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Development of an anti-angiogenic therapeutic model combining scAAV2-delivered siRNAs and noninvasive photoacoustic imaging of tumor vasculature development

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Abstract

Preclinical studies have established tumor angiogenesis as a potential therapeutic target for breast cancer. However, there is an urgent need to either improve existing anti-angiogenic agents or devise new anti-angiogenic therapy for a subset of breast cancer patients with resistance to current anti-angiogenic regimes. The purpose of this study is to develop an anti-angiogenic therapeutic model for breast cancer by a combination of 1) siRNA-based therapy intratumorally delivered by self-complementary adeno-associated virus serotype 2 (scAAV2) vector to target tumor vasculature, and 2) non-invasive monitoring for tumor response to anti-angiogenic agents by serial photoacoustic imaging. We have reported previously that under the stress conditions caused by tumor microenvironment or/and anti-VEGF therapies, endothelial cells adopt the up-regulation of IRE1 α /XBP-1 and ATF6. This in turn maintains VEGF intracrine signaling for endothelial cell survival. Here we identified that scAAV2 septuplet-Y-F mutant vector was able to transfect mice microvascular endothelial cells with high efficiency. scAAV2 septuplet-tyrosine mutant vectors encoding the siRNAs against IRE1 α or XBP-1 or ATF6 significantly inhibited breast cancer-

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[†]Conflict of Interest

W. W. H. and the University of Florida have a financial interest in the use of AAV therapies and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work.

induced angiogenesis *in vitro* by, in part, inhibiting endothelial cell survival. Acoustic-resolution photoacoustic microscopy (ARPAM) can provide non-invasive, label-free, high resolution vascular imaging. Utilizing ARAM, we showed that intratumoral delivering the siRNAs against IRE1 α or XBP-1 or ATF6 by scAAV2 septuplet-tyrosine mutant vector resulted in a significant decrease in tumor growth and tumor angiogenesis in breast cancer xenograft models. These data have generated a proof-to-concept model with important implications for the development of novel anti-angiogenic targeted therapies for patients with breast cancer.

Keywords

breast cancer; angiogenesis; unfolded protein response; siRNA; scAAV2; photoacoustic imaging

Introduction

Sustained aberrant tumor angiogenesis plays a central role in breast cancer carcinogenesis and metastatic potential (1). Members of vascular endothelial growth factor (VEGF) family are well-known angiogenesis activators. Despite the promising activity of anti-angiogenic drugs in preclinical tumor models, targeting VEGF signaling appears to be insufficient for permanently inhibiting tumor angiogenesis in patients with breast cancers. The reasons for this are likely to be multiple and complex. Nevertheless, there remains an urgent and unmet need for novel targeted therapies for patients with resistance to current anti-angiogenic agents. An ideal anti-angiogenic model for cancer treatment should consist of three elements: i) identification and validation of rationale-based anti-angiogenic targets; ii) an adequate drug form and delivery route; iii) advanced imaging modalities allowing noninvasive detection and monitoring tumor response to anti-angiogenic agents.

In rapidly growing tumors, vascular endothelial cells face a hostile microenvironments characterized by hypoxia, nutrient deprivation and acidosis. These environmental stressors induce endoplasmic reticulum (ER) stress, and cells respond by activating the unfolded protein response (UPR) pathway (2): i) to increase the protein-folding capacity of the ER, ii) to enhance the clearance of unfolded proteins from the ER, and iii) to inhibit general protein translation in the ER. Our recent study demonstrated that under the stress conditions caused by tumor microenvironment or/and anti-VEGF therapies, endothelial cells adopt the up-regulation of IRE1 α /XBP-1 and ATF6, which may contribute to the up-regulation of molecular chaperone and in turn increase the protein-folding capacity for misfolded/unfolded VEGF and maintain VEGF intracrine signaling for endothelial cell survival (3).

In the past decades, remarkable progress has been made using siRNA. They constitute a promising new class of drugs for targeting mutant or over-expression oncogenes. Several siRNA cancer therapies have entered early clinical trials (4). Delivery of siRNA into mammalian cells is usually achieved via the transfection of double-strand oligonucleotides or plasmid encoding RNA polymerase III promoter-driven small hairpin RNA (5). Recently, retroviral and lentiviral vectors have been used for siRNA delivery, which have overcome some of the problems of poor transfection efficiency seen with the plasmid-based systems (6). However, both retroviral and lentiviral vectors undergo random integration into host

chromosomal DNA, and there is persistent risk for insertion mutagenesis (7, 8). Non-integrating adenoviral vector has also been tested for vascular endothelial gene transfer (9), but many cell types express the adenoviral receptor preventing selective endothelial cell infection and precluding clinical use (10). Furthermore, adenoviral vectors are highly immunogenic and are therefore unsuitable for long term gene expression *in vivo* (11). In the present study, we have been pursuing that further development of alternative vector systems, such as self-complementary adeno-associated virus (scAAV) vectors for their potential to transfect microvascular endothelial cells with high efficiency, given the proven safety of AAV vectors in several clinical trials (12, 13).

Adequate non-invasive imaging can help physicians to determine whether to start and when to start anti-angiogenic therapies. In particular, such imaging is essential for monitoring the tumor response to anti-angiogenic therapies because tumor shrinkage may not occur within a short period of time even when anti-angiogenic treatment is effective. Several current non-invasive imaging modalities have differing limitations for monitoring vasculature development. For instance, X-ray computed tomography (CT) needs extrinsic contrast agent and exposures patients to ionization radiation (14), positron emission tomography (PET) screening often involves extrinsic contrast agents and magnetic resonance imaging (MRI) is limited by its low temporal/spatial resolution (15). Pure high-resolution optical imaging modalities such as single-photon, multi-photon fluorescence microscopy suffer from limited imaging depth (<1mm) (16). One potential non-invasive imaging modality is photoacoustic imaging (PA) consisting of the advantages of rich optical contrast in optical imaging and high ratio of imaging depth to spatial resolution in ultrasound imaging.

In the present study, we have identified that scAAV2 septuplet-tyrosine mutant vector, in which seven surface-exposed tyrosine residues of the capsid were changed to phenylalanine, was able to infect mouse microvascular endothelial cells with high efficiency. siRNAs against IRE1 α , or XBP-1 or ATF6 were effective in decreasing breast cancer-induced angiogenesis *in vitro*. Serial non-invasive photoacoustic imaging further confirmed that intratumoral delivering the siRNAs against IRE1 α , XBP-1 or ATF6 by the scAA2 septuplet-tyrosine mutant vectors resulted in a significant decreased in tumor growth and tumor angiogenesis in breast cancer xenograft models. These data have generated a proof-to-concept model with important implications for the development of novel anti-angiogenic targeted therapies for patients with breast cancer.

Materials and Methods

Construction of scAAV2 vector for delivering siRNAs against the UPR proteins

Self-complementary AAV serotype 2 (scAAV2) and their corresponding single and multiple tyrosine-to-phenylalanine (Y-F) mutants containing a ubiquitous, truncated chimeric CMV-chicken β -actin (smCBA) promoter (17) were generated and purified by previous described methods (18). Vectors were titered by quantitative real-time PCR and re-suspended in balanced salt solution (BSS: Alcon, Forth Worth, TX). siRNA has been widely used to knock down target gene expression for a variety of purpose. However, siRNAs can provoke unspecific degradation of all cellular RNAs through the induction of the interferon (IFN) response (19). It has been shown that short, <20 bp, siRNAs do not activate dsRNA-

activated protein kinase (PKR) or TLR2 and do not result in IFN response (20). We tested siRNAs of 19 bases in length against three pre-validated targets for each of mouse UPR proteins (IRE1 α , XBP-1 and ATF6) from Life Technologies Corporation (Life Technologies, Grand Island, NY) and selected one siRNA with highest knockdown efficiency for each protein (Supl. Fig. 2A). The cDNAs encoding IRE1 α (5'-GGATGTAAGTGACCGAATA-3'), XBP-1 (5'-CAGCTTTTACGGGAGAAAA-3'), ATF6 (5'-GGATCATCAGCGGAACCAA-3') and scramble (with the same nucleotide composition of IRE1 α cDNA) were ligated into scAAV2 backbones using *NotI* and *Sall* (Fig. 1C). The integrity of the siRNA coding region was confirmed by sequence analysis (data not shown).

scAAV2 infection of cells in vitro

Infection of scAAV2 vectors were carried out as previous described (21). Briefly, cells were plated in 14-mm microwells in a 35-mm Petri dish (MatTek, Ashland, MA) and reached up to 60-70% confluence. scAAV2 vectors were diluted in serum-free medium to achieve the desired multiplicity of infection (MOI). MOI was defined as the number of genome containing vector particles per targeting cell. The cells were rinsed in PBS and the virus dilutions were added. After 1 hour, complete medium was added to the cells. Infected cells were maintained at 37°C in 5% CO₂ for 3 days. All infections were performed in triplicate. Each vector was used to infect 24 wells in total. Each "sample" was pooled from eight wells, making a final sample count of three.

Microscopy and fluorescence activated cell sorting (FACS) analysis

Three days post infection, cells were observed using bright-field microscopy to ensure 100% confluence. Cells were then analyzed by an OlympusIX81-DSU Spinning Disk Confocal microscopy (Olympus America, Inc., Center Valley, PA) and digital photomicrographs were captured by imaging software-SlideBook™ (Intelligent Imaging Innovations, Denver, CO). For FACS analysis, the cells were dissociated with Accutase solution (MP Biomedicals, Solon, OH) and collected by centrifugation (200×g for 5 min). 10,000 cells per sample were counted and analyzed using a BD LSR II flow cytometer. Uninfected control cells were also counted and analyzed to establish transduction efficiency of baselines.

Cell culture

Mice microvascular endothelial cells (MMECs) were isolated from mouse brain tissue using modified methods as previous described (22). Briefly, freshly isolated mouse brains were homogenized, and after trapping on an 83- μ m nylon mesh, they were transferred into an enzyme mixture including 500 μ g/ml collagenase, 200 μ g/ml Pronase, and 200 μ g/ml DNase, at 37°C for 30 min. The resultant vessel fragments were trapped on a 53- μ m mesh, washed, and pelleted, and cells were plated in microvascular endothelial cell basal medium with growth supplement (Life Technologies™, Grand Island, NY) at 37°C in 5% CO₂ for 3 days. Purification of MMECs was achieved as previous described (23). Briefly, the cells collected from primary culture T25 cm² flask by trypsinization were reacted with rat anti-VE-cadherin antibody (Biolegend, Inc, San Diego, CA) for 10 min at 4°C. After washing, the cells were mixed with pan rat IgG beads (Life Technologies™, Grand Island, NY) for 20

min at 4°C with gentle tilting and rotation. The samples were placed in a magnetic particle concentrator (DynaMag™-15, Life Technologies™, Grand Island, NY) to immunoadsorbed cells for 2 min at room temperature. The bead-bound cells were then re-suspended in release buffer for 15 min. The purified cells were collected by centrifugation and seeded into T25 cm² flask in complete medium. Cells were subculture at a ratio of 1:2 on reaching confluence. The cells were used within three passages.

NeuT (a mice breast cancer cell line) was isolated from MMTV-c-neu transgene mice as described (24). NeuT EMTCL2 served as a malignant variant of NeuT and was developed by consecutive serial implantation of NeuT cells in athymic nude mice. The mouse breast cancer cells were grown in RPMI1640 supplemented with 10% fetal bovine serum and gentamycin (Gibco-BRL, MD) to 70-80% confluence subjected to sequential experiments or sub-cultured at ratio of 1:3 in fresh complete medium supplemented with 10% FBS.

For *in vitro* co-culture systems, NeuT or NeuTEMTCL2 cells were seeded onto 6-well Transwell inserts with 0.4 µm pores (Corning Life Sciences, MA) in a 6-well plate for 72 hr. MMECs were cultured in a separate 6-well plate. Confluent breast cancer cells on Transwell inserts were then transferred on top of MMECs and placed at 37°C for 48 hr prior to sequential experiments.

***In vitro* angiogenesis assay**

In vitro angiogenesis was measured by *in vitro* tube formation assay which reflects a combination of proliferation, migration and tube formation of microvascular endothelial cells (20). Briefly, MMECs were plated sparsely (2.5×10^4 /well) on 24-well plates coated with 12.5% (v/v) Matrigel (BD, Franklin Lakes, NJ) and left overnight. The medium was then aspirated and 250 µl/well of 12.5% Matrigel was overlaid on the cells for 2 hr to allow its polymerization, followed by addition of 500 µl/well of basal medium MCD131 with 10% fetal calf serum (FCS) for 48 hr. The culture plates were observed under a phase contrast microscope and photographed at random in five fields ($\times 10$). The tubule length (mm/mm²) per microscope field was quantified.

Apoptosis assay

Apoptosis was evaluated using FITC-conjugated annexin V/propidium iodide assay kit (R&D System, Minneapolis, MN) based on annexin-V binding to phosphatidylserine exposed on the outer leaflet of the plasma membrane lipid layer of cells entering the apoptotic pathway. Briefly, MMECs were collected by EDTA detachment and centrifuged (200×g for 5 min), washed in PBS and re-suspended in the annexin V incubation reagent in the dark for 15 min before flow cytometric analysis. The analysis of samples was performed by flow cytometric analysis on BD LSRII flow cytometer (BD Biosciences, MD). 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 forward light scanner and 90° light scatter before being analyzed using in-house software.

Proliferation assay

Crystal violet assay was conducted as previously described (25). Briefly, MMEC suspensions (100 μ l) were incubated in each well of 96-well plates at 1×10^5 cell/ml. The cells were fixed in 4% paraformaldehyde in PBS for 15 min. After being washing with distilled water, the plates were stained with 0.1% crystal violet solution for 20 min. The plates were washed with water and allowed to be air dry. Acetic acid (100 μ l of 33%) was added to each well for extraction of dye. Absorbance of the staining was measured by an automatic microtiter plate reader at 590 nm.

Mice breast cancer xenograft models

All animals used in this study were maintained at the animal facility of the University of Florida and handled in accordance with institutional guidelines. Athymic female nude mice (nu/nu) at 5 to 8 weeks were purchased from Charles River Laboratories (Charles River Laboratories, Inc., Wilmington, MA) and caged in groups of 5 or fewer. Mice breast cancer xenografts were established by subcutaneous injection of 1×10^5 NeuT or NeuT EMTCL2 cells into the mammary fat pads of the mice. Tumor volume was calculated from caliper measurements of the large (a) and smallest (b) diameters of each tumor using formula $a \times b^2 \times 0.4$. Three days after inoculation, most tumors had grown to $\sim 30 \text{ mm}^3$. All mice were euthanized when the tumor volume in the non-treated group reached $\sim 1000 \text{ mm}^3$.

For *in vivo* siRNA knockdown of the UPR proteins, mice with similarly sized tumors were divided into two groups (siRNA treatment and scrambled control). Mice were anesthetized with a mixture of ketamine (85mg/kg)/xylazine (4 mg/kg) and were intratumoral injected (10 μ l) with scAAV2 septuplet-tyrosine mutant vectors encoding siRNAs against IRE1 α or XBP-1 or ATF6 or scrambled siRNA at a titer of 2×10^{13} genome copies per ml.

Photoacoustic (PA) imaging systems and noninvasive monitoring in vivo tumor angiogenesis

In this study, photoacoustic microscopy (ARPAM) system was used to monitor vasculature change in breast cancer xenograft models. A short laser pulse of 6 ns duration was generated at 10Hz repetition rate and wavelength of 532 nm by a Nd:YAG laser (NL 303HT from EKSPILA, Lithuania, Supl. Fig.3). The laser beam was split into two sub-beams and coupled into separated optical fiber bundles with optimal illumination. A focused ultrasound transducer (50MHz, 3 mm aperture and 6 mm focal length) was used to receive induced photoacoustic waves. PA signals amplified by two different amplifiers (one was 17 dB from 100 kHz to 1 GHz and the other was 20 dB from 20 MHz to 3 GHz) were digitized by a 8-bit data acquisition board (NI5152, National Instrument, Austin, TX) at a sample rate of 250 MS/s. A 2D moving stage mounted with the optical fiber bundles and the transducer carried out 2D raster scanning with lateral resolution of 61 μ m and axial resolution of 15 μ m, triggered by laser pulse. The two-dimensional transverse scanning combined with the depth-resolved ultrasonic detection generated 3D PA images displayed in maximum amplitude projection (MAP). 3D PA signals were processed by Hilbert transform (26) and normalized the data to the same scale (0-256) with a threshold (-6dB level). The volumes of the blood vessels were calculated by integrating the corresponding image voxels (1 for blood vessel

and background being set to 0). Entropy was calculated from normalized MAP of each PA image at the same scale (0-256).

During longitudinal PA imaging (20 min/each time), mice were kept anesthetized and their body temperatures were maintained at 37°C by a temperature controlling pad. Mice skin at the tumor inoculation sites were gently depilated before tight contacting with the membrane-sealed imaging window in the bottom of the water tank with ultrasound gel for PA imaging.

Statistics analysis

All experiments were repeated at least 3 times. Analysis of variance (ANOVA) was used to assess the transduction efficiency of AAV2 vector infection. The unpaired Student *t*-test was to assess statistic significant for the rest data obtained from *in vitro* studies (*in vitro* angiogenesis assay, apoptosis assays and crystal violet assays) as well as *in vivo* animal experiments including tumor volume, entropy and normalized vessel volume. The data were expressed as mean±SEM. Statistical analysis was conducted by GraphPad Prism (version 5.01; GraphPad Software, Inc., La Jolla, CA) with *p*<0.05 considered statistically significant.

Results

scAAV2 septuplet-tyrosine mutant exhibits higher transduction efficiency in MMECs

AAV2 based vectors have, to date, exhibited higher transduction efficiency when to targeting transgene expression to vascular endothelium than other native AAV (27). A construct containing a truncated version of the hybrid chicken β-actin promoter/CMV promoter (smCBA) driving green fluorescent protein (GFP) was packaged into scAAV2 containing combinations of up to 7 surface-exposed tyrosine residues (252, 272, 444, 500, 700, 704 and 730F) in the capsid VP3 protein. Combinations tested for transduction of mouse microvascular endothelial cells (MMECs) included: triple (Y444+500+730F), quadruple (Y272+444+500+730F) and septuplet (Y252+272+444+500+700+704+730F). The transduction efficiency of each of the combination tyrosine-mutant vector at MOIs ranging from 100 to 10,000 was analyzed (as measured by GFP expression) and compared with unmodified scAAV2 in MMECs 72 hr later by flow cytometry. We present data generated from cells infected only at an MOI of 10,000 as it is representative of trends seen across all MOIs. We demonstrated that the transduction efficiency of the all tyrosine-mutant vectors was significantly higher compared with the unmodified scAAV2 (Fig. 1A; Supl. Fig. 1A). Specifically, the transduction efficiency of the septuplet-tyrosine mutant vectors was maximal, ~173-fold higher than the unmodified vector (Fig. 1A). Similarly, the triple-mutant and the quadruple mutant also had significant enhancement in GFP expression (~2- and 6-fold, respectively) (Fig. 1A).

We wanted to further confirm the scAAV-2 septuplet-tyrosine mutant transduction efficiency of tumor endothelial cells in breast cancer xenograft models. AAV2-smCBA-hGFP containing combination tyrosine-mutant vector was intratumoral injection (10 μl) at a titer of 2×10^{13} genome copies per ml. Very weak GFP expression was detected in the frozen sections of breast cancer xenografts treated with triple tyrosine-mutant vectors, but the

breast cancer xenografts injected with quadruple tyrosine-mutant vectors expressed moderate levels of GFP in the vasculature. As a comparison, the vasculature in the breast cancer xenografts injected with septuplet-tyrosine mutant vectors expressed high levels of GFP (Supl. Fig. 1B).

Septuplet tyrosine-mutations improve the transduction efficiency of scAAV2-mediated siRNAs infection in MMECs

Our previous study showed a crucial role of the unfolded protein response pathway in breast tumor-angiogenesis (3). Based on the observation (Fig. 1A; Supl. Fig. 1A) that scAAV2 septuplet-tyrosine mutant exhibited the most transduction efficiency in MMECs, we constructed a septuplet-tyrosine mutant AAV2 vector containing smCBA driving mCherry together with three siRNA sequences against IRE1 α , XBP-1, ATF6, respectively and a matched control containing a scrambled siRNA (Fig. 1B, C). To verify that vector containing the inserted siRNA sequence transduced MMECs, scAAV2 septuplet-tyrosine mutant vector containing the scrambled siRNA was used to infect MMECs at MOIs ranging from 400 to 12,000. As shown (Supl. Fig. 1C), mCherry expression was detected by fluorescence microscopy 72 hr post-infection. The transduction efficiency of scAAV2 septuplet-tyrosine mutant vector was elevated with the increased MOI of the vector (Fig. 1D).

siRNA knockdown of the UPR proteins decreased NeuT EMTCL2-induced *in vitro* angiogenic activity of endothelial cells

The balance between protein synthesis and protein proper folding in ER is essential for cellular homeostasis and survival. Many disease conditions that affect protein folding tip this balance and trigger an ER membrane-bound protein stress pathway known as the unfolded protein response (UPR). Our recent study demonstrated that malignant breast cancer cells significantly up-regulated three UPR proteins including IRE1 α , XBP-1 and ATF6 in the endothelial cells, implicating possible pro-angiogenic roles for UPR (3). To address the possible involvements of the UPR proteins for pro-angiogenic activity, MMECs were co-cultured with NeuT EMTCL2 as described in *Materials and Methods*. Since late stage of angiogenesis requires morphologic alterations of endothelial cells, leading to lumen formation, NeuT EMTCL2 cell-induced angiogenesis in MMECs was studied by measuring a network of capillary-like tubes in an *in vitro* 3-D Matrigel model which reflects a combination of proliferation, migration and tubule formation of endothelial cells (25). MMECs pre-treated with the scrambled siRNA exhibited a significant *in vitro* angiogenesis in contrast to non-treatment (Fig. 3A, B; Supl. Fig. 2B). As expected, the siRNAs against the UPR proteins markedly reduced the angiogenic responses of MMECs to the NeuT EMTCL2 cells' stimulation (Fig. 2A, B). When compared with scrambled siRNA, the siRNA against IRE1 α or XBP-1 or ATF6 caused a significant reduction of *in vitro* angiogenesis (Fig. 2A, B; Supl. Fig. 2B).

siRNA knockdown of the UPR proteins down-regulated the survival of endothelial cells

The effect of knockdown of IRE1 α , XBP-1 or ATF6 on the survival of MMECs was analyzed by apoptosis and proliferation. The knockdown of XBP-1 elicited a maximal

apoptotic response in MMECs, as evidenced by a significant 8-fold increase in the apoptotic cells compared to the scrambled siRNA (Fig. 3A, B). siRNAs against IRE1 α or ATF6 caused a lesser, yet still significant apoptotic response in MMECs (4-fold increase) (Fig. 3A, B). Consistently, crystal violet assay showed NeuT EMTCL2 stimulation significantly increased MMECs proliferation, which was inhibited by the knockdown of XBP-1 at maximal reduction of ~70% (Fig. 3C). Both of siRNAs against IRE1 α and ATF6 also caused a similar (~50%) reduction in NeuT EMTCL2-induced survival of MMECs (Fig. 3C).

Different malignant mouse breast cancer cells induced differential angiogenic responses in breast cancer xenograft models were monitored by serial photoacoustic (PA) imaging

To test the feasibility of noninvasively monitoring *in vivo* tumor vasculature development, we performed PA imaging for two mice breast cancer xenograft models. In one model, mice were injected with NeuT cells, while the other model was generated via injection of NeuT EMTCL2 cells. Serial PA imaging were carried out on day 3, 5, 7 and 9 after tumor inoculation, respectively. The inoculation of NeuT cells only caused a minimal tumor growth compared to a rapid tumor growth in NeuT EMTCL2 model which tumor volume reached ~250 mm³ by day 11 (Fig. 4A, D). Additionally, NeuT EMTCL2 cells induced a significant vasculature development evidenced as gradual splitting large host blood vessels to small ones. This was noticeable on day 5 and peaking on day 9. Using PA resolution and depth-section capability, we determined the kinetics of the vessel density changes surrounding tumor mass using the Entropy method (28). Entropy is a statistical measure of randomness that can be used to characterize the texture of the input image. In NeuT model, Entropy did not reveal a significant increase in vessel density. However, Entropy clearly indicated that NeuT EMTCL2 inoculation resulted in a increased vessel density by 20 % compared with NeuT implantation (Fig. 4B). Another PA image extraction analysis was normalized vessel volume changes that closely echo Entropy data. As shown (Fig. 4C), NeuT EMTCL2 implantation resulted in a steep increase in vessel volume, whereas there was no significant change in tumor vessel volume detected in the NeuT model (Fig. 4C).

Knockdown of the UPR proteins significantly inhibited NeuT EMTCL2-induced *in vivo* angiogenesis

To confirm the purported anti-angiogenic activities of the siRNAs against the UPR proteins (Fig. 5A, B), mice of NeuT EMTCL2 xenograft models were divided into four groups including one scrambled treatment, three siRNA treatments (IRE1 α , XBP-1 and ATF6) delivered by intratumoral injection of scAAV2 septuplet-tyrosine mutant vectors encoding the siRNAs against the UPR proteins. The tumor volume reached ~250 mm³ after 11 day of NeuT EMTCL2 inoculation in the scrambled siRNAs treatment group (Fig. 5D). Figure 3D also shows that the knockdown of IRE1 α and XBP-1 both exhibited significant decreased tumor growth to a similar extent (~45% on day 11) and the ATF6 siRNA was even more effective on inhibition of tumor growth (~54% by day 11). Serial PA imaging was employed to monitor and evaluate the development of tumor vasculature in the NeuT EMTCL2 xenograft models on day 3 after NeuT EMTCL2 inoculation followed on day 5, 7, 9 and 11. Entropy method delineated a significant decrease in vessel density (25%) by day 9 and sustained at that level thereafter in the IRE1 α siRNA treatment group compared with the

scrambled group (Fig. 5B). The NeuT EMTCL2 xenograft models treated with the siRNAs against XBP-1 or ATF6 evidenced a steady decrease in vessel density with statistic significant in comparison with the scramble group (Fig. 5B). All three siRNAs exhibited significantly steady decrease in vessel volume compared with the scrambled siRNA treatment (Fig. 5C).

Discussion

The UPR is generally considered to involve 3 signaling pathways from the ER membrane: IRE1 α (inositol-requiring protein 1 α), ATF6 (activating transcription factor 6) and PERK (PKR-like ER kinase). Upon activation, IRE1 α cleaves an intron of XBP-1 (X-box-binding protein 1) mRNA, resulting in a frame shift and the translation of the spliced form of XBP-1, a 41kDa basic leucine zipper (bZIP) family transcription factor that induces genes involved in UPR response (29). IRE1 α also cleaves many mRNAs, reducing the load of proteins in the ER, in favor of restoration of ER homeostasis (30). During ER stress, ATF6 is transported to the Golgi apparatus where it is cleaved and release a 50kDa bZIP to the nucleus to induced expression of ER chaperones (31). ATF6 also supports ER biogenesis independently of XBP-1 (32).

IRE1 α /XBP-1, ATF6 and PERK are aberrantly expressed in many types of cancers. The IRE1 α /XBP-1 pathway is important for tumor growth under deteriorating conditions of microenvironment. The levels of XBP-1 correlate with glucose starvation (33). XBP-1 splicing can be detected even in relatively small tumors in several genetic models for breast cancer, implicating that ER stress may occurs from the early stage of tumor development. Indeed, XBP-1 knockout caused cancer cell death and XBP-1 knockout cells produced smaller tumors in xenograft models. Over-expression of IRE1 α /XBP-1 restored tumor growth under these conditions. The significance of ATF6 in tumor development is less well characterized. However, ATF6 was found to be over-expressed and activated alone with increased XBP-1, suggesting a coordination and interdependency between IRE1 α /XBP-1-dependent and ATF6-dependent systems (34). ATF6 served as stress survival factor for dormant but not proliferative squamous carcinoma cells via GTPase and mTOR (35). A role for ATF6 pathway is further supported by an *in vitro* study in which activation of ATF6 resulted in apoptosis resistance of melanoma cells (36). The UPR pathway is a general mediator of vascular endothelial cell dysfunction in inflammatory disorders (37). Furthermore, our recent studies suggested that activation of IRE1 α /XBP-1 and ATF6 is closely related to angiogenic activity of endothelial cells, whereas endothelial PERK functions were not drastically affected by angiogenic stimulation (3). In the current study, we confirmed that selective knockdown of IRE1 α or XBP-1 or ATF6 can lead to inhibition of tumor angiogenesis and breast cancer growth using *in vitro* and *in vivo* models. However, it is plausible that selective disruption of UPR pathways (XBP-1, IRE1 α , ATF6) can suppress cancer progression. Recently, it is reported by our group that the knockdown of the UPR proteins led to attenuate the molecular chaperone- α B-crystallin. Our data suggested that α B-crystallin may be a key component in activation of the intracellular autocrine survival signaling, such as vascular endothelial growth factor (VEGF) pathway to promote cell survival under cellular stress conditions [3].

A challenge for RNAi based-therapies is the efficiency of delivery for the siRNA to the target endothelial cells. AAV vectors promoter long-term transgene expression via persistence as episomes in the nucleus, are minimally immunogenic and have an impressive safety record (38). In comparison to other cell types endothelial cells are relatively poorly transduced by AAV vector (39). Initial studies suggested sequestration of AAV2 within the extracellular matrix around endothelial cells, thereby preventing AAV2 cell surface binding and entry, was the key rate-limiting step to efficient endothelial cell transduction (40). Subsequent studies have aimed at targeting capsid to the endothelial cell surface by inclusion of peptide motifs with known affinity for vascular endothelial cells on the capsid surface of AAV2 (41). This approach has shown improvements in vector transduction. However, potential of such strategies remains to be tested in preclinical and clinical models.

Recent studies have revealed that EGF-PTK-induced tyrosine phosphorylation of AAV2 capsid proteins trigger ubiquitination-dependent degradation of AAV2 (42), leading to impairment of viral nuclear transport and decrease in transduction efficacy. Mutation of surface exposed targeting tyrosine residues of AAV2 capsid to phenylalanine (Y-F) results in AAV2 vectors with significantly increased transduction efficiency (21, 43). In this study, we tested AAV2 transduction efficacy in endothelial cells using a number of AAV2 based tyrosine to phenylalanine mutants and demonstrated the septuplet-Y-F mutants by far most effective in transducing microvascular endothelial cells, ~170-fold better than unmodified AAV2. Intriguingly, our current data is different from our previous published data which showed that the quadruple-Y-F mutant was the most efficient variant in bovine retinal microvascular endothelial cells (44), suggesting that AAV2 based Y-F mutants may have different levels of efficacy in different cell/tissue types. It is noteworthy that other studies have reported differences in the ordination of efficiencies for AAV2 Y-F mutants for the other cell types, perhaps this is due to differences in the intracellular milieu (components of the ubiquitin dependent degradation pathway and tyrosine kinase) found in those cells (45).

Our results demonstrated that knockdown of IRE1 α or XBP-1 or ATF6 exerts robust anti-angiogenic activities and suppresses tumor growth via local intratumorally administration of scAAV2 septuplet-tyrosine mutant vectors encoding the corresponding siRNAs. Although there are controversies surrounding the approaching of local intratumoral gene therapy, this strategy may offer several advantages for anti-angiogenic therapy: 1) intratumoral gene therapy can reduce the risk of widespread anti-angiogenesis resulting from systemic administration of an anti-angiogenic agent; 2) gene transfer can lead to a local accumulation of the anti-angiogenic agents; 3) anti-angiogenic gene therapy does not require that genes be transferred to all target cells.

Photoacoustic microscopy (PAM), a hybrid imaging technique combining high optical contrast and high ratio of imaging depth to spatial resolution of ultrasound, has multiple advantages (46). First, it can identify red blood cell perfused microvasculature supplying oxygen for tissues through the endogenous hemoglobin contrast. Second, unlike conventional optical microscopic, PAM is 100 time less acoustic scattering in biological tissues, thus greatly improving tissue transparency. Thirdly, the high contrast of hemoglobin at 532 nm (>100:1) enables using of low-level laser exposure. Recently, a study showed noninvasively label-free serial imaging of red blood cell perfused vasculature in ear of small

mice by using optical-resolution photoacoustic microscopy (ORPAM) (47). The authors demonstrated that the spatial resolution is high enough ($<2\ \mu\text{m}$) to image a single capillary and even a single red blood cell. However, the imaging depth of 400-700 μm may limit ORPAM application for monitoring neo-vascularization during tumor growth. In contrast, acoustic-resolution PAM with imaging depth reaching 3 mm is more adequate for monitoring tumor vascular development along with tumor growth (48). Using our established acoustic-resolution PAM systems, we were able to serially image dynamics of tumor neo-vascular network development in mouse breast cancer xenograft models. More importantly, it was first time that we successfully monitored and determined differential tumor response to siRNAs against IRE1 α , XBP-1 and ATF6. These were delivered using AAV2 septuplet-tyrsoine mutant vectors by intratumoral injection in murine breast cancer xenograft models. The acoustic-resolution PAM systems system used here equips a lower repetition rate (10Hz) pulse-laser resulting in relative longer scanning time (20 min) in comparison with some commercial high repetition rate ($>100\text{Hz}$) pulsed laser enabling completing each scanning within 2 min. To apply PA imaging in the clinic in the future, it will be greatly beneficial if we can develop quantitative PAM systems enabling extraction of more functional information, such as oxygen saturation, concentration of hemoglobin correlating to tumor growth and anti-angiogenic therapy.

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Figure. 1.

Comparative analysis of scAAV2-mediated transduction of MMECs. A, cells were infected with the WT or the triple-, quadruple- or septuplete-tyrosine mutant scAAV2-hGFP vectors at a of multiplicity infection (MOI) of 10,000 vgs/cell. Transgene expression was detected by fluorescence microscopy, 72 hr post-infection. Quantitative analysis of AAV2 transduction efficiency in MMECs is shown in arbitrary units calculated by multiplying the percentage of positive cells by the mean fluorescence intensity in each sample. B, shows a scAAV2 construct with smCBA driving expression of siRNAs against mice UPR proteins. C, transduction efficiency of scAAV2 septuplet-tyrosine mutant vectors in MMECs. D, a comparison of mCherry expression at different MOIs as indicated shown in arbitrary units calculated by multiplying the percentage of positive cells by the mean fluorescence

intensity. Each value represents the average of three samples (eight pooled wells of a 24-well plates/sample), based on 10,000 counted cells.

Figure. 2.

Pro-angiogenic role of the UPR proteins on endothelial cells. Mice microvascular endothelial cells (MMECs) were co-cultured with NeuT EMTCL2 for 48 hr followed by treated with scAAV2 encoding siRNAs against UPR proteins. A, MMECs were cultured between two layers of Matrigel for 48 hr. Morphometric analysis of the degree of tubule formation was then performed. B, representative photomicrographs of microscope fields showing tubule formation.

Figure. 3. Survival role of the UPR proteins on endothelial cells. A, annexin V-propidium iodide-positive cells were shown by flow cytometric analysis (top right, late stage apoptosis; bottom right, early apoptosis). B, cell apoptosis was expressed as a percentage of apoptotic cells in the total cell population. C, the cell proliferation was assessed by crystal violet staining.

Figure. 4.

Serial noninvasive photoacoustic (PA) imaging of the developing tumor vasculature and quantitative analysis for mice breast cancer xenograft models. Mice breast cancer xenografts were established by subcutaneous injection of 1×10^5 NeuT or NeuT EMTCL2 cells into the mammary fat pads of the mice. Serial PA imaging was performed on the same tumor inoculation site on day 3, 5, 7 and 9 post tumor inoculations. A, representative serial PA images of NeuT model (first and second panels) and NeuT EMTCL2 model (third and fourth panels). B, Entropy extraction for change in the vessel density over different time points as

indicated. C, shows comparative analysis of normalized vessel volumes at different time point as indicated. D, tumor volume was calculated from daily caliper measurements of the large (a) and smallest (b) diameters of each tumor using formula $a \times b^2 \times 0.4$. Representative images of H&E staining for breast cancer tissue sections of NeuT (top) and NeuT EMTCL2 (bottom) xenograft models.

Figure. 5.

Knockdown of the siRNAs against the UPR protein resulted in decreased tumor growth and tumor vasculature development in mice breast cancer xenografts. Mice breast cancer xenografts received intratumoral scAAV2 sept-mutant vector-delivered siRNAs against IRE1 α or XBP-1 or ATF6. PA imaging was performed on the same tumor inoculation site on day 3, 5, 7 and 9 post tumor inoculations. A, representative PA images of different treatments: the scrambled siRNA (top panel), IRE1 α siRNA (second panel), XBP-1 siRNA (third panel) and ATF6 siRNA (bottom panel). B, Entropy extraction for change in the

vessel density over different time points as indicated. C, shows comparative analysis of normalized vessel volumes at different time point as indicated. D, tumor volume was calculated from daily caliper measurements of the large (a) and smallest (b) diameters of each tumor using formula $a \times b^2 \times 0.4$.