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Contribution of the Unfolded Protein Response to VEGF Expression

Abstract

Tumor cells experience a limiting microenvironment due to inadequate vascularization that can affect the normal functioning of intracellular organelles. In the case of the endoplasmic reticulum, the limiting environment is further exacerbated by the high metabolic demands of the tumor cells, which together interfere with the proper maturation of nascent proteins synthesized there. The resultant accumulation of unfolded proteins activates a signal transduction pathway known as the Unfolded Protein Response, which serves primarily to protect the cell during stress and helps restore homeostasis to this organelle. As tumors expand resulting in regions that are a greater distance from functional blood vessels, they become increasingly hypoxic, which ultimately results in the activation of another stress pathway that is primarily regulated by the hypoxia inducible factor family of transcription factors (HIFs). This pathway protects cancer cells, in part, by up-regulating VEGFA, which stimulates blood flow to the tumor in a process known as angiogenesis. The interplay between these two stress pathways in tumor cell survival is relatively unexplored.

Microarray analysis of the unfolded protein response in a human medulloblastoma cell line revealed that, in addition to known targets, a large number of proangiogenic factors were upregulated. Real-Time PCR analyses confirmed that four of these factors, VEGFA, FGF2, angiogenin and IL8, were transcriptionally up-regulated in multiple cell lines by various ER stress inducers. Our studies on VEGFA regulation revealed that ATF4, a UPR-inducible transcription factor, bound to the mouse and human VEGFA promoters. Using a combination of mouse embryonic fibroblasts and human neuroblastoma cell lines that are deficient in this transcription factor, we demonstrated that ATF4 binds to the VEGFA promoter and contributes significantly to VEGFA expression in response to ER stress. We also found that VEGFA mRNA stability is increased in response to UPR activation, via activation of AMP kinase, demonstrating that increased mRNA levels occur at two regulatory points. In keeping with the increased mRNA levels, we found that VEGFA protein is secreted at levels as high as or higher than that achieved in response to hypoxia. Because the inadequate microenvironment experienced by solid tumors is expected to activate both UPR and HIF signaling pathways, we tested for possible interaction between them. Our studies show that VEGFA transcription rate and secreted protein levels are induced to a greater extent when both of these pathways are activated together as compared to each stress alone. Although we demonstrated a synergy between the two stress pathways experienced by tumor cells, surprisingly we found that this was not through the combined effects of the two different transcription factors, but instead that UPR activation can enhance HIF signaling, which has implications for other HIF targets that aid in tumor survival.

Our results indicate that the UPR plays a significant role in inducing positive regulators of angiogenesis. In the case of VEGFA expression this occurs at transcriptional, posttranscriptional and post-translational levels and is likely to have widespread implications for promoting angiogenesis in response to normal physiological cues as well as in pathological conditions like cancer.

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In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Ethel Rose Pereira
May 2012

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DEDICATION

To my father, who always told me to '*Be the Best of Whatever You Are*'
His smile will always be my inspiration.

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It has been six years of graduate school, a long and fulfilling journey that ironically, but correctly, brings me to write the first page of my dissertation at the end. This work, and more importantly my future as a scientist, would not be possible, without the continuous and endearing support of Dr. Linda Hendershot. Dr. Hendershot without doubt is the best advisor a graduate student could wish for and, in this regard, my appreciation I stress. Dr Hendershot has instilled in me values I will treasure throughout my future career as a scientist. She has always been my source of constant encouragement and taught me the value of focus and persistence. I thank Dr. Hendershot for having faith in me and giving me the liberties to pursue my project in her laboratory. I am so glad to have had the opportunity to have been part of her family at St. Jude (a.k.a. The Hendershot Lab). It has truly made living in Memphis, my home away from home, a wonderful experience.

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ABSTRACT

Tumor cells experience a limiting microenvironment due to inadequate vascularization that can affect the normal functioning of intracellular organelles. In the case of the endoplasmic reticulum, the limiting environment is further exacerbated by the high metabolic demands of the tumor cells, which together interfere with the proper maturation of nascent proteins synthesized there. The resultant accumulation of unfolded proteins activates a signal transduction pathway known as the Unfolded Protein Response, which serves primarily to protect the cell during stress and helps restore homeostasis to this organelle. As tumors expand resulting in regions that are a greater distance from functional blood vessels, they become increasingly hypoxic, which ultimately results in the activation of another stress pathway that is primarily regulated by the hypoxia inducible factor family of transcription factors (HIFs). This pathway protects cancer cells, in part, by up-regulating *VEGFA*, which stimulates blood flow to the tumor in a process known as angiogenesis. The interplay between these two stress pathways in tumor cell survival is relatively unexplored.

Microarray analysis of the unfolded protein response in a human medulloblastoma cell line revealed that, in addition to known targets, a large number of proangiogenic factors were up-regulated. Real-Time PCR analyses confirmed that four of these factors, *VEGFA*, *FGF2*, angiogenin and *IL8*, were transcriptionally up-regulated in multiple cell lines by various ER stress inducers. Our studies on *VEGFA* regulation revealed that *ATF4*, a UPR-inducible transcription factor, bound to the mouse and human *VEGFA* promoters. Using a combination of mouse embryonic fibroblasts and human neuroblastoma cell lines that are deficient in this transcription factor, we demonstrated that *ATF4* binds to the *VEGFA* promoter and contributes significantly to *VEGFA* expression in response to ER stress. We also found that *VEGFA* mRNA stability is increased in response to UPR activation, via activation of AMP kinase, demonstrating that increased mRNA levels occur at two regulatory points. In keeping with the increased mRNA levels, we found that *VEGFA* protein is secreted at levels as high as or higher than that achieved in response to hypoxia. Because the inadequate microenvironment experienced by solid tumors is expected to activate both UPR and HIF signaling pathways, we tested for possible interaction between them. Our studies show that *VEGFA* transcription rate and secreted protein levels are induced to a greater extent when both of these pathways are activated together as compared to each stress alone. Although we demonstrated a synergy between the two stress pathways experienced by tumor cells, surprisingly we found that this was not through the combined effects of the two different transcription factors, but instead that UPR activation can enhance HIF signaling, which has implications for other HIF targets that aid in tumor survival.

Our results indicate that the UPR plays a significant role in inducing positive regulators of angiogenesis. In the case of *VEGFA* expression this occurs at transcriptional, post-transcriptional and post-translational levels and is likely to have widespread implications for promoting angiogenesis in response to normal physiological cues as well as in pathological conditions like cancer.

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LIST OF ABBREVIATIONS

AMPK	AMP activated protein kinase
ARE	adenylate rich element
ATF4	activation transcription factor 4
ATF6	activating transcription factor 6
ATP	adenosine 5'-triphosphate
ECM	extracellular matrix
eIF2 α	eukaryotic initiation factor 2 α
ER	endoplasmic reticulum
FGF	fibroblast growth factor
HIF	hypoxia-inducible factor
HRE	hypoxia responsive element
HUVECs	human umbilical vein endothelial cells
IL6	Interleukin 6
IL8	Interleukin 8
Ire1	inositol requiring enzyme
JNK	c-Jun N-terminal kinase
MEFs	mouse embryonic fibroblasts
mRNA	messenger ribonucleic acid
NF κ B	nuclear factor kappa B
PDGF	platelet derived growth factor
PERK	PKR-like ER kinase
PKR	protein kinase R
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau tumor suppressor protein
XBP-1	X-box containing protein

CHAPTER 1. INTRODUCTION¹

Angiogenesis

The continued synthesis, sprouting and migration of blood vessels from pre-existing ones and remodeling of the vasculature is known as angiogenesis [1]. This process is required for normal development and homeostasis in a healthy organism. The complex network of tubular structures consisting of blood and lymphatic vessels that make up the vascular system are formed primarily by endothelial cells and serve to transport oxygen, nutrients, and cell signaling molecules between various tissues and organs in vertebrates. In addition to its role in normal physiology, increased angiogenesis contributes to the progression of several pathological conditions including cancer, macular degeneration, cardiac and inflammatory diseases [1]. Alternatively, the malformation or dysfunction of the vasculature can result in decreased blood supply to tissues causing ischemia [2]. Thus, a fine balance between proangiogenic factors that promote endothelial cell growth and antiangiogenic factors that limit it must be achieved to maintain a normal, functioning vasculature. Understanding the mechanisms by which this balance is maintained during normal physiology or how it is disrupted in disease conditions has been the focus of a large number of studies in recent years. [3]

Proangiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs), and IL8 are released by cells experiencing inadequate oxygen and nutrient supplies. These proangiogenic factors act as ligands that bind to specific receptors on endothelial cells causing them to release matrix metalloproteinases that degrade the extracellular matrix, which allows the endothelial cells to migrate toward the angiogenic stimulus and to proliferate in order to establish new blood vessels [4]. The predominant and best-studied proangiogenic factor is VEGFA, a homodimeric heparin binding glycoprotein that is produced in several isoforms due to alternative splicing (Figure 1-1). All VEGF isoforms are synthesized and processed in the endoplasmic reticulum (ER) and transported through the secretory pathway [5]. There are five different splice variants of the human VEGFA gene (206, 189, 165, 145 and 121) that have identical N-termini, including an ER targeting sequence, but different properties due to the inclusion of various exons at their C-termini that encode the ability to bind heparin and heparin-sulfate containing proteins. This is an important property that distinguishes the isoforms from each other as it impacts their ability to diffuse away from the cells or become associated with the extracellular matrix (ECM). As shown in Figure 1-1 the heparin-binding region for VEGFA is largely encoded by exons 6 and 7. Most cell types produce several VEGF isoforms simultaneously, although, 121, 165 and 189 are the predominant ones. VEGF₁₄₅ has

¹ Parts of this chapter were adapted with permission. Pereira, E., Preston, A. and Hendershot, L.M., UPR Activation in Cancer Cells: A Double-Edged Sword, in Endoplasmic Reticulum Stress in Health and Disease, 2012, Springer. In press [3].

Figure 1-1. Gene structure of human *VEGFA*. (A) *VEGFA* spans 16,272 bp of chromosome 6p12 and consists of eight exons. (B) Alternate 5' and 3' splice sites in exons 6, 7 and 8 generate multiple isoforms (C) Table indicating if the various VEGF isoforms can bind heparin or not. Adapted with permission. Nowak, D.G., J. Woolard, E.M. Amin, O. Konopatskaya, M.A. Saleem, A.J. Churchill, M.R. Lodomery, S.J. Harper, and D.O. Bates, 2008. Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors. *Journal of cell science*. **121**: 3487-95 [6].

restricted expression, and is produced mainly by cells derived from reproductive organs. There are two receptor tyrosine kinases (RTKs), VEGFR1 and VEGFR2 that can bind VEGFA on their immunoglobulin-like extracellular domains. Early studies identified VEGF binding to its receptors located on endothelial cells *in vitro* and *in vivo*, however, it is now clear that VEGF receptors are also found on bone marrow derived cells. The function of VEGFR1 signaling depends on the developmental stage of the animal and the cell type. Some of the functions of VEGFR1 signaling are to regulate monocyte and macrophage migration, induce expression of MMP9 thus facilitating tumor metastases and release growth factors like IL6. VEGFR1 can occur as an alternatively spliced, soluble form that negatively regulates the activation and signaling of VEGFR2. Soluble VEGFR1 can preferentially bind to VEGFA and thus act as a 'trap' and reduce the accessibility of VEGFA to bind to VEGFR2. VEGFR1 knockout animals are embryonic lethal at E8.5-9.0 due to increased hemangioblast and vascular disorganization. VEGFR2 signaling is very important in mediating mitogenic, angiogenic and permeability enhancing effects of VEGF. VEGFR2 signaling plays an important role in developmental angiogenesis, vasculogenesis and hematopoiesis. VEGFR2 knockout animals are also embryonic lethal at E8.5-9.5 due to defective blood-island formation and vasculogenesis [7].

Regulation of VEGF Expression

The HIF (hypoxia inducible factor) pathway is the best-characterized cellular stress pathway that leads to the up-regulation of proangiogenic factors in response to inadequate oxygen delivery [8]. Transcriptional responses to hypoxia are largely regulated by HIF, a heterodimeric protein composed of an oxygen-labile α subunit and a constitutively expressed β subunit [9]. There are two α subunit members, HIF1 α and HIF2 α , which are continuously synthesized and rapidly degraded under normal physiological conditions. The oxygen-dependent degradation of the HIF- α -subunit is mediated by hydroxylation of proline 402 and proline 564 by prolyl hydroxylase domain protein 2 [10]. Hydroxylated HIF- α binds to von Hippel Lindau tumor suppressor protein (VHL), which then interacts with the protein elongin C and recruits the E3 ubiquitin-protein ligase complex and targets the α subunit for degradation via the 26S proteasome [11]. In addition, hydroxylation of asparagine 803 inhibits the interaction of the α subunit with its transcriptional co-activators p300 and CREB binding protein [12]. However, when oxygen supplies are reduced, the activity of the hydroxylase is inhibited. As a result, the α subunit is not hydroxylated, does not bind VHL, and is stabilized. This allows it to dimerize with the β subunit and its co-activators, and the resulting complex (HIF-1 or HIF-2) binds to hypoxia-responsive elements (HRE) on the 5' flanking region of target genes like VEGF and increases its transcription [13]. In addition to the HIF pathway playing an essential role in some normal physiological processes like development of the embryo and erythropoiesis, there is also a vast amount of data demonstrating that it contributes to a number of disease states like cancer, ischemia and inflammation [13].

In addition to hypoxia leading to a HIF-dependent transcriptional up-regulation of VEGF, there are data demonstrating that the *VEGF* transcript can be stabilized by hypoxia [14]. This post-transcriptional mechanism of inducing VEGF mRNA is due to a hypoxia-induced protein complex bound to the VEGF 3'-UTR that mediates increased stability. Other HIF-independent post-transcriptional mechanisms for increasing VEGF expression have been reported. There are data showing that the stress inducible ER chaperone ORP150/GRP170 plays a role in post-translational processing/secretion of VEGFA [15]. Ectopic expression of ORP150 in C6, rat glioma cells, increased VEGFA secretion, whereas decreasing ORP150 levels with an antisense construct resulted in the retention of VEGFA in the ER. Furthermore, tumors arising from the antisense ORP150 C6 glioma transfectants demonstrate an initial phase of growth comparable to the wild-type glioma cells, which was followed by marked regression and decreased angiogenesis within 8 days [15].

The Unfolded Protein Response

In addition to inadequate vascularization of tissues impinging on oxygen delivery to cells, this condition can also inhibit the delivery of nutrients like glucose and the removal of acidic waste products. These changes in the extracellular environment of cells alter the normal homeostasis of the endoplasmic reticulum (ER), which disrupts folding and processing of secretory pathway proteins and leads to the accumulation of unfolded proteins in this organelle [16]. This activates the UPR; a complex signal transduction pathway that is largely cytoprotective and aims to decrease the detrimental effects of accumulated unfolded proteins. If normal homeostasis is not restored during prolonged stress conditions, the UPR can induce apoptosis in these cells in order to protect the organism [17].

The UPR was first delineated in yeast as a relatively simple signaling pathway [18]. In higher eukaryotes this cellular stress pathway is mostly conserved but greatly expanded and far more complex (Figure 1-2). In mammalian cells there are three transmembrane proteins that sense ER stress through their luminal domains and activate downstream responses through their cytosolic domains. Ire1 was the first to be identified based on its homology to the yeast ER kinase that is the single UPR transducer in this organism [19]. On sensing stress in the ER, Ire1's kinase activity is activated, which in turn activates an endonuclease activity in its C-terminus that excises 26 nucleotides from the X-box binding protein-1 (XBP-1) transcript. After religation, the resulting frameshift encodes an active transcription factor XBP-1(S) that regulates the expression of several downstream elements of the UPR [20, 21]. In addition to Ire1 signaling, mammalian cells also transiently inhibit cap-dependent translation through activation of a PKR-like ER localized kinase (PERK). Activated PERK phosphorylates eIF2 α , which inhibits loading of the 80s ribosomal complex on mRNAs. The resulting inhibition of protein translation leads to a loss of cyclin D1 from cells causing a G1 arrest [22], thus preventing the proliferation of cells experiencing stress. Contrary to the global inhibition of translation in PERK-activated cells, synthesis of the ATF4 transcription factor is

Figure 1-2. Components of the mammalian ER stress response. The accumulation of unfolded proteins in the ER leads to the activation of three ER membrane proteins Ire1, PERK, and ATF6 that act as signal transducers. These three arms of the response are largely cytoprotective and serve to regulate downstream targets which are ultimately responsible for the up-regulation of ER chaperones, inhibition of translation, cell cycle arrest, and a number of other transcriptional responses.

increased [23]. ATF4 transactivates expression of genes such as CHOP [24], which is proapoptotic [25], and GADD34, which is a regulatory subunit of the PP1 phosphatase that dephosphorylates eIF2 α allowing translation to resume [26]. PERK also activates NF κ B expression [27], which positively regulates anti-apoptotic proteins like Bcl-2 during ER stress, thus contributing to the balance between survival and death signals. ATF6, which is the third arm of the UPR pathway, is a bZIP transcription factor. ATF6 α and β are ER-associated transmembrane proteins with luminal stress sensing domains [28]. Upon activation, the ATF6 proteins are transported to the Golgi and cleaved by S1P and S2P proteases. The cytosolically oriented transcription factor domain is liberated and regulates the transcription of XBP-1 and other important ER chaperones [29]. These signal transducers collectively act to protect the cell during stress conditions by increasing the expression of chaperones that enhance the folding capacity of the ER, by transiently inhibiting protein translation to decrease the load of unfolded proteins, and by increasing the degradative capacity of the cell. If stress conditions do not subside, the UPR activates ER localized caspase-4/12 [30, 31] to eliminate cells experiencing prolonged stress and ultimately protecting the organism.

Cancer cell metabolism is regulated partly by cues from the tumor microenvironment

Once tumor cells accumulate mutations in genes that enable them to bypass cell cycle checkpoints, apoptotic pathways and in some cases alter cancer cell metabolism, increasing studies show that the heterogeneous tumor microenvironment also contributes to the metabolic phenotype of cancer cells [32]. Normally non-dividing, differentiated cells rely primarily on mitochondrial oxidative phosphorylation to generate energy required for various cellular processes, although under reduced levels of oxygen, or anaerobic conditions, they can convert glucose to lactate to supply their energy needs through a process known as glycolysis. However, proliferating cells, including cancer cells, usually metabolize glucose through aerobic glycolysis to produce energy even when oxygen levels are sufficient. Glycolysis is an inefficient way to generate energy as the metabolism of glucose to lactate generates only 2ATPs (adenosine 5'-triphosphate) per molecule of glucose whereas oxidative phosphorylation generates 36 ATPs from one molecule of glucose [33]. The reason for tumors and other proliferating cells to switch to an inefficient method for generating energy that would seem to be contrary to their requirements has been unclear. However, recent studies propose that this phenomenon known as the “Warburg effect” results in the production of metabolic byproducts from glycolysis, such as nucleotides, amino acids and lipids, that may be beneficial to cell proliferation [33]. Due to the highly proliferative nature of tumor cells, the ability to metabolize nutrients in a manner conducive to producing new cells rather than efficient energy production may provide an explanation of why cancer cells shift over to glycolysis. The shift in cancer cell metabolism is a powerful tool used in the clinic to image tumors with increased glucose uptake through the use of ^{18}F -deoxyglucose positron emission tomography (FDG-PET). Increased glucose uptake in tumors correlates with poor prognosis and higher metabolic rate, which is suggested to contribute to a malignant phenotype in several tumor types [34].

Role of HIF pathway in tumor angiogenesis

Angiogenesis is a complex process that requires multiple gene products regulated by different cell types to converge and stimulate blood vessel formation. Hypoxia plays a major role in regulating a large number of genes involved in different steps of angiogenesis including, upregulating expression of various growth factors like VEGF, fibroblast growth factor and angiopoietins. This stress pathway also has the ability to induce endothelial cell migration and tube formation in tissue culture. In addition to its role in regulating molecular determinants of angiogenesis in culture, the HIF pathway also plays an important role in tumor angiogenesis. Up-regulation of the HIF pathway in solid tumors is via a combination of limited oxygen supply in the microenvironment and genetic mechanisms. The HIF pathway can be induced by several growth-promoting stimuli and oncogenic pathways that are up-regulated in tumors like insulin, insulin-like growth factor-1, epidermal growth factor and mutant Ras and src kinase pathways. Mutation in several tumor suppressor genes like pVHL, PTEN, p53 and p14ARF activate the HIF pathway. Given that the HIF pathway plays a role in regulating multiple steps of the angiogenic process and this pathway is activated in tumors, it is plausible that the HIF pathway could be used as a potential anticancer target [35]. However, the effects of inactivating the HIF pathway in embryonic stem cell derived teratomas have been variable, with neither vascularity nor growth being correlated with an intact HIF system in every study conducted [36-38]. Another study engineered an inducible knockdown of HIF1 α in D54-a glioblastoma cell line and observed that inhibition of HIF1 α during the early-stages of tumor development is more efficacious at decreasing tumor burden when injected in the flank of the animal. Inhibition of HIF1 α at the late-stages in tumor development had no significant effect. The same study used a different tumor model and showed that HIF1 α plays no role in regulating the growth of tumors derived from MDA-MB435- cell line. On further investigation, the authors observed that the tumors derived from MDA cell line were still responsive to hypoxia, however, VEGF secretion in this line was through a HIF-independent mechanism making them unresponsive to inhibition of HIF1 α [39].

Is the tumor dependence on UPR activation due to it having a role in angiogenesis?

Once tumor cells acquire the necessary mutations to overcome cell cycle checkpoints and interfere with apoptotic pathways, limitations in their extracellular environment can pose the next hurdle to uncontrolled cell growth. The rapid division and high metabolic rate of many tumors increases their demand for essential nutrients and oxygen, which is further exacerbated as their growth increases the distance of some cells within the tumor mass from existing vasculature [4] as illustrated in Figure 1-3. Indeed, unless the tumor is able to rectify this situation, it enters a dormant state [40]. The presence of hypoxic cells in localized regions of solid tumors is well documented clinically and experimentally. Perhaps counter intuitively this is correlated with a more aggressive phenotype and a poorer prognosis [41], which in part stems from the fact that cytoprotective signal transduction pathways, including the UPR, are activated by these

Figure 1-3. Tumor angiogenesis is regulated by multiple stress pathways activated in cancer cells. Rapidly proliferating tumor cells have an increased demand for nutrients, ATP and oxygen due to a high metabolic rate (green). As the tumor mass grows in size, cells that are more distant from a functional blood vessel are deprived of nutrients and oxygen and become hypoxic (blue). Tumor cells adapt to these adverse conditions by activating stress signaling pathways like the UPR and HIF. The UPR has several protective functions that assist tumor cell survival, including up-regulation of several proangiogenic factors such as VEGF by UPR-inducible transcription factors XBP1(S) and ATF4. Activation of the HIF signaling pathway stabilizes HIF1 α and HIF2 α , which can transactivate VEGF gene expression during hypoxic/anoxic conditions. The UPR and HIF signaling pathways are thus both likely to contribute to angiogenesis and tumor growth.

conditions. Unlike HIF pathways that are only activated in regions of the tumor that are further than approximately 0.2 mm from blood vessels [1], the UPR appears to be activated throughout tumor masses due to their high metabolic rates, which might suggest that different subsets of cells within the tumor may experience different combinations of these two stress pathways. Our studies will assess the contribution of these pathways in regulating VEGF expression in cells experiencing both stresses.

To elucidate the role of Ire1 in regulating tumor growth and angiogenesis *in vivo*, two separate studies engineered U87 human glioma cells with either an Ire1 dominant negative mutant or a control empty vector and then orthotopically implanted them in mice [42, 43]. In both studies, tumors derived from cells expressing the Ire1-dominant negative mutant were significantly smaller, less vascularized but more highly infiltrative as compared to their control counterparts. Similar results were obtained using a chick chorio-allantoic membrane (CAM) assay [43]. Although blocking the expression of Ire1 in tumor cell lines introduced into animals resulted in the inhibition of neovascularization, somewhat counter-intuitively it also modified the growth characteristics of the cells and promoted tumor cell invasion. This highly infiltrative/avascular phenotype has been reported by several other laboratories in response to various methods of blocking angiogenesis, although the molecular mechanism for this striking phenotype is still unknown. Supporting data for the role of Ire1 in tumor growth came from a study where Ire1's downstream target XBP-1 was examined using transformed XBP-1 wild-type and null MEFs. In this case, the XBP-1 null cells severely impeded tumor growth. When extreme hypoxic conditions were used to activate the UPR in the two cell lines, the XBP-1 null cells had a higher rate of apoptosis and decreased survival, in keeping with their failure to grow in mice. However the secretion of the proangiogenic factors VEGF and FGF2 under these culture conditions at shorter times was not significantly different between the two lines, leading the investigators to conclude that failure to induce angiogenesis was unlikely to be the reason the XBP-1 null cells failed to form tumors. Instead, they hypothesized that the Ire1/XBP-1 axis contributed to tumor cell survival via a mechanism that remained to be elucidated [44]. It should be noted however that the extreme hypoxic conditions used in these cell culture assays would be expected to also activate the HIF pathway, which is not expected to be affected by deletion of XBP-1, although this has not been formally tested. These results suggest the possibility that in tumors, the UPR may play as large or larger role than the HIF pathway in promoting angiogenesis and survival, due to the fact that more cells within the tumor may have activated the UPR.

Cells cultured under hypoxic/anoxic conditions also activate the PERK branch of the UPR, which controls a translational inhibition program that is shared with other stress signaling pathways that use distinct eIF2 α kinases, and is therefore known as the integrated stress response (ISR). In addition to the transient inhibition of protein synthesis that is a hallmark of PERK activation, a number of transcription factors are up-regulated that are responsible for many of the changes observed in the ISR. One of these factors is ATF4/cyclic AMP (cAMP)-responsive element binding protein 2 that regulates the ISR and protects the cell against metabolic consequences of ER stress [45]. To determine if the PERK pathway might also contribute to tumor survival, K-ras

transformed PERK wild-type and null MEFs were injected into the flank of athymic Nu/Nu mice. These studies show that tumors derived from K-ras-transformed PERK null MEFs are smaller and exhibit less angiogenesis than tumors from cells that express PERK. These studies also revealed that PERK activation favors endothelial cell survival and functional blood vessel formation in a tumor microenvironment by preferentially up-regulating expression of proangiogenic factors like VCIP, an adhesion molecule that facilitates integrin binding, cellular adhesion and capillary morphogenesis [46].

UPR activation regulates other proangiogenic factors

Although VEGF is the most potent proangiogenic factor, there are other factors including FGF2, IL8, IL6, VCIP, and angiogenin that play a role in promoting angiogenesis [47]. Microarray analysis of polysome-bound RNA demonstrated that a subset of proangiogenic transcripts including VCIP, an adhesion molecule that promotes capillary morphogenesis, is preferentially translated in a PERK-dependent manner [46]. In a mouse model of pancreatic islet carcinoma, increased expression of several Fibroblast Growth Factor (FGF) family members including FGF2 is observed when the binding of VEGF to its receptor-VEGFR2 is inhibited by a monoclonal anti-VEGFR2 antibody [48]. Anti-VEGFR2 is currently used to inhibit angiogenesis in the treatment of some cancers. A clearer understanding of the compensation mechanisms that exist between proangiogenic factors is important for the development of therapeutic agents. IL8, FGF2 and angiogenin are induced by different stress conditions including, hypoxia and low glucose [49]. It has been reported that hypoxia can induce IL8 expression in a HIF-1 deficient colon cancer cell line. This induction was mediated by enhanced production of ROS and activation of NF κ B [50]. Since NF κ B can be activated downstream of PERK [27], it is conceivable that UPR activation can induce IL8 expression via this transcription factor.

In summary, when I began my dissertation studies, there were preliminary data from a microarray study conducted in our lab to suggest that UPR activation can promote the expression of a significant number of proangiogenic factors in cultured cells [49]. I wanted to understand how many of these factors are UPR targets. We then focused on understanding the regulation of the most potent and best characterized proangiogenic factor- VEGF by the UPR. It was important to determine which UPR-inducible transcription factor played a critical role in regulating its expression. The fact that tumors activate both HIF and UPR pathways raises the questions of which pathway is most critical in promoting angiogenesis (Figure 1-4), whether different cells within the tumor mass rely on distinct pathways, and what affect will dismantling the UPR have on VEGF production in a tumor model. These studies will determine if the UPR might represent a druggable pathway for inhibiting angiogenesis and treating cancer.

Figure 1-4. Regulation of angiogenic factors by stress-activated pathways.

Angiogenesis is regulated by a fine balance between proangiogenic factors (red circles) and antiangiogenic factors (blue circles) under normoxic conditions. The UPR is a positive regulator of angiogenesis, since its activation increases expression of proangiogenic factors and inhibits anti-angiogenic factors. The HIF signaling pathway also increases expression of proangiogenic factors under hypoxic conditions. Since certain regions of the tumor mass encounter environmental conditions that lead to the activation of both pathways, UPR and HIF, it is conceivable that they could either synergize or compete to increase expression of proangiogenic factors.

Specific Aims

Aim 1: To determine the role of the UPR in regulating the expression of proangiogenic factors and identify the mechanism(s) and critical signaling components of the response that do so. Preliminary microarray analysis on Daoy, a human medulloblastoma cell line, treated with thapsigargin, an UPR stress inducer, showed that in addition to increased expression of known UPR target genes there was an up-regulation of several proangiogenic factors including, VEGFA, IL-8, FGF2 and angiogenin. Studies outlined in this aim will:

- a) Determine the extent proangiogenic factors affected by ER stress.
- b) Confirm these findings by real-time PCR and extend them to other UPR inducers and other types of cell lines.
- c) Determine the mechanism(s) by which VEGF, the best-characterized and most important proangiogenic factor, is regulated by the UPR.
- d) Identify the UPR-induced transcription factors that contribute to VEGF expression.

Aim 2: To determine the relative importance of the UPR versus HIF signaling in regulating VEGF expression in cultured human cells. Solid tumors activate multiple signaling pathways simultaneously including the UPR and HIF. In order to determine the contribution of these two pathways to VEGF expression, we will:

- a) Establish the fold induction of VEGF with the two stresses alone and in combination.
- b) Identify the UPR-induced transcription factor(s) that regulate VEGF in response to ER stress.
- c) Determine the effects on pathway-specific transcription factors in binding to the VEGF promoter when both stresses are present.
- d) Assess to what extent each pathway contributes to VEGF expression under conditions of hypoxia and ER stress.

Aim 3: To determine the importance of the UPR in regulating tumor angiogenesis *in vivo*. Once we have identified the critical UPR-inducible transcription factor(s) that regulate expression of VEGFA in cultured cells, we wish to determine the relative effect of the UPR in contributing to angiogenesis in a xenograft model. To do so, we will:

- a) Engineer human cell lines in which the critical UPR-regulated transcription factor(s) can be conditionally knockdown.
- b) Determine the effects of inhibiting expression of the transcription factor on tumor cell line growth in culture and in animals.
- c) Perform xenograft studies to assess its contribution in mediating angiogenesis in an animal model.

CHAPTER 2. THE UNFOLDED PROTEIN RESPONSE POSITIVELY REGULATES PROANGIOGENIC FACTORS²

Introduction

Changes in the extracellular environment of a cell can adversely affect the normal homeostasis of the endoplasmic reticulum (ER), which disrupts the folding and processing of secretory pathway proteins. The resulting accumulation of unfolded proteins in the ER increases the demands for molecular chaperones and folding enzymes and activates a signal transduction cascade known as the unfolded protein response (UPR) [51]. This multi-component signal transduction pathway is largely cytoprotective; serving to decrease the detrimental effects of accumulated unfolded proteins by increasing molecular chaperones that bind to them, decreasing protein synthesis to limit the accumulation, and finally increasing the degradative capacity of the cell to eliminate them. However if normal homeostasis is not restored during prolonged stress conditions, the UPR can induce apoptosis in these cells in order to protect the organism [51, 52]. In mammalian cells, the UPR is controlled by three resident ER transmembrane proteins that “sense” ER stress and activate signals to downstream elements; Ire-1, PERK and ATF6. Ire-1 is an ER localized transmembrane protein, which has a kinase and endoribonuclease domain in its cytosolic tail. On sensing ER stress, Ire-1 is phosphorylated in *trans*, which in turn activates its endonuclease domain leading to the excision of 26 bases from the X-box binding protein (XBP-1) transcript [53]. The resulting frame shift encodes a fully active transcription factor XBP-1(S), which up-regulates expression of a number of resident ER proteins that contribute to folding or degradation of unfolded or misfolded proteins [21, 54]. In addition to Ire-1 signaling, mammalian cells also transiently inhibit cap-dependent protein translation and arrest cells in the G1 phase of the cell cycle through activation of the PKR-like ER kinase (PERK) [23, 55]. Contrary to this global inhibition in protein translation occurring in PERK-activated cells, synthesis of the ATF4 transcription factor is increased during ER stress [23]. ATF4 transactivates expression of a number of genes including CHOP [56], a pro-apoptotic protein, and GADD34 [26], which reverses the block in translation. PERK also activates NFκB [27], a pro-survival protein, thus contributing to the balance between survival and death signals. Lastly, activation of ATF6 results in its translocation to the Golgi and cleavage by the S1P and S2P proteases to release the cytosolically oriented active transcription factor that up-regulates expression of XBP-1, as well as folding enzymes and ER chaperones, such as PDI and BiP [57, 58].

In addition to protecting cells during physiological and chemical conditions that adversely affect protein folding in the ER, there is increasing evidence to show that the UPR also plays an important role in normal development and physiology. This includes

² This chapter was adapted with permission. Pereira, E.R., N. Liao, G.A. Neale, and L.M. Hendershot, 2010. Transcriptional and post-transcriptional regulation of proangiogenic factors by the unfolded protein response. *PloS one*. **5** [49].

liver development [59], plasma cell differentiation [60, 61], bone development [62, 63], and normal pancreatic homeostasis [64]. Mice that are null for either XBP-1 [59] or its upstream activator Ire1 α [65-68] die at day E12.5 due to hepatoinsufficiency. In both cases, this was later confirmed to be due to an inability to produce XBP-1(S), a major regulator of hepatic development. In addition to liver, pancreas, and muscle, XBP-1(S) is also highly expressed in the placenta [65], and Ire1 α null embryos show evidence of placental abnormalities. To determine the role of Ire1 in this tissue, a recent study generated mice lacking Ire1 α by crossing Ire1 α ^{+/-} mice with *Mox2*^{+/-Cre} transgenic mice [65]. *Mox2* is ubiquitously expressed except in the labyrinthine trophoblasts of the placenta. This allowed Ire1-deficient embryos to be produced that have normal levels of Ire1 in the placenta [65]. This study revealed that loss of Ire1 α in the placenta led to decreased vascular endothelial growth factor (VEGFA) production, which is a major inducer of angiogenesis, thereby resulting in severe dysfunction of this highly vascularized tissue.

Angiogenesis refers to the sprouting, migration and remodeling of existing blood vessels [69] and plays an important role in a number of normal physiological processes including embryonic development, wound healing, and the female reproductive cycle. It also plays a role in several pathological conditions including ischemia and cancer. Angiogenesis is regulated by a fine balance between factors that stimulate the formation of new blood vessels and those that inhibit it [70, 71]. Proangiogenic factors such as VEGF, fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs), and IL8 are released by cancer cells experiencing decreased oxygen and nutrient supplies [72-74]. These factors act as ligands that bind to specific receptors on endothelial cells, causing them to proliferate and to release matrix metalloproteinases that degrade the extracellular matrix, allowing them to migrate toward the angiogenic stimulus in order to establish new blood vessels [72]. The predominant and best studied proangiogenic factor is VEGFA, a homodimeric heparin binding glycoprotein that is produced in several isoforms due to alternative splicing. The different isoforms of VEGFA (206, 189, 165, 145 and 121) have varying expression patterns and contrasting properties [75]. Of these VEGF₁₆₅ is the predominant and best characterized isoform, and plays an important role in mediating angiogenesis [76]. All VEGF isoforms are synthesized and processed in the endoplasmic reticulum (ER) and transported through the secretory pathway [72, 77].

The HIF (hypoxia inducible factor) pathway is the best characterized cellular stress pathway that leads to the up-regulation of proangiogenic factors in response to inadequate oxygen delivery [78]. HIF-1 and HIF-2 are heterodimeric transcription factors consisting of an oxygen-labile α subunit and a constitutively expressed β subunit. Hypoxia stabilizes the α subunit, thereby activating the HIF complex, which in turn binds to the promoters of target genes such as *VEGF* and other proangiogenic factors and transactivates them [79]. Prolonged hypoxia can also increase *VEGFA* mRNA stability leading to further increases in VEGFA production [80]. In addition to the role of the HIF signaling pathway in up-regulating *VEGF* expression, several recent studies demonstrate that the UPR also contributes to *VEGF* transcription [81] and protein processing in the ER [15]. Using microarray analysis, we found that in addition to *VEGFA* a large number of proangiogenic factors were up-regulated by UPR inducers. The up-regulation of

several of these factors by ER stress was as robust as, or even greater than, that achieved with hypoxia. We found that two UPR-regulated transcription factors bound directly to the *VEGFA* promoter in response to ER stress and contributed to its transcription. In addition, activation of AMP kinase stabilized the *VEGFA* transcripts, further contributing to *VEGFA* mRNA levels. Our finding that a number of regulators of angiogenesis are targets of the UPR argues that this physiological process should be added to the growing list of normal homeostatic and developmental processes that this stress pathway controls.

Results

UPR activation results in the transcriptional up-regulation of a number of proangiogenic factors

We wished to characterize the UPR in a solid tumor cell line that could ultimately be used in xenograft studies to ensure that this stress response was fully active and that all branches were intact. To do so, we treated Daoy, a human medulloblastoma cell line with thapsigargin, a Ca^{2+} ATPase inhibitor and potent inducer of the UPR, and performed genome-wide microarray analyses. Overall, we identified 1069 probe sets with differential expression after either 3 or 8 hours of thapsigargin treatment compared to untreated cells. Further analysis of this data confirmed significant enrichment of the expected UPR target genes, including ER chaperones, folding enzymes, and proteins involved in ER associated degradation (ERAD), as well as the transcription factors that are known to up-regulate them in response to ER stress. In addition to UPR targets, somewhat unexpectedly, gene ontology analysis revealed a significant enrichment of genes associated with the regulation of angiogenesis. A total of 185 genes on the array are annotated as being associated with angiogenesis. As many of these encode endothelial cell-specific proteins or cell surface receptors on endothelial cells, we limited our further analysis to the 33 genes that are secreted proteins or transcription factors that either positively or negatively regulate angiogenesis. Of the 19 genes that are characterized as positive regulators of angiogenesis, 13 showed a greater than 2-fold increase in expression in at least one time point after thapsigargin-treatment (Table 2-1). Additionally expression of one negative regulator of angiogenesis, vasohibin (*VASH1*) was decreased with ER stress. These data suggest that regulating angiogenesis is likely to be a major function of the UPR.

Comparison of UPR inducers with hypoxia in the up-regulation of proangiogenic factors

To confirm the induction of proangiogenic factors by the UPR, we treated cell lines with UPR inducers and compared the magnitude of their induction to that achieved with conditions that activate the HIF pathway using quantitative Real-Time PCR (qRT-PCR) (Figure 2-1). We confirmed by western blot analyses that CoCl_2 and the

Table 2-1. UPR activation enhances expression of proangiogenic factors.

Gene Symbol	Gene Name	Angiogenic	Fold Change	
			3 Hour	8 Hour
ANG	Angiogenin	Positive	2.4	8.1
ANGPT2	Angiopoietin-2	Positive	-2.6	2.2
CTGF	Connective tissue growth factor	Positive	2.2	1.4
EPAS1	Endothelial Pas domain protein 1	Positive	1.8	2.6
EREG	Proepiregulin	Positive	2.3	6.3
FGF2	Fibroblast growth factor-2	Positive	1.5	3.1
F3	Thromboplastin	Positive	2.9	1.6
IL1A	Interleukin-1 α	Positive	4.4	10.8
IL6	Interleukin-6	Positive	4.8	7
IL8	Interleukin-8	Positive	54.25	27.9
KLF5	Kruppel-like factor 5	Positive	2.6	3.5
TGFB2	Transforming growth factor beta-2	Positive	4.1	2.9
VEGFA	Vascular endothelial growth factor A	Positive	1.7	2.7
VASH1	Vasohibin	Negative	-1.3	-3

Figure 2-1. Up-regulation of proangiogenic factor mRNA by the UPR and hypoxia. Daoy, NB1691, SKNAS, C6 and NIH3T3 cells were treated with 100 μ M CoCl₂, 1% O₂ hypoxia (Hy), 2.5 μ g/ml tunicamycin (Tm), 1 μ M thapsigargin (Tg), or no glucose media (No Glu) for 24 hours. RNA was extracted for qRT-PCR analysis and mRNA fold induction relative to the untreated control sample, which was set to 1, was determined for (A) VEGF (white bars) (B) angiogenin (striped bars) (C) FGF2 (chequered bars) and (D) IL8 (grey bars). Experiments were performed in triplicate (values are mean $\pm$ SD).

level of hypoxia (1% O₂) used in our experiments induced HIF1 α and BNIP3, its downstream target, but did not induce UPR targets. Importantly, the UPR inducing agents did not activate the HIF signaling pathway (Figure A-1). Thus the conditions we used in our analysis allowed us to specifically activate these two stress pathways independently. Five different cell lines were treated with a variety of UPR inducers (*e.g.*, tunicamycin, thapsigargin, and no glucose) and with two different inducers of the HIF pathway (*e.g.*, CoCl₂ and 1% oxygen) for 24 hours, and the induction of four of the best characterized proangiogenic factors: VEGFA, bFGF, angiogenin and IL8 was measured. We also confirmed that downstream UPR target genes like *CHOP* and *BiP* mRNA were up-regulated by ER stress in each of the cell lines tested (Figure A-2). As expected, all four factors were up-regulated by hypoxic conditions, although the magnitude varied dramatically between cell lines, largely due to differences in their basal levels of synthesis (Figure A-3). When UPR inducers were used, we found that in many cases the induction of the four proangiogenic factors was nearly as high as or even higher than that achieved with hypoxia, although there were some interesting differences. Hypoxia was a strong inducer of *VEGFA* mRNA in the NB1691 neuroblastoma cell line, while ER stress had little effect on *VEGFA* levels. Conversely ER stress induced *VEGFA* in the NIH3T3 fibroblast line, but hypoxia did not (Figure 2-1A). Similarly ER stress induced *FGF2* expression greater than hypoxia in the Daoy line, whereas neither stress condition stimulated its production in the C6 and NIH3T3 cell lines, perhaps due to the high levels of basal expression of *FGF2* in these two lines (Figure 2-1C). In keeping with the microarray data, angiogenin was modestly induced in the Daoy cell line and the NB1691 line, but in the other three lines there was very little effect with either hypoxia or ER stress inducers (Figure 2-1B), again in keeping with higher basal levels in these lines. Finally, increases in human-specific *IL8* expression were much more dramatic with ER stress than with hypoxia in all three human cell lines (Figure 2-1D).

UPR activation increases *VEGFA* mRNA stability via AMPK

In this study we focused on determining the mechanism by which the UPR regulates *VEGFA* expression, as VEGFA is the best characterized stimulator of angiogenesis and represents a therapeutic target for treating cancer as well as several ischemic, infectious and inflammatory disorders [82]. Additionally, we favored this target because in most of the lines we examined, including mouse cells, *VEGFA* was induced to higher levels with ER stress than with hypoxia. We chose the C6 cell line for these experiments, because it had a low basal expression of VEGF which was potently induced by ER stress, previous studies used this line to study VEGF gene regulation by hypoxia, and this line was used in xenograft studies to determine the role of ORP150/GRP170 in VEGF processing and secretion. *VEGFA* mRNA levels increase in response to hypoxic conditions via a combination of an enhanced transcription at early time points coupled with an increase in the stability of the mRNA at later times [83]. To investigate whether UPR activation might also increase *VEGFA* mRNA stability, we examined the turnover of *VEGFA* mRNA under control and various stress conditions (Figure 2-2A). Cells were pretreated with hypoxia or two different UPR

Figure 2-2. UPR activation stabilizes *VEGF* mRNA via AMPK. (A) C6 cells were pre-treated with normal culture conditions (NT-circle), 1% O₂ (Hy-square), thapsigargin (Tg-triangle), or no glucose media (No Glu-cross) for 6 hours. Actinomycin D (5 μg/ml) was added to the various cultures to block further transcription. At the indicated times, total RNA was extracted and subjected to qRT-PCR to determine *VEGF* mRNA levels. The mean values of data from duplicate experiments are presented (± SD). (B) C6 cells were either left untreated or treated as indicated in the figure in the presence or absence of Compound C for 9 hours or 14 hours. Western blot analysis was performed on cell lysates to determine levels of p-AMPK. Hsc70 was used as loading control. (C-D) C6 cells were pretreated with different stress inducers for 6 hours as indicated. No inhibitor (black) or compound C (AMPK inhibitor- white) was added to the cells as indicated for an additional 8 hours. Total RNA was extracted for qRT-PCR to determine *VEGF* mRNA (C) and *VEGF* hnRNA (D) levels. Experiments were performed in triplicate (values are mean ±SD).

inducers and then incubated with actinomycin D to inhibit further transcription. In control cells the low level of basal transcripts were rapidly degraded in keeping with previous studies [80, 84]. For all three stress inducers, there was a reproducible increase in *VEGFA* mRNA at 30 minutes after adding actinomycin D, which is compatible with an increase in transcription occurring before the inhibitor takes effect. We found that although hypoxic conditions led to an initial increase in *VEGFA* levels, the mRNA was rapidly degraded. This is consistent with a previous report using C6 cells, which showed that hypoxia had no significant effect on the half-life of *VEGFA* mRNA until much later time points [83]. When cells were pretreated with the two UPR inducers, we found that after the initial burst in *VEGFA* transcripts they decayed significantly slower than in control or hypoxia-treated cells. (Figure 2-2A), arguing that ER stress leads to increased *VEGFA* mRNA stability at relatively early times in the response.

The increase in *VEGFA* mRNA stability observed after prolonged exposure to hypoxic conditions is due to the binding of a hypoxia-inducible protein complex, such as HuR, to the ARE (adenylate-uridylylate rich elements) region in the 3'UTR region of *VEGFA* mRNA [80]. Additionally, stress activated protein kinases such as AMPK, p38MAPK, JNK, and PI3K have been implicated in increasing *VEGFA* mRNA stability through their action on the AU rich region of the 3'UTR [85-88]. We used a variety of kinase inhibitors to determine if any of their targets might play a role in increasing the stability of *VEGFA* mRNA during ER stress. When the UPR-activated cells were incubated with compound C, an AMP kinase inhibitor, there was a significant reduction in *VEGFA* transcripts (Figure 2-2C), suggesting that this kinase played a role in the UPR-induced stabilization of *VEGFA*. Activation of AMPK by ER stress was confirmed by western blotting, as was the efficacy of its inhibitor, Compound C (Figure 2-2B). We also co-incubated UPR activated cells with inhibitors of the PI3 and JUN kinases, but found that they had no effect on *VEGFA* mRNA levels in response to UPR activation (data not shown). As an additional control, the effect of the AMPK inhibitor on *VEGFA* mRNA levels was examined in cells pre-treated with hypoxia for 6h (Figure 2-2C), which was previously shown to be not long enough to stabilize *VEGFA* transcripts [83].

Unexpectedly, we found that *VEGFA* stability was actually increased in hypoxia treated cells that we incubated with Compound C, although we do not understand the basis for this effect. To verify that the effects of this inhibitor was specifically on *VEGFA* mRNA stability and did not alter transcription of the *VEGFA* gene, we treated cells with the various combinations of kinase inhibitor and stress inducers and examined heteronuclear *VEGFA* RNA (hnRNA) levels (Figure 2-2D), which can be used as a measure of transcription. We found that there was no indication that this inhibitor affected *VEGFA* transcription, thus confirming that *VEGFA* transcripts are stabilized during ER stress, which apparently is due to the activation of the AMP kinase.

UPR activation increases the transcription of *VEGFA*

Our analysis of *VEGFA* hnRNA in the experiment described above revealed that the unprocessed hnRNA levels were higher in cells treated with thapsigargin or no glucose than in control cells. This suggested that *VEGFA* might also be transcriptionally regulated in response to ER stress. Prior to using hnRNA levels as a measure of the transcription rate, we first confirmed that the splicing of *VEGFA* mRNA was not significantly affected by UPR activation. Cells were pretreated for 6 hours with the indicated stressors, and actinomycin D was added to inhibit further transcription. Heteronuclear RNA was then measured at the indicated time points. We found that *VEGFA* hnRNA decreased at a fairly similar rate in control and stress activated cells through at least eight hour of treatment, arguing that these stresses did not dramatically affect splicing up to this point (Figure 2-3A). Therefore, the measurement of *VEGFA* hnRNA could be used as an indication of the transcription rate of this gene in response to UPR activation (Figure 2-3B). Cells incubated in media containing no glucose, increased *VEGFA* transcription to a much greater extent than either thapsigargin or hypoxia at all time points measured, which is in keeping with the 30 minute time point in Figure 2-2A. Thapsigargin was as good as or better than hypoxia at inducing *VEGFA* transcription throughout the course of the experiment. Thus, the increased transcription rate, coupled with the stabilization of *VEGFA* transcripts, accounts for the higher steady state level of *VEGFA* mRNA in response to thapsigargin compared to hypoxia in this cell line (Figure 2-2A). Although the transcription rate of *VEGFA* appeared to be the highest in the presence of no glucose, this is not reflected in the steady state levels after 24 hours of treatment (Figure 2-2A), which may be due to some inhibition of splicing occurring at later time points.

XBP-1 binds to two regions in the rat *VEGFA* promoter

To identify potential binding sites for various UPR-inducible transcription factors, we analyzed the human, mouse and rat *VEGFA* promoters using the computer programs rVista and TRANSFAC (Figure A-4). In addition to HIF sites, the promoters of all three species have a number of potential binding sites for the UPR-induced transcription factors XBP-1 and ATF4, whereas only the mouse promoter has a single ATF6 site. We first assessed whether XBP-1(S) bound to any of the five potential sites in the rat promoter in response to ER stress using a chromatin immunoprecipitation (ChIP) assay, since the C6 rat glioma was used for both the mRNA stability and transcription assays. Indeed, XBP-1(S) could be detected at two different sites (i.e., one at ~1.9kb and one at ~5.2kb up-stream of the transcription start site) in response to both thapsigargin and no glucose treatment (Figure 2-4B). We were unable to detect XBP-1(S) binding to the remaining three potential sites in these cells upon UPR activation, suggesting that either they are not used or that the anti-XBP-1(S) antiserum used to immunoprecipitate the chromatin could not gain access to these sites. As a positive control, we showed that XBP-1 binds to the ERdj3 promoter (Figure 2-4C), as documented previously [89]. We detected XBP-1(S) protein in ER stressed but not in untreated C6 cell lysates that were used for the chromatin immunoprecipitation assays (Figure 2-4D).

Figure 2-3. UPR activation increases *VEGF* transcription rate. (A) C6 cells were pre-treated with normal culture conditions (NT-circle), 1% O₂ (Hy-square), thapsigargin (Tg-triangle), or no glucose media (No Glu-cross) for 6 hours as indicated. Actinomycin D (5 µg/ml) was added to block further transcription. At the indicated times, total RNA was extracted and subjected to qRT-PCR to determine the kinetics of the disappearance of *VEGF* hnRNA in control and stressed cells. **(B)** C6 cells were untreated (NT-black), treated with 1% O₂ (Hy-grey), thapsigargin (Tg-striped) or no glucose media (No Glu-white) for the indicated times. Total RNA was extracted, and *VEGF* hnRNA was quantitated by qRT-PCR. Experiments were performed in triplicate (values are mean ±SD).

Figure 2-4. XBP-1 binds to two regions in the rat *VEGF* promoter in response to ER stress and contributes to increasing *VEGF* transcription rate. (A) Potential XBP-1 sites in the rat *VEGF* promoter. (B) Cross-linked chromatin from C6 cells that were untreated (NT), thapsigargin-treated (Tg), or incubated in no glucose media (No Glu) for 8 hours were immunoprecipitated with anti-XBP-1 or with a control antiserum (anti-BiP). Ten-fold serial dilutions of precipitated chromatin and input controls were used for PCR amplification. (C) As a positive control, primers spanning the XBP-1 binding region on the ERdj3 promoter were used to PCR amplify the anti-XBP-1 precipitated chromatin (D) XBP-1(S) protein levels were detected in C6 cells treated with Tg and No Glu media using Western blot analysis. (E) XBP-1 wild-type (black) or null (white) MEFs were untreated (NT), thapsigargin-treated (1 μ M), treated with media lacking glucose (No Glu) or homocysteine-treated (10 mM) for 14 hours. Cell lysates were prepared and XBP-1(S) was detected by western blot analysis. (F-G) Cells were treated as in (E) and total RNA from the indicated samples was subjected to qRT-PCR to quantify *VEGF* hnRNA (F) or *VEGF* mRNA (G) at the indicated time points. RNA levels were expressed relative to the control untreated samples for each line, which was set to 1. Experiments were performed in triplicate (values are mean $\pm$ SD).

XBP-1 mediates increased expression of *VEGFA* following ER stress

To determine the contribution of XBP-1 to the up-regulation of *VEGFA* transcription, we made use of XBP-1 wild-type (XBP-1 wt) and null (XBP-1 ko) mouse embryonic fibroblasts (MEFs). Examination of these cells by western blotting confirmed that no XBP-1(S) protein could be detected in the XBP-1 null cells in response to UPR induction (Figure 2-4E). Next we compared the fold induction of both *VEGFA* hnRNA (Figure 2-4F) and *VEGFA* mRNA (Figure 2-4G) in both cell lines after treating with three different ER stress inducers; thapsigargin, no glucose media, and homocysteine. Our qRT-PCR analysis in the XBP-1 wild-type MEFs demonstrated an ~10 fold increase in *VEGFA* transcription rate after 8h of either thapsigargin or homocysteine treatment (Figure 2-4F); whereas no glucose media was a relatively poor inducer of *VEGFA* transcription in this cell line, perhaps in keeping with the reduced amount of XBP-1(S) produced by this stress condition (Figure 2-4E). The transcription rate was highest at 8h for both thapsigargin and homocysteine treatment, demonstrating that its induction is not sustained during UPR activation in the wild-type MEFs as was observed in the C6 cell line. This is mirrored in the total mRNA transcripts, which were also higher at 8 hrs of stress induction (Figure 2-4G). When the XBP-1 null cells were similarly examined, we found that there was little or no increase in *VEGFA* transcription with any of the treatments, suggesting that XBP-1 played a major role in the up-regulation of *VEGFA* in response to ER stress. However, closer analysis of the *VEGFA* hnRNA data from the two cell lines revealed that the untreated XBP-1 wild-type MEFs had a lower basal level of *VEGFA* hnRNA than the null cells, (Figure A-5). Hence, the exact contribution of XBP-1 in up-regulating *VEGFA* was somewhat complicated by the differences in basal transcription rates between the two lines.

ATF4 contributes to up-regulation of *VEGFA* expression following UPR activation

Inspection of the human, mouse and rat *VEGFA* promoters also revealed several potential ATF4 binding sites (Figures 2-5A and A-4). To determine if any of these sites was occupied by ATF4 in response to ER stress, we performed ChIP assays in ATF4 wild-type (ATF4 wt) and null (ATF4 ko) MEFs. We were unable to detect binding of ATF4 to any of the seven potential sites upstream of the transcription start site in response to thapsigargin treatment (data not shown). However, we did detect stress-inducible binding of ATF4 to a site at position +900 relative to the transcription start site in the wild-type ATF4 MEFs but not in the ATF4 null MEFs (Figure 2-5B). Similar to the wild-type MEFs, we were unable to detect ATF4 binding to any of the five upstream regions in the rat promoter (Figure A-6). However, unlike the human and mouse promoter, there does not appear to be a site downstream of the transcription start site in the rat promoter that corresponds to the one used in murine cells. To determine the contribution of ATF4 to *VEGFA* transcription in response to UPR stress inducers, we analyzed steady state *VEGFA* hnRNA (Figure 2-5C) and mRNA (Figure 2-5D) levels in ATF4 wild-type and null MEFs. Although the fold increase in total *VEGFA* mRNA in

Figure 2-5. ATF4 contributes to VEGF expression and binds to the mouse promoter in a stress inducible manner. (A) Potential ATF4 sites in the mouse VEGF promoter (B) Cross-linked chromatin from ATF4 wild-type or null MEFs that were untreated (NT) or thapsigargin-treated (Tg) for 6h was immunoprecipitated with anti-ATF4 or with a control antiserum (anti-BiP). Precipitated chromatin and input controls were used for PCR amplification. (C-D) ATF4 wild-type (black) or null (white) were cultured in normal media (NT), thapsigargin-treated (1 μ M), or tunicamycin-treated (2.5 μ g/ml) for 3 and 6 hours. Total RNA from the indicated samples was subjected to qRT-PCR, and *VEGF* hnRNA (C) or *VEGF* mRNA (D) levels were determined and represented as described above. Experiments were performed in triplicate (values are mean $\pm$ SD).

response to ER stress was not very dramatic in either the wild-type or null cells, it was consistently slightly higher in the ATF4 wt MEFs.

ATF6 does not significantly contribute directly to *VEGFA* expression

Although there were no obvious potential ATF6 binding sites in the human or rat *VEGFA* promoters, there was one potential ATF6 binding site in the mouse *VEGFA* gene at +1.4kb relative to the transcription start site. Thus, we also examined the potential contribution of ATF6 in regulating *VEGFA* transcription. qRT-PCR analysis of *VEGFA* mRNA in ATF6 wild-type (ATF6 wt) and null (ATF6 ko) MEFs revealed that ATF6 does not appear to play a significant role in up-regulating *VEGFA* expression in response to either tunicamycin treatment or incubation in media lacking glucose (Figure 2-6A). However, in response to thapsigargin treatment there was a modest, albeit significant, increase in total *VEGFA* mRNA in ATF6 wild-type cells compared to null cells. The difference between the stresses in inducing *VEGFA* transcripts was a bit puzzling. Because XBP-1 transcription is regulated by ATF6 and our analysis revealed that XBP-1(S) binds to the *VEGFA* promoter, we examined XBP-1(S) levels in the two lines in response to the various UPR inducers. Western blot analysis revealed a more dramatic increase in XBP-1(S) protein levels in the thapsigargin-treated ATF6 wild-type cells compared to the null cells (Figure 2-6B), whereas there was no obvious difference between the two tunicamycin-treated cell lines. Somewhat surprisingly, media lacking glucose did not induce XBP-1(S) in either cell line. These data imply that the higher levels of *VEGFA* mRNA observed in the thapsigargin-treated wild-type cells compared to the null cells might be partly due to a more robust increase in XBP-1(S) levels in the wild-type cells.

UPR activation increases *VEGFA* protein levels

Lastly, we measured the effects of the UPR and hypoxia signaling pathways on *VEGFA* protein levels in the cells (Figure 2-7A) and in the culture supernatant (Figure 2-7B). We could readily detect *VEGFA* in lysates obtained from cells treated with all three of the UPR inducers but not in cells cultured in the hypoxia chamber or treated with CoCl_2 (Figure 2-7A), in spite of the fact that hypoxia increased *VEGFA* mRNA in this cell line to about the same level as tunicamycin treatment. A faster migrating unglycosylated *VEGFA* was detected in cells treated with tunicamycin and no glucose media, as these stressors are known to inhibit protein glycosylation. In spite of our inability to detect *VEGFA* in hypoxia treated cells, when media from cells treated with the various UPR inducers and hypoxia were examined, we readily detected an increase in *VEGFA* secretion with all stressors (Figure 2-7B). However, the combination of cell-associated and secreted *VEGFA* with each of the treatments is not consistent with the mRNA levels. For example, although *VEGFA* mRNA levels were highest in cells treated with thapsigargin compared to any of the other four stress conditions (Figure 2-1A), it was not secreted as well from these cells as from tunicamycin or no glucose treated cells. Additionally, although both hypoxia and tunicamycin treatment resulted in similar

Figure 2-6. ATF6 does not significantly contribute to VEGF expression. (A) ATF6 wild-type (black) or null (white) MEFs were untreated (NT), thapsigargin treated (1 μ M), tunicamycin-treated (2.5 μ g/ml) or treated with media lacking glucose (No Glu) for 6 hours. Total RNA from the indicated samples was subjected to qRT-PCR, and the *VEGF* mRNA levels were determined. (B) XBP-1(S) was detected using western blot analysis on cell lysates from ATF6 wild-type and null MEFs that were untreated or treated as indicated. Experiments were performed in triplicate (values are mean $\pm$ SD).

Figure 2-7. UPR activation increases VEGF protein levels and secretion. (A) C6 cells were left untreated or treated as indicated for 24 hours. Western blot analyses on cell lysates were performed to detect VEGF protein levels. Hsc70 was used as a loading control. **(B)** Conditioned media from untreated and treated cells was analyzed for VEGF secretion by ELISA. **(C)** Total RNA from the indicated samples was subjected to qRT-PCR, and GRP170 mRNA levels were determined and expressed relative to the control cells.

increases in VEGFA transcripts, tunicamycin treatment resulted in the secretion of greater quantities of VEGFA (Figure 2-7B). Previous studies demonstrated that the ER stress-inducible chaperone GRP170/ORP150 plays an important role in VEGFA processing and secretion in the C6 cells [15]. Thus we examined the effects of the various stressors on GRP170 induction at both the mRNA and protein levels. We found that tunicamycin and no-glucose were potent inducers of GRP170 mRNA (Figure 2-7C) and protein (Figure 2-7A), whereas thapsigargin only modestly induced this chaperone and hypoxia had almost no effect on GRP170 levels. Thus the relatively high levels of VEGFA secretion in tunicamycin and no-glucose treated cells are consistent with the increased GRP170 levels in these cells.

Discussion

Angiogenesis is a normal physiological process that is important for embryonic development as well as wound healing [71, 82]. It is carefully controlled by a large number of secreted factors that bind to receptors on endothelial cells, as well as negative regulators that inhibit angiogenesis through direct effects on endothelial cells or indirect effects on growth factor mobilization and activation [70, 82]. Recent studies have shown that VEGFA, one of the major proangiogenic factors, is a target of the UPR. Our microarray analysis of UPR targets in thapsigargin-treated Daoy cells confirmed *VEGFA* induction, but unexpectedly revealed that there was a significant up-regulation of 12 additional positive regulators of angiogenesis, including secreted proangiogenic factors, cytokines, and transcription factors that positively regulate proangiogenic factors, as well as a decrease in one negative regulator of angiogenesis. This argues that the regulation of angiogenesis is likely to be an important function of the UPR. Of note, the UPR was a potent inducer of IL8 expression in multiple cells lines (Figure 2-2D). A recent report demonstrated that addition of IL8 to endothelial cells can induce *VEGFA* mRNA and protein levels in a HIF1 α -independent manner that requires NF κ B activation [90]. Because ER stress also leads to NF κ B activation, it is conceivable that IL8 contributes to VEGF induction during UPR activation in some of our lines. However, although the NB1691 cell line potently up-regulates IL8, it does not induce *VEGFA* mRNA levels on UPR activation. The reason for this is not known as other UPR targets are clearly activated in this line and *VEGF* is induced by hypoxia.

The major function of the UPR is thought to be restoring or maintaining ER homeostasis in response to an inadequate or toxic environment that adversely affects this organelle and its ability to fold and assemble proteins. Thus it is perhaps not surprising that one mechanism for doing this would be to increase the supply of blood flow to the affected cells so that more nutrients and oxygen can be delivered and waste and other toxic products could be taken away. In keeping with this possibility, physiological processes like wound healing require increased vascularization [91], and studies show evidence of UPR activation in the affected cells [92]. Similarly the placenta must be highly vascularized in order to supply adequate quantities of oxygen and nutrients to the developing fetus and to remove toxic waste products. Although there are not data to

demonstrate that the UPR is normally activated in the developing trophoblasts, a recent report found that Ire1 was required for normal placenta vascularization and fetal development [65]. This aspect of the UPR might also be used in pathological conditions like cancer [93, 94] and ischemia [95] to stimulate angiogenesis, as in both cases there is evidence of UPR activation [52]

We focused our further analysis on VEGFA because it is the best characterized and most potent endothelial growth factor that promotes angiogenesis and is a target of cancer therapy. Recently a study has shown that *VEGFA* mRNA can be up-regulated in cultured cells by the UPR inducers, thapsigargin and tunicamycin [81]. We confirmed the role of the UPR in up-regulating *VEGFA* expression in various cell lines and also shown that the UPR is a much better inducer of *VEGFA* mRNA than hypoxia in a number of transformed and non-transformed cell lines, further arguing that this represents a normal function of the UPR. We next focused our attention on the mechanism of increased *VEGFA* mRNA expression via UPR and hypoxia signaling pathways using the C6 rat glioma cell line. The UPR increases *VEGFA* mRNA stability as well as the transcription rate of the gene to an even greater extent than that achieved with hypoxia. Several stress-activated protein kinases, including AMPK, have been reported to increase *VEGFA* mRNA stability. The activation of the AMP kinase has been linked to low glucose levels that result in diminished ATP production [96]. In addition to nutrient deprivation, other metabolic stresses such as hypoxia, oxidative stress and exercise lead to activated AMPK [97]. In DU145 prostate carcinoma cells cultured without glucose, JNK was shown to act upstream of AMPK pathway to increase *VEGFA* mRNA stability [87]. However, in our analysis of the C6 cells treatment with SP600125 the JNK inhibitor had no effect on *VEGFA* mRNA stability, whereas treatment with the AMPK inhibitor enhanced its stability in response to both thapsigargin and no glucose, suggesting that conventional UPR inducing agents can also activate AMPK.

Next, we assessed the importance of the major UPR-induced transcription factors (*i.e.*, XBP-1(S), ATF4 and ATF6) in mediating *VEGFA* transcription. Most recently, Ghosh R *et al* [81], demonstrated that Ire1 null MEFs, which cannot splice XBP-1 and induce its downstream targets, have a significant reduction in *VEGFA* mRNA expression compared to the Ire1 wild-type cells when treated with thapsigargin. Using a VEGFA-promoter-luciferase assay and chromatin immunoprecipitation they also showed that exogenously supplied XBP-1 can bind to the VEGFA promoter and up-regulate *VEGFA* mRNA expression. Our studies reveal direct binding of endogenous XBP-1 to two distinct sites in the rat *VEGFA* promoter in response to ER stress. Our qRT-PCR data showed an increase in the *VEGFA* transcription rate in the XBP-1 wild-type cells treated with various UPR inducers compared with the XBP-1 null cells. The increased levels of *VEGFA* mRNA observed in the wild-type cells was primarily due to an increase in the transcription rate of the gene, since these inducers had little effect on the stability of *VEGFA* transcript in this cell line (data not shown). Previously published data showed that tumors derived from U87 cells expressing an Ire1 dominant negative construct developed smaller tumors with decreased vascularization as compared to tumors from control cells [98]. In addition to this study, two independent reports have also demonstrated a role for XBP-1 in tumor establishment, growth and angiogenesis [99,

100]. However, the former study found that when XBP-1 deficient cells are treated with extreme hypoxia/anoxia ($pO_2 < 0.02\%$) *in vitro*, there is no defect in secretion of the proangiogenic factors, VEGFA and bFGF, as compared to wild-type cells. As these extremely low O_2 conditions would be expected to induce both the UPR and the HIF pathways, it is conceivable that in the absence of XBP-1, HIF1 α and 2 α are able to compensate. In support of this possibility, there are HIF binding sites in close proximity with the XBP-1 “A” site occupied in response to ER stress (Figure 2-4B) in all three species. As tumor cells, ischemic tissue, and wounds are likely to activate both types of stress pathways, it will be important to understand the overlap and relative contribution of each factor in a physiological setting.

Several studies have demonstrated a role for ATF4 in mediating expression of *VEGFA* in response to various stimuli such as homocysteine [101], arsenite [102], oxidized phospholipids [103], and osteopontin [104]. Arsenite is an oxidative stressor that stimulates ATF4 binding to the *VEGFA* promoter in a human retinal pigment epithelial cell line at position +1767 relative to the transcription start site [102]. Similar observations by another group demonstrated that ATF4 binds to the same AARE site in the *VEGFA* promoter when a human umbilical vein endothelial cell line was stimulated with oxidized phospholipids [103]. These results are in accordance with data published by Ghosh et al reporting a PERK-ATF4 dependent up-regulation of *VEGFA* expression. Using both PERK and ATF4 null MEFs treated with thapsigargin, they showed that *VEGFA* mRNA levels were decreased as compared to the corresponding wild-type MEFs, and demonstrated binding of ectopically expressed ATF4 to the *VEGFA* promoter in cells treated with thapsigargin. Using ChIP assays, we confirmed that ATF4 contributes to *VEGFA* transcription and furthermore demonstrated that endogenous ATF4 binds to a region $\sim +0.9$ kb downstream of the transcription start site in mouse cells when treated with a UPR inducer.

Lastly, our data suggests that ATF6 does not play a significant role in directly mediating *VEGFA* mRNA expression. We observed a modest increase in *VEGFA* mRNA in the ATF6 wild-type MEFs compared to the ATF6 null cells when treated with thapsigargin but not the other stressors. This induction was most likely due to an increase in the XBP-1(S) protein levels observed only in thapsigargin-treated ATF6 wild-type cells. The *VEGFA* promoter-reporter assay performed by Ghosh et al, showed that over-expression of the transcription factor ATF6 increases luciferase activity ~ 6 fold compared to empty vector. The reporter construct used in this assay was derived by inserting ~ 1 kb of the sequence upstream of the mouse *VEGFA* transcription start site, in front of the luciferase gene. Using two different programs to identify transcription factor binding sites in this region of the mouse *VEGFA* promoter, we were unable to identify any potential ATF6 sites. However, this region contains a potential binding site for XBP-1, which this group reported could bind to ectopically expressed XBP-1 using ChIP assays. However, we were unable to detect binding of endogenous XBP-1 to this same site (data not shown).

In addition to the role of UPR-inducible transcription factors in mediating *VEGFA* expression, there are data showing that the stress inducible ER chaperone

ORP150/GRP170 plays a role in post-translational processing/secretion of VEGFA [15]. Ectopic expression of ORP150 in C6 cells increased VEGFA secretion, whereas decreasing ORP150 levels with an antisense construct resulted in the retention of VEGFA in the ER. Furthermore, tumors arising from the antisense ORP150 C6 glioma transfectants demonstrate an initial phase of growth comparable to the wild-type glioma cells which was followed by marked regression and decreased angiogenesis within 8 days. Our analysis of VEGFA secretion in C6 cells revealed that although hypoxia and tunicamycin lead to similar increases in *VEGFA* mRNA, that more VEGFA was secreted from the tunicamycin treated cells, which had higher levels of ORP150/GRP170 mRNA and protein levels. This correlation is further underscored by the finding that although thapsigargin was the strongest inducer of *VEGFA* mRNA, it caused a less robust up-regulation of GRP170 and less VEGFA was secreted from thapsigargin treated cells than from either tunicamycin or no-glucose treated cells.

In conclusion, using microarray analysis we found a significant up-regulation of a large proportion of positive regulators of angiogenesis in response to ER stress and verified four of these using qRT-PCR assays. Our studies revealed that in some cell lines UPR activation enhanced *VEGFA* mRNA and protein expression more potently than hypoxia and that this was achieved through a combination of transcriptional as well as post-transcriptional mechanisms. Thus, the UPR is likely to be an important regulator of angiogenesis in normal physiological settings as well as pathological conditions like cancer and ischemia and may synergize with the well-studied HIF pathway activated by hypoxia.

Materials and Methods

Cell culture and stress induction

Daoy human medulloblastoma cell line [105], C6 rat glioma cell line [106], XBP-1 wild-type and null MEFs, ATF6 wild-type and null MEFs, and NIH3T3 mouse fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% antibiotic-antimycotic at 37°C in a 5% CO₂ incubator. NB1691 and SKNAS human neuroblastoma cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine. Primary wild-type and ATF4 null MEFs were propagated in cell culture as previously described [26]. Cells were plated and left untreated (NT) or treated with thapsigargin (Tg, 1 µM), tunicamycin (Tm, 2.5 µg/ml), homocysteine (HCys, 10 mM), CoCl₂ (100 µM), media lacking glucose (No Glu) (DMEM cat.no.11966-GIBCO and RPMI cat. no.11879-GIBCO), or cultured in a hypoxia chamber containing 1% O₂ for the indicated periods of time.

mRNA and hnRNA quantification by qRT-PCR

Total RNA was extracted using the RNeasy Qiagen mini-prep kit according to the manufacturer's protocol. RNA samples were subjected to qRT-PCR, and reactions were done in duplicate using a TaqMan One-Step PCR Master Mix kit. Amplification of the corresponding genes was achieved using specific primers and probe set and measured continuously using an ABI 7900 HTI Detection System. Where indicated, VEGF hnRNA was measured using qRT-PCR primers and probe across intron 1 and exon 2, for the rat gene and across exon 3 and intron 3, for the mouse gene. The signal obtained for measuring both, mRNA and hnRNA, was compared relative to 18S rRNA internal control. A recent study detected down-regulation of ribosomal RNA by ER stress [107]. However, in our study the 18S rRNA cycle threshold (Ct) values obtained by qRT-PCR remained relatively unchanged in the presence of ER stress arguing that 18S rRNA levels were not changing with the conditions used. The value for untreated cells was set to 1 and the value for the various treatments was presented as a fraction of this number. In the case of wild-type and null cells, the untreated value for each cell type was set to 1 unless otherwise indicated.

mRNA stability assay

C6 cells were pre-incubated in normal complete media, media containing thapsigargin or no glucose, or in a hypoxia chamber for 6 hours. Actinomycin D (5 µg/ml- Sigma Aldrich) was added to each test set, and the cells were reincubated for the indicated times. Total RNA was extracted and VEGF mRNA was analyzed by qRT-PCR.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed using a ChIP kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, cells were incubated for the indicated times with or without different stress inducers. Formaldehyde was then added (final concentration, 1 %), and cells were incubated for 10 minutes at room temperature to stabilize DNA-protein interactions. Cross-linking was stopped by the addition of glycine (final concentration, 0.125 M). Cell extracts were sonicated with a Branson Sonifier 250 (VWR) for 5 bursts at 10 seconds each at 20 % power output to shear DNA to 1kb or less. Extracts from 10^7 cells were incubated overnight with antibodies against ATF4 generously provided by Dr. David Ron (University of Cambridge, U.K.), rabbit anti-XBP-1(S) polyclonal antiserum (Santa Cruz Biotechnology sc-7160X) or rabbit anti-BiP polyclonal antiserum [108], which served as a negative control. Two percent of the extract volume was removed before immunoprecipitation and served as input control. DNA fragments from immunoprecipitated complexes and input controls were released by heating at 65°C overnight and purified using the PCR purification kit (QIAquick, Qiagen) according to the manufacturer's protocol. Purified immunoprecipitated DNA and input DNA were then analyzed by PCR.

Western blotting

For Western blotting cells were lysed in Nonidet P-40 lysing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and 0.5 % deoxycholic acid) for 30 minutes on ice. The proteins were electrophoresed under reducing conditions, transferred to a PVDF membrane and probed with the indicated primary antibodies: Rabbit anti-XBP-1(S), rabbit anti-ATF4, goat anti-Hsc70, and goat anti-Lamin B1 were purchased from Santa Cruz Biotechnology, mouse anti-HIF1 α and mouse anti-BNIP3 from Abcam, and rabbit anti-phospho-AMP kinase from Cell Signaling. Rabbit anti-CHOP has been described previously [56]. Isolation of nuclear and cytosolic fraction for detecting HIF1 α by Western blotting was performed as previously described [109]. Blots were incubated with the appropriate HRP-conjugated secondary antibody, and proteins were visualized using the Pierce enhanced chemiluminescent substrate (Thermo Scientific).

Microarray gene expression analysis

Total RNA (5-10 μ g) was processed according to the Affymetrix eukaryote one-cycle target labeling protocol at the Hartwell center microarray core at St Jude Children's Research Hospital. Biotin-labeled cRNA (15 μ g) was hybridized overnight at 45°C to the human HG-U133 Plus 2.0 GeneChip array, which interrogates more than 54,000 human transcripts and ESTs. After staining and washing, arrays were scanned and expression values summarized using the MAS5 algorithm as implemented in the GCOS v1.4 software (Affymetrix, Santa Clara, CA). Signals were normalized for each array by scaling to a 2% trimmed mean of 500. Detection calls (Present, Absent and Marginal) were determined using the default parameters of the software. Signal values were log₂-transformed prior to analysis. Differential expression between thapsigargin-treated and untreated cells was determined from two independent experiments using the Local Pooled Error t-test(1) (S-Plus 6.2, TIBCO, Palo Alto, CA). False discovery estimates were calculated as described [110]. Hierarchical clustering was performed using the Spotfire Decision Site 9.0 software (TIBCO). Probe set annotations were obtained from the Affymetrix website. Gene ontology and network analysis was performed using Metacore from GeneGo Inc. (St. Joseph, MI). All data is MIAME compliant, and the raw data has been deposited in GEO, a MIAME compliant database, accession number: GSE21979.

Statistical analysis

All results are expressed as mean values plus or minus SD from triplicate measurements performed in 2 to 4 independent experiments producing similar results.

CHAPTER 3. ROLE OF THE UPR AND HIF SIGNALING PATHWAYS IN REGULATING *VEGF* EXPRESSION

Introduction

Solid tumors contain regions that are poorly oxygenated due to a limited accessibility to functional blood supply. These areas are extremely heterogeneous and very dynamic in an individual tumor and between tumors in a given patient. Hypoxia is an important factor that determines prognosis for patients, because it has the ability to alter the cellular metabolism and cause resistance to radiotherapy and chemotherapy. Hypoxic cells are more resistant to radiation therapy compared to well-oxygenated cells, because oxygen is required for the induction of DNA damage by radiation [111]. The poorly vascularized, hypoxic regions of the tumor are also resistant to chemotherapeutic agents due to inefficient drug delivery by distantly located blood vessels. Clinical evidence suggests that hypoxia not only alters the therapeutic outcome of the patient but also contributes to a more aggressive tumor phenotype. The use of mouse models in tumor biology has enabled us to understand some important aspects of tumor oxygenation. First, the severity of hypoxia can vary from completely anoxic (extreme hypoxia) to mildly hypoxic or even normoxic regions in the tumor. The variations observed in the pO_2 in different regions of the tumor is largely due to the transient changes in blood flow and the inability of blood vessels to fuel the rapidly expanding tumor mass. Second, the oxygenation status of any tumor cell is highly dynamic due to the disorganized architecture of tumor blood vessels and rapid vasoconstriction and dilation of the vasculature. These patterns of oxygenation have very different biological and clinical implications, as varying amounts of oxygen levels in the cell have the ability to activate cytoprotective stress signaling pathways [112, 113].

The normal atmospheric concentration of oxygen (normoxia) is 21 % or 160 mm Hg. Measurements of oxygen levels in animal models have demonstrated that the pO_2 in regions of the tumor at a distance of greater than 100 μm from a vessel can vary from 10 mm Hg (hypoxia) to nearly 0 mm Hg (anoxia). Studies with cultured cells have revealed that when oxygen levels drop below 5% O_2 or ~ 38 mm Hg, the hypoxia-inducible factor (HIF) pathway is activated [114]. Transcriptional responses to hypoxia are largely regulated by HIF, a heterodimeric protein composed of an oxygen-labile α subunit and a constitutively expressed β subunit. There are two α subunit members, HIF1 α and HIF2 α , which are continuously synthesized and rapidly degraded under normal physiological conditions [115]. The oxygen-dependent degradation of the HIF- α -subunit is mediated by hydroxylation of proline 402 and proline 564 by prolyl hydroxylase domain protein 2. Hydroxylated HIF- α binds to von Hippel Lindau tumor suppressor protein (VHL), which then targets the α subunit for degradation via the 26S proteasome [10]. The decrease in oxygen levels that occurs with increasing distance from a blood vessel also correlates with a decrease in the environmental pH. The decreased pH observed in tumor tissues is due to an increase in the glycolytic rate of cancer cells. As cancer cells shift their metabolism to glycolysis from oxidative phosphorylation, due to the decreased availability of oxygen, there is an increase in the production of lactate,

which is secreted into the extracellular space making the tumor microenvironment more acidic [116].

Several more recent studies have demonstrated that extreme hypoxia (<0.02 % O₂) can also activate the UPR [117, 118], which enables the cell to adapt to the suboptimal conditions for protein folding by decreasing the overall rate of protein synthesis. Secreted and membrane proteins are processed in the endoplasmic reticulum where they encounter molecular chaperones and folding enzymes, like protein disulfide isomerases, that catalyze the formation of disulphide bonds. These bonds serve to stabilize both folding intermediates and the mature protein, but the enzymatic reaction that catalyzes their formation requires molecular oxygen. Hence, limiting amounts of oxygen in the cell can impinge on the protein folding capacity in the ER, leading to the accumulation of unfolded proteins and activation of the UPR [16]. The change in the pH of the tumor microenvironment that occurs in response to the shift to glycolytic metabolism also has the ability to contribute to UPR activation. The reduced pH can change the net charge on the side chains of amino acids, which play a major role in defining folding pathways. The high metabolic rate of many tumor cells put additional demands on the folding capacity of the ER and provides yet another stimulus for UPR activation. Thus, within a tumor mass it is likely that individual cells are likely to have different compliments of these two stress signaling pathways, with some cells having only one of them activated and others having both.

The HIF pathway is well documented to play a significant role in the production of VEGF in cultured cells and in promoting angiogenesis in animal models. VEGF is a potent proangiogenic factor required for proliferation and migration of endothelial cells that enables formation of new blood vessels. *In vivo* studies reported that HIF1 α ^{-/-} embryonic stem cell derived tumors had fewer blood vessels and impaired vasculature within the tumor mass as compared to the control wild-type counterparts [119]. The importance of the hypoxia-inducible transcription factors, HIF1 α and HIF2 α , in regulating VEGF expression was further emphasized from complete knockout experiments. Both, HIF1 α and HIF2 α knockout animals are embryonic lethal due to lack of functional vasculature [119, 120]. More recently, we [49], and others [121], have shown that the UPR also plays an important role in regulating VEGF expression at both transcriptional and post-transcriptional levels in cultured cells. While we wish to ultimately assess the contribution of the UPR to tumor angiogenesis, due to the heterogeneous nature of tumors, it is critical that we first understand the relative contribution of the UPR and HIF signaling pathways when experienced alone and in combination, in regulating VEGF expression using carefully defined conditions that are specific to each of these stress pathways.

Due to the large variety of knockout lines available, in our previous study we used a combination of rodent cell lines to identify critical elements of the UPR. We demonstrated that both XBP-1(S) and ATF4 bound to the VEGF promoter and contributed to VEGF expression. However, the exact contribution of these two factors could not be determined, because the corresponding controls for the various mouse knockout cell lines had different basal and stress induced levels of VEGF mRNA and

protein. To remedy this problem and to determine the significance of the UPR in regulating VEGF expression in human tumor cell lines, we identified two human neuroblastoma cell lines, SKNAS and NB1691, which have low basal levels of VEGF expression that are significantly induced by ER stress. Similar to the studies in rodent cells, we were able to detect both XBP-1(S) and ATF4 bound to the human VEGFA promoter in a stress-inducible manner. Using siRNA in transient expression experiments, we reduced the expression of both XBP-1(S) and ATF4 independently and found that ATF4 plays the predominant role in regulating VEGF expression in response to UPR activation in human cells. Thus, we have engineered these two neuroblastoma lines to inducibly express shRNA to ATF4. In addition, clones that stably express shRNA to HIF1 α have been obtained for both cell lines. In preliminary experiments, we found that there is a synergy between the two pathways, as VEGF expression is significantly higher when cells are exposed to both stresses simultaneously. However, although ATF4 plays a major role in regulating VEGF expression in cells experiencing only ER stress, it does not contribute significantly to its expression when the UPR and HIF pathways are activated together in cell culture experiments. Instead, the UPR appears to enhance HIF1 α activity and result in HIF1 α -dependent increases in the expression of VEGF and other HIF targets via an as yet undetermined mechanism. The neuroblastoma cell lines expressing inducible stable ATF4 shRNA will be useful in animal experiments to determine the contribution of this factor at various time points during the course of tumor development.

Results

Defining culture conditions that are specific to HIF and UPR pathways

To determine the contribution of the UPR versus the HIF pathway in regulating VEGF expression in human neuroblastoma cell lines, it was critical to identify conditions that would activate each pathway without affecting the other. This was particularly important, since extreme hypoxia has been reported to activate not only the HIF pathway but also the UPR [117, 122]. In addition, we wished to use more physiological activators of the UPR instead of the more traditional pharmacological activators, like thapsigargin or tunicamycin. SKNAS and NB1691 human neuroblastoma cells were treated with media containing decreased concentrations of glucose to mimic a physiological stress experienced by tumors. Under non-stressed conditions, regular tissue culture media contains 25 mM glucose. A variety of concentrations were tested, but we chose 1 mM, a concentration that readily activated the UPR, was not toxic to cells, and is likely to mimic conditions encountered by tumor cells [123]. The two lines were treated with low glucose for 8 and 16 h and the effects on UPR targets and HIF targets were assessed by real-time PCR. The cell lines were also incubated in a hypoxia chamber containing 1% O₂ to activate the HIF signaling pathway. In both cell lines, low glucose lead to the up-regulation of GADD34, a transcriptional target of ATF4 that is activated downstream of PERK (Figure 3-1A and C), whereas incubating the cells in 1% O₂ had no effect on this

Figure 3-1. Low glucose activates the UPR but not the HIF signaling pathway and 1% O₂ (hypoxia) activates the HIF pathway but not the UPR. Cells were either left untreated (NT), incubated in media containing 1 mM glucose (Lglu), or treated in a hypoxia chamber containing 1% O₂ for 8 hours and 16 hours as indicated. Total RNA from the indicated samples was subjected to qRT-PCR to quantify *Gadd34* mRNA (A{SKNASluc} and C{NB1691luc}) or *BNIP3* mRNA (B{SKNASluc} and D{NB1691luc}) at the indicated time points. RNA levels were expressed relative to the control untreated samples for each line, which was set to 1. Experiments were performed in duplicate (values are mean $\pm$ SD).

target. Similarly, when the cells were cultured in 1% O₂, the HIF target, BNIP3, was readily induced, but there was no up-regulation of the UPR target, GADD34 (Figure 3-1B and D). Thus, it was possible to define conditions that were specific for each of the two stresses.

UPR and HIF activation induces VEGF secretion

We next measured the effect of these pathways on VEGF secretion using the stress conditions described above that activated either the HIF or UPR alone without affecting the other pathway. Although there was a detectable amount of VEGF secreted from untreated cells, we observed an increase of VEGFA in the media from cells treated with either of the stress inducers (Figure 3-2). Culturing cells in low glucose media for 16 hours caused a ~3-fold increase in VEGF secretion as compared to the untreated control sample in the SKNASluc line. Hypoxia seemed to be a somewhat more potent inducer of VEGF secretion in this line, as 16 hours of treatment in the hypoxia chamber resulted in a ~5-fold increase as compared to the control sample. When the cells were treated with a combination of both stresses; hypoxia and low glucose media, we observed an increase in VEGF secretion that was somewhat greater than what was seen with either of the stress conditions alone (Figure 3-2).

UPR and HIF signaling pathways have a synergist effect on VEGF transcription

Since both pathways are known to regulate VEGF at the transcriptional level, we determined the relative contribution of the UPR and HIF signaling pathways in regulating both the transcription rate and steady state mRNA levels. VEGF hnRNA levels were used as a measure of the transcription rate of the gene. The VEGF transcription rate was increased ~3-fold in SKNASluc cells treated with low glucose media for 8 hours, which was increased further after 16 hours (Figure 3-3A). Hypoxia induces VEGF transcription rate ~4 fold at both, 8 and 16-hour time points. The combination of both UPR and HIF activation results in an increase in VEGF hnRNA levels to ~7-fold at 8 hours, and after 16 hours the combination of these two stress pathways appeared synergistic and induced the VEGF transcription rate ~12-fold. The VEGF mRNA levels were induced 2 fold in SKNASluc cell line after 8 and 16 hours of treatment in media lacking glucose (Figure 3-3B). In keeping with the increased transcription rate observed with the combination of UPR and HIF activation, we found an additive effect on up-regulation of VEGF mRNA levels in the line at both time points. The NB1691luc cell line had a similar pattern of VEGF hnRNA and mRNA induction with UPR and HIF activation at both time points. VEGF expression was induced ~4 fold with each of these pathways activated alone at both time points as indicated in Figure 3-2C and D. However, the combination of both stresses had a more potent and synergistic effect on up-regulating VEGF expression, particularly in the NB1691 cell line.

Figure 3-2. VEGF secretion is increased with UPR and HIF activation.

SKNASluc cells were either left untreated (NT), incubated in media containing 1 mM glucose (Low Glu), treated in a hypoxia chamber containing 1% O₂ (Hypoxia 1% O₂) or treated with a combination of low glucose media and incubated in a hypoxia chamber (Low Glu 1 mM+Hypoxia 1% O₂) for 8 hours and 16 hours as indicated in the figure. Conditioned media was collected from the indicated samples at the given time points and analyzed for VEGF secretion by ELISA. Experiments were performed in duplicate (values are mean $\pm$ SD).

Figure 3-3. VEGF mRNA and hnRNA levels are up-regulated with the UPR and HIF signaling pathways alone and more potently with the combination of both stresses together. Cells were either left untreated (NT), incubated in media containing 1 mM glucose (Low Glu), treated in a hypoxia chamber containing 1% O₂ (Hypoxia 1% O₂) or treated with a combination of low glucose media (1 mM) and incubated in a hypoxia chamber (Low Glu 1 mM+Hypoxia 1% O₂) for 8 hours and 16 hours as indicated in the figure. Total RNA from the indicated samples was subjected to qRT-PCR to quantify *VEGF* hnRNA (**A** {SKNASluc} and **C** {NB1691luc}) or *VEGF* mRNA (**B**{SKNASluc} and **D**{NB1691luc}) at the indicated time points. RNA levels were expressed relative to the control untreated samples for each line, which was set to 1. Experiments were performed in duplicate (values are mean $\pm$ SD).

Human VEGF promoter has potential binding sites for both UPR-inducible and HIF transcription factors

We next analyzed 10kb upstream and 3 kb downstream of the transcription start site of the human VEGF promoter for potential binding sites of UPR-inducible transcription factors, XBP-1(S) and ATF4 and hypoxia-inducible HIF. A number of potential binding sites were identified for each of these factors as illustrated in Figure 3-4. Under hypoxic conditions, HIF1 α rapidly accumulates, dimerizes with HIF1 β , and binds to the core DNA binding sequence 5'-RCGTG-3' (R, purine (A or G)) on target genes. On the other hand, upon UPR activation XBP-1(U) is spliced to form the activated form; XBP-1(S), which translocates to the nucleus and binds to core DNA sequence 5'-ACGTG-3'. Upon UPR activation, ATF4 is translated and binds to its target genes containing the core 5'-CGTXA-3' (X= C or A) sequence. Inspection of the human VEGF promoter indicated that several of the potential UPR-inducible transcription factor sites overlapped with possible HIF binding sites. Thus, we first tested occupation of these sites when each pathway was activated alone.

ATF4 and XBP-1 bind to the human VEGF promoter in response to UPR activation and HIF1 α binds in response to hypoxia

To determine if either of the UPR induced transcription factors binds to the human VEGFA promoter in response to ER stress, we performed ChIP assays in the SKNAS, NB15 and NB7 cell lines. We were unable to detect binding of ATF4 to any of the six potential sites upstream of the transcription start site in response to low glucose treatment (data not shown). However, we did detect UPR-stress-inducible binding of ATF4 to a site at position +1.2 kb relative to the transcription start site (Figure 3-5D), which is analogous to where ATF4 binds in the mouse promoter. We also performed ChIP assays in the human neuroblastoma cell lines, NB7 and NB15, and were able to detect binding of XBP-1(S) at a region 0.5 kb upstream of the transcription start site after treatment with the UPR inducer, thapsigargin (Figure 3-5E). Promoter analysis of the human *VEGFA* gene revealed several potential HIF binding sites (Figure 3-5C). It has been reported in the literature that HIF1 α binds to the human VEGF promoter -900 bp relative to the transcription start site in response to hypoxia [8]. We performed ChIP assays in the SKNAS cell line to determine if this site and the other potential HIF binding sites are occupied with hypoxia (1% O₂). We detected a slight but reproducible binding of HIF1 α to a region on the human VEGF promoter -900bp relative to the transcription start site in response to hypoxia (Figure 3-5F), but were unable to detect HIF1 α to any of the other potential binding sites on the promoter.

ATF4 plays a major role in regulating UPR-induced VEGF secretion in SKNASluc cells

Chromatin immunoprecipitation studies performed in the human neuroblastoma cell lines demonstrated that ATF4 and XBP-1(S) bind to the human VEGF gene on UPR

Figure 3-4. Potential binding sites for XBP-1, ATF4 and HIF1 on the human VEGF promoter. Potential binding sites of UPR downstream transcription factors in human VEGF promoter. The online software rVista was used to identify potential binding sequences of transcription factors, XBP-1 (cyan), ATF4 (green) and HIF (red) in a region 10 kb upstream and 3 kb downstream of the transcription start site (TSS) on the human VEGFA promoter.

Figure 3-5. UPR-inducible transcription factors, ATF4 and XBP-1(S) bind to the human VEGF promoter in response to UPR activation and HIF1 α binds in response to hypoxia. Potential ATF4 (A), XBP-1(S) (B) and HIF (C) sites in the human *VEGF* promoter. Cross-linked chromatin from SKNAS cells that were untreated (NT), incubated in media containing 1 mM glucose (Lglu) or treated in a hypoxia chamber containing 1% O₂ for 8hours were immunoprecipitated with anti-ATF4 (D) or anti-HIF1 α (F). Human neuroblastoma cell lines, NB7 and NB15 either left untreated or treated with thapsigargin, were used in ChIP assays with anti-XBP-1(S) antibody (E). Immunoprecipitated chromatin and input controls were used for PCR amplification.

activation. Our previous study revealed that ATF4 also binds to the mouse VEGF gene using mouse embryonic fibroblasts deficient in this factor. XBP-1(S) was also shown to bind to the rat VEGF promoter and using knockout cell lines, and we were able to show that it plays a role in regulating VEGF expression in cell culture experiments. We were unable to determine the exact contribution of ATF4 and XBP-1 in regulating VEGF expression as the untreated wild-type MEFs had different basal levels of VEGF and induced its expression to different levels [49]. To overcome this problem, we performed transient siRNA experiments to determine the contribution of these two UPR transcription factors in the same human cell line. Since it is important to obtain ~100% of the cell population expressing the siRNA, we co-transfected the indicated siRNAs with FITC-labeled non-targeting RNA and then sorted the samples after 48 hours of transfection to obtain only the viable FITC positive cells. Cells transfected with siRNA targeting human ATF4 significantly decreased UPR-induced expression of this factor and had no significant effect on XBP-1(S) protein levels (Figure 3-6B). In a similar manner, cells transfected with XBP-1(S) siRNA had significantly decreased expression of UPR-induced XBP-1(S) protein. Of note, hypoxia (1% O₂) did not induce either ATF4 or XBP-1(S) protein levels (Figure 3-6 B). The culture supernatant from each group was obtained and used to measure VEGF protein levels by ELISA. VEGF secretion was measured from the negative control cells, as well as cells treated with ATF4 siRNA or XBP-1(S) siRNA. Knocking down ATF4 expression significantly reduced VEGF secretion to the basal level, where as knocking down XBP-1(S) only decreased VEGF secretion ~25% as compared to the negative control-thapsigargin treated sample. These results suggest that ATF4 is the major regulator for UPR-induced VEGF secretion in the SKNASluc cell line.

Inducible stable knockdown of ATF4 inhibits VEGF transcription rate in response to low glucose but not hypoxia or the combination

To further test the role of the UPR-inducible transcription factor, ATF4, in regulating VEGF expression, we engineered doxycycline-inducible knockdown SKNASluc lines, and are in the midst of selecting clones for the NB1691 cell line. This strategy will enable us in the future to manipulate the expression of ATF4 in xenograft studies at various time points during the course of tumor growth. Single cell clones were isolated after transfecting with a single shRNA specific for ATF4. The cells were incubated with dox for 0, 24, 48hr and then treated with thapsigargin for 16 hours. Cell lysates were prepared and blotted for ATF4 to examine the effects of the shRNA on ATF4 expression. We also examined cell lysates for XBP-1 expression as a negative control. Two representative clones were isolated that showed a significant inhibition of stress-induced ATF4 expression and decreased Gadd34 mRNA levels-a downstream target of ATF4 (Figure 3-7A and B). When these clones were then examined for the effects on UPR-induced VEGF transcription, we found that the rates were reduced to basal levels. As anticipated, decreasing ATF4 expression had no effect in regulating hypoxia-induced VEGF expression, keeping with the results obtained from the transient knockdown experiments. Although we started isolating clones with inducible XBP-1shRNA expression, given the fact that decreasing ATF4 appeared to block the

Figure 3-6. ATF4 plays an important role in regulating VEGF secretion in response to UPR activation. SKNASluc cells were either transiently co-transfected with FITC-labeled non-targeting RNA and siRNA specific for human XBP-1(S) or ATF4 as indicated in the figure. As a control, cells were transfected with only FITC-labeled non-targeting RNA. After 48hours, cells were sorted and the FITC positive viable population was re-plated. 24 hours post-sorting, the cells from each group were either left untreated, treated with 1 μ M thapsigargin or incubated in a hypoxia chamber (1% O₂). **(A)** Conditioned media was obtained from each treatment as indicated in the figure and analyzed for VEGF secretion by ELISA. **(B)** Western blot analysis was performed on cell lysates as indicated in the figure. ATF4 and XBP-1(S) levels were analyzed from nuclear fractions and Lamin-B1 was used as a loading control.

Figure 3-7. ATF4 regulates UPR-induced VEGF transcription rate but has no affect in response to hypoxia or the combination of both the pathways. Two clones with stable expression of Tet-repressor and pSuperior.puro-ATF4shRNA vectors in the SKNASluc cell line were obtained. **(A)** The ATF4shRNA clones were treated with doxycycline (1 μ g/ml media) for 24 hours or 48 hours after which the cells were either left untreated or treated with thapsigargin for 16 hours. Cells were harvested and ATF4 protein levels were measured using western blot analysis. **(B)** The negative control cell line and ATFshRNA clones were treated with Dox for 48 hours following which they were either left untreated or treated as indicated in the figure for 16 hours. Cells were harvested and RNA was extracted to measure Gadd34 levels- a downstream target of ATF4. **(C)** The negative control cell line and ATFshRNA clones were treated with Dox for 48 hours following which they were either left untreated or treated with low glucose media (1 mM glucose), incubated in a hypoxia chamber (1% O₂) or treated with a combination of low glucose media and hypoxia for 16 hours. Post-treatment cells were harvested, RNA was extracted and total RNA from the indicated samples was subjected to qRT-PCR to quantify *VEGF* hnRNA. RNA levels were expressed relative to the control untreated sample, which was set to 1.

UPR-induced expression of VEGF completely, we did not continue with these.

We next designed an experiment to determine the relative contribution of ATF4 to VEGF expression when cells have activated both the UPR and HIF pathways simultaneously. To our surprise, we found that decreasing ATF4 expression had no perceivable effect in regulating VEGF transcription rate when both, the HIF and UPR signaling pathways were activated together (Figure 3-7C).

Thus, we first examined ATF4 levels in these clones and assessed the binding ability of this factor in the presence low glucose or both stresses together. As observed in the parental line, ATF4 binds to the human VEGF promoter 1.2 kb downstream of the transcription start site when treated with low glucose media (Figure 3-8B). A somewhat fainter PCR product for ATF4 binding was detected with a combination of both stresses together. We also analyzed ATF4 protein levels in the SKNASluc (Figure 3-8C) and NB1691 (Figure 3-8D) by Western blot analyses. ATF4 protein levels were induced both with low glucose media, which activates the UPR, as well as with a combination of low glucose media and hypoxia in both cell lines. However, it must be noted that ATF4 protein levels were decreased in both lines when the two pathways are activated together as compared to UPR activation alone, particularly at later time points. The ChIP assay was performed after 8 hours of treatment as indicated in the figure, and at this time point ATF4 levels appear to be more similar when either the UPR is activated alone or in combination with hypoxia (Figure 3-8C).

HIF1 α binding to the human VEGF promoter appears greater with the combination of hypoxia and low glucose

We repeated ChIP assays in the SKNASluc to determine if this site and the other potential HIF binding sites are occupied with hypoxia alone and the combination of hypoxia and low glucose together. We detected hypoxia-inducible binding of HIF1 α to the region on the human VEGF promoter -900bp relative to the transcription start site (Figure 3-9B). We were also able to detect binding of HIF1 α with the combination of hypoxia and low glucose, which appeared to be somewhat stronger, although this has not been repeated using quantitative PCR analysis, which we will do in our follow-up studies. We also analyzed HIF1 α protein levels in the SKNASluc (Figure 3-9C) by Western blot analyses. HIF1 α protein levels were induced with hypoxia, however, unlike ATF4, the combination of low glucose media and hypoxia reproducibly lead to somewhat similar levels of HIF1 α .

HIF1 α regulates VEGF transcription rate in response to hypoxia and the combination of both stresses together

Our ATF4 knockdown experiments argued that ATF4 did not appear to contribute to VEGF expression with the combination of low glucose and hypoxia. This could suggest that another UPR-induced transcription factor played a bigger role with the

Figure 3-8. UPR-inducible transcription factor, ATF4, binds to the human VEGF promoter. (A) Potential ATF4 sites in the human *VEGF* promoter. (B) Cross-linked chromatin from SKNASluc cells that were untreated (NT), incubated in media containing 1 mM glucose (Lglu), treated in a hypoxia chamber containing 1% O₂ or treated with a combination of low glucose media and incubated in a hypoxia chamber (Hyp+Lglu) for 8hours were immunoprecipitated with anti-ATF4. Immuno-precipitated chromatin and input controls were used for PCR amplification. As a positive control, primers spanning the ATF4 binding region on the CHOP promoter were used to PCR amplify the anti-ATF4 precipitated chromatin. ATF4 protein levels were detected in SKNASluc (C) and NB1691luc (D) cells treated with low glucose (1 mM), hypoxia or Low glucose + Hypoxia using Western blot analysis.

Figure 3-9. HIF1 α binds to the VEGF human promoter when the HIF signaling pathway is activated alone and in combination with the UPR. (A) Potential HIF sites in the human *VEGF* promoter. **(B)** Cross-linked chromatin from SKNASluc cells that were untreated (NT), incubated in media containing 1 mM glucose (Lglu), treated in a hypoxia chamber containing 1%O₂ or treated with a combination of low glucose media and incubated in a hypoxia chamber (Hyp+Lglu) for 8 hours were immunoprecipitated with anti-HIF1 α . Immuno-precipitated chromatin and input controls were used for PCR amplification. **(C)** HIF1 α protein levels were detected in SKNAS cells treated with Hyp and Hyp+Lglu using Western blot analysis.

combination of stresses. Thus, we first wished to determine the contribution of HIF1 α in regulating VEGF transcription rate. To do so, we analyzed SKNAS cells that have stable expression of HIF1 α shRNA. As anticipated, the VEGF transcription rate was decreased to basal levels in the cells expressing HIF1 α shRNA were incubated in a hypoxia chamber compared to the control line, whereas there was no effect on UPR induced expression. However, we found that the VEGF transcription rate was reduced to near basal levels in the HIF1 α shRNA cells when they were treated with the combination of hypoxia and low glucose as compared to the negative control (Figure 3-10A). This argued that instead of both stress-regulated transcription factors playing a joint role in increasing VEGF transcription that the UPR was somehow increasing either HIF expression or activity.

As this had not been reported previously to our knowledge, we wished to determine how the UPR was affecting HIF. Although HIF is not induced at the transcriptional level in response to hypoxia, we reasoned that if the UPR either led to increased HIF mRNA by either increasing its transcription or stability, that there might be a larger pool of HIF protein to be stabilized by ER stress. Therefore, we first measured HIF transcription rate with both stresses alone and the combination. Although HIF transcription rate was not induced with hypoxia alone at either of the time points we analyzed, there was a modest (~2 fold) but reproducible increase in the HIF transcription rate when both stress pathways were activated together (Figure 3-10B). These results suggest that the UPR might synergize with the HIF pathway by up-regulating HIF1 α transcription. Further studies in other lines and at additional time points will be required to determine if indeed HIF1 α is a transcriptional target of the UPR.

Lactate production is inhibited in SKNAS_{luc} cells treated with low glucose and the combination of low glucose + hypoxia

A second possibility for the observed synergy relates to the mechanism used by tumor cells to produce energy. Due in part to limited availability of oxygen, cancer cells generate ATP through a less efficient mechanism compared to normal differentiated cells by increasing glycolysis and decreasing oxidative phosphorylation. This switch in metabolism is known as the Warburg effect. By decreasing glucose, which is necessary for glycolytic energy production, we reasoned that perhaps when oxygen levels were also limited in our experimental set up, it was possible that the cells would be forced to continue using oxidative phosphorylation and thus become more hypoxic than when similar levels of oxygen were encountered alone, which could result in more HIF activity. As a somewhat indirect method of testing the possibility that the combination of these stresses could increase the hypoxic state of the interior of the cells, we measured lactate production from the SKNAS_{luc} treated with various UPR inducers, with hypoxia alone, and with the combination (Figure 3-11). Lactate secretion gives us a measure of the amount of glycolysis occurring in the cell, because glucose is broken down via multiple steps to pyruvate and finally converted to lactate as the end product. We observed a relatively high basal level of lactate secretion in the untreated cells, suggesting that they were already using some glycolysis to produce energy. This is a common feature of

Figure 3-10. HIF1 α regulates VEGF transcription rate in response to HIF signaling and the combination of UPR and HIF signaling. (A) SKNAS cells that have stable expression of either HIF1 α shRNA or control shRNA were either left untreated (NT), incubated in media containing 1 mM glucose (Low Glu), treated in a hypoxia chamber containing 1% O₂ (Hyp) or treated with a combination of low glucose media (1 mM) and incubated in a hypoxia chamber (Hyp+Lglu) for 8 hours as indicated in the figure. Total RNA from the indicated samples was subjected to qRT-PCR to quantify *VEGF* hnRNA. RNA levels were expressed relative to the control untreated samples for each line, which was set to 1. (B) SKNASluc cells were treated with low glucose media, hypoxia and the combination of both stresses for 8 and 16 hours as indicated in the figure. Total RNA was extracted and subjected to qRT-PCR to quantify HIFhnRNA levels.

Figure 3-11. Lactate production from SKNASluc cells treated with UPR inducers and hypoxia. SKNASluc cells were either left untreated or treated with thapsigargin (Tg1 μ M), low glucose media (1 mM), hypoxia (1% O₂) or a combination of Tg and hypoxia or Low glucose media and hypoxia for various time points as indicated in the figure. Post-treatment cells were harvested, media was collected, de-proteinized using a 10 kD spin column and used to measure lactate production. Lactate is represented as mmol secreted per 1x10⁶ cells.

cancer cells. As anticipated, lowering the glucose levels resulted in decreased secretion of lactate at all time points, whereas treating cells with thapsigargin, another UPR inducer which was not expected to affect cellular glucose levels, had no effect on lactate production. Although we anticipated that incubating cells in hypoxia would increase lactate production, we observed only a modest increase at the latest time point in our experiment. Two interesting observations did come from this experiment. First, in all cases, treating the cells with low glucose simultaneous with low oxygen, radically decreased the production of lactate, indicating that indeed glycolysis was not possible without sufficient availability of glucose. Thus it is likely that the cells are more reliant on oxygen for energy production and might become more hypoxic at a given concentration of oxygen or at an earlier time after switching to low oxygen conditions. A second interesting result was found with cells that were subjected to the combination of thapsigargin and hypoxia. As expected, in this case there was no effect of the UPR on lactate production. Based on this hypothesis, we would not have expected a synergy with thapsigargin and hypoxia on VEGF production. However, inspection of VEGF transcription rates with this combination of stresses reveal higher levels of VEGF induction than with either stress alone (data not shown).

Discussion

A limiting microenvironment can activate cellular stress pathways like the UPR and HIF signaling when nutrients and oxygen levels are suboptimal. These pathways synergize to positively regulate proangiogenic factors, which stimulate growth of new blood vessels toward the affected cells to restore homeostasis. Our tissue culture studies revealed that the UPR and HIF signaling pathways induce VEGF expression to a greater extent than each of the stress pathways alone. In the case of a rapidly proliferating tumor mass, there should be a subpopulation of tumor cells that activate both stress pathways together, which would result in greater secretion of VEGF as compared to tumor cells that have only one pathway activated. The mechanism for this synergy in the case of a pharmacological UPR activator like thapsigargin is unknown, although in the case of low glucose our data allows us to speculate. Tumor cells have an altered metabolism that enables them to produce significant amounts of ATP from glycolysis rather than oxidative phosphorylation. By decreasing the oxygen supply to a cell we would expect to see a further increase in its glycolytic potential. The synergistic induction in VEGF expression when observed when both stresses were present together led us to speculate that when glucose was limited the cell would be forced to rely more on oxidative phosphorylation, which might further decrease cellular oxygen levels and result in more hypoxic conditions in the cell for a given concentration of oxygen. Indeed, we found that the levels of glucose in our “low glucose” conditions was not sufficient to support glycolysis as a means of energy production. This would imply that the cells were either producing less ATP or producing it via oxidative phosphorylation. To distinguish between these possibilities, it would be necessary to measure cellular ATP levels or oxygen consumption by the mitochondria, as this is where oxidative phosphorylation takes place. If the synergy on VEGF transcription that we observed with low glucose and hypoxia was due to increasing the intracellular level of hypoxia, we would not have

expected to see a synergy when thapsigargin was used to activate the UPR with hypoxia, since this did not reduce lactate production. However, we also found that VEGF mRNA levels were significantly higher when these two activators were combined. Thus, there must be another mechanism for synergy between these responses. It is formally possible that in this case ATF4 still binds to the VEGF promoter to increase transcription. Experiments with this combination of stresses will be conducted in the ATF4 deficient cells. Importantly, these data have been obtained with a single line and must be reproduced in another line before we can draw conclusions. However, if they hold, these results have very important implications in tumor angiogenesis, as they might argue against a need to target the UPR pathway to inhibit VEGF production in cells where both stress pathways are activated due to both limited oxygen and glucose.

The importance of HIF1 α in regulating VEGF expression is well documented in the literature. To the best of our knowledge, HIF1 α has not been shown to be regulated by UPR activation. Experiments designed to understand the mechanism by which the UPR increases the activity of this factor in regulating VEGF and at least one other HIF target, BNIP3 expression when both stresses are applied together revealed an interesting finding. We found that these dual conditions appeared to modestly increase HIF1 α binding to the human VEGF promoter, although importantly the experiment was not conducted in a way to measure quantitative increases in binding. Interestingly, we did observe a modest induction (~2 fold) in the HIF1 α transcription rate (hnRNA) with the combination of both stresses together, but this was not sufficient to noticeably increase VEGF mRNA or protein levels. Thus it is possible that at later time points we would begin to see an increase in VEGF transcripts, but within the time frame of our experiments, this was not the cause of increased HIF1 α binding to the promoter. On preliminary inspection of the HIF1 α promoter we were able to identify potential binding sites for XBP1(S), ATF4 and NF κ B, which are activated downstream of the UPR, but further experiments will be needed to determine the significance of this slight increase in HIF hnRNA, particularly given the fact that we did not see this increase with UPR activation alone. It is possible to speculate that the UPR has the ability to modify the chromatin structure of the VEGF promoter at this site, or enhance the transcriptional machinery or HIF1 α co-factors, like p300/CBP, to facilitate HIF1 α binding to the VEGF promoter and thus indirectly contributing to VEGF expression. Further studies will be required to determine if any of these changes occurs. Lastly, and perhaps more likely in the case when low glucose is used to activate the UPR, it is conceivable that cells become hypoxic at an earlier time point, which might result in the stabilization of HIF1 α even before ATF4 is synthesized. Although the two sites are not particularly close on the linear sequence of the gene, it is possible that the binding of the HIF1 α complex to its site on the promoter precludes the binding of ATF4 to its site. The experiments where thapsigargin and hypoxia are used together might shed light on this possibility.

From these experiments one thing we can definitely conclude is that the interplay between these two pathways in regulating VEGF expression is complex and further experiments will be required to determine the exact mechanism of how they synergize with one another. Our future experiments will elucidate the importance of ATF4 in regulating tumor angiogenesis in orthotopic xenograft studies.

Materials and Methods

Cell culture and stress induction

SKNASluc and NB1691luc human neuroblastoma cell line stably expressing luciferase were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% antibiotic-antimycotic at 37°C in a 5% CO₂ incubator. Cells were plated and left untreated (NT) or treated with thapsigargin (Tg, 1μM), media containing 1 mM glucose (Low Glu) (RPMI cat. no.11879-GIBCO), or cultured in a hypoxia chamber containing 1% O₂ for the indicated periods of time.

mRNA and hnRNA quantification by qRT-PCR

Total RNA was extracted using the RNeasy Qiagen mini-prep kit according to the manufacturer's protocol. RNA samples were subjected to reverse transcription using High Capacity cDNA synthesis kit from ABI Life technologies. The cDNA was diluted and Real-time PCR reactions were done in duplicate using a SyBr Green PCR Master Mix kit. Amplification of the corresponding genes was achieved using specific primers and measured continuously using an ABI 7900 HTI Detection System. Where indicated, VEGF hnRNA was measured using qRT-PCR primers across exon 1 and intron 1, for the human gene. The signal obtained for measuring both, mRNA and hnRNA, was compared relative to GAPDH internal control. The value for untreated cells was set to 1 and the value for the various treatments was presented as a fraction of this number. The primers used in this study were as follows: VEGFhnRNA Fwd 5'GCTGTCTTGGGTGCATTGGAG3' Rev 5'CCATGGACCCGCGTGG5' VEGFmRNA Fwd 5'GTAGCTCGGAGGTCGTGGCGC3' Rev 5'GCGAGAACAGCCCAGAAGTTGGACGA3' Gadd34mRNA Fwd 5'GCCAGAAAGGTGCGCTTCTC3' Rev 5'CTCAGCTCCTCCTGGGCC3' BNIP3mRNA Fwd 5'TTAAACACCCGAAGCGCACGG3' Rev 5'GACTCCAGTTCTTCATCAAAAGGT3' HIFhnRNA Fwd 5'GTGCCCTTTTATAGGTGATTTG3' Rev 5'GTGCATTTTACCTGAGTTAATCCC 3'

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed using a ChIP kit (Millipore) according to the manufacturer's instructions. Briefly, cells were incubated for the indicated times with or without different stress inducers. Formaldehyde was then added (final concentration, 1%), and cells were incubated for 10 minutes at room temperature to stabilize DNA-protein interactions. Cross-linking was stopped by the addition of glycine (final concentration, 0.125M). Cell extracts were sonicated with a Branson Sonifier 250 (VWR) for 5 bursts at 10 seconds each at 20% power output to

shear DNA to 1kb or less. Extracts from 10^7 cells were incubated overnight with antibodies against ATF4 generously provided by Dr. David Ron (University of Cambridge, U.K.), rabbit anti-XBP-1(S) polyclonal antiserum (Santa Cruz Biotechnology sc-7160X), anti-HIF1 α (Abcam) or rabbit anti-BiP polyclonal antiserum, which served as a negative control. Two percent of the extract volume was removed before immunoprecipitation and served as input control. DNA fragments from immunoprecipitated complexes and input controls were released by heating at 65°C overnight and purified using the PCR purification kit (QIAquick, Qiagen) according to the manufacturer's protocol. Purified immunoprecipitated DNA and input DNA were then analyzed by PCR. The primers used to amplify the regions on the human VEGF promoter are as follows:

"HIF" binding site Fwd 5'GGCTTGGGGAGATTGCTCTACTTCC3' Rev
5'GCGAGAACAGCCCAGAAGTTGGACGA3'

"ATF4" binding site Fwd 5'GGTCGGGCCTCCGAAACCATGAACT3'
Rev 5'GCAGCGGCAACGCAAGCCCAGC3'

"XBP-1(S)" binding site Fwd 5'GGTGGGAGCTCTGGGCAGCTGG3'
Rev 5'CCAGGGGAGAAGAATTTGGCACCAA3'

Western blotting

For Western blotting cells were lysed in Nonidet P-40 lysing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and 0.5% deoxycholic acid) for 30 minutes on ice. The proteins were electrophoresed under reducing conditions, transferred to a PVDF membrane and probed with the indicated primary antibodies: Rabbit anti-XBP-1(S), rabbit anti-ATF4, goat anti-Hsc70, and goat anti-Lamin B1 were purchased from Santa Cruz Biotechnology and mouse anti-HIF1 α from BD Biosciences. Blots were incubated with the appropriate HRP-conjugated secondary antibody, and proteins were visualized using the Pierce enhanced chemiluminescent substrate (Thermo Scientific).

Vascular endothelial growth factor ELISA

The quantikine human VEGF ELISA kit (R&D Systems) was used to measure the VEGF levels in treated and untreated culture supernatants. VEGF secreted in the media was determined based on a standard curve and was expressed in pg/ 1×10^6 cells.

Generation of dox-inducible ATF4 shRNA in the SKNASluc cell line

Two separate shRNA sequences targeting human ATF4, one specific for the 3'UTR of the gene and a second sequence that targets exon 4, were cloned into the pSuperior-puro vector (Oligoengine), which expresses shRNA molecules from a doxycycline-inducible H1 promoter. Both these sequences were validated previously using transient siRNA knockdown experiments in the neuroblastoma lines. Two separate

vectors; one containing the tetracycline repressor and the other encoding the ATF4 shRNAs were stably transfected in the SKNASluc cell line. Blasticidin (3µg/ml media) and Puromycin (1µg/ml media) were used to select for clones stably expressing the tet-repressor and p-Superior vectors respectively. After obtaining antibiotic-resistant double transfectants, a number of individual clones were picked using cloning cylinders and screened for regulated knock-down of ATF4 expression. Cells were treated with or without doxycycline for 24 or 48 hrs and then treated with thapsigargin for 16 hrs as indicated, and the level of ATF4 expression was determined using Western blot analysis. The ATF4 shRNA sequence used to clone in the pSuperior vector with BglIII and XhoI overhangs is as follows: Fwd
5'GATCCCCCCTTCTGACCACGTTGGATTTC AAGAGAATCCAACGTGGTCAGA
AGGTTTTTA3' and Rev
5'TCGATAAAAACCTTCTGACCACGTTGGATTCTCTTGAAATCCAACGTGGTCA
GAAGGGGG3'

Lactate assay

The SKNASluc neuroblastoma cell lines were treated as indicated above. Post-treatment cells were harvested for Western blot analysis and media was collected to measure lactate production. Media was deproteinized using a 10 kd spin column (Biovision) following which the media was used to determine lactate amounts using a Lactate Assay Kit (Biovision).

CHAPTER 4. ROLE OF THE UPR-INDUCIBLE TRANSCRIPTION FACTOR ATF4 IN REGULATING ANGIOGENESIS *IN VIVO*

Introduction

After acquiring genetic changes that result in a neoplastic phenotype, highly proliferative cancer cells survive in a limiting environment that activates cytoprotective stress pathways including the UPR, which has been documented in a number of tumor types including breast, hepatocarcinoma, gastric cancer and gliomas in both patient samples and xenograft studies.

The three signal transducers of the UPR: Ire1, PERK and ATF6 have reported to play roles in regulating tumor angiogenesis in xenograft studies. To elucidate the role of Ire1 in regulating tumor growth and angiogenesis *in vivo*, two separate studies engineered U87 human glioma cells with either an Ire1 dominant negative mutant or a control empty vector and then orthotopically implanted them in mice [42, 43]. In both studies, tumors derived from cells expressing the Ire1-dominant negative mutant were significantly smaller, less vascularized but more highly infiltrative as compared to their control counterparts. Cells grown in hypoxic/anoxic conditions in tissue culture and in hypoxic areas of the tumor activate a translational control program called the Integrated Stress Response (ISR), which adapts cells to endoplasmic reticulum stress. Tumors derived from K-ras transformed PERK^{-/-} MEFs were much smaller and had decreased microvessel density as compared to the tumors derived from PERK wild-type MEFs when injected in the flank of the animals. The decreased angiogenic phenotype observed in PERK^{-/-} MEFs was analyzed by microarray and was attributed to down-regulation of proangiogenic factor VCIP, an adhesion molecule required for capillary tube formation [46].

Using microarray analysis in a human medulloblastoma line, we found that a large number of proangiogenic factors including VEGF were up-regulated by thapsigargin. Four proangiogenic factors, VEGF, FGF2, angiogenin and IL8 were further analyzed using Real-time PCR. The up-regulation of these factors by ER stress was as robust as, or even greater than, that achieved with hypoxia in several different cell lines tested. We found that two UPR-regulated transcription factors, ATF4 and XBP-1(S) bound directly to the *VEGFA* promoter in response to ER stress and contributed to its transcription. In addition, activation of AMP kinase stabilized the *VEGFA* transcripts, further contributing to *VEGFA* mRNA levels. At the same time our study was published another group reported the importance of the UPR transcription factors, ATF4, XBP-1(S) and ATF6 in regulating VEGF mRNA using different knockout MEFs.

An intact UPR is required for various aspects of tumor growth, proliferation and angiogenesis. Targeting various components of the UPR is a promising strategy for treatment of cancer. A recent study identified STF-083010, a novel small-molecule inhibitor of Ire1 endonuclease activity but has no effect on its kinase activity. The efficacy of this molecule was tested in a human model of multiple myeloma and was

shown to decrease disease burden as compared to untreated controls [124]. Another study identified a small molecule, guanabenz that disrupted the stress-induced dephosphorylation of the α subunit of the translation initiation factor-eIF2 by direct binding to the protein phosphatase 1 PPR15A/Gadd34. Treatment of stressed-cells with this inhibitor enabled them to cope with the increased burden of unfolded proteins by inhibiting production of new protein and thus restoring proteostasis during stressful conditions [125]. Inhibiting critical components of the UPR is a promising strategy as treatment options for several disease conditions. To assess the contribution of ATF4 in regulating angiogenesis *in vivo* we will use an inducible knockdown system to inhibit expression of this factor in a neuroblastoma tumor model.

Neuroblastoma is the most common solid childhood tumor of the peripheral sympathetic nervous system and accounts for 7-10% of all pediatric cancers [126]. The tumors arise typically in the adrenal medulla or paraspinal ganglia that can present as lesions in the neck, chest, abdomen or pelvis. The clinical presentation of the disease is highly variable and can range from a mass that can regress spontaneously, especially in infants, to a primary tumor that can locally invade surrounding tissues or a distantly disseminated disease observed in children of greater than one year of age. The common genetic changes associated with neuroblastoma include *N-Myc* oncogene amplification [127], loss of caspase-8 expression either through gene methylation or deletion [128], near triploid karyotype [129] and deletion of short arm of chromosome 1 [130], these changes have been correlated with the clinical prognosis of the disease. For example, a near-triploidy change in the karyotype of the patient is associated with favorable outcome whereas MYCN amplification or allelic loss at chromosome 1p is associated with more aggressive tumors and poor prognosis. Studies by different groups have identified three discrete sites that are deleted on chromosome 1p36 in neuroblastoma. These regions are being mapped intensively to identify putative tumor suppressor genes that are deleted in neuroblastoma patients. Based on the phenotypic and genetic features of the disease, neuroblastoma is stratified as low, intermediate and high risk [131]. Low risk tumors are localized, can be treated by surgery alone and the survival rate is >98%. Intermediate risk neuroblastomas most often occur in infants and are characterized by a localized tumor with regional lymph node invasion and metastases to bone marrow and bone. The treatment approach is moderate-intensity chemotherapy and surgery that results in a 90-95% survival rate. High-risk tumors predominantly occur in children older than one year of age and are characterized by metastases to bone marrow and bone. These patients are treated with intensive multimodal therapy that includes dose-intensive chemotherapy, surgery, radiotherapy to primary tumor and resistant metastatic sites, myeloablative chemotherapy and immunotherapy. Despite aggressive treatment options, patients older than one year and having advanced stage of the disease have a poor survival rate. Only 40% of neuroblastoma patients that after the age of 4 years survive for 5 years, making it necessary to develop more effective and innovative therapy for treatment of malignant neuroblastoma.

Neuroblastoma is a highly vascularized solid tumor that depends on the tumor vasculature for growth, invasion and metastases. Although the biological mechanisms regulating the clinical heterogeneity of neuroblastoma is not completely understood,

several recent reports have implicated the importance of using anti-angiogenic therapy as a successful treatment strategy for this pediatric cancer. There are three kinds of angiogenic inhibitors described: direct, indirect and mixed inhibitors. Direct inhibitors like endostatin, angiostatin and thrombospondin target microvascular endothelial cells that are required for the proliferation, growth and formation of new blood vessels. Indirect inhibitors are agents that are used to block the activity of growth factors like VEGF or PDGF that are produced by tumor cells. Lastly, mixed inhibitors are multipotent tyrosine kinase inhibitors or interferon- α that affect both tumor cells and endothelial cells. The Food and Drug Administration (FDA) have approved several anti-angiogenic agents for use in clinical trials including bevacizumab (Avastin), a monoclonal antibody that binds to VEGFA and blocks the subsequent activation of its receptor signaling [132]. This agent was shown to slow tumor growth and extend the life of patients with cancers like colorectal, non-small cell lung cancer and colon cancer. Bevacizumab has been used in several xenograft studies to assess its efficacy as treatment strategy for neuroblastoma. One study reported that established orthotopic neuroblastoma xenografts treated with this drug, altered the physiology of the tumor blood vessels, which improved delivery and efficacy of chemotherapeutic agents to the tumor [133]. Another study reported that SKNAS, IMR32 and SH-SY5Y xenografts treated with bevacizumab were much smaller than their untreated counterparts due to reduced angiogenesis [134]. A third study assessed the efficacy of anti-VEGF therapy in disseminated disease using the NB1691 cell line. Although they observed a decrease in tumor burden and prolonged survival of tumor-bearing mice treated with bevacizumab, there was an increase in basic fibroblast growth factor and VEGF expression most likely due to inhibition of VEGF signaling [135]. Taken together, these results emphasize that VEGF blockade in combination with chemotherapy can be an effective strategy to treat neuroblastoma patients.

Validating our tissue culture results in an animal model is a critical step toward delineating the contribution of the UPR-inducible transcription factor ATF4 in mediating angiogenesis *in vivo* and determining whether the UPR might represent a druggable target for the treatment of neuroblastoma. Because the PERK pathway, which is responsible for ATF4 induction during the UPR, has been shown to be required for growth of *K-Ras* transformed MEFs [46], we have chosen to use a regulated shRNA system for knocking down expression of ATF4 in human neuroblastoma cell lines. This will give us the flexibility to turn off expression of this factor during various stages of tumor development to allow the evaluation of target inhibition on tumor initiation, maintenance and metastases.

Results

Neuroblastoma tumors show evidence of UPR activation

A large number of primary tumor types from St. Jude patients have been adapted for xenograft studies and were available for our studies. However, before choosing a

model system for these studies, we wished to identify one that might have relevance to a human cancer. Using an affinity purified, anti-CHOP polyclonal antiserum for immunohistochemistry staining, we found that multiple pediatric tumor types expressed this UPR-induced transcription factor. We chose neuroblastoma as our model system, since a significant number of the pediatric tumor samples were positive for UPR activation (Figure 4-1A), there are numerous well-established xenograft lines, and genetic mouse models are available. *In situ* hybridization analyses of tumor sections obtained from TH-*MYCN* hemizygous mice, a transgenic mouse model of neuroblastoma [136], revealed up-regulation of *BiP* mRNA expression, a molecular chaperone that is a UPR target, in tumor cells (Figure 4-1B), demonstrating evidence of UPR activation in the mouse model similar to that observed in the patient samples. Together, these data provide us with evidence that neuroblastoma is a good model system to study the role of UPR activation in regulating angiogenesis.

Neuroblastoma cell lines induce VEGF secretion on UPR activation

A large number of patient neuroblastoma cell lines have been established at St. Jude for use in xenograft studies. These represent the variety of distinct molecular signatures that are observed in patient tumors, including deletion or inactivation of caspase-8, a cysteine protease regulated in both a death receptor-dependent and independent manner during apoptosis, and/or amplification of the *MYCN* oncogene [137]. We obtained 22 different neuroblastoma (NB) cell lines from Dr. Jill Lahti and Dr. Andrew Davidoff at St. Jude and analyzed them by ELISA for their ability to induce VEGF secretion upon UPR activation. The lines were treated with or without thapsigargin for 24 hrs and culture supernatants were collected and analyzed. We found that VEGF secretion was significantly induced in all cell lines after treatment with thapsigargin, however, the fold induction was quite variable (Figure 4-2). Some lines, such as NB14 and NB16, have a very high basal level of VEGF secretion, making them poor candidates for our studies even though VEGF production was further increased with ER stress. Whereas, several others, including SKNAS, NB7 and NB15 lines have a very low basal level of VEGF secretion and potently induce VEGF on UPR activation making them particularly good candidates for determining the role of the UPR in regulating VEGF expression. The VEGF secretion data was further categorized based on two known genetic features associated with this cancer (Figure 4-3). Cell lines were first grouped into three categories for *MYCN* protein expression; high, intermediate and low. We observed no correlation in any of the groups for either basal levels of VEGF expression or the fold increase in VEGF secretion after UPR activation. Next, the cell lines were subgrouped according to caspase-8 protein expression. Once again, we found cell lines with both high and low basal level of VEGF expression that showed varying levels of induction. Thus, UPR-induced VEGF secretion appeared to be independent of both the *MYCN* protein levels and caspase-8 status.

Based on these analyses, we decided to choose lines the SKNASluc and NB1691luc cell lines to perform our animal xenograft experiments, as these lines were potent inducers of VEGF on UPR activation and both lines have been engineered to

A.

B.

Figure 4-1. UPR activation in neuroblastoma tumors. (A) Slides from the tumor tissue arrays were stained with rabbit anti-CHOP antiserum. Portions of two slides are shown at low magnification at the left. A single tumor sample from each slide is enlarged. The top panel is a sample from a ganglioneuroblastoma. CHOP expression is detected in the large ganglion-like cells and in the smaller more poorly differentiated neuroblastoma cells that form this tumor. **(B)** Tumor sections were obtained from a genetic mouse model of neuroblastoma (TH-MYCN). The left panel shows Hematoxylin and Eosin staining; the darker stained area is the tumor sample with highly proliferative cells. The right panel shows expression levels of BiP mRNA by in situ hybridization.

Figure 4-2. UPR activation potently induces VEGF secretion in neuroblastoma cell lines. 22 different Neuroblastoma cell lines were either left untreated or treated with thapsigargin, a UPR inducer, for 24 hours. Media was collected and VEGF secretion was measured using ELISA assay. Results are represented as amount of VEGF secreted in pg/ml per 1 million cells.

A.

B.

Figure 4-3. UPR-induced VEGF secretion in neuroblastoma lines is independent of MYCN amplification and caspase-8 protein. Neuroblastoma lines were either left untreated or treated with thapsigargin for 24 hours. Media was collected and VEGF secretion was analyzed using ELISA. The NB lines were categorized based on (A) MYCN amplification-high, moderate or low and (B) caspase-8- presence or absence.

express luciferase, which makes it possible to do live cell imaging of tumor growth in either orthotopic and flank xenograft studies. In addition, these two lines were selected based on their genotype; SKNAS expresses caspase-8 protein, whereas NB1691 does not, and the SKNAS cell line has low MYCN protein expression, whereas NB1691 has high MYCN expression. Thus, we have a representative cell line from each of these major categories, which should make our studies more clinically relevant.

Engineering ATF4 inducible stable knockdown neuroblastoma lines

Since previous studies have shown that full activation of the UPR, including the PERK pathway that regulates ATF4 expression, is critical for tumor growth and survival, we will use an inducible system to knockdown ATF4 before and after the tumor has been established in animal. This design is also critical for determining potential feasibility of manipulating the PERK-ATF4 branch of the UPR to treating existing patient tumors. Two separate shRNA sequences targeting human ATF4, one specific for the 3'UTR of the gene and a second sequence that targets exon 4, were cloned into the pSuperior-puro vector (Oligoengine), which expresses shRNA molecules from a doxycycline-inducible H1 promoter. Both these sequences were validated previously using transient siRNA knockdown experiments in the neuroblastoma lines. Two separate vectors; one containing the tetracycline repressor and the other encoding the ATF4 shRNAs were stably transfected in the SKNAS cell line. After obtaining antibiotic-resistant double transfectants, a number of individual clones were obtained and screened for regulated knock-down of ATF4 expression. Cells were treated without or with doxycycline for 24 or 48 hrs and then treated with thapsigargin for 16 hours as indicated, and the level of ATF4 expression was determined using Western blot analysis (data not shown). Two representative clones for a single ATF4shRNA sequence are shown here. While both clones showed robust induction of ATF4 in response to thapsigargin, pretreatment with dox for 24 or 48 hour was sufficient to significantly block its expression in response to thapsigargin (Figure 4-4A). These two clones were further analyzed for the effects of shRNA expression on the UPR-induced VEGF transcription rate using Real-time PCR (Figure 4-4B). VEGF hnRNA levels, an indication of transcription rate, were significantly reduced in response to both thapsigargin and low glucose in both of the two SKNAS-ATF4 shRNA clones after dox pretreatment as compared to the control lines, strongly supporting the role for ATF4 being a major regulator of UPR-induced VEGF expression. As a control, Gadd34 up-regulation, a well-characterized ATF4 target, was inhibited in the shRNA-expressing lines and induced in the control lines on UPR activation (Figure 4-4C).

Cell cycle analysis of SKNASluc ATF4 shRNA lines

Before initiating xenograft studies, we wish to determine if the genetic manipulations and clonal selection have any gross effects on cell growth. To determine this, we will perform two analyses. The first was to assess cell growth of these two clones before and after dox treatment compared to the control line. We found that the

Figure 4-4. SKNASluc lines with Dox-inducible expression of ATF4- an important factor regulating VEGF transcription rate. (A) Western blot analysis of SKNAS-ATF4 shRNA clones were either left untreated or treated with Dox for 24hours or 48hours. The cells were further treated with thapsigargin for 16hours to induce the UPR and ATF4 levels were determined. Real-time PCR analysis of (B) VEGF hnRNA and (C) Gadd34, an ATF4 downstream target, was measured in the control and ATF4-shRNA lines in untreated samples or samples treated with the UPR inducers thapsigargin (Tg) or media lacking glucose (Lglu) after 48 hours of pretreatment with dox.

two clones expressing ATF4shRNA grew at a similar rate with the control SKNASluc cell line in the absence of Doxycycline (Figure 4-5A). In the presence of Doxycycline (Figure 4-5B) the control parental line grew slightly faster than the lines having stable expression of ATF4shRNA. We are in the process of cloning the negative control shRNA (*Renilla luciferase*) in the parental SKNASluc line, which will be used in future orthotopic xenograft studies. We expect that after obtaining stable *Ren luc*-shRNA clones they would also have the same proliferation rate as our ATF4shRNA clones. We are currently analyzing cell cycle distribution of the two clones and the control line before and after induction of shRNA expression with dox.

Future Experiments/Discussion

Orthotopic xenograft experiments to analyze effects on tumor size and vascularization after knocking down ATF4

The next critical aspect of characterizing these clones was to insure that both would still produce tumors in nude mice. It was possible that in our genetic manipulations, we would have isolated clones that no longer were strongly tumorigenic. Alternatively, it was possible that the shRNA would be somewhat leaking even in the absence of dox administration and interfere with tumor growth. To test for these possibilities, we performed flank injections in three SCID mice each with either the control SKNASluc line or the two clones that inducibly expressed ATF4 shRNA. Although in our initial experiment the control line grew up slightly faster, all of the clones resulted in tumor growth within 3-4 weeks. The tumors resulting from the two ATF4 shRNA clones were visualized using the Xenogen imaging machine as these lines have stable luciferase expression (Figure 4-6). The animals were sacrificed and the tumors were either flash frozen or formalin-fixed and paraffin embedded for further analysis. This will include analyzing the tumor sections for UPR target genes like ATF4 and XBP-1. We will also perform IHC analysis to visualize HIF staining in the tumors.

Now that we have preliminary data showing that the clones expressing inducible ATF4 shRNA are able to form well-vascularized tumors in nude mice, we will next perform orthotopic xenograft studies, in order to establish tumors from the conditional knockdown cell lines in a clinically relevant microenvironment. The dox-inducible neuroblastoma cell lines will be injected orthotopically in the retroperitoneal cavity in SCID mice.

The animals receiving retroperitoneal injections of the genetically manipulated SKNASluc cells will be randomized into 2 main groups, *i.e.*, those with shRNA specific for ATF4 and those with shRNA specific for *Renilla luciferase* as negative control. Prior to knocking down ATF4 expression, we will confirm its presence in tumors that reach a size of ~100 mm³ by sacrificing two animals in each group, harvesting the tumor and performing western blot analysis to detect ATF4 expression and its downstream targets.

Figure 4-5. Cell proliferation rate in the SKNASluc control and ATF4shRNA clones. The SKNASluc cell line and two clones with stable expression of ATF4shRNA were either (A) untreated or (B) treated with Doxycycline for 2 or 3 days as indicated in the figure. Post-treatment the cells were trypsinized and counted.

Figure 4-6. Inducible ATF4shRNA clones in the SKNASluc cell line form tumors when injected in the flank of SCID mice. SKNASluc cells expressing Dox-inducible ATF4shRNA were injected into the flank of SCID mice at 1×10^7 cells per site. Two clones expressing the same shRNA targeting ATF4 were tested with 3 animals per group. 28 days later tumor growth was visualized by injecting mice with luciferase substrate D-luciferin and was imaged with a Xenogen CCD camera.

Once expression is confirmed, each group will be further divided into 4 subgroups (8 animals/group), one group will receive doxycycline 7 days after surgery, as the animals need to recover from the invasive procedure. The second group will receive doxycycline when tumors reach an average size of at least 100 mm³ and the third group will receive doxycycline at a late stage in tumor development when the average size is about 250 mm³. The fourth group will be time-matched animals expressing these two clones without receiving any dox. In the former case, animals will receive doxycycline hyclate at a concentration of 25 mg/kg per day by oral gavage, four times a week for the duration of the study. This will induce the knockdown of ATF4 in the SKNAS ATF4 shRNA group but should have no effect on ATF4 expression in the control shRNA group. Time-course experiments with doxycycline will be performed to determine the number of days required for administration of the drug to obtain efficient knockdown of ATF4 and to assess the optimum time at which tumors will be harvested after doxycycline administration. In this way we can determine the short term and long term effects of knocking down ATF4 on tumor angiogenesis, as well as to assess vascularization in the various tumor samples prior to administering doxycycline. The animals in the control group (SKNAS Ren-luc negative control and receiving doxycycline) will enable us to assess the effects, if any, doxycycline will have on the general health of the animals. Once the tumors are established, the animals will be monitored twice a week using the Xenogen luminometer, to assess tumor size at the implantation site. Animals in all groups will be regularly monitored for signs of weight loss and neurological symptoms. In our efforts to determine the effects of knocking down the UPR-inducible transcription factor, ATF4, on tumor vascularization, we will perform a combination of histological, vascular function and phenotype analysis on treated and untreated tumors. We will first examine the effect of knocking down ATF4 on microvessel density, pericyte coverage and tumor oxygenation status using the methods outlined below. In addition to formalin-fixed tissue for in situ hybridization and immunohistochemistry analyses, snap frozen tumor samples will be obtained to assess the knockdown efficiency in the SKNAS ATF4 shRNA group and determine the extent of ATF4 expression in the control groups.

Tumor endothelial cell staining: Formalin fixed, paraffin embedded tumors from each group of animals will be obtained. 4µm thick sections will be analyzed by immunohistochemistry for CD34, an endothelial cell marker and α-smooth muscle actin (αSMA), a pericyte marker, as described previously. Four independent fields will be analyzed using a light microscope. Positive staining will be quantified using Image J software and will be reported as the mean number of positive pixels/tumor section. In situ hybridization will also be performed to analyze mRNA levels of UPR target genes like CHOP (an ATF4 target), BiP and ERdj3. We will also analyze activation of the HIF pathway by probing for BNIP3 mRNA levels, a downstream target for HIF1 activity.

RNA and protein analysis: RNA will be extracted from tumors expressing ATF4shRNA and Renilla luciferase-shRNA. Real time PCR analysis will be performed to determine expression of VEGF. We will also determine expression of other proangiogenic factors like FGF2, IL8 and angiogenin. If ATF4 proves to be an important regulator of VEGF from our *in vitro* experiments, then we will check if inhibiting VEGF

in vivo has any effect on expression of the other proangiogenic genes important for angiogenesis. In addition to analyzing expression levels of the proangiogenic factors, we will perform Real-time PCR to determine levels of *CHOP*, a direct target of ATF4 and *ERdj3*, an off-target control. We expect CHOP expression to be down-regulated in the ATF4shRNA tumors as compared to tumors expressing Renilla luciferase shRNA. *ERdj3* expression levels should be similar in the tumors expressing targeted shRNA and negative control shRNA. We will also perform Western blot analyses to determine protein expression of ATF4 in knockdown and control tumors. In a similar manner described for the *in situ* hybridization, we will also measure BNIP3 mRNA levels in tumor tissue derived from control and ATF4 shRNA samples. Since both pathways, the UPR and HIF, are simultaneously activated in tumors we will determine if knocking down ATF4 expression has any effect on the HIF pathway.

Tumor oxygenation and intratumoral hypoxia: In a separate group of animals we will examine the effects of inhibiting angiogenesis, by knocking down a UPR transcription factor, on the oxygenation status of tumors. . We will determine tumor oxygenation using a pO₂ monitor. This apparatus measures oxygen based on the principle of oxygen quenching of fluorescence using a small optical sensor for monitoring rapid temporal oxygen changes in a given tissue micro region. We hypothesize that tumors expressing ATF4 shRNA may have transient vessel normalization and increased oxygen delivery to the tumor compared to control tumors. We would expect to observe a transient increase in the pO₂ in the ATF4 shRNA tumors as compared to control tumors. Finally, to determine intratumoral hypoxia, mice will be injected i.p. with Hypoxyprobe-1 (Chemicon International) 90 min prior to sacrifice. Tumors will be excised, fixed in formalin and embedded in paraffin. Tumor sections will then be stained using the Hypoxyprobe-1 Plus kit. Hypoxia (HIF) positive staining will be quantified using the Image J software as described previously. Because tumor hypoxia can be affected by tumor volume, size-matched tumors will be used for comparison. In a similar manner described above, we anticipate decreased intratumoral hypoxia staining in the ATF4 shRNA tumors as compared to control tumors.

Functional analyses of the tumor vasculature

If experiments described above suggest that knocking down ATF4 in established tumors inhibits tumor angiogenesis we will proceed to perform detailed functional analyses of the tumor vasculature.

Evans Blue dye assay: VEGF is known to be a potent proangiogenic factor and increases vessel permeability. To examine vessel permeability we will inject 100 µl of 2 % Evan's dye via tail vein in the ATF4 shRNA and control group that will be allowed to circulate for 20 min. Animals will be sacrificed and tumors will be excised and placed in formamide for 72 hours to extract the dye. Levels of dye in the extract will be quantified (µg/mg) using a spectrophotometer at 620 nm.

Contrast Enhanced Ultrasonography: As a measure of functional tumor perfusion we will perform contrast enhanced ultrasonography. Briefly, we will inject Optison ultrasound contrast agent (Amersham Health, Inc.), which is a suspension of human serum albumin microspheres encapsulating octafluoropropane gas. The mean microsphere size ranges from 2-4.5 μm and particles remain in the intravascular space. A region of interest will be drawn within the ultrasound to encompass the entire tumor, which will then be analyzed for precontrast baseline signal intensity, change in signal intensity from baseline to initial peak (in dB) and rate of signal intensity increase (in dB/s). Using this imaging modality we can evaluate intratumoral perfusion by assessing the two variables described above, the change in contrast enhancement and the rate of this change. We expect ATF4 shRNA tumors to have improved tumor perfusion and consequently these two variables will be much greater than those of the control tumors. Finally, we can confirm the functional consequence of the improved tumor perfusion by showing a decrease in the intratumoral hypoxia as described above.

Materials and Methods

Cell culture and stress induction

SKNASluc human neuroblastoma cell line stably expressing luciferase were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum, 2 mM glutamine and 1 % antibiotic-antimycotic at 37°C in a 5 % CO₂ incubator. Cells were plated and left untreated (NT) or treated with thapsigargin (Tg, 1 μM), media containing 1 mM glucose (Low Glu) (RPMI cat. no.11879-GIBCO), or cultured in a hypoxia chamber containing 1% O₂ for the indicated periods of time.

mRNA and hnRNA quantification by qRT-PCR

Total RNA was extracted using the RNeasy Qiagen mini-prep kit according to the manufacturer's protocol. RNA samples were subjected to reverse transcription using High Capacity cDNA synthesis kit from ABI Life technologies. The cDNA was diluted and Real-time PCR reactions were done in duplicate using a SyBr Green PCR Master Mix kit. Amplification of the corresponding genes was achieved using specific primers and measured continuously using an ABI 7900 HTI Detection System. Where indicated, VEGF hnRNA was measured using qRT-PCR primers across intron 1 and exon 2, for the human gene. The signal obtained for measuring both, mRNA and hnRNA, was compared relative to GAPDH internal control. The value for untreated cells was set to 1 and the value for the various treatments was presented as a fraction of this number.

Western blotting

For Western blotting cells were lysed in Nonidet P-40 lysing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 % Nonidet P-40, and 0.5 % deoxycholic acid) for 30 minutes on ice. The proteins were electrophoresed under reducing conditions, transferred to a PVDF membrane and probed with the indicated primary antibodies: Rabbit anti-XBP-1(S), rabbit anti-ATF4, goat anti-Hsc70, and goat anti-Lamin B1 were purchased from Santa Cruz Biotechnology and mouse anti-HIF1 α from BD Biosciences. Blots were incubated with the appropriate HRP-conjugated secondary antibody, and proteins were visualized using the Pierce enhanced chemiluminescent substrate (Thermo Scientific).

Vascular endothelial growth factor ELISA

The quantikine human VEGF ELISA kit (R&D Systems) was used to measure the VEGF levels in treated and untreated culture supernatants. VEGF secreted in the media was determined based on a standard curve and was expressed in pg/1x10⁶ cells.

Generation of dox-inducible ATF4 shRNA in the SKNASluc cell line

Two separate shRNA sequences targeting human ATF4, one specific for the 3'UTR of the gene and a second sequence that targets exon 4, were cloned into the pSuperior-puro vector (Oligoengine), which expresses shRNA molecules from a doxycycline-inducible H1 promoter. Both these sequences were validated previously using transient siRNA knockdown experiments in the neuroblastoma lines. Two separate vectors; one containing the tetracycline repressor and the other encoding the ATF4 shRNAs were stably transfected in the SKNAS cell line. Blasticidin (3 μ g/ml media) and Puromycin (1 μ g/ml media) were used to select for clones stably expressing the tet-repressor and p-Superior vectors respectively. After obtaining antibiotic-resistant double transfectants, a number of individual clones were picked using cloning cylinders and screened for regulated knock-down of ATF4 expression. Cells were treated with or without doxycycline for 24 or 48 hrs and then treated with thapsigargin for 16 hrs as indicated, and the level of ATF4 expression was determined using Western blot analysis.

CHAPTER 5. DISCUSSION

In normal tissues, an appropriate balance between proangiogenic and antiangiogenic factors is required to regulate the formation and maintenance of the vasculature. A functional network of blood vessels fuels normal tissues and organs with nutrients, growth factors, signaling molecules and oxygen. The growth of blood vessels or angiogenesis is essential from the early stages of embryonic development, organ morphogenesis and throughout adult life. During adulthood, blood vessels are mostly quiescent, except in the cycling ovary, during wound healing and in the placenta during pregnancy. Its importance has also been implicated in several disease conditions, like ischemia, macular degeneration and cancer. Over the past two decades, genetic studies in different animal models including mice, zebrafish and tadpoles have provided insights into the molecular mechanisms and signaling molecules involved in the regulation and growth of new blood vessels. VEGF is the most potent proangiogenic factor required for angiogenesis. Other proangiogenic factors like bFGF, and cytokines like IL8 and IL6 also contribute to stimulating the development of new blood vessels. Proangiogenic factors like PDGF and angiopoietin-1 play important roles in recruitment of supporting mural cells around endothelial tubes. Angiogenesis is a complex process that requires a fine-tuned balance of several stimulatory and inhibitory signals, chemokines, growth factors, oxygen sensors and endogenous inhibitors. The remarkable ability of endothelial cells to divide and proliferate in response to physiological stimuli like hypoxia, which leads to subsequent activation of the HIF pathway, has been the focus of numerous studies. In addition, recent data from several labs, including ours, have implicated the UPR in the production of proangiogenic factors, which suggested that it might also contribute to blood vessel formation.

UPR Activation Regulates Angiogenesis in Normal Physiological Processes

The UPR is constitutively activated in certain normal tissues and organs like the pancreas and muscle that have a high metabolic rate or secrete large amount of proteins. It is well established that this cytoprotective response increases ER chaperones, folding enzymes and ERAD machinery to enable the ER to cope with increased metabolic demands. However, in the past several years increasing evidence obtained primarily from experiments with cultured cells, suggests that activation of the UPR might also play an important role as a global regulator of angiogenesis by preferentially increasing expression of proangiogenic factors and down-regulating antiangiogenic factors [49]. Data to support a role in normal angiogenesis came from a recent study where the *Ire1* gene was disrupted. Ire1 is an up-stream UPR transducer that is conserved from yeast to humans and plays a critical role in regulating membrane biosynthesis, molecular chaperones, components of the ER degradation machinery, and even cell death [19]. Ire1 is expressed throughout mouse embryogenesis and adult development. Studies with an Ire1 reporter mouse revealed that Ire1 is constitutively active in the pancreas and muscles of adult animals [138], arguing that it contributes to the normal physiology of these organs. Disruption of the *Ire1* gene in mice results in embryonic lethality after 12.5 days

of gestation [19, 139, 140], demonstrating that it has an essential function early in mouse development. *In vivo* imaging analysis found that indeed Ire1 is highly activated in the placenta of embryos. More high-resolution studies revealed that loss of Ire1 resulted in a severely dysfunctional labyrinth layer of the placenta [139], which is normally highly vascularized and is the site of nutrient and oxygen exchange between the mother and fetus. The placenta phenotype in the *Ire1* null mice correlated with a significant decrease in VEGFA expression in this tissue, which was independent of HIF signaling, as HIF protein levels were unchanged between the wild-type and null embryos. To confirm that the early embryonic lethality in the *Ire1* null mice was due to inadequate placenta vascularization, mice in which the *Ire1* gene had been floxed were crossed with *Mox2*^{+/-Cre} transgenic mice [139]. *Mox2* is ubiquitously expressed in all tissues except in the labyrinthine trophoblasts of the placenta, thus allowing *Ire1* null embryos to be produced that have normal levels of Ire1 in the placenta. This strategy rescued the embryonic lethality observed in the conventional *Ire1* null mice and the placentas in the rescued mice were well vascularized. Since the UPR is the only known mechanism for activating Ire1, these data argue that the UPR plays a critical role in embryonic development and survival by controlling angiogenesis. In addition to the role of the UPR in regulating placental angiogenesis, its activation has been detected in highly vascularized normal tissues like the human endometrium. Expression of BiP, the master regulator of the UPR is significantly induced in the early proliferative and late secretory phases of the menstrual cycle [141]. Although the function of this induction has not been investigated, it is reasonable to hypothesize that the UPR also plays a role in the regulation of angiogenesis in this tissue. Although angiogenesis is known to be critical in wound healing [142], there are currently no studies to determine if the UPR plays a role in this process.

Identifying the UPR-Induced Transcription Factor that Up-Regulates *VEGFA* has not been Straightforward

In spite of a number of recent studies implicating the UPR in VEGF regulation, identifying the responsible transcription factor(s) has been contradictory. Although the mouse genetic studies, strongly implicated Ire1 in regulating VEGF in the placenta, they argued that this was not via XBP-1. Examining placenta in mice that were deficient in XBP-1 revealed only mild morphological and histological phenotypes [139]. There was no obvious explanation for this finding as XBP-1 has long been considered to be the only known target of Ire1's activity. Indeed, studies from the Urano lab [121] and ours [49] demonstrated direct binding of XBP-1 to the VEGF promoter in humans and mice, which results in an increase in VEGF mRNA in human HepG2 and mouse MEFs respectively. A separate study injected transformed XBP-1 deficient MEFs into the flanks of SCID mice [44]. The resulting tumors were much smaller than the wild-type controls, however there was no description of the effects on vascularization. Cell culture experiments with XBP-1 deficient MEFs using extreme hypoxia to activate the UPR showed no difference in VEGF secretion compared to wild-type MEFs [44]. This might be interpreted to be supportive of the placenta data, however it is critical to note that extreme hypoxia would also activate the HIF pathway, which of course would up-regulate HIF. Two others

studies led further support to Ire1 playing a role in regulating VEGFA in a tumor model [42, 43]. In both cases a dominant negative construct of Ire1 were expressed in the U87 human glioma tumor line. Orthotopic studies revealed reduced growth and vascularization of tumors, which was linked to decreased VEGFA, as well as other proangiogenic factors [42]. From these studies, one might conclude that Ire1, perhaps independent of XBP-1, is a major regulator of VEGF.

However, the importance of ATF4 in regulating VEGF expression was suggested in cell culture studies by Roybal et. al. using ATF4 null MEFs. Several thiol-containing reductive compounds, as well as thapsigargin, were shown to activate the UPR and transcriptionally up-regulate VEGF mRNA via an ATF4-dependent mechanism [143]. However, similar to our studies, the level of VEGF induction with these stresses was very modest, which we found was due to a particularly high basal level of transcription in these particular cells [49], making it difficult to assess the contribution of ATF4 using knockout MEFs. Two other studies supported the role of ATF4 in regulating VEGF expression. In the first case cells were treated with oxidized phospholipids, a common lipid component of atheroma that was shown to induce ER stress. They demonstrated direct binding of ATF4 to the human VEGF promoter in human HUVEC and HAEC cells after treatment with oxidized phospholipids and demonstrated ~50% reduction in VEGF mRNA when ATF4 levels were reduced with siRNA [103]. The second study use siRNA to reduce ATF4 levels in human HepG2 cells and found a similar reduction in VEGF transcripts [121]. These data are in keeping with our studies in two neuroblastoma cell lines where we observed an even greater reduction (~ 90%) in the VEGF transcription rate and VEGF secretion using siRNA to VEGFA (Chapter 3). It is unclear if the differences in the effects on VEGF are due to variability in the efficiency of ATF4 depletion or to the cell types used. Two studies [49, 121], ours included, examined whether ATF6 might also contribute to ER stress induced VEGF up-regulation as there are putative ATF6 binding sites on the VEGF promoter, but the effects of this transcription factor appear to be quite modest.

Thus on face value, it would seem that there are contradictory data to demonstrate a significant, and in cases sole, contribution of a single UPR component in regulating VEGF transcription; Ire1 and ATF4. Importantly ATF4 is downstream of the PERK arm of the UPR not the Ire1 arm. Although these branches are thought to signal independently of each other it is feasible that there is some interplay between them. For example, most recently an XBP-1-independent activity for Ire1 has been identified. In addition to cleaving XBP-1 mRNA, Ire1 was shown to have a rather promiscuous RNase activity, known as Regulated Ire1-dependent decay of messenger RNA (RIDD), which serves to more globally reduce the expression of RNA transcripts [144]. Although this has not been directly examined, it could impact other branches of the UPR. Alternatively it is possible that distinct UPR components or combinations of them are dominant in different tissues or cell types.

Why should angiogenic factor(s) be a target of the UPR?

A major function of the UPR in cell culture and *in vivo*, is thought to be restoring or maintaining ER homeostasis in response to an inadequate or toxic environment that adversely affects this organelle and its ability to fold and assemble proteins. Thus it is perhaps not surprising that one mechanism for taking care of this problem *in vivo* would be to increase the supply of blood flow to the affected cells so that more nutrients and oxygen can be delivered and waste and other toxic products could be taken away. In keeping with this possibility, physiological processes like wound healing require increased vascularization, and studies show evidence of UPR activation in the affected cells. Although a major focus of my studies and other recent ones was on VEGF, it is important to emphasize that our microarray data revealed a 2-fold or greater up-regulation of 13/19 genes that are positive regulators of angiogenesis. In addition to VEGF, the transcriptional up-regulation of three of these, angiogenin, FGF-2, IL-8 was confirmed in a variety of cell lines representing different tissues and species using several UPR inducers [49]. The balance in angiogenesis is tightly regulated by both proangiogenic and antiangiogenic factors, and interestingly we found that one anti-angiogenic factor, vasohibin, was down regulated. Another recent study found several other antiangiogenic factors like SPARC, decorin and TIMP-2 to be up-regulated when Ire1 activity was inhibited in a human glioma cell line [42].

However, since inadequate vascularization leads to decreased oxygen in tissues, which would induce the HIF pathway, a potent inducer of VEGF, it is reasonable to ask why this isn't sufficient to regulate angiogenesis. Perhaps the first reason comes from the fact that the UPR appears to regulate a multitude of proangiogenic factors, whereas the HIF pathway is more focused on VEGF. Although VEGF is apparently the most potent inducer of angiogenesis, in studies where VEGF inhibitors were used therapeutically, treatment failure was linked to increased expression of the fibroblast growth factor family members [145], which we have shown is a target of the UPR. Thus the ability to regulate so many factors may provide a benefit to the UPR having this capability. A second reason may stem from the stimuli that activate these pathways. Whereas the HIF pathway is uniquely regulated by decreased oxygen availability, the UPR is induced by low glucose levels, decreased pH, some heavy metals, and even by extreme hypoxia giving it a broader number of potentially toxic conditions to respond to in which increased blood flow might benefit the cells. While all of these conditions have the potential to damage the cell, there is another UPR inducer with less toxic characteristics. Studies have shown that the UPR can be activated in response to the increased trafficking of proteins through the ER. This can be observed in the secretory tissues, like the pancreas, liver and plasma cells, where activation of the UPR increases the capacity of these cells to synthesize large quantities of proteins. This latter inducer of the UPR might be particularly important in tissues like the placenta. The placenta must be highly vascularized in order to supply adequate quantities of oxygen and nutrients to the developing fetus and to remove toxic waste products. Since HIF1 α protein is stabilized only in response to limiting levels of oxygen, the ability to vascularize this tissue via the HIF pathway would result in intermittent periods of low oxygen in the placenta, which could be quite deleterious to the developing fetus. Indeed, the highly vascularized

placenta does not have any detectable levels of HIF1 α protein but instead shows strong evidence of UPR activation. This suggests that the cues to activate angiogenesis in this tissue might be less dependent on an inadequate microenvironment but instead rely on the high secretory nature of the placenta. This raises an important point of how normal physiological processes like angiogenesis can be controlled by alternate compensatory pathways in different organs or cell types. This aspect of the UPR in regulating angiogenesis in a HIF-independent manner might also be used in pathological conditions like cancer, and ischemia to stimulate angiogenesis, as in both cases there is evidence of UPR activation.

Does the UPR contribute to angiogenesis in tumor cells that also activate HIF?

In contrast to normal differentiated cells that rely on mitochondrial oxidative phosphorylation to produce energy for most cellular processes, cancer cells rely on glycolysis, a phenomenon known as the “Warburg effect”. The reason for cancer cells to shift to a more inefficient method for producing energy is still under investigation but some studies suggest that this facilitates the production of nucleotides and other metabolic intermediates that are conducive to cell proliferation. A hypoxic environment stabilizes HIF, which significantly contributes to the conversion of glucose to lactate, the end product of glycolysis. Adaptation to the tumor microenvironment results in increased glucose uptake and lactate production. However, the tumor microenvironment often encounters decreased nutrients and glucose supply in addition to hypoxia. Thus, it is important to assess the contribution of other pathways like the UPR to tumor cell metabolism.

Studies are required to determine the percent of cells in a tumor that activate the UPR and HIF signaling pathways alone and the percent that activate both pathways simultaneously. Our studies show that activation of both pathways together are potent inducers of VEGF expression compared to when each pathway is activated alone. Thus identifying the expression profile of these pathways in tumors will be important. The mechanism of how these two pathways influence each other to potentially induce VEGF expression is still unknown. It is possible that the UPR could enhance the activity of HIF by yet unknown post-transcriptional mechanisms. The UPR could also have the ability to enhance binding of HIF to its target genes like VEGF, by modifying the chromatin structure and making it more accessible for HIF transcriptional machinery to bind and transactivate its target genes.

Studies from our lab show that the UPR is activated throughout the tumor tissue, this is largely due to the rapid proliferation and high metabolic rate of tumor cells. Thus, areas that do not have the HIF pathway activated could still have the UPR activated with subsequent activation of downstream transcription factors like ATF4. We would thus expect ATF4 to be a critical regulator of VEGF expression in most parts of the tumor. The follow up studies for this project in the orthotopic neuroblastoma tumor model will enable us to determine the contribution of the UPR-inducible transcription factor ATF4 in regulating tumor angiogenesis during different stages of tumor growth. The

Dox-inducible regulation of ATF4 will give us the flexibility to turn off its expression during early and late stages of tumor development. In this way we can determine the relative importance of the UPR and HIF signaling in regulating tumor angiogenesis and thus identify a druggable target for inhibiting angiogenesis.

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APPENDIX. SUPPLEMENTAL FIGURES FOR CHAPTER 2

Figure A-1. HIF signaling pathways are not activated by UPR inducers nor are UPR targets activated by hypoxia. C6 cells were treated with 100 μM CoCl₂, 1% O₂ hypoxia (Hy), 2.5 μg/ml tunicamycin (Tm), 1 μM thapsigargin (Tg), or no glucose media (No Glu) for 24 hours. Western blot analysis was performed to measure **(A)** BNIP3 and CHOP protein levels in the cytosolic fraction. Hsc70 was used as a loading control. **(B)** HIF1α levels were determined in the nuclear fraction using Lamin B1 as control and CHOP levels were determined in the cytosolic fraction using Hsc70 as control.

Figure A-2. UPR inducing agents up-regulate CHOP and BiP mRNA. Daoy, C6, NB1691, SKNAS and NIH3T3 cells were treated with 100 μM CoCl₂, 1% O₂ hypoxia (Hy), 2.5 μg/ml tunicamycin (Tm), 1 μM thapsigargin (Tg), or no glucose media (No Glu) for 24 hours. RNA was extracted for qRT-PCR analysis and expression levels of CHOP mRNA (black bars) and BiP mRNA (white bars) were determined relative to 18SrRNA. Experiments were performed in triplicate (values are mean ± SD).

Figure A-3. Basal levels of proangiogenic factor expression in different cell lines. Daoy, NB1691, SKNAS, C6 and NIH3T3 cells were treated with 100 μ M CoCl_2 , 1% O_2 hypoxia (Hy), 2.5 μ g/ml tunicamycin (Tm), 1 μ M thapsigargin (Tg), or no glucose media (No Glu) for 24 hours. RNA was extracted for qRT-PCR analysis and basal levels of expression for (A) VEGF (B) angiogenin (C) FGF2 and (D) IL-8 were determined relative to 18SrRNA. Experiments were performed in triplicate (values are mean $\pm$ SD).

Figure A-4. Potential binding sites of UPR downstream transcription factors in human, mouse and rat VEGF promoter. Two online softwares, rVista and TRANSFAC were used to screen potential binding sequences of transcription factors, XBP-1 (cyan), ATF4 (green), HIF (red) and ATF6 (yellow) in 9k upstream promoter region of human, mouse and rat VEGF gene.

Figure A-5. Basal levels of VEGF hnRNA in XBP-1 wild-type and null MEFs. XBP-1 wild-type MEFs (black) and null MEFs (white) were untreated (NT), Thapsigargin-treated (1 μ M), treated with media lacking glucose (No Glu) or Homocysteine-treated (HCys, 10mM) for 8h and 14h. Total RNA from the indicated samples was subjected to qRT-PCR and VEGF hnRNA/18S ratios were determined relative to the control untreated samples.

Figure A-6. ATF4 does not appear to bind to the rat VEGF promoter. (A) Potential ATF4 sites in the rat VEGF promoter. **(B)** Cross-linked chromatin from C6 cells that were untreated (NT), Thapsigargin-treated (Tg), or incubated in No glucose media (No Glu) for 8h were immunoprecipitated with anti-ATF4. As positive control, primers spanning the ATF4 binding region on the CHOP promoter were used to PCR amplify the anti-ATF4 precipitated chromatin **(C)** CHOP protein levels were determined using Western blot analysis in the C6 cells that were used in the ChIP assays.

VITA

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