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Cross-Talk Between Endothelial and Breast Cancer Cells Regulates Reciprocal Expression of Angiogenic Factors In Vitro

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ABSTRACT

Reciprocal growth factor exchange between endothelial and malignant cells within the tumor microenvironment may directly stimulate neovascularization; however, the role of host vasculature in regulating tumor cell activity is not well understood. While previous studies have examined the angiogenic response of endothelial cells to tumor-secreted factors, few have explored tumor response to endothelial cells. Using an in vitro co-culture system, we investigated the influence of endothelial cells on the angiogenic phenotype of breast cancer cells. Specifically, VEGF, ANG1, and ANG2 gene and protein expression were assessed. When co-cultured with microvascular endothelial cells (HMEC-1), breast cancer cells (MDA-MB-231) significantly increased expression of ANG2 mRNA (20-fold relative to MDA-MB-231 monoculture). Moreover, MDA-MB-231/HMEC-1 co-cultures produced significantly increased levels of ANG2 (up to 580 pg/ml) and VEGF protein (up to 38,400 pg/ml) while ANG1 protein expression was decreased relative to MDA-MB-231 monocultures. Thus, the ratio of ANG1:ANG2 protein, a critical indicator of neovascularization, shifted in favor of ANG2, a phenomenon known to correlate with vessel destabilization and sprouting in vivo. This angiogenic response was not observed in nonmalignant breast epithelial cells (MCF-10A), where absolute protein levels of MCF-10A/HMEC-1 co-cultures were an order of magnitude less than that of the MDA-MB-231/HMEC-1 co-cultures. Results were further verified with a functional angiogenesis assay demonstrating well-defined microvascular endothelial cell (TIME) tube formation when cultured in media collected from MDA-MB-231/HMEC-1 co-cultures. This study demonstrates that the angiogenic activity of malignant mammary epithelial cells is significantly enhanced by the presence of endothelial cells. J. Cell. Biochem. 113: 1142–1151, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: TUMORIGENESIS; VASCULAR ENDOTHELIAL GROWTH FACTOR; ANGIOPOIETIN

The reciprocal interactions between tumor and stromal cells such as smooth muscle cells, pericytes, myofibroblasts, and vascular endothelial cells [Park et al., 2000], are considered to be an integral part of tumor formation, progression and the evolution of metastasis. Tumor cells recruit stromal cells and promote neovascularization through production of stimulatory growth factors and cytokines, thereby sustaining the proliferative and invasive activity of the tumor [Wernert, 1997; Liotta and Kohn, 2001; Tlsty, 2001]. Angiogenesis, the expansion and remodeling of the primitive blood vessel network into a complex network, is critical to meet the demand of the growing tumor mass [Carmeliet, 2000; Nikitenko, 2009]. This process requires a precise balance of stimulatory and inhibitory signals that modulate endothelial growth and organization at sites of neovascularization [Hanahan and Folkman, 1996; Nikitenko, 2009]. Perhaps the most important microenvironmental interaction with regard to angiogenesis is the tumor cells' ability to

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cross-talk with endothelial cells [Jung et al., 2002]. Several angiogenic molecules and pathways responsible for stimulating endothelial cell activity have been implicated in tumorigenesis. These include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1), angiopoietins (ANG), chemokines, and integrins [Carmeliet and Jain, 2000; Korah et al., 2000; Liotta and Kohn, 2001; Metheny-Barlow and Li, 2003; Tait and Jones, 2004; Joyce, 2005; Nikitenko, 2009]. Discovery of these tumor-derived factors has provoked an interest in developing cancer therapies that target these molecules and their receptors. However, little is known about the mechanisms by which endothelial cells influence expression of angiogenic growth factors within the tumor microenvironment.

Emerging evidence suggests that endothelial cells may have an active role in regulating angiogenic factor expression by tumor cells [Carmeliet and Jain, 2000; Dias et al., 2000; Calabrese et al., 2007; Kaneko et al., 2007; Butler et al., 2011]; however, this phenomenon has not been directly investigated. Among these angiogenic factors is VEGF, an endothelial cell survival factor that functions to stimulate angiogenesis and induce vessel permeability [Carmeliet and Jain, 2000; Carmeliet, 2005]. Also critically involved in angiogenesis are the angiopoietins, a family of extracellular ligands that recognize and bind to the endothelial cell-specific tyrosine kinase receptor Tie2 [Tait and Jones, 2004]. Specifically, angiopoietin-1 (ANG1) acts as an agonist to activate the Tie2 signaling pathways and has been shown to induce endothelial migration, tubule formation, and survival in vitro [Witzenbichler et al., 1998; Hayes et al., 1999]. Angiopoietin-2 (ANG2) specifically blocks the ANG1 phosphorylation of Tie2, rendering vessels more amenable to sprouting [Jones, 2003; Tait and Jones, 2004].

Research by Maisonpierre et al. [1997], which analyzed expression of ANG1, ANG2, and VEGF in a rat ovary model, demonstrated a relationship between the angiopoietins and VEGF in physiological angiogenesis. These studies led to the current understanding that ANG1 and VEGF promote vessel maturation and stability in vivo, whereas ANG2 serves to antagonize this relationship. In the presence of VEGF, ANG2 induces vessel destabilization and neovascularization; however, it promotes endothelial cell death and vessel regression when VEGF activity is inhibited [Maisonpierre et al., 1997; Lobov et al., 2002]. In addition, a wide range of malignant tumors exhibit up-regulation of ANG1 and ANG2, favoring a shift in the ANG2:ANG1 ratio towards ANG2 that correlates with tumor angiogenesis [Tait and Jones, 2004]. Therefore, the ratio of ANG1 and ANG2 in the presence of VEGF has important implications regarding both physiologic and pathogenic angiogenesis.

We hypothesize that bidirectional cross-talk between endothelial and tumor cells stimulates reciprocal growth factor exchange that directly influences the angiogenic response and metastatic potential of the tumor. Currently, the role of tumor vascular endothelial cells in balancing the expression of angiogenic factors to modulate intratumoral angiogenesis remains unknown [Butler et al., 2011]. Therefore, understanding reciprocal tumor-endothelial cell signaling within the tumor microenvironment may allow for more selective inhibition of tumor growth.

This study examines the influential role of endothelial cells on the angiogenic phenotype of breast cancer cells using an in vitro co-culture system. We observed that co-culture of an immortalized human endothelial cell line HMEC-1, with a human breast carcinoma cell line MDA-MB-231, results in increased expression of angiogenic factors in vitro. In contrast, no angiogenic activity was observed in the nonmalignant breast epithelial cell line MCF-10A when co-cultured with HMEC-1 cells. This highlights an important disparity in cell signaling between malignant and non-malignant breast epithelial cells and the endothelium. The direct influence of endothelial cells on tumor cell proliferation and expression of angiogenic factors has significant therapeutic implications in regulating tumorigenesis.

MATERIALS AND METHODS

CELL CULTURE

A human breast carcinoma cell line MDA-MB-231 (American Type Culture Collection (ATCC)) and a nonmalignant human mammary epithelial cell line MCF-10A (ATCC) were used in this study. A human microvascular endothelial cell line, HMEC-1, was provided by Dr. Edwin Ades and Mr. Fransisco J. Candal of the Center for Disease Control and Prevention (Atlanta, GA) and Dr. Thomas Lawley of Emory University (Atlanta, GA). Telomerase-immortalized human microvascular endothelial cells labeled with red fluorescent protein (TIME-RFP) were provided as a generous gift from Dr. Shay Soker at the Wake Forest Institute for Regenerative Medicine (Winston-Salem, NC). A lentiviral vector system was used to genetically modify the cells to stably express RFP for visualization of the cells during the tube formation assay.

MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), and 1% penicillin–streptomycin (Invitrogen). MCF-10A cells were cultured in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 20 ng/ml epidermal growth factor (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 1% penicillin–streptomycin (Invitrogen). HMEC-1 cells were cultured in MCDB 131 medium (Mediatech, Herndon, VA) supplemented with 1% antibiotic/antimycotic (MediaTech, Inc., Manassas, VA), 10 mM L-glutamine (Fisher, Pittsburgh, PA), 1 μg/ml hydrocortisone (Sigma-Aldrich), 10 ng/ml epidermal growth factor (Sigma-Aldrich), and 10% FBS (Sigma-Aldrich). TIME cells were cultured in EBM-2 (Lonza, Rockland, ME) media supplemented with a growth factor BulletKit (Lonza CC-4176). All cell cultures were maintained in a humidified 5% CO_2/95% air atmosphere at 37°C within an incubator.

All cell cultures were grown to 70–80% confluence in tissue-culture treated polystyrene (TCPS) flasks before being passaged for use in the experiment. Co-culture of MDA-MB-231/HMEC-1 cells and MFC-10A/HMEC-1 cells was conducted using Millicell 0.4 μm pore size hanging PET 6-well culture inserts (Millipore, Bedford, MA) in which breast epithelial cells were seeded in the bottom of a 6-well dish (25,000 seeding density) and microvascular endothelial cells were seeded (25,000 seeding density) in the hanging culture

insert, similar to methods described by Spector et al. [2002]. Breast epithelial cells and microvascular endothelial cells were cultured separately for 48 h to establish attachment. After 48 h, HMEC-1 seeded inserts were moved over either MDA-MB-231 or MCF-10 cell cultures in the 6-well plates to create the hanging co-culture setup. Due to the membrane pore size and diffusional distance between cells within this set-up, cell-to-cell contact is prevented but paracrine signaling can occur between epithelial cells in the 6-well plate and endothelial cells on the insert. The working volume of media was 2,750 μl in each 6-well and 2,000 μl in the hanging inserts. Every 3–4 days, 1 ml of culture media was removed from the 6-well compartment and 1 ml from the insert compartment. Then, 1 ml of fresh MDA-MB-231 culture media was added to the 6-well compartment and 1 ml fresh HMEC-1 culture media was added to the insert compartment. MDA-MB-231 and MCF-10A cells were each cultured alone (monoculture) on TCPS without HMEC-1 seeded inserts as controls. HMEC-1 cells were also grown as monocultures on Millicell inserts over empty 6-well TCPS plates as controls. Each cell type was fed every 3–4 days with their respective media over the 4-week duration of the experiment.

PROLIFERATION AND CELL VIABILITY

Proliferation of MDA-MB-231, MCF-10A, and HMEC-1 cells was evaluated at each experimental time point using an alamarBlue™ assay (Invitrogen) according to the manufacturer's protocol. This assay incorporates an (REDOX) indicator that undergoes colorimetric change in response to cellular metabolic reduction. Metabolic activity, corresponding to a measure of cell proliferation and/or cell viability, was assessed by percent reduction of alamarBlue™ [Al-Nadiry et al., 2007]. Growth media were removed and replaced with 1 ml media containing 10% alamarBlue™ reagent. Cells were incubated for 1 h at 37°C, then 100 μl aliquots of the alamarBlue™ medium were removed (in triplicate) and the absorbance was read at 570 and 600 nm using SpectraMax M2^{e} microplate reader (Molecular Devices, Sunnyvale, CA). Viability of cells was measured using a Vi-CELL™ cell viability analyzer (Beckman Coulter, Brea, CA).

OXYGEN AND pH MEASUREMENT

Environmental conditions, including oxygen tension and pH, were monitored over the duration of the experiment to ensure that expression of angiogenic growth factors was not stress-induced. Hypoxia is a potent inducer of VEGF expression [Bos et al., 2001]. Therefore, uncontrolled variations in media oxygenation would significantly complicate interpretation of results. Oxygen tension in the culture media was measured using an ExStik dissolved oxygen meter (Extech Instruments, Waltham, MA) to monitor hypoxic conditions. Culture media pH was also measured over the duration of the experiment using an Orion Star pH meter (Thermo Fisher Scientific, Waltham, MA).

QUANTITATIVE REAL-TIME RT-PCR

Expression levels of target genes in MDA-MB-231, MCF-10A, and HMEC-1 cells for all experimental conditions were determined quantitatively by real-time RT-PCR. Cells were removed from their respective culture surface (breast epithelial cells from the 6-well TCPS and endothelial cells from the PET insert) for molecular analysis. At each time point (1, 7, 14, 21, and 28 days), total RNA was isolated and purified by spin protocol using the RNeasy Mini kit (Qiagen, Inc., CA) and QIAshredder (Qiagen, Inc.) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed at 25°C for 15 min, 42°C for 45 min, and 99°C for 5 min using components of a Reverse Transcription System (Promega Corporation, WI) with random hexamers as primers. Following reverse transcription, quantitative PCR amplification was performed on an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan Universal PCR Master Mix (Applied Biosystems), and gene-specific TaqMan PCR primers: VEGF-A (NM_001025366.2), ANGPT-1 (NM_001146.3), ANGPT-2 (NM_001118887), and GAPDH (NM_002046.3) (Applied Biosystems). A standard thermal cycler protocol was followed (reaction initiation at 50°C for 2 min followed by 95°C for 15 s and 60°C for 1 min, repeated 45 times). Relative quantification, which represents the change in gene expression from quantitative RT-PCR experiments between co-culture and monoculture (control) groups, was calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method [Livak and Schmittgen, 2001; Lee et al., 2004]. The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold. The C_T from each sample was determined using the Applied Biosystems Sequence Detection Software v1.2.3. Evaluation of $2^{-\Delta\Delta C_T}$ indicates the fold change in gene expression, normalized to GAPDH housekeeping gene and relative to the control group.

ENZYME-LINKED IMMUNOSORBENT ASSAY

At each time point (1, 7, 14, 21, and 28 days), protein levels of VEGF, ANG1, and ANG2 in co-cultures of MDA-MB-231/HMEC-1 and MCF-10A/HMEC-1 and monocultures of MDA-MB-231, MCF-10A and HMEC-1 (control) were determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine Human Immunoassay kits (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol. Media samples were collected assuming homogenous distribution of growth factors within the co-culture or monoculture.

ENDOTHELIAL CELL TUBE FORMATION ASSAY

To demonstrate a functional endothelial response to increased angiogenic factors in MDA-MB-231/HMEC-1 co-cultures, an endothelial tube formation assay was conducted on three dimensional (3D) type I collagen gels as described previously [Pepper et al., 1992; Benndorf et al., 2003]. Briefly, type I collagen was isolated from excised rat tail tendon and dissolved in a pH 2.0 HCl solution. Type I collagen gels (1.5 mg/ml) were prepared by neutralization with 1N NaOH and 10X M199 media and allowed to gel in 24-well culture dishes. TIME-RFP cells were seeded on solidified collagen gels (300 μl) at a density of 100,000 cells. After 30 min, the media were replaced with conditioned cell culture supernatant collected from either MDA-MB-231/HMEC-1 co-culture or MDA-MB-231 monoculture (control) on days 1, 7, 14, 21, and 28. The assay was conducted for 3 days in which endothelial tube formation was qualitatively examined every 12 h by visualization with a fluorescence microscope (Leica AF6000). A quantitative comparison of experimental groups was conducted by

measuring the total endothelial tube length using ImageJ software (National Institutes of Health, Bethesda, MD). Lengths were measured in several random view-fields and the values were then averaged.

STATISTICAL ANALYSIS

The experiment was conducted with a total of six samples per group (n = 6). Experimental groups were tested and analyzed independently and the data are expressed as mean value ± standard error of the mean. Significance of results was verified using Student's *t*-test. A 95% confidence criterion was used to determine statistically significant differences between co-cultures and monocultures (control).

RESULTS

PROLIFERATION AND CELL VIABILITY

Cell viability in all cultures remained >90% viable for the duration of the study. Growth exhibited a characteristic exponential phase during the first week of culture, followed by a plateau of growth. Overall, there was no difference in proliferation between the co-culture and control groups (data not shown).

OXYGEN AND pH

Trends for change in oxygen saturation or pH of the culture media remained similar over the duration of the experiment, indicating that these environmental conditions were not influential factors of the angiogenic response (Fig. 1).

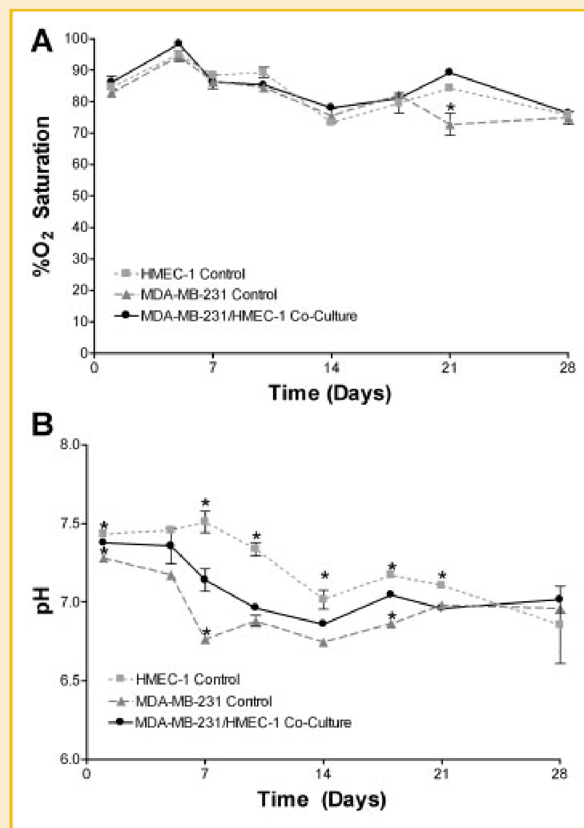

Fig. 1. Oxygen and pH measurements. Similar trends in (A) O_2 saturation and (B) pH fluctuations of culture media over the duration of the experiment indicate that a stress-induced cellular response due to environmental conditions was not occurring. *Co-culture statistically significant as compared to monoculture (control) ($P < 0.05$).

mRNA EXPRESSION OF ANGIOGENIC FACTORS

Expression of angiogenic growth factor mRNA in MDA-MB-231 cells was significantly influenced by co-culture with HMEC-1 cells (Fig. 2). ANG2 mRNA expression was up-regulated during MDA-MB-231/HMEC-1 co-culture relative to MDA-MB-231 monoculture (control) for all time points, with a significant 2-, 2.5-, 5-, and 20-fold induction on days 7, 14, 21, and 28, respectively (Fig. 2C). In general, ANG1 and VEGF-A mRNA were not consistently increased due to co-culture, although a modest but statistically significant induction of VEGF-A on day 1 (twofold) and ANG1 on day 28 (twofold) was observed. The increase in ANG2 relative to ANG1 during co-culture suggests that a potential feedback system may exist which regulates expression of the angiopoietins [Hashimoto et al., 2004].

Expression of angiopoietins in normal breast epithelium has not been well defined [Djonov et al., 2001; Rudolph et al., 2003]. Angiogenic mRNA expression in the nonmalignant MCF-10A breast epithelial cell line, in the presence of endothelial cells, was also assessed. In general, VEGF-A and ANG1 mRNA was down-regulated during co-culture with HMEC-1 cells and ANG2 mRNA was undetected (Fig. 3). However, a modest, but statistically significant up-regulation of VEGF-A mRNA (1.5-fold relative to MCF-10A monoculture) was observed on day 14 (Fig. 3A).

Significant up-regulation of VEGF-A mRNA (2-fold on days 14, 21, and 28) and ANG1 mRNA (1.5- and 2-fold on days 21 and 28) occurred in the HMEC-1 cells during co-culture with MDA-MB-231 cells relative to HMEC-1 monoculture (Fig. 4).

PROTEIN EXPRESSION OF ANGIOGENIC FACTORS

Significantly increased secretion of VEGF and ANG2 proteins was observed in MDA-MB-231/HMEC-1 co-cultures, while ANG1 protein was decreased compared to both HMEC-1 and MDA-MB-231 monocultures (control) (Fig. 5). VEGF protein concentration was increased for all time points after 7 days (5,300, 16,100, 38,400, and 31,700 pg/ml on days 7, 14, 21, and 28, respectively) in the MDA-MB-231/HMEC-1 co-culture (with up to a 7-fold increase) relative to MDA-MB-231 monoculture and (with up to a 1000-fold increase) relative to HMEC-1 monoculture (Fig. 5A). ANG2 protein concentration was significantly higher in the MDA-MB-231/HMEC-1 co-culture (200, 515, and 580 pg/ml on days 14, 21, and 28, respectively) corresponding to a 1.5-, 5-, and 3-fold induction relative to MDA-MB-231 monoculture and a 2-, 1.5-, and 1.1-fold induction relative to HMEC-1 monoculture (Fig. 5C). This corresponds with the significant up-regulation of ANG2 mRNA expression in the MDA-MB-231/HMEC-1 co-culture. Most importantly, the relative protein concentrations of each angiogenic growth factor represent a balance that would induce tumor vascularization and growth in vivo. VEGF protein is high (5,000–30,000 pg/ml) relative to the angiopoietins, and ANG2 protein concentration (200–600 pg/ml) is greater than ANG1 (100–300 pg/

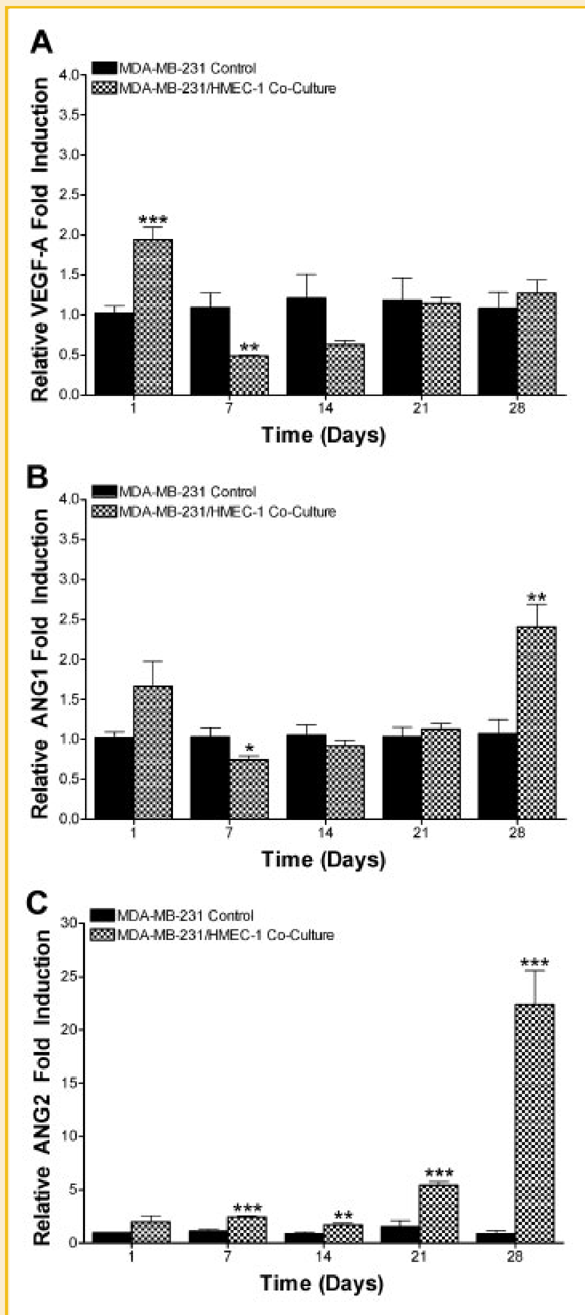

Fig. 2. Expression of VEGF-A, ANG1, and ANG2 mRNA in MDA-MB-231 cells co-cultured with HMEC-1 cells as compared to MDA-MB-231 monoculture (control). The relative mRNA to GAPDH mRNA of (A) VEGF-A, (B) ANG1, and (C) ANG2 as determined by quantitative real-time PCR indicate a significant fold induction of VEGF-A mRNA on day1 and ANG2 mRNA on days 7, 14, 21, and 28 during co-culture. No significant fold induction of ANG1 mRNA was observed until day 28. Co-culture statistically significant as compared to monoculture (control) denoted by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

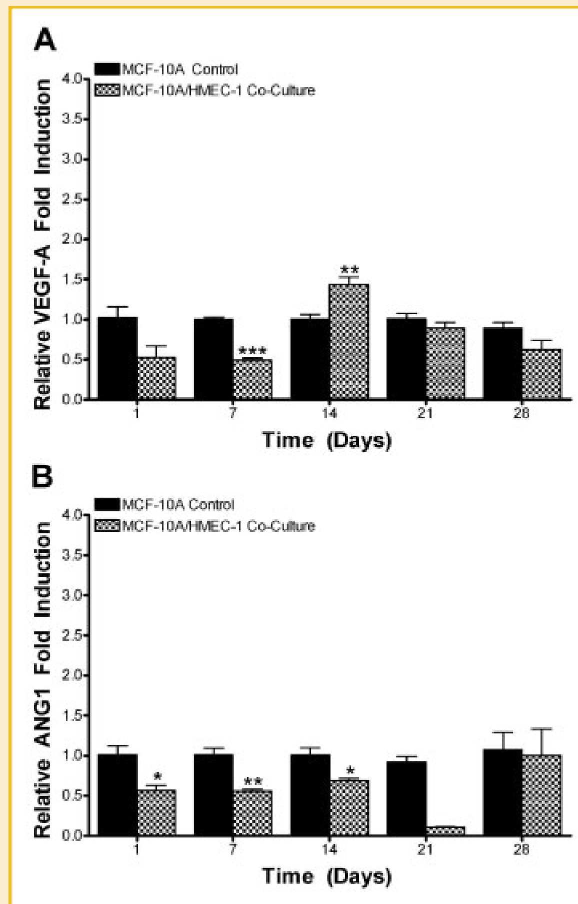

Fig. 3. Expression of VEGF-A, ANG1, and ANG2 mRNA in MCF-10A cells co-cultured with HMEC-1 cells as compared to MCF-10A monoculture (control). The relative mRNA to GAPDH mRNA of (A) VEGF-A and (B) ANG1 as determined by quantitative real-time PCR indicate no significant fold induction of ANG1 or VEGF-A mRNA with the exception of VEGF-A on day 14, while ANG2 mRNA was undetected. Co-culture statistically significant as compared to monoculture (control) denoted by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

ml) protein concentration. This expression profile demonstrates a time-dependent pattern of angiogenic protein secretion.

Although a modest, but statistically significant increase in secretion of VEGF (up to 700 pg/ml) and ANG2 (up to 15 pg/ml) protein was observed in MCF-10A/HMEC-1 co-culture supernatants (Fig. 5D,F), the absolute levels of these angiogenic proteins are low (an order of magnitude less) in comparison to the MDA-MB-231/HMEC-1 secreted protein concentrations. It is also likely that the presence of VEGF and ANG2 protein in the supernatant can be attributed to secretion of these growth factors by the HMEC-1 cells. ANG1 protein was also decreased in the MDC-10A/HMEC-1 co-culture relative to the MCF-10A monolayer (control) (Fig. 5E).

The increased gene and protein expression observed in this study reveals a potentially significant influence of endothelial cells on the angiogenic potential of breast cancer cells, and highlights the importance of their role in regulating vascularization of the tumor microenvironment.

ENDOTHELIAL TUBE FORMATION ASSAY

To test whether the increased expression of angiogenic factors during MDA-MB-231/HMEC-1 co-culture conditions affected endothelial angiogenesis, a tube formation assay was conducted to measure endothelial cell response in vitro. Endothelial tube

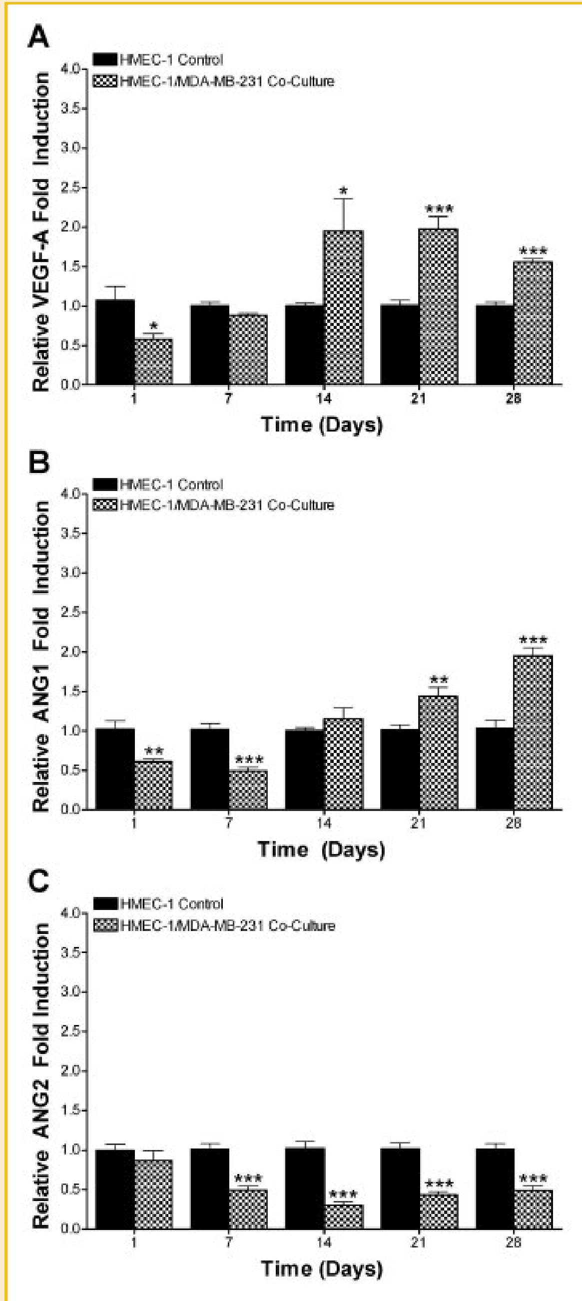

Fig. 4. Expression of VEGF-A, ANG1, and ANG2 mRNA in HMEC-1 cells co-cultured with MDA-MB-231 cells as compared to HMEC-1 monoculture (control). The relative mRNA to GAPDH mRNA of (A) VEGF-A and (B) ANG1, and (C) ANG2 as determined by quantitative real-time PCR indicate a significant fold induction of VEGF-A mRNA on days 14, 21, and 28 and ANG1 mRNA on days 1, 7, 21, and 28, while ANG2 mRNA was down-regulated. Co-culture statistically significant as compared to monoculture (control) denoted by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

formation was induced in cells grown in conditioned supernatant collected from MDA-MB-231/HMEC-1 co-culture compared to cells grown in conditioned supernatant collected from MDA-MB-231 monoculture (control). Interconnecting networks of endothelial cell-lined tubes were first observed at 12 h with increased proliferation and migration into organized endothelial tubes occurring over time (24 h) for all timepoints (Fig. 6A). The increased proliferative response can be attributed to the increased VEGF protein concentration (ranging from 5 to 30 ng/ml), which is much greater than typical TIME cell culture media, which is supplemented with 2 ng/ml VEGF. Qualitative assessment of endothelial sprouting and formation of lumenal structures on collagen gels after 24 h indicates well defined, interconnecting cellular networks (in endothelial cells cultured in MDA-MB-231/HMEC-1 co-culture supernatant from day 14, 21, and 28 timepoints) due to migration, alignment, and development of endothelial tubes. This endothelial response was quantitatively assessed by measuring the total length of all capillary tubes in the view-field after 24 h, in which the average tubule length was statistically increased in endothelial cells grown in MDA-MB-231/HMEC-1 conditioned supernatant (from days 14, 21, and 28 timepoints) relative to MDA-MB-231 monoculture conditioned supernatant (Fig. 6B). Weakly enhanced tube formation was observed for some controls (days 7 and 14); however, the endothelial cells grown in MDA-MB-231 monoculture conditioned supernatant overall did not form tubes as well defined as the endothelial cells grown in MDA-MB-231/HMEC-1 co-culture conditioned supernatant.

DISCUSSION

Normal endothelial and epithelial cells, as well as tumor cells, communicate through a complex network of interactions to drive cellular differentiation and formation of tissue structures. One mechanism of this communication is exchange of both soluble and insoluble signaling molecules [Bissell and Radisky, 2001; Warner et al., 2008]. Stromal cells, such as fibroblasts and infiltrating inflammatory cells, have the capacity to modulate both epithelial and tumor cell morphogenesis [Shekhar et al., 2000, 2001]. In vivo, tumor cells have been observed to preferentially align toward and associate with blood vessels, even prior to the initiation of angiogenesis [Li et al., 2000]. While previous studies have examined angiogenic changes in endothelial cells in response to tumor cells or conditioned media [Hewett et al., 1999; Khodarev et al., 2003], our data, using a long-term (28-day) in vitro co-culture system, suggests that the tumor endothelium can also exert potent influence on tumor cells.

Results from the alamarBlue™ assay indicate that the presence of endothelial cells in co-culture with cancerous or nonmalignant epithelial cells does not enhance tumor cell proliferation. Also, despite the high protein concentration of VEGF, a potent endothelial cell mitogen [Brown et al., 1999], increased HMEC-1 proliferation was not observed when co-cultured with the MDA-MB-231 cells as compared to MCF-10A cells. This response may be due to the growth limitations of 2D cell-culture monolayers. Although endothelial cell proliferation is required for angiogenesis, remodeling and migration of the existing vasculature may be more critical in developing a functional vascular supply within the solid tumor [Fox et al., 1993]. In vitro, ANG1 and ANG2 have also been shown to have little effect on endothelial cell proliferation, and instead function as apoptosis survival factors [Kim et al., 2000ab]. Future investigation into

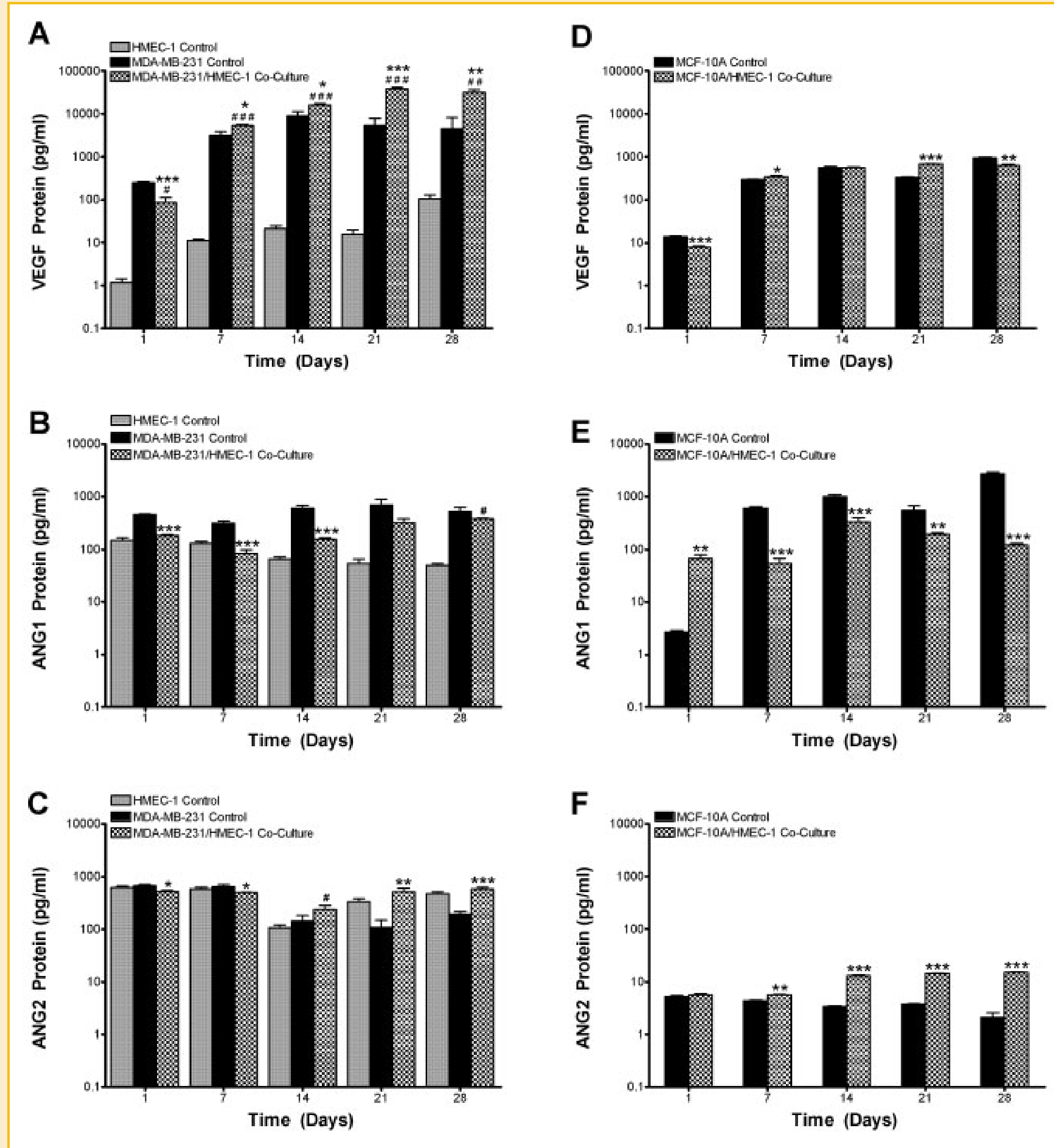

Fig. 5. Secretion of VEGF, ANG1, and ANG2, and proteins by MDA-MB-231/HMEC-1 and MCF-10A/HMEC-1 co-cultures compared to MDA-MB-231, MCF-10A, and HMEC-1 monocultures (control). Cell culture supernatants for each experimental group were collected for ELISA. A: A significant increase in VEGF protein secretion (pg/ml) was observed in MDA-MB-231/HMEC-1 co-cultures compared to MDA-MB-231 monocultures (after day 7) and HMEC-1 monocultures (all timepoints). B: A significant decrease in ANG1 protein secretion (pg/ml) was observed in MDA-MB-231/HMEC-1 co-cultures compared to MDA-MB-231 monocultures (on days 1, 7, 14, and 28) while no difference in ANG1 protein secretion (pg/ml) was observed compared to HMEC-1 monocultures (with the exception of a significant increase on day 28). C: A significant increase in ANG2 protein secretion (pg/ml) was observed in MDA-MB-231/HMEC-1 co-cultures compared to MDA-MB-231 monocultures (after day 21) while no difference in ANG2 protein secretion (pg/ml) was observed compared to HMEC-1 monocultures (with the exception of a significant increase on day 14). D: A modest but significant increase in VEGF protein secretion (pg/ml) was observed in MCF-10A/HMEC-1 co-cultures compared to MCF-10A monocultures (after day 7); however, these levels are two orders of magnitude lower than VEGF-A protein (pg/ml) in MDA-MB-231/HMEC-1 co-cultures. E: A significant decrease in ANG1 protein secretion (pg/ml) was observed in MCF-10A/HMEC-1 co-cultures compared to MCF-10A monocultures (on days 7, 14, 21, and 28) while an increase in ANG1 protein secretion (pg/ml) was on day 1. F: A significant increase in ANG2 protein secretion (pg/ml) was observed in MCF-10A/HMEC-1 co-cultures compared to MCF-10A monocultures (after day 7); however, these levels are an order of magnitude lower than ANG2 protein (pg/ml) in MDA-MB-231/HMEC-1 co-cultures. In conclusion, protein analysis in MDA-MB-231/HMEC-1 co-cultures indicate VEGF > ANG2 > ANG1 protein (pg/ml) relative to monocultures (control), and VEGF and ANG2 protein (pg/ml) in MDA-MB-231/HMEC-1 co-cultures are an order of magnitude greater than VEGF and ANG2 protein (pg/ml) in MCF-10A/HMEC-1 co-cultures. Co-culture statistically significant as compared to MDA-MB-231 or MCF-10A monoculture (control) denoted by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Co-culture statistically significant as compared to HMEC-1 monoculture (control) denoted by $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$.

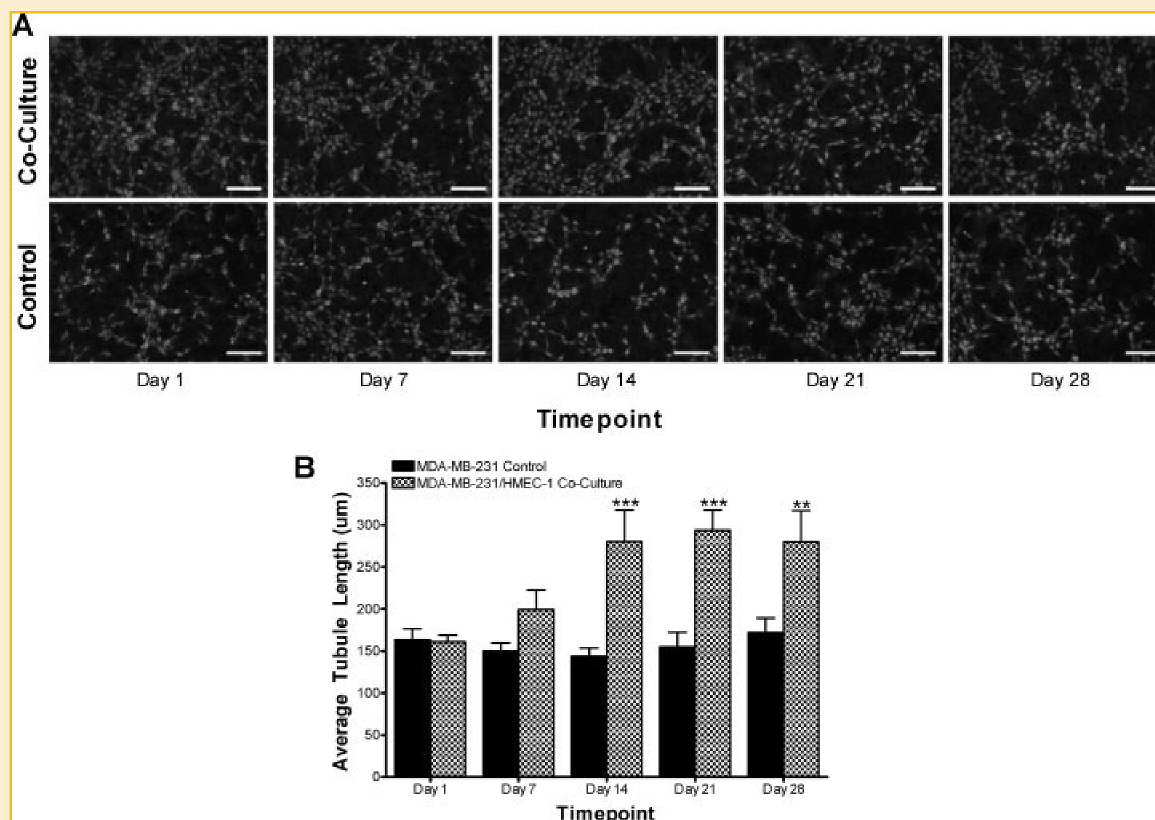

Fig. 6. Tube formation assay on collagen gels. Endothelial cells stably expressing red fluorescent protein (TIME-RFP) were seeded on type 1 collagen gels (1.5 mg/ml) and grown in conditioned cell culture supernatant collected from either MDA-MB-231/HMEC-1 (Co-Culture) or MDA-MB-231 monoculture (Control) experimental groups on days 1, 7, 14, 21, and 28. A: Tube formation visualized by fluorescence microscopy at 24 h (representative images shown) indicate increased migration and alignment into organized, interconnected cellular networks of endothelial cells grown in co-culture conditioned supernatant compared to monoculture conditioned supernatant (control). B: Quantification of average tubule length indicate a statistically significant increase in average tubule length in cells grown in co-culture conditioned supernatant (from days 14, 21, and 28 timepoints) compared to monoculture conditioned supernatant (control). Co-culture statistically significant as compared to monoculture (control) denoted by $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Scale bar = 200 μm.

receptor expression of angiogenic ligands (Tie2 and VEGFR-2) under co-culture conditions may provide further information on the cellular response to increased secretion of angiogenic factors. Furthermore, utilization of a 3D co-culture system, now under development in our laboratory, may allow for observation of more subtle, spatial changes in tumor and endothelial cell growth due to these secreted mitogens.

MDA-MB-231 cells in the presence of endothelial cells during co-culture demonstrated a time-dependent and significant increase in ANG2 gene expression as determined by real-time RT-PCR. While the mRNA levels of ANG2 were low at early time points when cells were in an exponential growth phase, mRNA levels were increased when the cells reached plateau phase. In contrast, ANG1 and VEGF-A gene expression in the nonmalignant MCF-10A cells was typically decreased during co-culture with endothelial cells, while ANG2 mRNA was undetected. This study demonstrates that only the MDA-MB-231 cells respond to endothelial cells with increased expression of angiogenic cues, perhaps highlighting a fundamental difference in tumor responsiveness between the malignant breast epithelial cell line compared to the nonmalignant breast epithelial cell line (MCF-10A).

Increased gene expression of VEGF-A and ANG1 in HMEC-1 cells during co-culture with MDA-MB-231 cells further provides evidence that reciprocal cross-talk between the two cell types influences expression of angiogenic factors. Results from angiogenic gene expression analysis highlight an important role for endothelial cells in creating and maintaining the tumor vasculature.

Similar to the mRNA expression trends, the ANG2 protein concentration in the MDA-MB-231/HMEC-1 co-culture was greater than ANG1, with the ratio of ANG2:ANG1 increasing over time. It has been suggested that it is not the absolute levels of ANG1 and ANG2, but rather the ratio of these opposing factors, that modulates angiogenesis. ANG1 is known to play a role in stabilizing the tumor vasculature, while ANG2 promotes tumor angiogenesis in vivo [Hayes et al., 2000; Kim et al., 2000b]. In a study using the mouse corneal micropocket assay, both ANG1 and ANG2 failed to stimulate an angiogenic response when administered alone; however, when co-administered with VEGF, both ANG1 and ANG2 augmented the formation of neovessels [Asahara et al., 1998]. In our study, the presence of high VEGF protein concentration, with an increased ANG2 protein concentration in MDA-MB-231/HMEC-1 co-cultures, provides evidence that endothelial cells have the ability to enhance

the angiogenic potential of breast cancer cells in vitro. Due to the fact that no significant changes in cell viability, oxygen tension, or pH were observed over the duration of the experiment, we can assume the observed angiogenic response is not stress (hypoxia) induced. Rather, the response is primarily due to the close proximity of endothelial cells and cancer cells in culture.

The profile of these angiogenic factors during MDA-MB-231/HMEC-1 co-culture represent a highly pro-angiogenic state, as increased VEGF and ANG2 levels are associated with increased plasticity of the vasculature and angiogenic sprouting [Vajkoczy et al., 2002]. The functionality of this increased angiogenic activity was confirmed using an endothelial tube formation assay, in which endothelial cells readily and rapidly formed well-defined tubes and interconnecting cellular networks when grown in media collected from MDA-MB-231/HMEC-1 co-cultures in vitro. By comparison, MDA-MB-231 monocultures (control) secreted less VEGF protein than the co-culture with the ratio of ANG1:ANG2 favoring ANG1. These conditions are characteristic of a more stabilized and mature tumor vasculature in vivo. This angiogenic profile was also evaluated using the endothelial tube formation assay, demonstrating little sprouting with weakly enhanced to no tube formation in endothelial cells grown in control supernatants in vitro. This provides further supportive evidence that the angiogenic activity of tumor and endothelial cells in co-culture is enhanced compared to tumor cells alone.

Research has shown tumor cell-enhanced up-regulation of ANG2 in endothelial cells [Zhang et al., 2003], though few reports have examined tumor response to endothelial cells [Tsai et al., 1995; Spring et al., 2005]. Other studies have investigated the signaling pathways implicated in cross-talk between lymphoblastic leukemia cells and endothelial cells [Indraccolo et al., 2009], as well as cross-talk between squamous epithelial cells and endothelial cells in tumor angiogenesis [Zeng et al., 2005]. Such reports suggest the existence of molecular exchanges between tumor cells and stromal compartments that effect tumor growth. However, few studies have examined how endothelial cells directly mediate the angiogenic phenotype of neoplastic mammary epithelial cells; this is an important area for further research.

CONCLUSIONS

Our results demonstrate that in vitro, the angiogenic activity of malignant mammary epithelial cells, but not nonmalignant mammary epithelial cells, is significantly enhanced by the presence of endothelial cells. Overall, the most striking finding from our study is an endothelial-induced change in MDA-MB-231 expression of ANG2 over ANG1 with absolute protein levels of VEGF $>$ ANG2 $>$ ANG1 during MDA-MB-231/HMEC-1 co-culture compared to MDA-MB-231 or HMEC-1 monocultures. This phenomenon reveals therapeutically and biologically significant interactions between cancer cells and endothelial cells for tumor angiogenesis. Identifying the role of endothelial cells within the tumor microenvironment and their effect on the temporal release of angiogenic factors may lead to novel methods to overcome tumor resistance to current anti-angiogenic therapies.

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