EMMPRIN Expression is Required for Response to Bevacizumab Therapy in HNSCC Xenografts

J. Robert Newman[1], **Emily E. Helman**[1], **Seena Safavy**[1], **Wenyue Zhang**[1], and **Eben L. Rosenthal**[1]

1 *Department of Surgery, Division of Otolaryngology – Head and Neck Surgery, University of Alabama at Birmingham, Birmingham, Alabama*

Abstract

The HNSCC cell line, FaDu was stably transfected with control vector (FaDu) or with plasmid expressing small interfering RNA against EMMPRIN (FaDu/siE). Tumor cells were treated with bevacizumab (0, 25, 50, and 75 ng/mL) *in vitro*, and then cell counts were performed at 72 hours. For *in vivo* analysis, tumor cells were xenografted onto the flank of SCID mice, and were treated with 100 μg bevacizumab twice weekly for three weeks. Xenograft samples from the control and treatment groups were analyzed for microvessel density. Escalating doses of bevacizumab had no effect on the growth of tumor cells *in vitro (P ≥ 0.086)*. However, tumor xenografts expressing EMMPRIN responded to bevacizumab treatment (*P* = 0.0013), whereas the EMMPRIN knockdown cell line did not (*P* = 0.7942). Immunohistochemical analysis demonstrated that microvascular density was reduced in the treated FaDu tumors (*P* = 0.005), but not in the FaDu/siE tumors (*P* = 0.48). Currently there is limited information on biomarkers to predict response to bevacizumab. By demonstrating effectiveness of bevacizumab therapy in tumors that express EMMPRIN, but not in tumors with silenced EMMPRIN expression, this study suggests that EMMPRIN may serve as a biomarker for response to bevacizumab treatment.

Keywords

1. Introduction

Tumor growth beyond 1–2 mm is highly dependant upon the complex process of angiogenesis [1]. Vascularization and tumor growth are stimulated by cytokines elicited by endothelial cells and stromal cells surrounding the tumor, as well as the tumor itself. Vascular endothelial growth factor (VEGF) and other angiogenic growth factors cause endothelial cell proliferation and migration from existing blood vessels [2,3,4]. During endothelial migration through the tumors, matrix metalloproteases (MMPs) degrade the extracellular matrix, which then allows for growth of these newly formed vessels as they move into the growing neoplasm [5,6,7].

Corresponding Author: Eben L. Rosenthal, MD, Division of Otolaryngology, BDB Suite 563, 1808 7th Avenue South, Birmingham, AL 35294-0012, Tel: (205) 934-9767, Fax: (205) 934-3993, oto@uab.edu.

Extracellular matrix metalloprotease inducer (EMMPRIN) has been shown to induce MMPs and is believed to stimulate VEGF expression [5,8].

Extracellular matrix metalloprotease inducer (EMMPRIN, also known as CD147 or basigin) is a membrane bound glycoprotein found on the surface of tumor cells [9]. EMMPRIN overexpression is exhibited to varying degrees in most tumor types, with head and neck squamous cell carcinoma (HNSCC) having some of the highest levels [10]. Elevated EMMPRIN expression has been shown to correlate with metastasis and tumor progression in tumors of the oral cavity [11] and larynx [12], along with multiple other tumor types [13,14].

Although the exact mechanisms by which EMMPRIN promotes HNSCC growth is unknown, it has been well established that EMMPRIN promotes expression of matrix metalloproteases (MMPs) and modulates the tumor microenvironment by stimulation of MMP-1, MMP-2 and MMP-3 production by stromal cells [15,16,17]. Evidence has recently begun emerging that EMMPRIN promotes neovascularization not only through the elaboration of MMPs, but also by stimulation of VEGF expression [18,19]. The relationship between EMMPRIN and VEGF has been illustrated in breast cancer [18,20], hepatocellular carcinoma [19], gastric carcinoma [21], pancreatic carcinoma [22], melanoma [23], and glioblastoma [24]. We have previously demonstrated that EMMPRIN expression in HNSCC increases growth of tumor xenografts and can increase VEGF secretion [25].

Bevacizumab (Avastin; Genentech USA, Inc., San Francisco, CA) is a humanized recombinant IgG antibody that targets and neutralizes VEGF activity, causing inhibition of angiogenesis and tumor growth. It is currently indicated for use in metastatic colorectal cancer [26], non-small cell lung cancer (NSCLC) [27], and metastatic breast cancer [28]. Although there is no good biomarker for determining which patients will respond, bevacizumab is typically used in combination with traditional cytotoxic agents, which improves patient outcomes without overlapping toxicities. Preclinical evidence has recently emerged that bevacizumab may have a role in treatment of head and neck squamous cell carcinoma (HNSCC) [29] and Phase I–II trials are underway. A significant percentage of patients do not respond to bevacizumab. Due to the possibility of significant toxicities and the costs involved with therapy, it is important to identify a marker that would predict which patients will respond to treatment.

Although many stimuli have been identified that upregulate VEGF expression [30] and VEGF expression has been shown to predict poor outcomes in cancer patients [31,32], levels of VEGF stimulators and levels of VEGF itself have failed to predict which patients will respond [33]. Although the relationship between EMMPRIN expression and VEGF levels has been demonstrated, to our knowledge no one has shown that EMMPRIN mediated VEGF expression affects efficacy of anti-VEGF tumor therapy. In this study, we investigate how EMMPRIN expression affects VEGF targeted therapy of head and neck squamous cell carcinoma xenografts using *in vivo* and *in vitro* models.

2. Materials and methods

2.1. Cells and culture conditions

FaDu (ATCC, Manassas,VA) cells were maintained in Dulbecco's modified Eagle medium (DMEM; Mediatech, Manassas,VA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin-streptomycin solution (10,000 units/mL penicillin and 10,000 μg/mL streptomycin, Mediatech) in a humidified atmosphere containing 5% CO_2 at 37°C. Because tumor cells interact with stromal cells *in vivo*, tumor cells were co-cultured with and without normal dermal fibroblasts to better evaluate EMMPRIN's role in cell growth. Normal dermal fibroblasts (NDFs) were isolated from primary culture and maintained in DMEM with 10% FBS and antibiotics as described previously [34].

To obtain an EMMPRIN stably silenced cell line, oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and cloned into pSilencer 4.1 CMV puro vectors (Ambion Inc., Austin, Texas) as described in the manufacturer's instruction manual. Vectors containing the inserts were transformed into NovaBlue Singles (EMD Biosciences Inc, La Jolla, CA) competent cells. After PCR and DNA sequencing, confirmed plasmids were propagated and transfected into FaDu cells. Cells resistant to puromycin (1.0μg/ml) selection were analyzed by Western blot for EMMPRIN expression and then selected by flow cytometry. The target sequence selected for EMMPRIN silencing is GAGCTACACATTGAGAACCTG. The expression levels of EMMPRIN were routinely monitored using Western blot analysis using anti-EMMPRIN monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA). Mouse monoclonal antibody to β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was then used to confirm equal loading.

2.2. Reagents

We used bevacizumab (Avastin, Genentech USA Inc., San Francisco, CA), a recombinant humanized monoclonal IgG1 antibody that binds to and inhibits the biologic activity of human VEGF. Bevacizumab contains human framework regions and the complementarity-determining regions of a murine antibody that binds to VEGF. Bevacizumab is specific for human VEGF, and has been shown to have no activity against murine VEGF [35].

2.3. Effects of bevacizumab *in vitro*

To measure the effects of bevacizumab on tumor cell growth and VEGF expression *in vitro*, tumor cells were seeded at 50,000 per well on a 96 well plate were in DMEM with 0.1% BSA and 1% penicillin/streptomycin. Normal dermal fibroblasts were (NDFs) were seeded at 12,000 per well. All treatments were done in triplicate. Bevacizumab (0, 25, 50, and 75 ng/mL) was added 24 hours after seeding. Media was changed at 48 hours and cells were counted at 72 hours.

2.4. Animal models

Severe combined immunodeficient (SCID) male mice, age 4–6 weeks (Charles River Laboratories, Wilmington, MA and NCI-Frederick, Frederick, MD) were obtained and housed in accordance with The University of Alabama's Institutional Animal Care and Use Committee (IACUC) guidelines. FaDu cells transfected with the control vector or FaDu cells carrying the EMMPRIN silencing construct (FaDu/siE) were used to generate subcutaneous flank tumors. Mice were divided into 4 groups: FaDu (n = 4), FaDu + bevacizumab (n = 5), FaDu/siE (n = 4), and FaDu/siE + bevacizumab (n = 8). The variable number of mice per group resulted from inconsistent tumor growth. Once tumors were established to a mean of 23 mm^2, treatment with bevacizumab began. Animals received 100 μg bevacizumab (i.p.) biweekly for three weeks. Tumors in all four groups were measured on treatment days; tumor area was calculated by multiplying length and width. Measurements continued for one week beyond treatments, at which time mice were sacrificed according to our IACUC guidelines.

2.5. Immunohistochemistry

Formalin fixed paraffin embedded 5μm tissue sections mounted on Bond-Rite (Richard-Allan Scientific, Kalamazoo, MI) slides were heated to 60° for two hours. After three baths of xylenes and graded alcohols to rehydrate the sections, antigen retrieval was performed with high temperature treatment with pH 10 Tris buffer 0.5M. H_2O_2, avidin and biotin solutions and 3% goat serum were used to quench peroxidases, block endogenous biotin and block nonspecific binding. Rabbit polyclonal antibody to CD31 (Abcam Inc., Cambridge, MA) was diluted 1:200 and applied to the tissue at room temperature for one hour. The secondary antibody was goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and the label was

avidin-HRP (Signet Pathology Systems, Inc., Dedham, MA). After the DAB chromagen (BioGenex Laboratories, Inc., San Ramon, CA) was applied the tissues were counterstained with hematoxylin and the cover slips mounted with Permount.

2.6. Statistical analyses

For FaDu and FaDu/siE tumors treated with bevacizumab, the response to treatment underwent the following analysis: the tumor area data for each animal were transformed to represent % change from untreated tumor, i.e. (average tumor area treated group/average tumor area untreated group). A two-sample t-test was used to compare the two groups across all time points using GraphPad Prism software.

Statistical significance was measured using an unpaired t-test. Data analysis for immunohistochemistry and *in vitro* cell growth was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered significant in t test analysis.

3. Results

3.1. Silencing EMMPRIN results in decreased cell growth

Western blot analysis was performed to verify decreased extracellular matrix metalloprotease inducer (EMMPRIN) expression in the silenced FaDu cell lines (Fig. 1A). Results verified knockdown of EMMPRIN expression in the FaDu/siE cell line and intermediate levels of expression were seen in the control vector transfected line (FaDu). To ensure silencing of EMMPRIN functionality (in cell growth), cells were placed in media both with and without normal dermal fibroblasts (NDFs) and allowed to grow for 72 hours, at which time cells were trypsinized and counted (Fig. 1B). Control vector cells plated with NDFs demonstrated higher growth rates compared to silenced cells (FaDu vs. FaDu/siE, $P = 0.0009$), whereas the differences seen between cell lines plated without NDFs did not reach significance ($P = 0.0861$). Though these differences did not reach significance, the apparent trend warrants further investigation.

3.2. Bevacizumab does not effect tumor cell growth *in vitro*

To measure the effects of bevacizumab on tumor cell growth *in vitro*, FaDu and FaDu/siE cells were seeded with and without normal dermal fibroblasts (NDFs) in a 96 well plate and treated with 0, 25, 50, and 75 ng/mL bevacizumab. After 72 hours, tumor cells were counted. Bevacizumab treatment had no effect on the growth rate of either of the cell lines (*P ≥ 0.086*, Fig. 2).

3.3. Silencing EMMPRIN inhibits the effects of bevacizumab *in vivo*

To determine the role of EMMPRIN in VEGF mediated tumor growth, mice xenografted with FaDu and FaDu/siE cells were treated with and without bevacizumab (100 μg i.p./biweekly) for three weeks. Tumors were allowed to reach a mean size of 23 mm^2 prior to initiating treatment. At the end of treatment, the FaDu tumors treated with bevacizumab were 53% smaller than untreated control (Fig. 3A; $P = 0.0013$). Average tumor size in the FaDu/siE group treated with anti-VEGF antibody did not differ from the untreated control ($P = 0.7942$).

3.4. Reduced microvascularization in treated FaDu tumors

To investigate the effects of anti-VEGF therapy on vascularization, xenografts of each tumor line treated with bevacizumab were analyzed for microvessel density (CD31). The percentage of cells staining positively for CD31 in treated FaDu xenografts was 44% of untreated control

FaDu xenografts (Fig. 3B; $P = 0.005$). No statistical difference was found between the treated and untreated FaDu/siE tumors ($P = 0.48$).

4. Discussion

We have demonstrated that xenografted tumors expressing EMMPRIN responded to anti-VEGF therapy, while those without EMMPRIN did not. Although VEGF is widely expressed in most tumor types, including HNSCC, there are still many patients that do not respond to anti-VEGF therapy [36,37]. Studies to date have failed to demonstrate a correlation between levels of plasma angiogenic factors (such as VEGF or basic fibroblast growth factor (bFGF)) and response to bevacizumab [33]. Determining which patients will respond could help guide therapy, which could increase success rates and decrease costs and toxicity related to unsuccessful therapy.

We have previously shown decay of microvessel density and VEGF expression with loss of EMMPRIN [25]. Furthermore, we have shown in HNSCC xenografts that silencing EMMPRIN results in suppression of tumor growth and increased survival [25]. It has also been shown that tumor growth and vascularization can be inhibited by suppressing EMMPRIN expression in both breast cancer [18] and malignant melanoma [23].

Because EMMPRIN modulates VEGF expression, we sought to determine whether levels of EMMPRIN expression would affect response to anti-VEGF therapy. While previous studies have shown correlations between the two molecules and that loss of EMMPRIN does diminish VEGF production [5,20], it has not been demonstrated if EMMPRIN would affect response to VEGF targeted therapy. In the current study, we found that EMMPRIN expression was required for response to bevacizumab in HNSCC xenografts.

Consistent with the findings of other authors, we found no effect of bevacizumab on tumor cell growth *in vitro* [29,38], regardless of EMMPRIN expression. Analysis of the xenograft samples from our treated and untreated mice demonstrated that microvascular density was decreased in tumors that expressed EMMPRIN, but not in those with low EMMPRIN expression. A possible explanation is that without the influence of EMMPRIN, less VEGF is available to stimulate angiogenesis, and therefore bevacizumab is less effective. However, EMMPRIN is certainly not the only factor known to upregulate VEGF expression. A range of stimuli have been shown to upregulate VEGF gene expression, including: epidermal growth factor (EGF), fibroblast growth factor-4, platelet derived growth factor (PDGF), interleukin (IL-1) and IL-6, among others [30]. Furthermore microvessels were still seen within the tumor samples of the FaDu/siE xenografts, but they weren't affected by treatment with bevacizumab. Although further investigation is required to fully explain these observations, it is possible that upregulation of other vascular growth factors (such as bFGF) is responsible for the vascularization of the FaDu/siE xenografts [39].

From this pilot study of HNSCC xenografts, EMMPRIN appears to be required for tumors to respond to bevacizumab therapy. The precise understanding of how EMMPRIN activity is involved in angiogenesis and anti-VEGF therapy requires further study and has significant implications for cancer treatment. By demonstrating effectiveness of bevacizumab therapy in tumors that express EMMPRIN, but not in tumors with silenced EMMPRIN expression, this study suggests that EMMPRIN levels may aid in determining which patients will respond to bevacizumab treatment.

Acknowledgements

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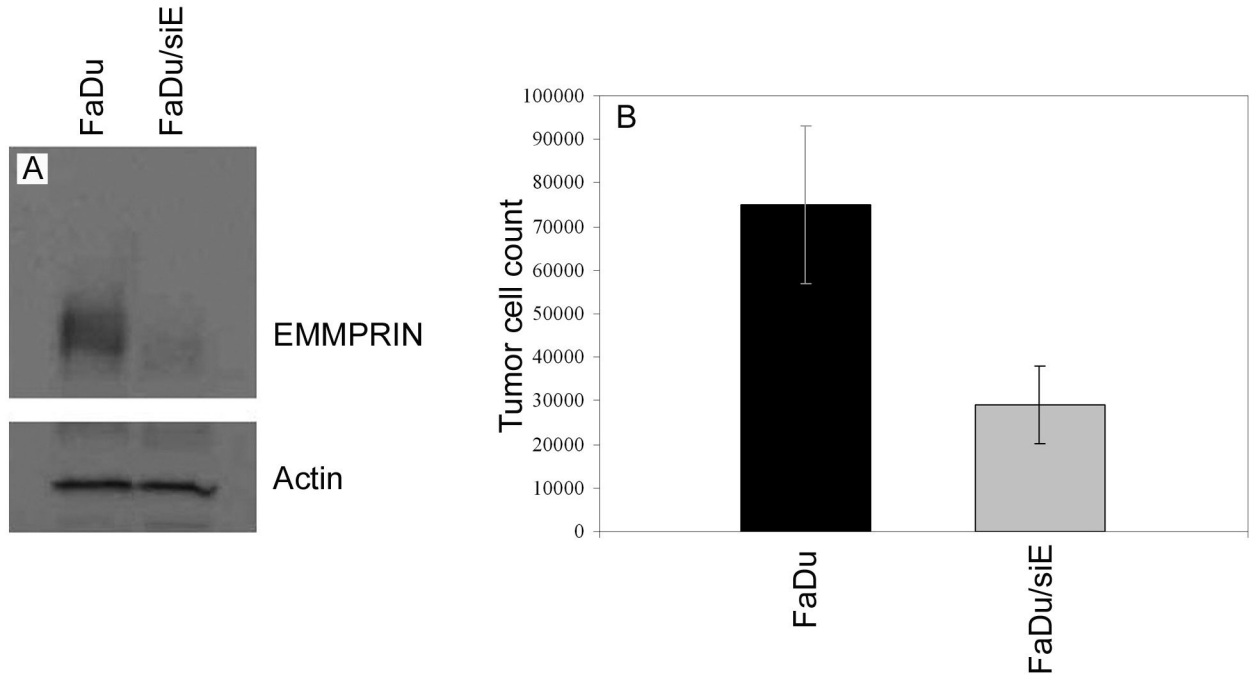
Fig. 1.
Extracellular matrix metalloprotease inducer (EMMPRIN) expression in transfected FaDu cell lines. (A) Western blot analysis confirms that EMMPRIN expression was reduced in the FaDu/siE cell lines, whereas control vector transfected cells (FaDu) expressed intermediate basal levels of EMMPRIN. Equal protein loading was confirmed with β-actin. (B) To confirm that EMMPRIN functionality was suppressed as well, tumor cells were plated with and without normal dermal fibroblasts (NDFs). After 72 hours, control vector cells plated with NDFs demonstrated higher growth rates compared to silenced cells ($P = 0.0009$).
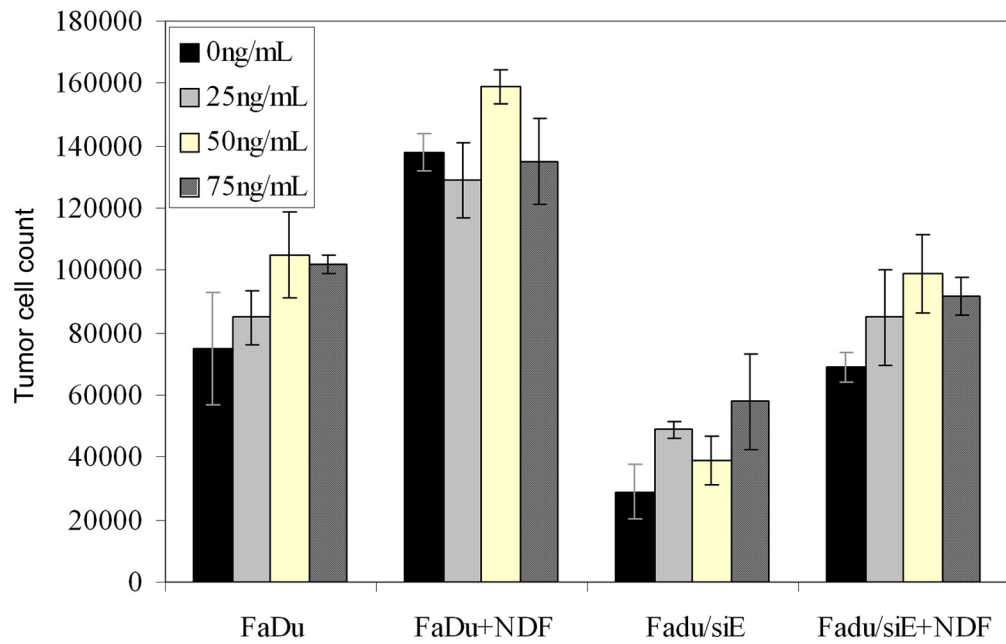
Fig. 2.
Bevacizumab has no effect on tumor cells *in vitro.* Tumor cells from the FaDu and FaDu/siE cell lines were plated with and without normal dermal fibroblasts were treated with 0, 25, 50 and 75 ng/mL of bevacizumab. After 72 hours, cells were trypsinized and counted. Bevacizumab had no effect on cell growth, regardless of EMMPRIN expression ($P \geq 0.086$).
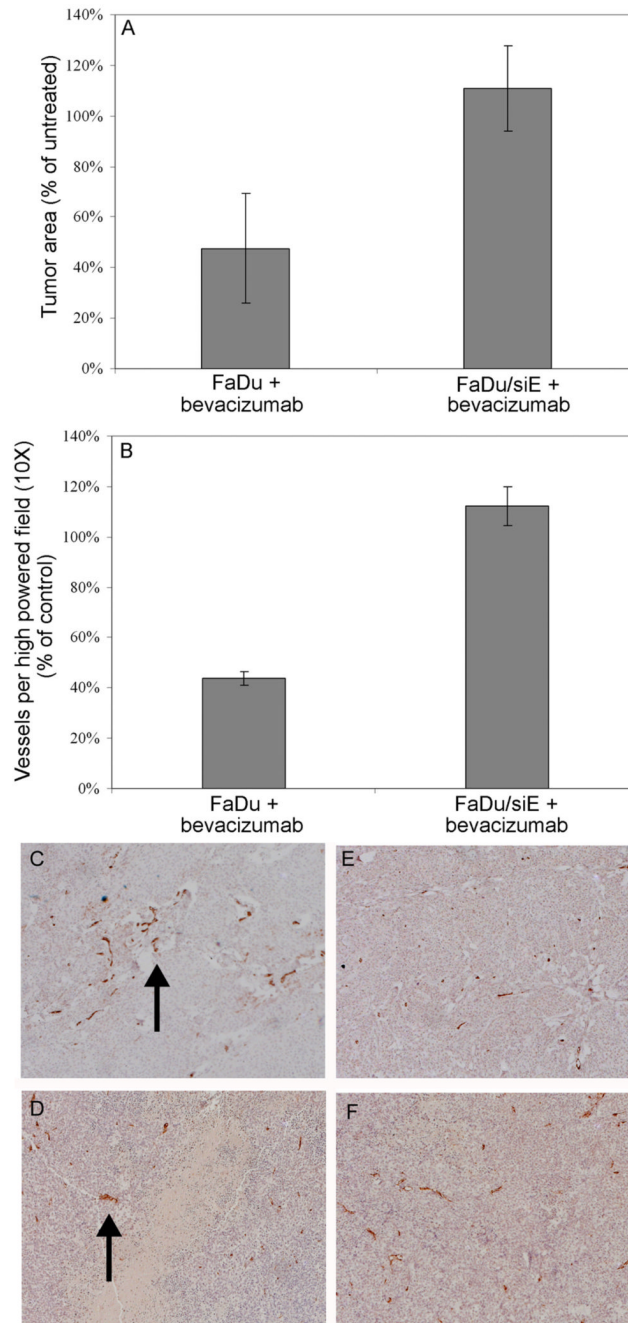
Fig. 3.
EMMPRIN expression required for bevacizumab response *in vivo.* (A) bevacizumab was
effective in treating HNSCC xenografts in EMMPRIN expressing FaDu tumors ($P = 0.0013$),
but response was not seen in tumors with knockdown EMMPRIN expression (FaDu/siE, $P =
0.7942$). Analysis of xenograft samples by immunohistochemistry staining for CD31 (B)
revealed that bevacizumab decreased microvascular density of tumors that expressed
EMMPRIN ($P = 0.005$), but had no effect on the vascularity of FaDu/siE tumors ($P = 0.48$).
Immunohistochemistry of CD31 expression by FaDu control tumors (C) and tumors treated
with bevacizumab (D) compared to FaDu/siE control (E) and treated (F) tumors. Data are
normalized as percentage of untreated controls. Arrows indicate vascularity.