenchymal cancer cells back to a more differentiated, epithelial cell state (35). We speculate that once the SCC cells have moved through the connective tissue, and EMT is no longer a favorable trait, these cells default back to their original epithelial phenotype.

ALDH is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome for patients with cancer (36, 37). CD44-positive cells have the properties of cancer stem cells in human HNSCC (20). Notably, CD44 is a strong predictor of local recurrence after radiotherapy in larynx cancer (38). We have successfully used the combination of ALDH activity and CD44 expression to identify a small subpopulation of highly tumorigenic cancer stem cells (24). Here, we observed that endothelial cell-secreted factors (EGF) are likely involved in metastatic spread by attracting epithelial tumor cells toward blood vessels, allowing their transit through connective tissue via EMT, and enhancing their tumorigenic potential by endowing tumor cells with a stem cell-like phenotype.

We observed here that endothelial cell-secreted factors, and particularly EGF, activate major signaling pathways in SCC cells, that is, STAT3, ERK, and PI3K/Akt. We have previously shown that these pathways regulate head and neck tumor cell proliferation, migration, and anoikis resistance (23). The induction of cell survival may be responsible, at least in part, for the ability of epithelial tumor cells to leave their nests and migrate through connective tissue. Notably, EGF signaling through PI3K/Akt seems to be the primary pathway for the induction of the Snail. This observation suggests that EGF-induced EMT is mediated by PI3K/Akt signaling and induction of Snail activity, a major transcriptional regulator of EMT and metastases (40, 41). Collectively, these data suggest that EGF enables epithelial tumor cell invasion through the connective tissue via EMT while protecting these cells against anoikis.

The current paradigm for antiangiogenic therapies for cancer is focused on the induction of endothelial cell death and disruption of the tumor microvascular network. The benefits of this approach to the survival of patients with cancer have remained largely evasive. Tumor cells are...
Figure 7. Endothelial cell-secreted EGF enhances tumor growth and the fraction of cancer stem-like cells. 

A, pooled primary human endothelial cells (HDMEC) were stably transduced with scramble vector control (shRNA-C) or with shRNA-EGF and selected with 1 μg/mL puromycin. Graph depicting the expression of EGF, as determined by ELISA. Clone 2 showed a significant reduction in EGF expression and was used for the remaining of the experiments of this figure. 

B, a total of $1 \times 10^5$ UM-SCC-22B cells were seeded with either $9 \times 10^5$ HDMEC-shRNA-EGF (clone 2) or $9 \times 10^5$ HDMEC-shRNA-C in biodegradable scaffolds that were transplanted in the dorsum of SCID mice (10 mice per experimental condition). Graph depicting tumor volume over time. 

C–E, mice were euthanized 50 days after transplantation, and tumors were removed and weighed (C). Single cell suspensions were prepared from each tumor and ALDH activity was analyzed by flow cytometry (D and E). F, a slice of each tumor was fixed, analyzed by hematoxylin and eosin staining and by immunohistochemistry for ALDH1 (brown). * $P < 0.05$. 

Endothelial EGF and Cancer Stem Cells

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frequently able to overcome the effect of current antiangiogenic therapies by: (i) activating alternative proangiogenic signaling pathways that reconstitute the tumor microvascular network; (ii) inducing expression of potent proangiogenic molecules in response to the hypoxic conditions generated by effective disruption of tumor blood vessels; and/or (iii) developing a process of evasive resistance (42), in which tumor cells migrate away from the primary tumor site in response to the unfriendly environment generated by antiangiogenic drugs. Here, we showed a prominent role for endothelial cells as the source of signaling events that induce EMT and empower epithelial cells to move through connective tissues while endowing these cells with stem-like properties. These data suggest that endothelial cells actively initiate signaling events that enable the movement of tumor-initiating cells toward the blood vessel, that is, the first steps of the metastatic cascade. Collectively, these observations suggest a new treatment paradigm that is focused on the blockade of endothelial cell-initiated signaling mediating tumor cell dissemination, rather than on the killing of the endothelial cell. This could readily be tested by optimizing treatment regimens with existing targeted drugs to enable effective and sustained blockade of signaling events initiated by endothelial cells, likely by using a metronomic regimen (43, 44) based on low-dose/high-frequency dosing. Such approach would not create the hypoxic and nutrient-deprived states that enhance the aggressiveness of the tumor cells that is frequently observed with standard antiangiogenic therapies. Rather, it would inhibit tumor cell motility toward the blood vessel and metastatic spread, which is typically associated with poor patient outcome.

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

Authors’ Contributions
Conception and design: Z. Zhang, J.E. Nör Development of methodology: Z. Zhang, M.S.A Filho, J.E. Nör Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Zhang, Z. Dong, I. Lauzen, M.S.A Filho, J.E. Nör Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Zhang, J.E. Nör Writing, review, and/or revision of the manuscript: Z. Zhang, J.E. Nör Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Zhang, I. Lauzen, J.E. Nör Study supervision: M.S.A Filho, J.E. Nör

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