**Angiogenesis, Metastasis, and the Cellular Microenvironment**

**αB-Crystallin, an Effector of Unfolded Protein Response, Confers Anti-VEGF Resistance to Breast Cancer via Maintenance of Intracrine VEGF in Endothelial Cells**

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**Abstract**

Effective inhibition of angiogenesis targeting the tumor endothelial cells requires identification of key cellular and molecular mechanisms associated with survival of vasculatures within the tumor microenvironment. Intracellular autocrine (intracrine) VEGF production by endothelial cells plays a critical role on the vasculature homeostasis. *In vitro* breast cancer cell–stimulated activation of the unfolded protein response (UPR) of the endothelial cells contributes to maintenance of the intracrine VEGF levels in the endothelial cells through the upregulation of a previous undescribed downstream effector-αB-crystallin (CRYAB). siRNA-mediated knockdown of two major UPR proteins—inositol requiring kinase 1 and ATF6, led to attenuated CRYAB expression of the endothelial cells. Finally, inhibition of CRYAB blocked the breast cancer cell–stimulated increase in the endogenous VEGF levels of the endothelial cells. A VEGF limited proteolysis assay further revealed that CRYAB protected VEGF for proteolytic degradation. Here, we report that the molecular chaperone-CRYAB was significantly increased and colocalized with tumor vessels in a breast cancer xenograft. Specifically, neutralization of VEGF induced higher levels of CRYAB expression in the endothelial cells cocultured with MDA-MB-231 or the breast cancer xenograft with a significant survival benefit. However, knockdown of CRYAB had a greater inhibitory effect on endothelial survival. These findings underscore the importance of defining a role for intracrine VEGF signaling in sustaining aberrant tumor angiogenesis and strongly implicate UPR/CRYAB as dichotomous parts of a crucial regulation pathway for maintaining intracrine VEGF signaling. *Mol Cancer Res;* 9(12); 1632–43. ©2011 AACR.

**Introduction**

Breast cancer is the most frequently diagnosed cancer in women in developed countries and responsible for almost half a million deaths per year in women worldwide. Breast cancers represent a heterogeneous group of tumors that are diverse in progression and response to therapy. Although traditional clinical indices such as tumor size and grade and auxiliary lymph node metastasis are still useful prognostic factors in breast cancer, recent expression profiling has revealed 5 molecular subtypes of breast cancer, including luminal A and B, normal breast like, basal like, and HER-2 positive (1). Luminal A and B are derived from estrogen receptor–positive tumors, whereas normal breast like, basal like, and HER-2 positive are derived from estrogen receptor–negative tumors. Among these newly recognized subtypes, basal-like breast cancer expresses genes characteristic of basal epithelial cells and 80% to 90% of them are triple-negative breast cancers (TNBC) lacking the expression of estrogen receptor, progesterone receptor, and HER-2 (2). TNBC represents 10% to 17% of all breast cancers and also tends to occur in younger women. Clinically, they have increased frequency of distant metastasis formation (visceral and brain metastases) and poor overall survival compared with patients with non-TNBC. Despite the fact that TNBCs exhibit a higher pathologic response rate to diverse neoadjuvant chemotherapies, patients with TNBC tend to have a worse clinical outcome compared with those with non-TNBC if a complete pathologic response has not been achieved (3).

Numerous studies have shown that enhanced angiogenesis with high levels of VEGF supports rapid growth and early metastases in patients with advanced breast carcinoma (4–6). A previous retrospective study further indicated that augmented VEGF levels were correlated with negative estrogen receptor/progesterone receptor status (7). As a result, bevacizumab, a monoclonal antibody that blocks the activity of VEGF, is highly recommended for TNBC treatment (8). However, a more recent meta-analysis of 5 randomized controlled trials containing 3,163 eligible patients showed that the addition of bevacizumab to chemotherapy had no additional benefit over chemotherapy alone (9). As a result, bevacizumab is not recommended for the treatment of TNBC in the National Comprehensive Cancer Network guidelines (10).

The vascular system of the tumor microenvironment is self-sustaining and represents one of the most important factors determining the progression and metastasis of cancer (11). A significant amount of literature has indicated that the expression of key genes involved in the regulation of the angiogenic process is associated with breast cancer progression and recurrence (12). Vascular endothelial growth factor (VEGF) is an essential regulator of tumor angiogenesis and plays a critical role in the stimulation of the proliferation and survival of the endothelial cells (13, 14). In the tumor microenvironment, autocrine (intracrine) VEGF production by endothelial cells plays a critical role on the vasculature homeostasis (15, 16). In breast cancer, a previous study reported that the autocrine production of VEGF in endothelial cells and the expression of the VEGF receptor VEGFR-2 is related to the clinical outcome of breast cancer patients (17). The overexpression of VEGF in breast cancer cells is associated with increased tumor cell invasion and angiogenesis (18, 19). Therefore, the regulation of VEGF production and its signaling pathways is an important target for breast cancer therapy.

**Materials and Methods**

*In vitro* assays were used to evaluate the role of VEGF in breast cancer angiogenesis. MDA-MB-231 cells were used as the breast cancer cell lines. The cell viability was determined using a colorimetric assay based on the reduction of MTT to formazan by a mitochondrial enzyme in viable cells. The expression of CRYAB and VEGF in the endothelial cells cocultured with MDA-MB-231 cells was evaluated using Western blotting. The VEGF levels in the endothelial cells were measured by ELISA. The VEGF production by endothelial cells was assessed using a VEGF limited proteolysis assay. Finally, the VEGF levels in the endothelial cells were measured after siRNA-mediated knockdown of CRYAB.

**Discussion**

The results of this study indicate that CRYAB is a novel downstream effector of the unfolded protein response (UPR) in endothelial cells. Specifically, the upregulation of CRYAB expression in endothelial cells cocultured with breast cancer cells is associated with increased VEGF levels. This finding suggests that CRYAB may play a critical role in the regulation of VEGF expression in endothelial cells. The results of this study also indicate that CRYAB may be a potential target for the treatment of breast cancer angiogenesis.

**Conclusion**

In conclusion, CRYAB is a novel downstream effector of the UPR in endothelial cells. The upregulation of CRYAB expression in endothelial cells cocultured with breast cancer cells is associated with increased VEGF levels. This finding suggests that CRYAB may play a critical role in the regulation of VEGF expression in endothelial cells. The results of this study also indicate that CRYAB may be a potential target for the treatment of breast cancer angiogenesis.

**Abbreviations**

CNBP = cellular nuclear binding protein; CRYAB = crystallin, alpha B; EGF = epidermal growth factor; ELISA = enzyme-linked immunosorbent assay; ER = estrogen receptor; HGF = hepatocyte growth factor; KCl = potassium chloride; MTT = 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide; NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells; PBS = phosphate-buffered saline; PMA = phorbol 12-myristate 13-acetate; siRNA = small interfering RNA; TNBC = triple-negative breast cancer; UPR = unfolded protein response; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor; VS = vesi
cular storage.
patients shows that the addition of bevacizumab to chemotherapy regimens does not benefit overall survival (9). The fraction of nonresponsive patients in these clinical trials is significant. Even to the most responsive patients, the benefits of bevacizumab seem to be transitory. Bevacizumab produces initial response manifested as tumor stasis or shrinkage, followed almost inevitably by the tumor regrowth. Such refractoriness to bevacizumab reflects possible mechanisms of intrinsic and/or acquired resistance (10).

There is a clear need to identify more specific targeted therapies for TNBC. Recent studies reported the presence of αB-crystallin (CRYAB) in various types of solid tumors as a novel protein as well as a prognostic marker (11, 12). A gene profiling study identified that overexpression of CRYAB is more highly correlated with TNBC breast cancer (45%) than with non–basal-like tumors (6%; ref. 13). CRYAB is primarily found as a major structural protein for the maintenance of ocular lens transparency. Many studies also showed that in nonlenticular tissues CRYAB can be induced as a principal member of the mammalian small heat shock protein (sHsp) super family (14, 15). The sHsp family is characterized by a conserved, approximately 90 amino acids of the α-crystallin C-terminal domain. α-Crystallin functions as a cytoprotective molecular chaperone, renatures a wide range of structurally unrelated, unfolded, or misfolded proteins by keeping these aggregation-prone proteins in reservoirs of nonnative, refoldable intermediates within large, soluble, and multimeric structures (16). Until recently, the importance of CRYAB in the pathology of cancer has been mainly focused on the tumor cells as a novel oncoprotein. There is little information about the expression of CRYAB in the tumor endothelium and whether high levels of CRYAB affect the progression of tumor angiogenesis, particularly in the face of anti-VEGF treatment. Nevertheless, data from previous studies implicate that CRYAB can modulate VEGF expression (17) and vascular tube formation (18). In this article, we provide the first evidence that CRYAB is an important chaperone of the unfolding protein response pathway (UPR). Our data suggest that CRYAB may be a key component in the activation of the intracellular autocrine VEGF pathway by protecting VEGF from intracellular degradation in the tumor endothelial cells.

**Materials and Methods**

**Coculture of human microvascular endothelial cells with breast tumor cell lines**

Human mammary gland is composed of 2 main cellular compartments, the terminal duct lobular units—an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells, the surrounding stroma-fibroblast, adipocytes, immune cells, endothelial cells, and extracellular matrix. The tumor stroma accounts for approximately 80% of total tissue volume. Tumor cells are suggested to affect endothelial cells by secreting soluble factors such as VEGF. To determine whether the tumor cell–stimulated endothelial cells also contribute to the elevated levels of CRYAB detected in the breast cancer tissues, in vitro coculture was employed. A panel of breast cancer lines were used to represent increased malignancy from nontumorigenic lines (MCF-10A) to tumorigenic nonmetastatic (MCF-7, T-47D, and BT-20) to tumorigenic metastatic lines (SK-BR-3, Hs-578T and MDA-MB-231; ref. 19). Briefly, to prevent direct cell–cell contact, the breast cells were seeded on 6-well Transwell inserts with 0.4 μm pores (Corning Life Science) and cultured in a 6-well plate for 72 hours. Human microvascular endothelial cells (HMEC; Invitrogen) were cultured in a separate plate. Confluent breast cancer cells on Transwell inserts were then transferred on top of the endothelial cells and placed at 37°C for 24 hours.

For neutralization of VEGF, 100 ng/mL neutralizing VEGF165 antibody (R&D System) was added and HMECs cocultured with MDA-MB-231 for 24 hours.

In experiments of blocking de novo protein synthesis, HMECs cocultured with MDA-MB-231 were treated with 60 μg/mL of cycloheximide for indicated times.

**Western blotting analysis and immunoprecipitation**

Cells were rinsed in PBS and lysed in 4°C radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors (Sigma) and phosphatase inhibitors (Roche Applied Science) for 30 minutes. After lysates were centrifuged for 15 minutes at 15,000 × g at 4°C, the supernatants were collected and their total protein concentrations were measured by the MicroBCA reagent (Pierce). Western blot analysis was conducted after SDS-PAGE (equal aliquot of total proteins per lane) and transferred onto membranes. The proteins were hybridized with primary antibodies as indicated, and the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies, which were detected with the Amersham ECL system (GE).

For immunoprecipitation, total proteins were immunoprecipitated with 10 μg relevant antibodies for 2.5 hours at 4°C followed by the addition of 20 μL of protein A/G-agarose overnight at 4°C. The resultant pellet was dissolved in 2× Laemml buffer (Bio-Rad Laboratories) and subjected to Western blot analysis.

**In vitro tube formation assay**

In vitro tube formation assays were carried out as previously described (20). Briefly, HMECs were plated sparsely (2.5 × 10^4 cells per well) on 24-well plates coated with 12.5% (v/v) Matrigel (BD) and left overnight. The medium was then aspirated, and 250 μL per well of 12.5% Matrigel was overlaid on the cells for 2 hours to allow the polymerization of Matrigel, followed by addition of 500 μL per well of basal medium MCD131 with 10% fetal calf serum (FCS) for 24 hours. The following day, the culture plates were observed under a phase contrast microscope and photographed at random in 5 fields (10×). The tubule length (mm/mm^2) per microscope field was quantified.

**siRNA knockdown**

HMECs were seeded in 6-well tissue culture dishes for 24 hours before transfection with prevalidated Stealth Select siRNA from Invitrogen targeting human CRYAB.
(HSS102316 and negative control 12935-300) or inositol
requiring kinase 1 (IRE1; HSS140846 and negative control
12935-400) or ATF6 (HSS177036 and negative control
12935-300) or protein kinase-like extracellular signal-regu-
lated kinase (PERK; HSS114409 and negative control
12835-200) with Lipofectamine 2000 (Invitrogen) accord-
ing to the manufacturer’s protocol. The medium was
changed to endothelial cell basal medium with growth
supplement (Invitrogen) after 24 hours. At 48 hours after
transfection, the cells were subjected to different treatments.

**VEGF ELISA measurement**

HMECs were cultured in 6-well plates. After 24 hours
coculture of breast tumor cells, the endothelial cells were
rinsed in PBS and fresh microvascular endothelial basal
medium with 5% FCS was added. The cell numbers of
each well were counted. After 24 hours, the endothelial cell
lysates were prepared as described (20), whereas the super-
natant was harvested by centrifugation at 2,000 × g for 10
minutes. VEGF concentrations were measured in duplicate
in each sample with either RayBio human VEGF ELISA
(RayBiotech) for cell lysate or human VEGF DuoSet ELISA
(R&D system) for cell supernatant according to the manu-
facturers’ instructions. The results were expressed as VEGF
(pg) per 10⁶ cells.

**VEGF limited proteolysis assay**

The VEGF limited proteolysis assay was modified from a
previous study (21). Briefly, the cells at T75 cm² were
trypsinized and centrifuged at 200 × g for 5 minutes. The
resultant pellets were dissolved in 500 μL of denatured buffer
(20 mmol/L Tris-HCl, pH 8.0, 6 mol/L guanidine HCl, 5
mmol/L EDTA, 4 mmol/L dithiothreitol) at room temper-
ture for 2 hours. Total protein concentration estimated by
measuring the ultraviolet absorbance at 280 nm was diluted
with denature buffer to final concentration of 1 mg/mL.
After centrifugation (46,000 × g, 4°C, 30 minutes), the super-
natant was concentrated to 100 μL with Millipore
Ultrafree centrifugal devices (molecular weight cutoff: 3.5
kDa; Millipore). After transferring the samples into fresh
tubes, 2 μL of a subtilisin solution (0.05 mg/mL; Sigma) was
added to each tube, resulting in a protease to substrate ratio
of 1:100. The proteolysis was aborted after 10, 50, and 100
minutes by adding 10 μL of 10 mmol/L phenylmethylsul-
fonylfuoride solution (Sigma). The samples were mixed
with standard sample buffer (1:2) and analyzed by Western
blot analysis using antibody against the C-terminal of human
VEGF (Abcam).

**Proliferation assays**

Crystal violet assay was conducted as previously described
(20). Briefly, 100 μL HMECs were incubated in each well of
96-well plates at 1 × 10⁵ cell/mL in endothelial basal
medium with growth supplement (Invitrogen). The cells
were fixed in 4% paraformaldehyde in PBS for 15 minutes.
After being washing with H₂O₂, the plates were stained with
0.1% crystal violet solution for 20 minutes. The plates were
washed with H₂O₂ and allowed to be air dry, followed by
adding 100 μL 33% of acetic acid to each well. Absorbance
of the staining was measured by an automatic microtiter
plate reader at 590 nm.

**Apoptosis assay**

Apoptosis was evaluated using fluorescein isothiocyanate–
conjugated annexin V–propidium iodide assay kit (R&D
System) based on annexin V binding to phosphatidylserine
exposed on the outer leaflet of the plasma membrane lipid
bilayer of cells entering the apoptotic pathway. Briefly,
HMECs were collected by EDTA loosening, pelleted by cen-
trifugation (200 × g for 5 minutes), washed in PBS, and
resuspended in the annexin V incubation reagent in the dark
for 15 minutes before flow cytometric analysis. The analysis
of samples was conducted by a fluorophotometric flow
cytometer. An excitation wavelength of 488 nm was used
with fluorescence emission measured at 530 ± 15 nm
through fluorescence channel one. A minimum of 10,000
cells per sample were collected using log amplification for
fluorescence channel one and linear amplification for for-
ward light scanner and 90 degrees light scatter before being
analyzed using in-house software.

**Human breast cancer xenograft**

Animal experiments were carried out in the animal facility
of The University of Florida in accordance with institutional
guidelines. Athymic female nude mice (nu/nu), 3 to 5 weeks
of age, purchased from Charles River Laboratories, Inc. and
acclimated and caged in groups of 5 or fewer. Breast cancer
xenografts were established by subcutaneous injection of 1 ×
10⁶ MDA-MB-231 cells into the mammary fat pads of the
mice. Tumor volume was calculated from caliper measure-
ments of the largest (a) and smallest (b) diameters of each
tumor using formula a × b² × 0.4. Three days after
inoculation, most tumors had grown to approximately 20
to 23 mm³. Mice with similarly sized tumors were divided
into 2 groups. One group was treated by intratumoral
injection (10 μg) of neutralizing VEGF₁₆₄ antibody (R&D
System) every other day for a total of 4 times. The other
group was treated with an irrelevant isotype-matched
antibody.

**Immunohistochemistry detection**

The breast cancer xenograft tissues were fixed in 4% paraformaldehyde. Frozen sections were double stained for
the presence of mice vascular endothelial cells [using rabbit
Anti-von Willebrand Factor (Millipore) in 1:800 followed by
anti-rabbit-Alexa Fluor 568 conjugate in 1:300], and mice
αB-crystallin [using anti-mice Alpha B-Crystallin
(Abcam) in 1:800 following anti-mouse-Alexa Fluor 488
conjugate 1:300]. The tissue sections were examined, and
photomicrographs were obtained using a DSU-Olympus
IX81 confocal microscope.

**Real-time PCR assays for CRYAB expression in human
breast specimens**

A total of 152 tissue samples were analyzed from a
prospective series of breast carcinoma patients (33
background normal breast and 119 breast cancer tissues) who had undergone complete mastectomy as initial treatment (i.e., without prior radiotherapy or chemotherapy). All protocols were reviewed and approved by the ethical committee of the University Hospital of Wales. A written informed consent was obtained. Shortly after surgery, tissue samples were snap frozen in liquid nitrogen and selected by a pathologist. Background normal mammary tissues were made anonymous before the study, and clinical information about lymph node status, distant metastasis, disease-free and overall survival were collected for analysis. For patients clinical data, see Table 1.

Total RNA was extracted from all tissue samples by total RNA isolation reagent (Abgene UK) according to the manufacturer’s instructions and as previously described (22). Total RNA yield and purity were determined by spectrophotometry and only samples with an A260/A280 ratio above 1.6 were kept for further examination. PCR primers were designed by Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). A 416-bp PCR amplicon was designed for human β-actin gene and a 230-bp amplicon was designed for CRYAB gene. For human β-actin, the primers were forward 5‘-CTCCATCTCTGCGCCTGGGTG-3’ and reverse 5‘-GCTGCTACCTTCACCGTTCC-3’. For CRYAB gene, forward primer 5‘-CTTTGACCTCCATCCCTGGA-3’ and reverse 5‘-CTTTGACCTCCATCCCTGGA-3’. Quantitative PCR (qPCR) was carried out on the iCycler IQ system (Bio-Rad) with the above primers, the Amplifluor system (Intergen Inc), and a universal probe (Uniprimer; Chemicon). Real-time conditions were set to 12 minutes at 95°C, followed by 65 cycles at 95°C for 15 seconds, 55°C for 60 seconds, and 72°C for 20 seconds. The levels of CRYAB transcript from a given sample was automatically calculated by the software from an internal standard, a method previously described (23).

Statistics
Experiments were conducted at least 3 times. The Mann–Whitney U test was used to determine statistical significance in the laser densitometry data of Western blot analysis. Tubular formation was analyzed with the unpaired Student t test. VEGF ELISA and apoptosis assay were also analyzed with unpaired Student t test. The significance of the association between CRYAB mRNA levels and clinicopathologic variables was assessed by the Kruskal–Wallis test except that the difference among Nottingham prognosis index (NPI) groups was analyzed by multivariate analysis of variance (MANOVA). The data of Results section are expressed as mean ± SEM. Statistical analysis was conducted by MINITAB (version 13.32; Minitab Inc.) with P < 0.05 considered statistically significant.

Results
Breast cancer cell lines differentially upregulated CRYAB protein expression in HMECs
Expression levels of CRYAB were compared in lysates of HMECs cocultured with various breast cancer lines. Equal protein samples were separated by SDS-PAGE and immunoblotted. All the breast cancer cell lines stimulated CRYAB expression in HMECs (Fig. 1A). There was a gradual elevation in the abundance of CRYAB with the increased malignancy of breast cancer lines. The results of the densitometric analysis showed that the tumorigenic, metastatic lines (SK-BR-3, Hs-578T, and MDA-MB-231) exerted the great expression as compared with the tumorigenic non-metastatic lines (MCF-7, T-47D, and BT-20). qRT-PCR analysis showed a similar pattern of mRNA levels of CRYAB in HMECs stimulated by breast cancer cell lines (Supplementary Fig. S1A).

Interestingly, Supplementary Fig. S1B shows that the quickly proliferating breast cancer cells resulted in a significant decrease in the glucose concentrations of the supernatants. As shown in Supplementary Fig. S1C, glucose starvation caused an increase in CRYAB expression in HMECs at dose-dependent manner with the greatest effect at 0.03 mmol/L of glucose in the medium, which represented the lowest glucose levels detected in the supernatants of the breast cancer cell lines.

To exclude that CRYAB protein is directly delivered to endothelial cells by exosomes released by the tumor cells, we isolated and purified microvesicle (MV) from the supernatants of MDA-MB-231 cells. CRYAB protein was undetectable in the MV by Western blot analysis (Supplementary Fig. S1D).

Table 1. Breast cancer patients’ clinical data details

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Figure 1. Differential expression of CRYAB induced by breast cancer cell lines regulates tubule formation and cellular levels of VEGF in endothelial cells. A, HMECs cocultured with different breast cancer cell lines were described in Materials and Methods. The overall expression of CRYAB in HMECs was examined by Western blot analysis using an anti-CRYAB antibody (top); Blots probed for overall expression of CRYAB were stripped and reprobed with anti-α-tubulin antibody (bottom); densitometric analyses are presented as the relative ratio of CRYAB to α-tubulin. The ratio relative to control is arbitrarily presented as 1. Vertical bars are SEM. B, HMECs were treated with the CRYAB siRNA or scramble siRNA. The cells were cocultured with different breast cancer cell lines as indicated for 48 hours before being seeded between 2 layers of Matrigel for 24 hours. Morphometric quantitative analysis of in vitro tube formation of tubule length (mm/mm²) per microscope field was carried out. Each experiment was repeated a minimum of 3 times. C, HMECs were cocultured with different breast cancer cells. The overall expression of UPR proteins was examined by Western blot analysis with antibodies against XBP-1, ATF6, IRE1, and PERK, respectively. D, HMECs were transfected with one scramble siRNA and 3 prevalidated siRNAs against IRE1, ATF6, and PERK, respectively. The HMECs were cocultured with different breast cancer cell lines. CRYAB protein expression was analyzed by Western blot using an antibody against CRYAB. E, VEGF protein expression was measured by ELISA. The breast cancer cell lines significantly induced VEGF expression in both intracellular and extracellular compartments of HMECs, which was attenuated by knockdown of CRYAB. The error bars show the SEM.
siRNA knockdown of CRYAB decreased breast cell line–induced in vitro angiogenic activity of endothelial cells

Later stages of angiogenesis require morphologic alterations of endothelial cells, which results in lumen formation. To address the involvement of CRYAB for the proangiogenic activity of endothelial cells, we next employed an in vitro tubule formation assay in which the breast cancer cell–stimulated endothelial cells were induced to form a network of capillary-like tubes in a 3-dimensional Matrigel. HMECs pretransfected with scrambled siRNA (Supplementary Fig. S2A) stimulated a significant tubule formation that correlated with the increased malignancy of the breast cancer lines (Fig. 1B and Supplementary Fig. S2B). In contrast, endothelial cells transfected with CRYAB siRNA showed little response to the stimulations from the breast cancer cell lines. Quantitative evaluation of tubule formation revealed that coculture with the tumorigenic metastatic lines increased the length of the endothelial tubular structures by approximately 15% compared with that seen with the tumorigenic non-metastatic lines (Fig. 1B and Supplementary Fig. S2B). These results show that CRYAB overexpression in endothelial cells is involved in breast tumor–induced angiogenesis in vitro, which is inhibited by treatment with CRYAB siRNA.

Breast cancer cells differentially regulated endoplasmic reticulum stress proteins in the endothelial cells

Homeostasis of endoplasmic reticulum (ER), as defined by the dynamic balance between the protein synthesis and the ER capacity to process protein proper folding, is essential for normal cellular functions (24). Many pathologic conditions can affect this balance and cause ER stress with the elevation of 3 ER membrane–bounded protein-regulated stress pathways—IRE1, ATF6, and PERK. Of them, processed XBP-1 is a key transcriptional regulator activating genes involved in protein folding. To determine whether breast cancer cells stimulating endothelial angiogenic activities perturb ER homeostasis in the endothelial cells, HMECs were cocultured with breast cancer cell lines of different malignancy, and the expressions of these 3 ER stress proteins were analyzed by Western blot. We found that the processed XBP-1 was significantly upregulated in the endothelial cells by the breast cancer cell lines. As shown in Fig. 1C and Supplementary Fig. S3A, a 200% to 800% of upregulation of processed XBP-1 was observed among the endothelial cells cocultured with breast cancer cells of different malignancy compared with those cocultured with MCF-10A. As IRE1 is a key upstream regulator for XBP-1 mRNA splicing, it was shown that the breast cancer cell lines cause a differential increase in IRE1 expression in the endothelial cells at tumor malignancy–dependent manner (Fig. 1C and Supplementary Fig. S3A). Similar to IRE1, ATF6 transcriptionally regulates ER stress chaperon proteins, which may be responsible for the proper folding of proteins. We next examined levels of ATF6 proteins in the endothelial cells cocultured with the breast cancer cells. Treatment of HMECs with the breast cancer cells triggered ATF6 expression with the greatest effects at those cocultured with the tumorigenic metastatic lines (Fig. 1C and Supplementary Fig. S3A). To our surprise, coculture of endothelial cells with the breast cancer cells did not induce significant changes in the protein level of PERK compared with those cocultured with MCF-10A (Fig. 1C and Supplementary Fig. S3A), suggesting that PERK functions were not drastically affected in the endothelial cells stimulated by the breast cancer cells. A previous study has shown that PERK activation results in inhibition of overall mRNA translation, which was believed to reduce ER protein load and relieve ER stress (25).

Breast cancer cells upregulated CRYAB via the activation of the unfolded protein response pathway in the endothelial cells

To rectify ER homeostasis, cells evolve an adaptive response termed the UPR pathway initiated by the 3 ER stress proteins—IRE1, ATF6, and PERK. These responses result in 2 distinctive outputs: upregulation of molecular chaperones which prevent further accumulation of unfolded proteins and reduction of protein translation which lowers ER protein load and enhances protein folding activity. It is believed that CRYAB acts as a chaperone protein in response to cellular stress (26). As described above, the breast cancer cells significantly upregulated CRYAB expression in the endothelial cells. Therefore, we speculated a possibility that the activation of the UPR pathways leads to the upregulation of CRYAB. To test this idea, we measured CRYAB expression in the endothelial cells treated with the siRNAs against CRYAB and ER stress proteins—IRE1, ATF6, and PERK followed by coculture of the breast cancer lines. Either knockdown of IRE1 or ATF6 significantly attenuated the breast cancer cell–induced expression of CRYAB protein in the endothelial cells with an approximately 40% to 60% reduction compared with those treated with the scrambled siRNA (Fig. 1D and Supplementary Fig. S3B). The PERK siRNA, however, had little impact on the breast cancer cell–induced upregulated expression of CRYAB protein in the endothelial cells.

Breast cancer cell line–induced VEGF upregulation in the endothelial cells is dependent on CRYAB

One of the important mechanisms by which breast cancer cells contribute to tumor angiogenesis is through upregulation of VEGF in endothelial cells. Furthermore, autocrine VEGF production in endothelial cells was recently found to be triggered by tumor cells (27). We reasoned that the autocrine VEGF production in endothelial cells could be significantly amplified by the sustained tumor stimulation. Indeed, our ELISA assays showed that HMECs exposed to the breast cancer cell lines elevated amounts of intracellular and secretion, which were correlated with the breast cancer of malignancy–dependent manner (Fig. 1E). Comparison between the levels of intracellular VEGF and those secreted extracellular space, the latter was approximately 2-fold greater than the former, further indicating that the
tumor-induced autocrine VEGF production in endothelial cells may more significantly contribute to sustain tumor vasculature than we expected. To determine whether CRYAB is important in autocrine VEGF production, knockdown of CRYAB expression in HMECs were achieved by transfection with siRNA against CRYAB. VEGF ELISA assay further showed that the treatment of CRYAB siRNA significantly blocked the breast cancer cell–induced autocrine VEGF production in the endothelial cells with the greater impact on the extracellular secretion than intracellular VEGF (Fig. 1E).

In our studies, breast cancer cells also upregulate CRYAB expression in the endothelial cells. Thus, we first checked the interaction between CRYAB and VEGF with immunoprecipitation method. As shown in Fig. 2A, VEGF protein was coprecipitated with CRYAB, showing the association between CRYAB and VEGF in the endothelial cells. To further test whether the association between CRYAB and VEGF increases the stability of VEGF protein, we measured the half-life of VEGF protein after blocking de novo protein synthesis with cycloheximide. Fig. 2B shows that in HMECs cocultured with MDA-MB-231, the half-life of VEGF was prolonged to 100 minutes. However, the half-life of VEGF was 60 minutes in the presence of CRYAB siRNA. This result suggests that CRYAB enhanced VEGF protein stability in the endothelial cells.

CRYAB protects endogenous VEGF from proteolytic degradation

As endogenous VEGF reflects the balance between VEGF synthesis/extracellular secretion and intracellular degradation, we detected overall VEGF expression of the cell lysates to evaluate the hypothesis that CRYAB may exert a protective effect on proteolytic degradation of VEGF. Western blot

Figure 2. CRYAB protects VEGF from proteolytic degradation. A, HMECs were cocultured with different breast cancer cell lines as described in Materials and Methods. Total cell lysates were immunoprecipitated with antibody against VEGF, followed by Western blot analysis of CRYAB. B, HMECs were transfected with siRNA against CRYAB or a scrambled siRNA, and the cells were cocultured with MDA-MB-231. The cells were then incubated with cycloheximide for the indicated time. Total cellular proteins were subjected to Western blot analysis to determine the CRYAB protein level. C, VEGF protein expression in total cell lysates from HMECs was analyzed by Western blot using an antibody against VEGF. D, limited proteolysis of VEGF was carried out as described in Materials and Methods. Total cell lysates were incubated with subtilisin for increasing time periods as indicated. VEGF degradation was detected by Western blot analysis using antibody against the C-terminal of VEGF.
analysis of VEGF in HMECs after coculture with breast cancer cell lines showed a protein band with an approximate molecular weight of 42 kDa. The intensity of the band correlated with the increased malignancy of tumors (Fig. 2C). In contrast, when the endothelial cells were treated with CRYAB siRNA, the 42-kDa band underwent migration to multiple bands of approximately 38 kDa suggesting VEGF protein degradation. In addition, the tumorigenic metastatic line treatment resulted in the highest degree of the VEGF migration in the endothelial cells (Fig. 2C). These observations strongly suggest that CRYAB prevents endogenous VEGF from intracellular degradation. Proper folding conditions of proteins could be identified on the basis of the ability of the protein to withstand proteolytic degradation by the protease subtilisin (21). As shown in Fig. 2D, the time course of the degradation showed that the VEGF from all HMECs cocultured with breast cancer cells, apart from MCF-10A, did not exhibit degradation over 100 minutes. However, after knockdown of CRYAB, VEGF appeared to be degraded by 50 minutes for all tested breast cell lines except of HS-578T, further confirming that CRYAB plays a major role in protection of unfolded/misfolded VEGF from degradation within the tumor cell–stimulated endothelial cells.

**Anti-VEGF treatment exerts synergistic effect on the upregulation of CRYAB in endothelial cells induced by breast cancer cells**

Because many cancer clinical studies have implication possible resistance to anti-VEGF treatment, we investigated the effect of neutralization of VEGF on CRYAB expression in endothelial cells. As shown in Fig. 3A, Western blotting showed an increase in expression of CRYAB in HMECs cocultured with MDA-MB-231, which could be further elevated by approximately 25% by addition of neutralizing VEGF antibody. This was confirmed by immunofluorescent staining of the frozen sections of breast cancer xenografts with an anti-CRYAB antibody. As expected, the breast cancer xenografts exhibited an accumulated CRYAB in juxtaposition to the cell nucleus, whereas CRYAB protein was undetectable in normal breast tissues (Fig. 3B). The immunoreactivity of CRYAB seemed to be stronger in the breast cancer xenografts intratumoral injected with neutralizing VEGF antibody than those of non–anti-VEGF treatment (Fig. 3B).

To elucidate association of CRYAB with tumor vasculature in breast cancer tissues, second immunofluorescent staining for von Willebrand factor was also carried out on the frozen sections of the breast cancer xenografts. Microvessels were homogeneously distributed throughout the normal breast tissues, whereas the border between the tumor and the normal host breast tissues was highly vascularized with the central regions of the tumors being mostly devoid of vessels (data not shown). Within the highly vascularized regions, the 2 patterns of staining for CRYAB and von Willebrand factor mostly overlapped, suggesting that CRYAB seemed to locate in the endothelial cell layer of the tumor blood vessels (Fig. 3B).

Although 10 days of anti-VEGF treatment led to significant damage of tumor vasculature with increasing dilatation, CRYAB expression level was more pronounced in xenografts treated with neutralizing VEGF antibody (Fig. 3B and Supplementary Fig. S4A). In addition, a decrease in the levels of VEGF and VEGF receptor 2 was observed in the xenograft tissues with neutralizing VEGF antibody as compared with those without neutralizing VEGF antibody (Supplementary Fig. S4B).

**CRYAB promotes the proliferation and the survival of endothelial cells**

To address whether overexpression of CRYAB may lead to anti-VEGF resistance, the proliferation and the survival of HMECs were analyzed with crystal violet staining and apoptosis assay, respectively. The knockdown of CRYAB elicited a marked apoptotic response in HEMCs, as evident by a significant 5-fold increase in the apoptotic cells compared with control (Fig. 3C and D). Intriguingly, treatment with neutralization of VEGF alone induced a very mild HMEC apoptotic response in HEMCs compared with control (Fig. 3C and D). Consistently, crystal violet staining revealed that MDA-MB-231 coculture significantly increased HMECs proliferation which was inhibited by neutralizing VEGF treatment by 50%. However, a 75% reduction in HMEC proliferation was observed with CRYAB siRNA treatment regardless of neutralization of VEGF (Fig. 3E).

**Quantification of CRYAB expression in human breast tissues**

mRNA derived from the breast tissues (tumor, n = 119; background, n = 33) was subjected to CRYAB gene–specific real-time qPCR (all values are displayed as mean transcript copies) to determine whether tumor-specific changes in the mRNA levels of CRYAB mRNA represents a potential cause for the high levels of CRYAB expression in the breast tumor tissues. We show that the CRYAB expression value in tumors was 3.34 ± 1.4 compared with 0.56 ± 0.36 in normal tissue. Therefore, our results reveal that the CRYAB expression was significantly increased in the breast cancer (P = 0.024) compared with that in the normal background breast tissue (Fig. 4A).

**CRYAB expression in relation to prognosis**

We examined CRYAB levels in patients with different prognosis, with the NPI as an indicator. The NPI-1 group (n = 67) represents patients with a good prognosis (NPI less than 3.4). The NPI-2 group (n = 37) containing patients with a moderate prognosis, and an NPI of 3.4 to 5.4. NPI-3 patients (n = 15) had a poor prognosis with a NPI more than 5.4. The patients assigned to the NPI-1 group had a CRYAB transcript value of 0.36 in C6/C6 and 0.11. A MANOVA showed that there was a significant association between increased levels of CRYAB transcript and high NPI status (P < 0.05; Fig. 4B). We also assessed the degree of CRYAB expression in relation to the node status of the breast
Figure 3. Anti-VEGF enhances CRYAB expression and survival role of CRYAB on endothelial cells. A, HMECs were cocultured with MDA-MB-231. The overall expression of CRYAB in HMECs was examined by Western blot analysis utilizing an anti-CRYAB antibody (top). Blots probed for overall expression of CRYAB were stripped and reprobed with anti-α-tubulin antibody (bottom). Densitometric analyses are presented as the relative ratio of CRYAB to α-tubulin. The ratio relative to control is arbitrarily presented as 1. Vertical bars are SEM. B, dual immunofluorescent stainings for CRYAB (green) and von Willebrand factor (red). Merged images were shown with colocalization of CRYAB and von Willebrand factor appearing as yellow. Scale bar, 25 μm. C–E, HMECs were pretransfected with an siRNA against CRYAB or a scramble siRNA, and the cells were cocultured with MDA-MB-231 in the presence of or the absence of neutralizing VEGF antibody. C, annexin V–propidium iodide–positive cells were shown (top right, late stage apoptosis; bottom right, early apoptosis). D, cell apoptosis was expressed as a percentage of apoptotic cells in the total cell population. E, the cell proliferation was assessed by crystal violet staining.
cancer patients (patients with negative nodes, $n = 67$; patients with positive nodes, $n = 51$). The patients without node involvement ($0.307 \pm 0.209$) had a significant difference in CRYAB expression compared with those patients with node involvement ($1.596 \pm 0.3508$; Fig. 4C).

**CRYAB and breast tumor grade**

There was no apparent difference in CRYAB transcript levels between the well-differentiated grade 1 tumors ($0.808 \pm 0.08$; $n = 23$), the modestly differentiated grade 2 classed tumors ($0.737 \pm 0.45$; $n = 40$), and the poorly differentiated grade 3 tumors ($1.056 \pm 0.558$; $n = 40$; $P = 0.3769$) even though mean CRYAB values in grade 2 seemed to increase compared with grade 1 tumors (Fig. 4D).

**CRYAB expression and survival status**

We assessed the survival status of patients with breast cancer in association with CRYAB levels, with an average of a 6-year follow-up period. Patients were divided into 2 groups, the patients who remained disease free were assigned to the disease-free group ($n = 89$), whereas the patients who had recurrence, metastasis to a distant site, or had died as a result of breast cancer were designated to the poor prognosis group ($n = 30$). The quantity of CRYAB from each tumor specimen was assessed, and we revealed that patients with a poor prognosis had dramatically increased levels of CRYAB ($0.56 \pm 0.298$) compared with the statistically low levels observed in the disease-free group ($1.15 \pm 0.53$; Fig. 4E).

**Tumor estrogen receptor classification of patients**

We assessed the degree of CRYAB expression in relation to the ER status of the breast cancer patients (patients with negative estrogen receptor, $n = 71$; patients with positive estrogen receptor, $n = 48$). We report that patients without ER expression ($0.748 \pm 0.32$) had significant differences in CRYAB expression compared with those patients with estrogen receptor expression ($1.61 \pm 0.811$; Fig. 4F).

**Discussion**

It is commonly believed that regulation of growth factor signaling in cellular functions is mainly attributable to a paracrine mechanism—that the recipient cell expresses cell-surface receptors and responds to growth factors excreted by...
adjacent cells. In our case, an in vitro coculture system is designed to keep tumor and endothelial cells in separate chambers and avoid direct cell–cell contact between the 2 cell types. Here, our data apparently indicate that tumor cells are able to effectively stimulate endothelial cells via soluble factors by a paracrine mode alone, particularly during the initial stage of tumor angiogenesis. After stimulation by exogenous growth factors including VEGF, endothelial cells are believed to synthesize and secrete active VEGF on their own in the extracellular space and activate the VEGF receptors on its own cell surface which leads to amplification of VEGF signaling (27). This external loop is classified as autocrine signaling. Here, our ELISA analysis of the intracellular and extracellular VEGF levels show that VEGF in both cellular and extracellular compartments is significantly elevated even 24 hours after tumor cells are withdrawn from the coculture systems, consistently suggesting that endothelial VEGF is an important autocrine effector of the tumor-induced angiogenesis.

However, prevailing evidence suggests that endogenous VEGF and its receptors in endothelial cells may internally transduce VEGF signaling without VEGF secretion (28, 29). To distinguish from classic autocrine signaling, the paradox of cell autonomous signaling has been dubbed as the internal autocrine signaling or simply intracrine. Previous studies show that VEGF intracrine signaling is crucial for vascular homeostasis (29). It has been shown that the lack of intracrine VEGF signaling results in systemic vascular pathologies even with normal total VEGF in vivo. Intriguingly, the defects in the vasculature of mice lacking endothelial VEGF could not be rescued by exogenous VEGF.

The microenvironment of human tumors is unlike that of any normal tissue and is characterized by nutrient deprivation and limited oxygen availability (30). These unique features develop as a consequence of inadequate perfusion as the primary tumor rapidly outgrows its initial blood supply and dramatic structural alterations lead to the highly irregular vascular supply. Within such an environment, not only do tumor cells suffer from nutrient deprivation, hypoxia, and acidosis but endothelial cells also are under similar stress. Our results from increased levels of ER stress proteins in the endothelial cells cocultured with breast tumor cells indicate that the biological stress on endothelial cells imposed by tumor cells can trigger the UPR activation.

The molecular chaperone role of sHSPs has been shown in many cells including microvascular endothelial cells (31, 32). CRYAB is the archetype for sHSPs and UPR that respond to protein unfolding/misfolding (33). It has been reported that CRYAB interacts with a number of growth factors, and there are interactive sequences for VEGF in human CRYAB (34). Previous studies clearly showed that CRYAB overexpression in the basal-like group of TNBC is a molecular mechanism responsible for the aggressive behavior in this group of breast cancer (13, 35). Furthermore, we identified that CRYAB highly colocalizes with the tumor vessels in the breast cancer xenografts. Notably, elevated CRYAB expression was induced in endothelial cells by breast tumor cell lines, correlating with increased malignancy of breast cancer cells, thereby suggesting that CRYAB overexpression observed in breast cancer tissues may be partially due to the elevated CRYAB levels in the tumor endothelial cells, which to our knowledge was previously unrecognized.

Almost a decade ago, it had been suggested that hematopoietic cells can produce several growth factors including VEGF, but not secret, constituting an internal autocrine loop–intracrine signaling (36, 37). The intracrine pathway only recently has been identified to involve VEGF signaling of endothelial cells (38). Subsequent study further revealed that the absence of intracrine VEGF leads to endothelial cell apoptosis, and exogenous VEGF fails to rescue the loss of endogenous VEGF in endothelial cells, strongly suggesting that the intracrine VEGF signaling in endothelial cells is crucial for vascular homeostasis (29). Due to its independence on extracellular VEGF, VEGF intracrine signaling may provide tumor endothelial cells with the ability to escape from the blocking exogenous VEGF such as bevacizumab therapy. In this study, we addressed the question of whether UPR/CRYAB was responsible for proper folding of endogenous VEGF of endothelial cells, thereby maintaining VEGF intracrine signaling. Recent ELISA-based protein pin array assays have led to the identification of sequences involved in interaction with regulatory proteins including VEGF with CRYAB (34). Our findings that selective disruption of UPR pathways (IRE1/XBP-1, ATF6) or knockdown of CRYAB significantly increased VEGF proteolytic degradation are strongly suggestive of a crucial role of UPR/CRYAB in maintaining endothelial VEGF. In light of recent findings from the laboratory and the clinic that a substantial number of cancer patients are refractory to anti-VEGF therapy, 2 types of mechanisms of resistance to anti-VEGF therapies have been proposed: intrinsic and acquired. Primary resistance may be due to the current anti-VEGF therapies targeting at paracrine VEGF or the classic autocrine signaling pathway, leaving the intracrine VEGF untouched. We propose that intracrine VEGF signaling acts as the primary intrinsic resistance. Under the stress conditions caused by tumor microenvironment and/or anti-VEGF therapies, tumor endothelial cells adopt the upregulation of UPR/CRYAB as required resistance to maintain the intracrine VEGF signaling. In this regard, we propose that intracellular disruption of VEGF signaling by targeting UPR/CRYAB in tumor endothelial cells may represent a powerful addition to current antiangiogenic arsenal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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