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Characterization of Heat Shock Protein A12B as a Novel Angiogenesis Regulator

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A dissertation

presented to

the faculty of the Department of Biochemistry and Molecular Biology

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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August 2008

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Keywords: Angiogenesis, AKAP12, Hsp70, HspA12B, Hypoxia, Migration, VEGF

ABSTRACT

Characterization of Heat Shock Protein A12B as a Novel Angiogenesis Regulator

by

Rebecca J. Steagall

Previously, we cloned Heat shock protein A12B (HspA12B), the newest member of a recently defined subfamily of proteins distantly related to the Hsp70 family that are enriched in atherosclerotic lesions. We have found that HspA12B is predominantly expressed in vascular endothelium, and that it is involved in angiogenesis which we probed by *in vitro* angiogenesis assays (Matrigel), migration assays and Directed *In Vivo* Angiogenesis Assay (DIVAA). Hsp70s are molecular chaperones that are inducible by stress and have been found to be anti-apoptotic (Li et al. 2000; Nylandsted et al. 2000; Garrido et al. 2001). Because of its homology to Hsp70, we propose that it is the first endothelial-specific chaperone that is required for angiogenesis and interacts with known angiogenesis regulators. To begin to understand the molecular mechanisms underlying the role of HspA12B in angiogenesis, we turned our attention to identifying proteins that are involved in angiogenesis and also interact with HspA12B. Through the use of a yeast two-hybrid (Y2H) system HspA12B was found to interact with a known angiogenesis regulator, A Kinase Anchoring Protein 12 (AKAP12). This interaction was confirmed by co-immunoprecipitation and by colocalization. In primary human umbilical vein endothelial cells (HUVECs), shRNA mediated HspA12B knockdown increased AKAP12 levels and decreased VEGF by more than 75%, whereas HspA12B over-expression decreased AKAP12 and more than doubled VEGF levels. We further identified a 32-Amino Acid (32-AA) domain in AKAP12 that mediates interaction with HspA12B. Over-expression of this 32-AA domain in HUVECs disrupted the HspA12B-AKAP12 interaction and decreased VEGF expression suggesting the importance of the HspA12B-AKAP12 interaction in regulating VEGF. This is the first evidence

that HspA12B promotes angiogenesis resulting in up-regulation of VEGF by suppressing AKAP12. Consistent with the proposed role in angiogenesis, HspA12B was also found to be increased in endothelial cells (ECs) by angiogenic stresses including hypoxia and shearing stress while knockdown of HspA12B abolished hypoxia-induced tubule formation. This work provides new insight into the mechanisms controlling angiogenesis by providing the first example of an EC-specific molecular chaperone that acts as a regulator of angiogenesis and lays the foundation for future studies of HspA12B-derived therapeutics for angiogenesis related diseases.

DEDICATION

This manuscript is dedicated to my husband, Monta Steagall, and my three beautiful daughters, Brittany, Victoria and Jessica. They have been my main source of support and encouragement, and I am forever grateful for their love, sacrifice, and patience throughout my graduate studies.

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Completion of this dissertation could not have been possible without the support and guidance of many people. I wish to thank my parents, Dr. Theodore G. Kottke and Bernice Kottke, for their unwavering source of support, love, and guidance during all of these years. In addition, special thanks go to my Mother and Father-in-law, Hazel and Monta Steagall, for everything they have done for my family. Without their help, I cannot imagine the completion of my graduate program at E.T.S.U.

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CHAPTER 1

INTRODUCTION

Cardiovascular Disease

Atherosclerosis and Myocardial Infarction

The primary pathological expression of cardiovascular disease is myocardial infarction (MI) resulting from an ischemia/reperfusion (I/R) insult. Often MI injury is associated with atherosclerotic plaque rupture and thrombosis in coronary arteries. When a weakened plaque ruptures, it causes a thrombosis (or clot) to form. If the clot is big enough, it will halt the flow of blood to the heart, producing a heart attack. The ischemic environment created is deficient of oxygen and nutrients and results in apoptotic and necrotic myocardial cell death (Bonavita et al. 2003; Tantini et al. 2006). In an attempt at self preservation the cells at risk induce the synthesis of a family of heat shock proteins which enhances the ability of the stressed cells to cope with increased concentrations of unfolded or denatured proteins (Nollen et al. 1999). Reperfusion occurs when the blood supply and nutrients are restored to the injured myocardium through the release of the occlusion or reestablishment of the vessel network by bypass or new vessel formation.

Epidemiology

According to the American Heart Association's (AHA) National Heart, Lung and Blood Institutes Atherosclerotic Risk in Communities Study (ARIC) and the Cardiovascular Health Study (CHS) (1994-2004) approximately 1.2 million heart attacks occur each year in the United States, with about 450,000 resulting in death (AHA 2006). Currently available treatments include opening the blockage in the artery with angioplasty or rerouting the blood supply with bypass surgery. However, some patients are not candidates for these conventional treatments, thus considerable amount of attention and interest has also been directed towards the use of

angiogenic stimulators to induce newly created vessels that could serve as a biological bypass of the atherosclerotic vessel.

Angiogenesis

The three major processes of neovascularization that contribute to the growth of new blood vessels are; vasculogenesis, angiogenesis, and arteriogenesis. They are natural processes of vessel formation and are key processes involved in normal development, the reproduction cycle, and wound healing. During development of the vascular system, vasculogenesis refers to the process in which endothelial progenitors differentiate, proliferate, multiply, and migrate to give rise to a primitive vascular network of arteries and veins; angiogenesis refers to the formation of new blood vessels from pre existing endothelial cells (EC) in adult or embryonic tissue through proliferating, sprouting (Figure 1.1), pruning, and remodeling. Pericytes and smooth muscle cells are recruited to cover nascent endothelial channels, which provide strength and regulation of vessel perfusion, a process termed arteriogenesis (Carmeliet 2005).

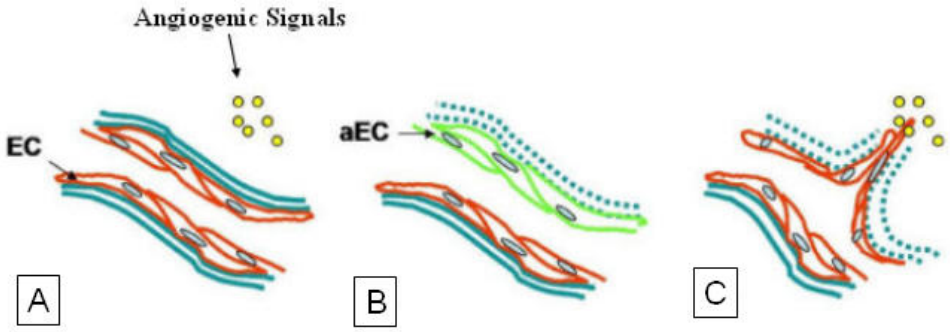


Figure 1.1 (continued on next page)

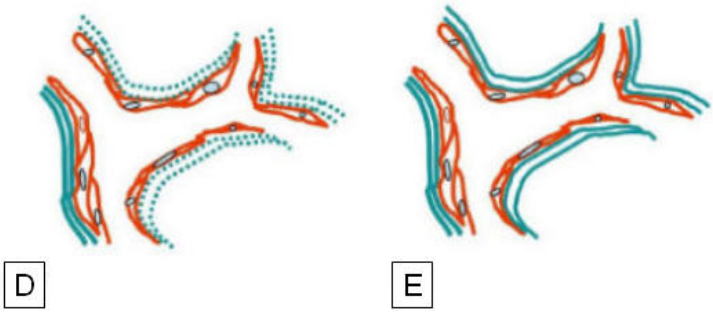


Figure 1.1 Sprouting angiogenesis. This multi-step process begins with A, endothelial cells (ECs) reception of angiogenic signals (yellow spot) from the surrounding; B, detachment of pericytes from the adluminal surface of capillary, secretion of protease from activated endothelial cells (aECs) and proteolytic degradation of extracellular membrane (green dash-line); C, chemotactic migration of ECs under the induction of angiogenic stimulators; D, proliferation of ECs and formation of lumen by fusion of formed vessels with formation of cell-cell tight junctions; E, recruitment of pericytes, deposition of new basement membrane and initiation of blood flow. (Adapted from Yue, P, Chin Med., 2007 (Yue et al. 2007))

The major triggers of angiogenesis can be grouped into three main categories: mechanical, chemical, and molecular factors. Mechanical influences involve the two factors, hemodynamic and shear stress. Alterations in hemodynamic forces (blood flow, blood vessel diameter, wall shear stress) stimulate vascular sprouting (Hudlicka et al. 1995) and result in hemodynamic remodeling. When exposed to physiological levels of shear stress endothelial cells elongate, align in the direction of flow, and maintain barrier function. In contrast, low, oscillating, and disordered shear stress promotes the development of atherosclerosis (Wang et al. 2006). The chemical influences of angiogenesis can occur through hypoxia or the production of nitic oxide [NO]. Hypoxia causes the induction of genes that initiate angiogenesis and enhance blood vessel permeability including; vascular endothelial growth factor (VEGF) (Shweiki et al. 1992; Ladoux and Frelin 1993), the VEGF receptors -2 and -3 (VEGFR), the angiopoietin receptor Tie2, platelet-derived growth factor-B (PDGF-B), and inducible nitric oxide synthase (iNOS) (Kuwabara et al. 1995; Melillo et al. 1995; Yuan et al. 2000; Nilsson et al. 2004). Nitric oxide (NO), the factor responsible for vasodialation, plays a role in both physiological and pathological angiogenesis (Ziche and Morbidelli 2000). The role of NO in VEGF induced angiogenesis has been shown in eNOS knockout mice resulting in reduction of blood vessel formation (Lee et al. 1999). The molecular influences of angiogenesis include glucose (Sone et

al. 1996), inflammation (Sunderkotter et al. 1991a; Sunderkotter et al. 1991b) and growth factors including VEGF/VEGFR, angiopoietin/Tie families, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and placental growth factor (PLGF) (Coultas et al. 2005). With so many influences on angiogenesis, a delicate balance must exist to maintain a state of health that can be tipped in either direction to switch angiogenesis on or off.

Therapeutic Angiogenesis

In health, angiogenesis is normally in the quiescent state but is susceptible to both rapid activation and inactivation. Angiogenesis plays a prominent pathological role in a number of diseases. Cancer tumors stimulate angiogenesis to supply the growing tumor with blood and nutrients. Alternatively, beneficial angiogenesis is critical for supplying the healing infarcted myocardium with O_2 and nutrients necessary to sustain metabolism. When pro-angiogenic proteins are administered in order to seek a clinical benefit it is termed "therapeutic angiogenesis." The goal is to stimulate the creation of new blood vessels in ischemic organs or tissue. This serves to increase the level of oxygen and nutrient-rich blood necessary to sustain metabolism (Simons 2005). Candidates for pharmacological stimulation of angiogenesis include the growth factors; basic fibroblast growth factor (bFGF) and VEGF administered as recombinant proteins or by gene therapy (Rosinberg et al. 2004). Growth factor administration has resulted in significantly higher vessel counts (Kawasuji et al. 2000; Yanagisawa-Miwa et al. 1992) and collateral flow (Banai et al. 1994) in animal models. Other candidate therapeutic angiogenic factors include angiopoietin and PDGF, interleukin (IL) 8 and macrophage inflammatory proteins (MIP) (Nossuli et al. 2001), transcription factors including hypoxia inducible factor (HIF) (Shyu et al. 2002), and compounds such as resveratrol (Das and Maulik 2006). Despite the great progresses in finding key regulators in angiogenesis, characterizing new genes is still necessary and greatly beneficial for a full understanding of the process. The precise and delicate coordination, combination, and collaboration of the molecular players of angiogenesis in the right time, space, and dose, so critical for the formation and maintenance of

functional blood vessels, would be greatly aided by molecular chaperones. But to date, no endothelial-specific molecular chaperone has been identified. The characterization of HspA12B provides new insight on angiogenesis and may lead to a new target for disease intervention.

Heat Shock Protein A12B (HspA12B)

Expression

Dr. Zhihua Han (Han et al. 2003) cloned and characterized HspA12A and B, the newest members of the Hsp70 family of proteins. The first member of this subfamily, HspA12A, is a candidate gene for atherosclerosis susceptibility, based on evidence of genetic linkage and expression profiling (3, 4). The second member of this family, HspA12B, was cloned through sequence homology (60% identical to HspA12A) and is also enriched in atherosclerotic lesions. Dr. Han reported that both heat shock protein A12A and A12B had 87- and 6-fold higher mRNA levels, respectively, in portions of the thoracic aorta from 10 month old chow-fed C57BL6 apoE knockout mice containing lesions than non-lesional areas (Han et al. 2003).

Structure

An alignment search for HspA12A and HspA12B against the National Center for Biotechnology Information database (nonredundant) revealed homology with the Conserved Domain database for Hsp70 (pfam00012.5, Hsp70). Hsp70 family members consist of two distinct domains; a highly conserved 44-kDa N-terminal ATPase domain and a more divergent 25-kDa C-terminal substrate-binding domain (Figure 1.2 A). Prototypic Hsp70s such as DnaK are 638 aa long, with amino acids 1–385 specifying the ATPase domain and 393–537, the substrate-binding domain. mHspA12A amino acids 58–244 align with the pfam00012.5, Hsp70 consensus sequence amino acids 1–175, showing 28% identity and 38% homology. The mHspA12A amino acids 312–542 align with the pfam00012.5, Hsp70 consensus sequence amino acids 189–398, showing 26% identity and 44% homology (Figure 1.2 B). The results for

mHspA12B are almost identical (Figure 1.2 C). Thus, HspA12A and HspA12B appear to have an atypical Hsp70 ATPase domain, which is in two parts separated by spacer amino acids 175–189 (Han et al. 2003).

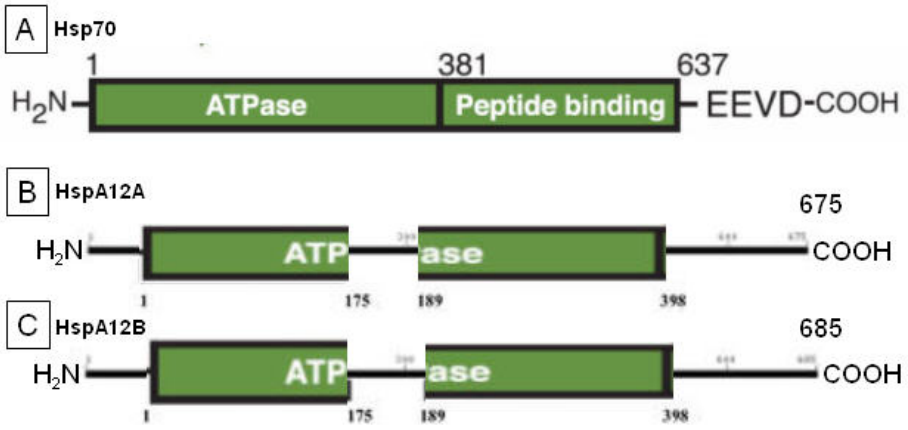


Figure 1.2 Atypical ATPase domains. Schematic representation of A, Hsp70, B, mHspA12A and C, mHspA12B illustrating the atypical Hsp70 ATPase domains of HspA12A and B which are in two parts separated by spacer amino acids 175–189. (Adapted from Hartle, F *et al*., Science 2002; 295; 1852 (Hartl and Hayer-Hartl 2002))

Function

Hsp70 proteins are chaperones that recognize and bind to short stretches of exposed hydrophobic peptides. These stretches arise when proteins are denatured under stress or in the processes of synthesis, folding, assembly, and translocation to an appropriate subcellular compartment. When bound to ATP, Hsp70 assumes an open form in which an exposed hydrophobic pocket transiently binds to exposed hydrophobic regions of unfolded proteins and prevents them from irreversible aggregation (Hartl and Hayer-Hartl 2002). When released from Hsp70, the misfolded proteins have a chance to resume correct folding; however, repeated misfolding increases the likelihood of degradation by the ubiquitin-proteasome system (Meacham et al. 2001). Hsp70 proteins protect cells against apoptosis induced by heat shock,

oxidative stress, toxins, and cellular stress. In addition to its chaperone functions, it has increasingly recognized that Hsp70s also act as signaling molecules (Pratt and Toft 2003; Li et al. 2005). We hypothesize that HspA12B may function as an endothelia-specific chaperone that regulates angiogenesis by directly regulating the synthesis, trafficking, interaction, and degradation of proteins required for angiogenesis.

SPECIFIC AIMS

The overall goal of the present study is to characterize HspA12B's regulation of angiogenesis. We investigate the hypothesis that HspA12B is an endothelial cell specific molecular chaperone acting as a regulator of angiogenesis. The specific aims of this study are as follows: 1) to prove that HspA12B is predominantly expressed in endothelial cells and is required for angiogenesis *in vitro*, 2) to define interacting proteins and molecular pathways by which HspA12B induces angiogenesis, and 3) to investigate HspA12B's molecular chaperone functions including; its induction by stress (angiogenic stresses such as hypoxia and shear stress) and its role in protein turnover of an angiogenesis regulating protein, AKAP12, through the proteasome pathway. This analysis of HspA12B may provide a better understanding of the mechanistic events that occur during angiogenesis and may lead to treatment or prevention of angiogenic disorders.

CHAPTER 2

HSPA12B IS PREDOMINANTLY EXPRESSED IN ENDOTHELIAL CELLS AND REQUIRED FOR ANGIOGENESIS

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Body of text: ~4845 words

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12 figures and 2 tables

Keywords: angiogenesis, endothelial cells, HspA12B, HSP70 family, migration

Running title: HspA12B function in angiogenesis

Abstract

Objective—HspA12B is the newest member of Hsp70 family of proteins and is enriched in atherosclerotic lesions. This study focused on HspA12B expression in mice and its involvement in angiogenesis.

Methods and Results—The expression of HspA12B in mice and cultured cells was studied by: (1) Northern blot; (2) in situ hybridization; (3) immunostaining with HspA12B-specific antibodies; and (4) expressing Enhanced-Green-Fluorescent-Protein under the control of the HspA12B promoter in mice. The function of HspA12B was probed by an *in vitro* angiogenesis assay (Matrigel) and a migration assay. HspA12B is predominantly expressed in vascular endothelium and induced during angiogenesis. *In vitro* angiogenesis and migration are inhibited in human umbilical vein endothelial cells in the presence of HspA12B-neutralizing antibodies.

Conclusions—We provide the first evidence to our knowledge that the HspA12B is predominantly expressed in endothelial cells and is required for angiogenesis. We postulate that HspA12B provides a new mode of angiogenesis regulation and a novel therapeutic target for angiogenesis-related diseases. **(*Arterioscler Thromb Vasc Biol*. 2006;26:2012-2018.)**

Introduction

Blood vessel development and formation are essential for organ growth and repair, wound healing, and reproduction cycle, and an imbalance of functional vessels contributes to diseases such cancer and ischemia (1). During development of the vascular system, vasculogenesis refers to the process in which endothelial progenitors differentiate, proliferate, multiply, and migrate to give rise to a primitive vascular network of arteries and veins; angiogenesis refers to the process of blood vessels expansion/remodeling from the existing endothelial cell (EC) network through proliferating, sprouting, pruning, and remodeling. Pericytes and smooth muscle cells are recruited to cover nascent endothelial channels, which provide strength and regulation of vessel perfusion, a process termed arteriogenesis (2). The formation and maintenance of functional blood vessels is a complex process involving the interplay of multiple genes. These genes include members of many signaling pathways such as vascular endothelial growth factors/vascular endothelial growth factor receptors, angiopoietin/Tie families, platelet-derived growth factor, transforming growth factor-β, Notch pathways, certain integrins, neuronal axon guidance molecules such as ephrin, semaphorins, netrins, and robo, transcriptional factors, and many other genes (3). In adults, many of the embryonic and early pathways are reactivated in situations of neoangiogenesis.

Despite great progresses in finding key regulators in angiogenesis, characterizing new genes is still necessary and greatly beneficial for a full understanding of the process. The precise and delicate coordination, combination, and collaboration of these molecular players in the right time, space, and dose, so critical for the formation and maintenance of functional blood vessels, would have been greatly aided by molecular chaperones. For example, an endothelial-specific molecular chaperone might explain the endothelial-specific effects of many transcriptional factors that are expressed rather broadly (4). But to date, no endothelial-specific molecular chaperone has been reported.

Heat shock proteins (Hsp) are a group of proteins that are abundant in cells, highly conserved among species, and associated with stress responses. Hsp70 is the largest and most conserved family of Hsp (5). Hsp70s function as molecular chaperones, assisting in protein synthesis, folding, assembly, trafficking between cellular compartments, and degradation (6). Hsp70s participate in cellular stress response by binding and refolding misfolded protein, or removing the consistently-misfolded proteins through the ubiquitin–proteasome system by interacting with CHIP (carboxyl terminus of Hsp70-interacting protein), an Hsp70-associated ubiquitin ligase (7, 8). In addition to its chaperone functions, it has been increasingly recognized that Hsp70s also act as signaling molecules (9) and regulators in cellular processes such as apoptosis (10). Expression of Hsp70 family members has been considered to be ubiquitous and not restricted to any specific cell type.

We have previously cloned a new subfamily of Hsp70 proteins, the HspA12 subfamily (11). The first member of this subfamily, HspA12A, is a candidate gene for atherosclerosis susceptibility, based on evidence of genetic linkage and expression profiling (11, 12), The second member of this family, HspA12B, was cloned through sequence homology (60% identical to HspA12A) and is also enriched in atherosclerotic lesions. We set out to characterize the expression and function of this gene and its role in angiogenesis.

Materials and Methods

Endothelial Cell Cultures

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Cambrex (Rockland, ME) and were propagated through passage 6 in F-12K medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Cambrex), 0.1 mg/ml porcine heparin, and 0.05 mg/ml bovine EC growth medium from Sigma (St Louis, MO).

HspA12B Expression Constructs

The *mHspA12B* full-length coding region was RT-PCR amplified and subcloned into: 1) *pCMV-Tag2A* (Stratagene, La Jolla, CA), 2) *pIRES2-EGFP*, 3) *pEGFP-C1* (Clontech, Mountain View, CA), and 4) *pEGFP-N1* to generate 1) N-terminus Flag-tagged *HspA12B*, 2) wild type *HspA12B*, 3) N-terminus Enhanced-Green-Fluorescence-Protein (EGFP)-tagged *HspA12B* (*EGFP-HspA12B*), 4) C-terminus EGFP-tagged *HspA12B* (*HspA12B-EGFP*). Figures of expression constructs are shown in Appendix A.

Experimental Animals

All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care and were in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals published by National Institute of Health (Publication Number NIH 85-23, revised 1985).

Northern Blot and *In Situ* Hybridization

Mouse multiple-tissue blots and human heart-tissue blots were purchased from Clontech (Mountain View, CA); the rat brain tissue blot and time-course blot were purchased from Seegene (Rockville, MD). Northern Blots and *in situ* hybridizations experiments were performed

as previously described (11). Northern blot probes were generated by polymerase chain reaction and in situ hybridization probes were generated with a Dig RNA labeling kit from Roche (Indianapolis, IN). Primers sequences are listed in Table 2.1. Mice were housed in East Tennessee State University Animal Care Facility and fed normal chow diet. Twelve-week-old C57BL/6 atherosclerosis susceptible mice were from Jackson Laboratories. *HspA12B-BAC-EGFP* transgenic mice were generated in the Rockefeller University (www.gensat.org).

TABLE 2.1 Northern Probe, *In Situ* Hybridization and Cloning Primers

PCR Primer	Name	Sequence
Northern Probe Primers	**mHspA12B forward**	GCGGCGTGTGCTCATCAATCTGTA
	mHspA12B reverse	GAAAGGGGTGCTGCTGTGAAAACT
	hHspA12B forward	AGGCGCGGGACTGGCTCTACTTC
	hHspA12B reverse	ACGTGCGGCTGTGGCGGGACCTTC
	rHspA12B forward	AGGAAGCTCTAGGCAATCGT
	rHspA12B reverse	CTTCAGGCTCAAGCTCCAGG
***In Situ* Hybridization Primers**	**mHspA12B forward**	gagagaattcGCGGGCAGCCATCGACTTTCTTTC
	mHspA12B reverse	gagaaagcttAACCCCCGCCCCCTTGCCACAGTA
Cloning Primers∗		
N-Flag-tagged HspA12B	**mHspA12B forward**	atatgaattcaATGCTGACTGTCCCGGAAATGGGC
	mHspA12B reverse	gagaaagcttAACCCCCGCCCCCTTGCCACAGTA
N-EGFP-tagged HspA12B (EGFP-HspA12B)	**mHspA12B forward**	atatgaattcaATGCTGACTGTCCCGGAAATGGGC
	mHspA12B reverse	atatggtaccttGTTGGAAAGAAAGTCGATGGCTGC
C-EGFP-tagged HspA12B (HspA12B-EGFP)	**mHspA12B forward**	atatgaattcaATGCTGACTGTCCCGGAAATGGGC
	mHspA12B reverse	atatggtaccttGTTGGAAAGAAAGTCGATGGCTGC
Bi-cistronic wild type HspA12B	**mHspA12B forward**	atatgaattcaATGCTGACTGTCCCGGAAATGGGC
	mHspA12B reverse	atatgtcgacTCAGTTGGAAAGAAAGTCGATGGC

∗See Appendix A for figures of expression constructs.

Antibody Production and Western Blot Analysis

Two antibodies, the N-terminus-specific Ab4110 and the C-terminus-specific Ab4112, were generated by immunizing rabbits with peptides MLTVPEMGLQGLYISSC (mHspA12B

amino acid (AA1 to 17) and CVDVSTNRSVRAAIDFLSN (mHspA12B AA667–685), respectively (ProSci Inc, Poway, CA). Enzyme linked immunosorbent assay-positive sera were further purified by affinity column using the same peptides. The Western blots were performed using standard procedures. For membranes probed with primary antibodies Ab4110 or Ab4112, a goat-anti-rabbit IgG/horseradish peroxidase conjugate (Pierce, Rockford, Ill) was used as the secondary antibody and signals were developed using a chemiluminescent substrate (WesternDura; Pierce, Rockford, IL). As a control for loading and normalization, membranes were reprobed with an anti-GAPDH monoclonal antibody (MMS-580S; Covance, Berkeley, CA). Chemiluminescence signal was scanned with a FLA-500 phosphorimager (Fujifilm, Tokyo, Japan) and quantified by Image-Gauge software.

HspA12B-BAC-EGFP Transgenic Mice and Confocal Imaging

To generate the transgenic mice expressing EGFP under the promoter of *mHspA12B* (HspA12B-BAC-EGFP mice), a bacterial artificial chromosome (BAC) containing *HspA12B* was engineered so that the EGFP replaced the *HspA12B* coding region at the starting ATG. Transgenic mice were generated from the engineered BAC and were of FVB/N crossed with Swiss-Webster background (13). Two independent founder lines were studied and gave identical results. Tissue sections (20 μm) from 12- to 16-week-old transgenic mice and their wild-type littermates were imaged using a Leica TCS SP2 confocal microscope system. Z-series were collected and maximum intensity projection images were created from the series.

Immunohistochemistry

Mice were anesthetized with ketamine/xylazine and euthanized by cardiac perfusion with 4% paraformaldehyde. The organs were removed, post-fixed in 4% paraformaldehyde for 1 hour, incubated in 25% sucrose at 4°C for 96 hours, and stored in -80°C. Tissue sections were generated with a Leica CM1850 cryostat. Immunofluorescent stainings were performed by

standard protocols with AlexaFluor-488 goat-anti-rabbit IgG (H+L) (green) and AlexaFluor-555 goat-anti-rat IgG (H+L) (red) from Molecular Probes (Carlsbad, CA). Briefly, sections were blocked in 10% bovine serum albumin (BSA) blocking solution for 1 hour, incubated with primary antibodies (1:200) for 4 hours at room temperature, washed 4 times with 1× phosphate-buffered saline (PBS) for 15 minutes each, incubated with secondary antibodies-conjugates (1:200) for 1 hour, and washed 4 times with 1× PBS for 15 minutes each before mounting. The anti-enhanced-Green-Fluorescent-Protein (EGFP) was purchased from Abcam (Ab290; Cambridge, MA), and rat-anti-CD31 (PECAM-1) from Pharmingen (San Diego, CA).

Endothelial Cell Transfections

Transfection of the antibodies into HUVECs was performed with the BioPORTER kit from Sigma (St. Louis, MO) following manufacture's instructions. Exponentially growing HUVECs were seeded at a density of 3×10^5 cells in gelatin coated 6-well plates (BD Biosciences, San Jose, CA) for 18-24 hours until ~60-80% confluence. The cells were incubated with transfection reagents and antibodies (10 μg/well) for 4 hours before Matrigel or migration assays. 1 μg of AlexaFluor-555-labeled non-specific antibodies (red) were spiked into each transfection to monitor the transfection efficiency.

For plasmid transfection, exponentially growing HUVECs were seeded at a density of 2×10^5 cells in gelatin coated 6-well plates for 18-24 hours until ~40-60% confluence, and 2 μg of plasmids plus 12 μl Lipofectin (Invitrogen, Carlsbad, CA) were added for transfection. 24 hours later, cells were analyzed for protein expression or plated for Matrigel assay.

Matrigel-Based *In Vitro* Angiogenesis Assay

Wells of a 24-well plate were coated with 250 μL Matrigel (BD Biosciences) and incubated at 37°C for 30 minutes; 5×10^4 HUVECs in complete EC medium were added to Matrigel-coated wells and incubated at 37°C with 5% CO_2 for 24 hours. When required, cells

were recovered from Matrigel with Cell Recovery Solution (BD Biosciences) in the presence of 1× protease inhibitors cocktail (Sigma) for making cell extracts.

Migration Assay

Four hours after antibody transfection with BioPORTER reagent (Sigma), HUVECs were starved for 1 hour in M199 containing 1% FBS, trypsinized, pelleted, and resuspended in M199 media with 1% FBS. 5×10^4 cells per well were placed in the upper chambers of 8-μm cell culture inserts (Falcon HTS, BD Biosciences) coated with 50 μg/ml collagen. The lower compartment contained growth media with 10 ng/ml vascular endothelial growth factor (VEGF) or M199 medium with 1% serum (background control). After incubation for 2 hours at 37°C, cells on the upper compartment were washed off, and cells that had migrated through the filters were fixed in formalin, stained with propidium iodide (2 μg/ml in PBS, overnight at 4°C), and counted on photographs under a fluorescent microscope. Migration was quantified by counting the cells that had migrated through the inserts in 10 randomly selected fields (original Magnification 10×). The number of cells that had migrated through the inserts towards M199 medium with 1% serum was considered as background and was arbitrarily set at 1. Data were gathered from three independent assays.

Statistical Analysis

All results were expressed as the mean ± standard deviation of the mean (± SD). Significance of differences were tested for with the Student *t* test with significance at $P<0.05$.

Results

HspA12B mRNA is expressed in Multiple Organs and in Blood Vessels Specifically

In mouse multi-tissue Northern blot; a single ~3.3-kb *HspA12B* mRNA transcript was detected at the highest level in heart, followed by lung (Figure 2.1A). Longer exposure revealed *HspA12B* was expressed in all tissues examined, as confirmed by reverse-transcription polymerase chain reaction (not shown). *HspA12B* mRNA of similar size was detected in rat brain (Figure 2.1B) and human heart (Figure 2.1C). In mouse brain, *HspA12B* was present at 17.5 day postcoitum embryo, peaked at day 3, and decreased gradually (Figure 2.1D).

In situ hybridization experiments revealed that, compared with sense-probe control, the antisense probe recognized a distinctly vessel pattern, indicating *HspA12B* was expressed specifically in blood vessels (Figure 2.2).

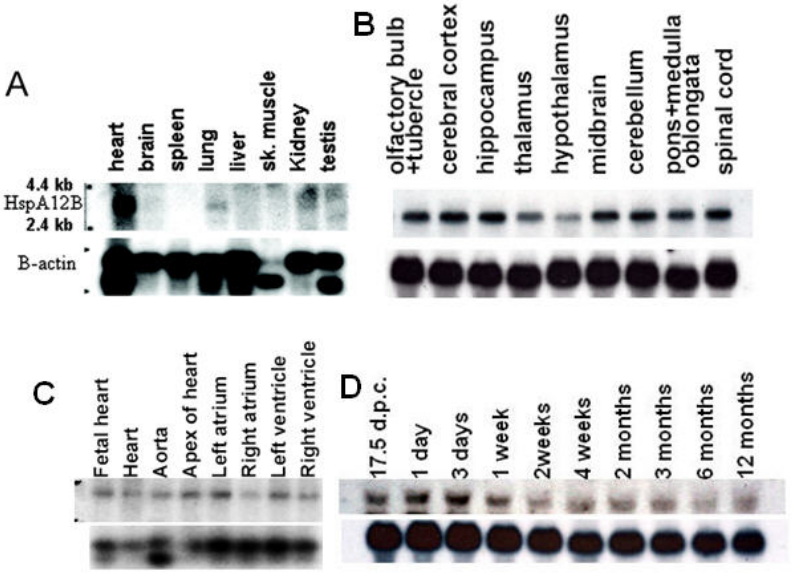


Figure 2.1 *HspA12B* mRNA expression in multiple tissues and preferentially in blood vessels, detected by Northern blots (A to D). A, Mouse multi-organs. B, Rat brain tissues. C, Human heart tissues. D, Mouse brain developmental time course. Northern blots were performed as previously described (11).

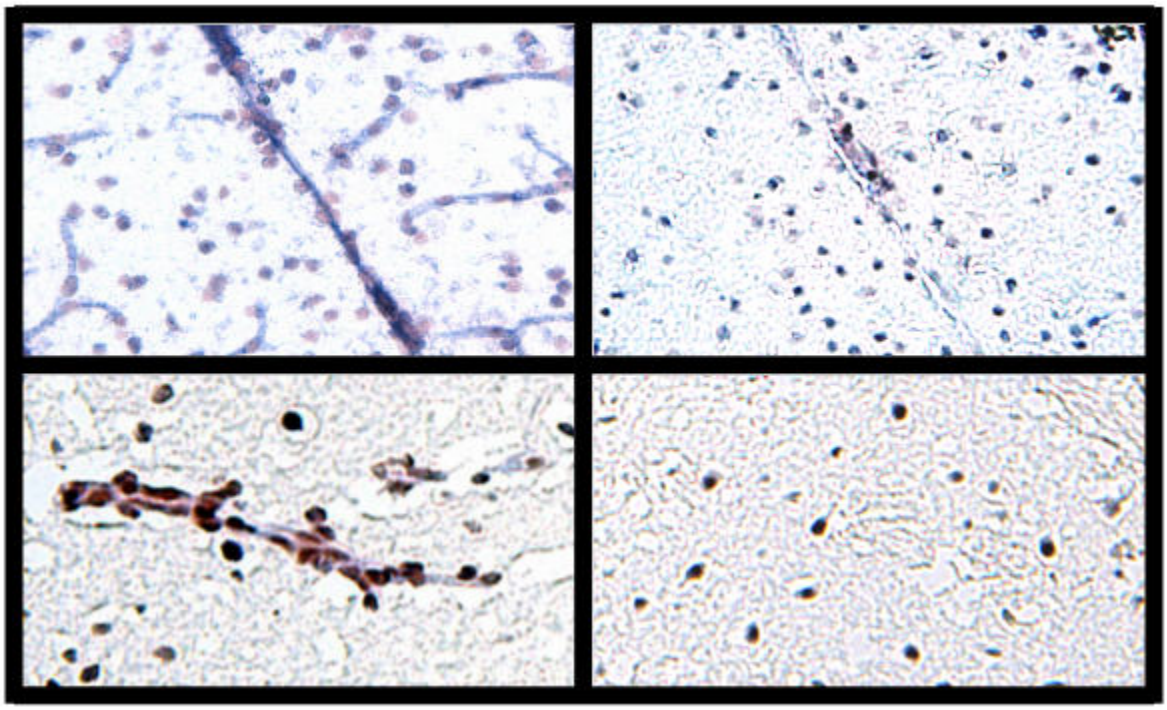


Figure 2.2 HspA12B *In situ* hybridization on mouse brain sections. Left panels are antisense probe showing *HspA12B* signal, and right panels are sense-strand probe as negative control showing background. *In situ* hybridization was performed as previously described (11).

HspA12B Proteins are Expressed in Endothelial Cells

Two HspA12B-specific antibodies, Ab4110 (N-terminus specific) and Ab4112 (C-terminus-specific), recognized ~76-kDa protein (predicted MW 76-kDa) in HEK293 cells overexpressing HspA12B and in HUVEC cells, and ~80-kDa protein in HEK293 cells overexpressing Flag-tagged-HspA12B (Figure 2.3A). The interaction was specific because blocking peptides used to elicit the antibodies abolished the signals.

We then measured HspA12B protein levels in multiple mouse organs by Western blots. The microvessel-rich lung, instead of heart, expressed the highest level, suggesting a post-transcriptional regulation mechanism (Figure 2.3B). In a panel of randomly selected cells, HspA12B proteins were present in HUVEC cells at the highest level (Figure 2C), and the order

of expression in human primary endothelial cell lines was HMVC≥HUVEC>HAEC (Figure 2C), and not detected in human primary fibroblasts and human peripheral blood mononuclear cells (not shown).

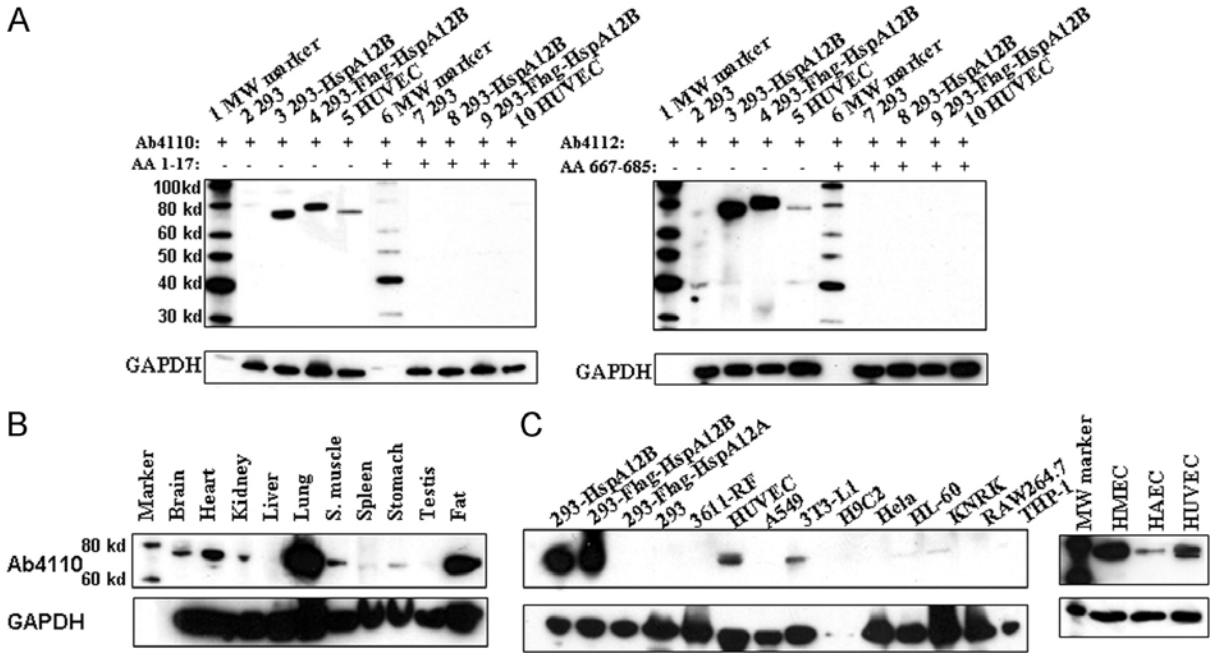


Figure 2.3 HspA12B proteins expression in multiple tissues and preferentially in HUVEC, detected by Western blots (WB). A to C, Blots were stripped and re-probed with anti-GAPDH monoclonal antibody to assess protein loading. A,Validation of the antibodies. Western blots with Ab4110 (left) and Ab4112 (right) as the primary antibodies on whole cell lysates (10 µg) were performed. lane 2, 293 cells; lane 3, 293 cells overexpressing HspA12B; lane 4, 293 cells overexpressing Flag-tagged HspA12B; lane 5, HUVEC; lane 6 to 10 were exact replica of lane 1 to 5, except that 10 µg/mL of the peptide AA 1 to 17 (left) or the peptide AA 667 to 685 (right) were included during primary antibody incubation. The expected MW for HspA12B is 76 kd. B, western blot of mouse tissues with Ab4110. Mice were euthanized by cardiac perfusion with 1x phosphate-buffered saline, and lysates were made in RIPA buffer in the presence of 1x protease inhibitor mix (Sigma). 30 µg lysates were loaded. C, Western blot of multiple cell lines with the Ab4110; 20 µg whole cell lysates were loaded. Ln 1 and 2 are positive controls with 293 cells expressing HspA12B and Flag-tagged HspA12B, respectively; Ln 3, 293 cells expressing Flag-tagged-HspA12A, showing that Ab4110 did not cross-react with HspA12A. HMEC, human microvessel endothelial cells; HAEC, human aortic endothelial cells. See Table 2.2 for the rest of the cell lines details.

Table 2.2 Cell Lines Used in Figure 2.3

Cell lines	**Description**
HEK293	human kidney embryonic cell line
3611-RF	rat fibroblast cell line
HUVEC	human umbilical cord endothelial cell
A549	human lung carcinoma epithelial cell line
3T3-L1	mouse embryonic fibroblast
H9C2	rat cardiomyocyte
Hela	cervix adenocarcinoma epithelial line
HL60	human promyeloblast
KNRK	rat kidney cell line
RAW 264.7	mouse monocyte/macrophage
THP-1	human monocyte

We next characterized HspA12B cell-type specificity by immunohistochemistry. HspA12B proteins were detected specifically in blood vessels in mouse heart and brains (Figure 2.4A), consistent with *in situ* hybridization. Double staining with anti-HspA12B antibody Ab4110 and endothelial cell-specific anti-CD31 (PECAM-1) revealed complete colocalization (Figure 2.4B), indicating that HspA12B was specifically expressed in endothelial cells. Immunostaining with a colorimetric reagent instead of fluorescent reagents produced the same endothelial cell-specific patterns (Figure 2.5).

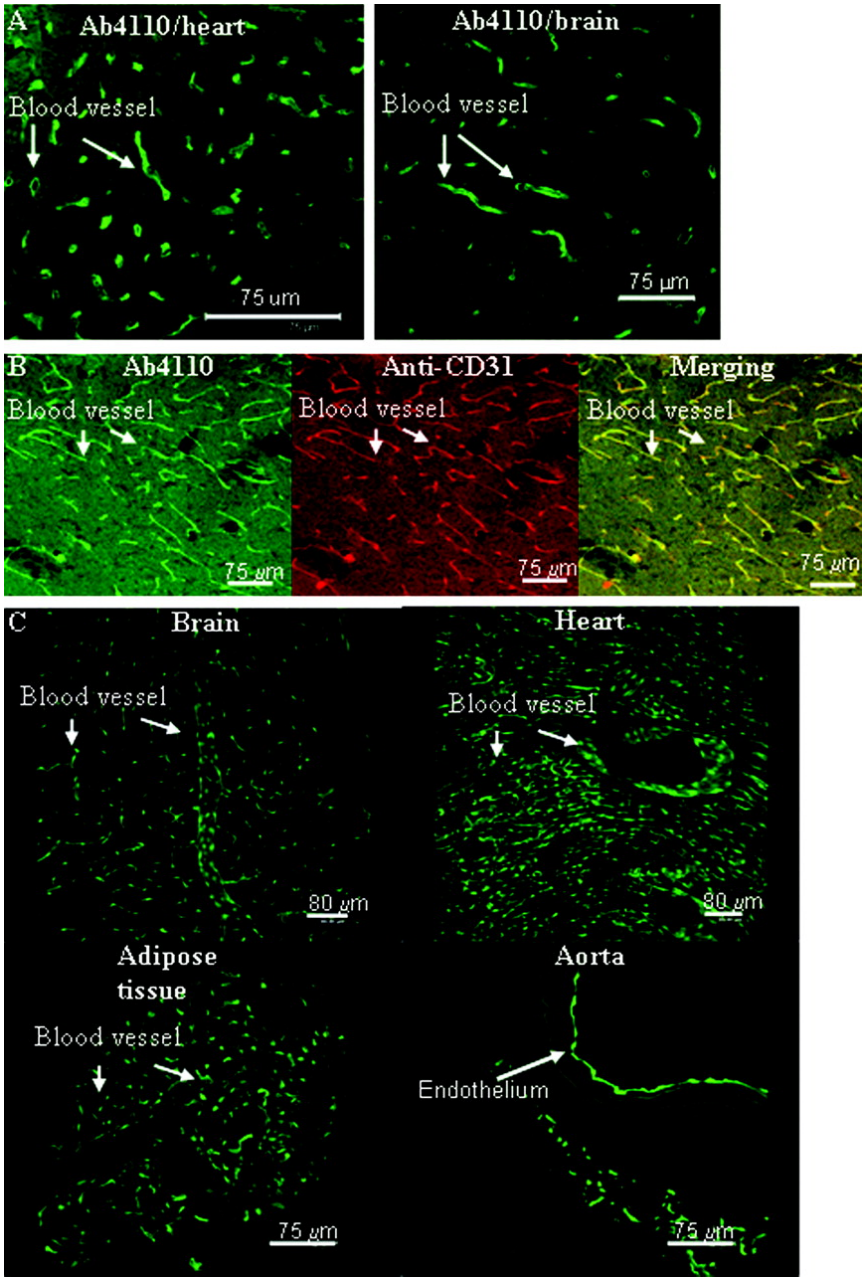


Figure 2.4 HspA12B expression in vascular endothelium by immunohistochemistry and confocal microscopy. A, Mouse heart and brain frozen sections were stained with Ab4110, followed by the fluorescent reagent (AlexaFluor-488 goat-anti-rabbit IgG (H+L) and visualized under confocal microscopy. B, Double staining of mouse brain sections with Ab4110 and vessel specific rat-anti-CD31, visualized by fluorescent reagents (AlexaFluor-488 goat-anti-rabbit IgG (H+L) for HspA12B, green; and AlexaFluor-555 goat-anti-rat IgG (H+L) for CD31, red) and confocal microscopy. Note: there was no leakage between the green (AlexaFluor-488) and red (AlexFluor-555) signals. C, Fluorescent confocal microscopy pictures were taken directly on tissue sections of *HspA12B-BAC-EGFP* mice, showing strong and specific endothelium patterns of EGFP driven by *HspA12B* promoter.

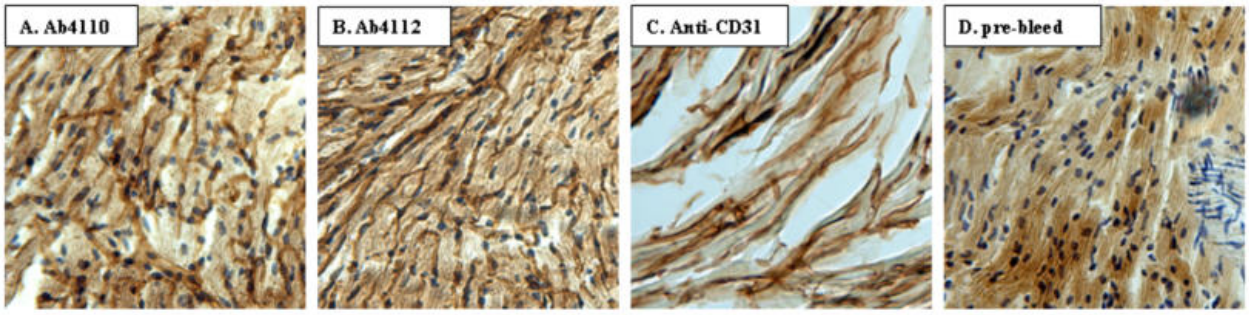


Figure 2.5 HspA12B protein expression in vascular endothelium by immunohistochemistry. Mouse heart sections were stained with HspA12B-specific antibodies and visualized with ABC Vectastain kit and DAB substrate (Vector laboratories, Burlingame, CA). The antibodies are **(A)** Ab4110, showing blood vessel-specific pattern. **(B)** Ab4112, showing blood vessel-specific pattern. **(C)** blood vessel-specific CD31 (PECAM-1) as positive control, showing blood vessel staining pattern. **(D)** pre-immune sera as negative control.

Finally, we tracked the HspA12B expression in mice expressing EGFP under the control of the *HspA12B* promoter (*HspA12B-BAC-EGFP* mice). This method takes advantage of the observation that bacterial artificial chromosome (BAC) commonly preserves the expression pattern of the gene it encodes, presumably because of its length (13). Fluorescent EGFP signals were observed specifically in vessels of various calibers in brain and heart, adipose tissue capillaries, endothelial cells lining the aorta (Figure 2.4C), as well as lung alveolar capillaries, skeletal muscle capillaries, renal glomeruli and intertubular capillaries, and endothelial cells lining the aortic root and aortic valve epithelium (Figure 2.6). By contrast, inside liver, only the portal veins in the periportal area were fluorescent, whereas the fenestrated sinusoidal endothelial cells were consistently negative (Figure 2.6). Again, the fluorescent signals localized closely to CD31, as shown in fluorescent microscopy with anti-CD31 staining (Figure 2.7). Thus, evidence from in situ hybridization, Western blot, immunohistochemistry, and *HspA12B-BAC-EGFP* transgenic mice is consistent with HspA12B being predominantly expressed in endothelial cells lining blood vessels. These results suggest that HspA12B may play a role in angiogenesis.

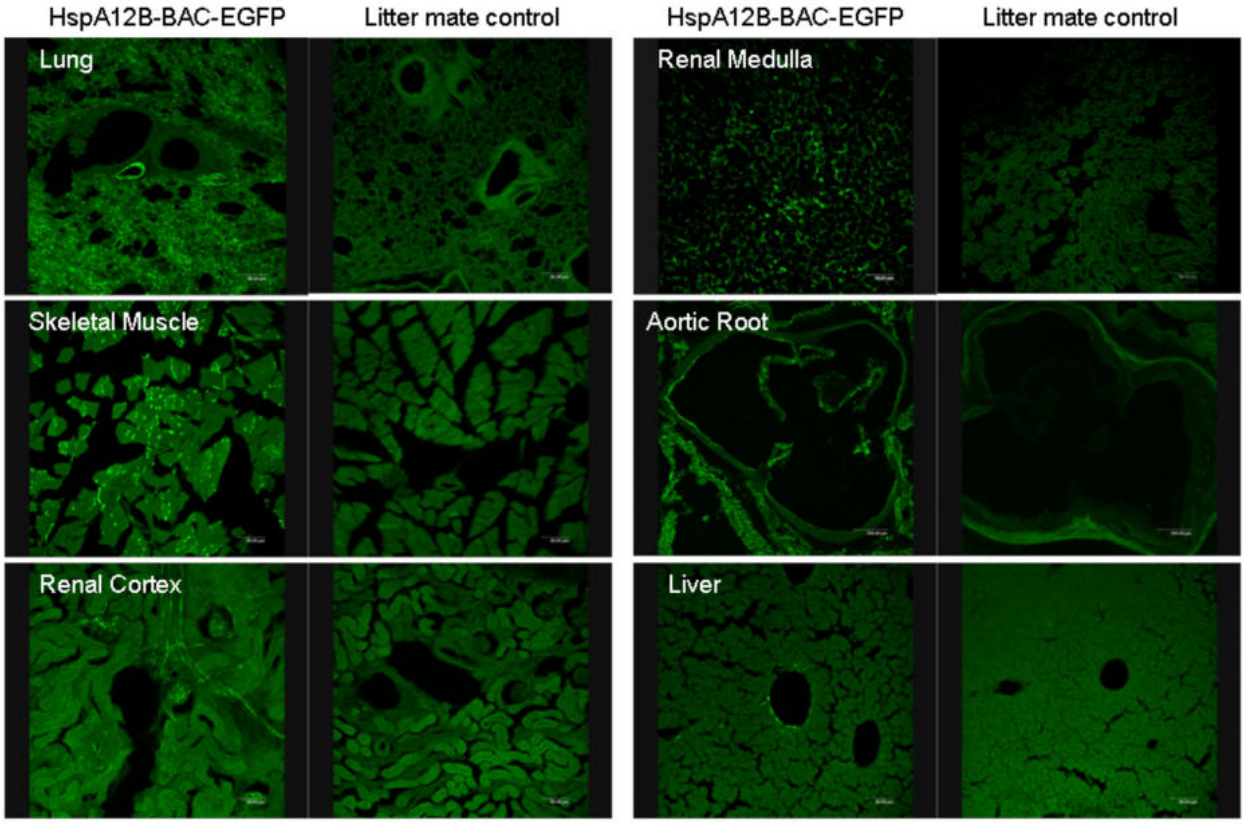


Figure 2.6 Confocal microscopy of frozen sections of *HspA12B-BAC-EGFP* mice, showing endothelial cell-specific expression of HspA12B. Transgenic mice that expressed EGFP under *HspA12B* promoter (left) and their littermates (right) were anaesthetized with ketamine and xylazine and perfused with 4% paraformaldehyde. The organs were post-fixed with 4% paraformaldehyde for 1 hour) and frozen. 20 µm Sections were generated with a Leica CM1850 cryostat and used directly for confocal imaging.

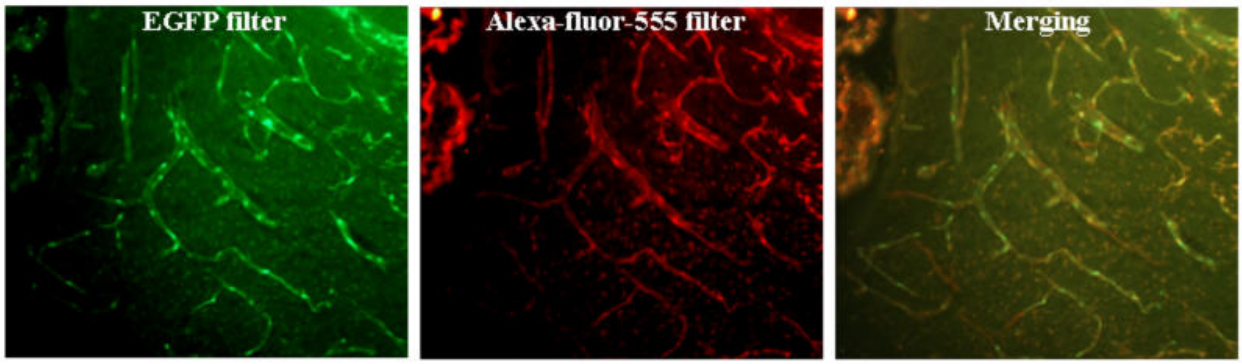


Figure 2.7 Immunostaining of brain sections from *HspA12B-BAC-EGFP* mouse with rat anti-CD31 and goat-anti-rat AlexaFluor-555 (red). Left: EGFP signal driven by *HspA12B* promoter (green); middle: CD31 signal; right: the merged image (yellow). Pictures were taken with a UV-fluorescent microscope.

HspA12B Expression Increases in Confluent Endothelial Cells when Cells Come into Contact and Reduces in Quiescent Cells

Proteins functioning in angiogenesis often display upregulation during cell proliferation/alignment/movement. Therefore, we examined HspA12B expression in actively growing versus quiescent endothelial cells. Exponentially growing, subconfluent HUVECs (Expo) were grown to confluence and were maintained in complete endothelial cell specific medium for an additional 8 hours (Conf). Thereafter, the medium was replaced by M199 medium containing only 5% fetal bovine serum without endothelial cell-specific growth factors for 8 hours (M199–8 hour). The HspA12B protein levels increased significantly in confluent, active HUVECs, and decreased with the withdrawal of endothelial-specific growth factors (Figure 2.8A).

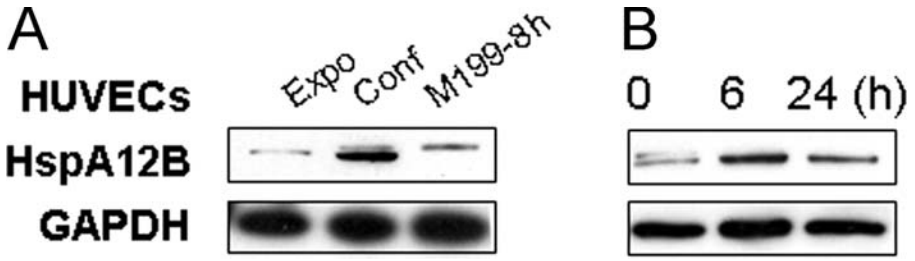


Figure 2.8 HspA12B upregulation in confluent cells and during *in vitro* angiogenesis. A, Western blot, proliferating HUVECs (Expo) were grown to confluence and maintained in complete EC-specific medium for an additional 8 hours (Conf). Thereafter, the medium was replaced by M199 medium containing only 5% fetal calf serum without EC-specific growth factors for 8 hours (M199–8 hour). Whole cell lysates were prepared and HspA12B expression was monitored by Western blot using the Ab4112 antibody. B, Cells that underwent angiogenesis assays were recovered from Matrigel at the indicated times to prepare whole cell extracts and HspA12B expression was monitored by Western blot using Ab4112. The blots were re-probed with an anti-GAPDH monoclonal antibody for normalization of protein levels. Representative chemofluorescent detection of three independent experiments are shown.

Modulation of HspA12B Protein Expression during *In Vitro* Angiogenesis

In vitro angiogenesis operates on the principle that endothelial cells form capillary structures when cultured on a supportive matrix derived from a murine tumor (Matrigel). This assay reproduces the angiogenic processes of migration, alignment, and cell differentiation and has proved to be an important tool for studying the mechanisms of angiogenesis (14). A typical *in vitro* angiogenesis experiment is shown in Figure 2.9. Endothelial cells plated on Matrigel underwent alignment and elongation within 1 to 3 hours after seeding, establishing the pattern for further capillary networking. By 6 hours, formation of cords had begun and by 12 hours virtually all cells had fused into continuous cords. Stabilization and refinement of the cords progressed until 24 hours. We studied the kinetics of endogenous HspA12B protein expression during *in vitro* angiogenesis of HUVECs, and found it increased within 6 hours and leveled off at 24 hours (Figure 2.8B). Thus HspA12B expression is modulated during *in vitro* angiogenesis: upregulated during tubule formation.

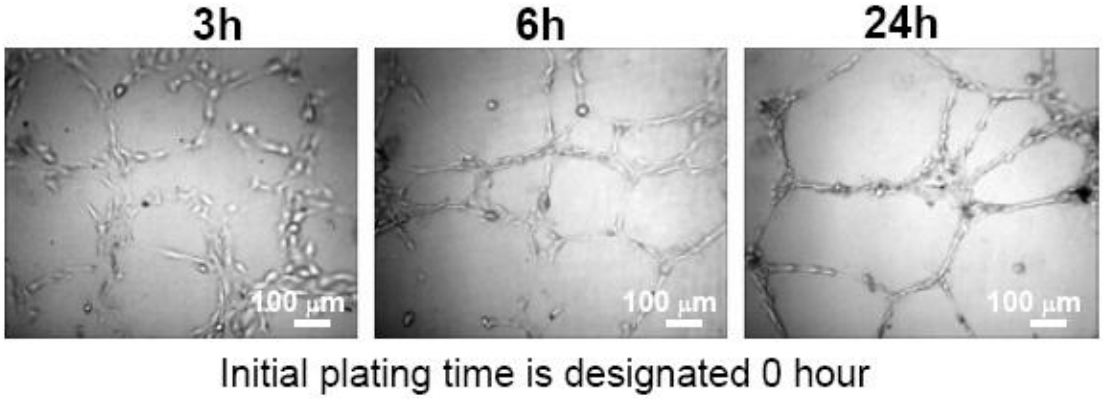


Figure 2.9 Photomicrographs of two-dimensional cultures of HUVECs on Matrigel demonstrating the progression of the tubular network.

N Terminus-Specific Anti-HspA12B Antibodies Block Angiogenesis

A transient increase in HspA12B levels might be functionally important in the regulation of tubule formation. Thus, we tested the effect of neutralizing HspA12B on angiogenesis. A representative experiment is shown in Figure 2.11A. Delivery of the preimmune isotypic control caused no gross deformation in the tubule network formation. In contrast, the N-terminusspecific Ab4110 inhibited tubule formation, indicating HspA12B was required for *in vitro* angiogenesis. The Ab4110 was capable of quantitatively immunoprecipitating HspA12B proteins from cell lysates overexpressing HspA12B (Figure 2.10), thus may serve as neutralizing reagents once delivered inside the cells. The transfection efficiency achieved was consistently >95% as indicated by the addition of a nonrelevant AlexaFluor-555-labeled antibody (Figure 2.11A, top panel).

Expression of EGFP-HspA12B but not HspA12B-EGFP Chimeras in HUVECs Interferes with Angiogenesis

The previous experiment suggested that masking the N-terminus of HspA12B with Ab4110 inhibited angiogenesis. To corroborate this, we tested the effect of overexpressing an N-terminus–tethered form of HspA12B chimera (*EGFP-HspA12B*) on angiogenesis, using EGFP as well as a C-terminus-tethered HspA12B chimera (*HspA12B-EGFP*) as controls. To do this, we transiently transfected HUVECs with: (1) *EGFP*; (2) the *HspA12B-EGFP* fusion; and (3) the *EGFP-HspA12B* fusion (Figure 2.11B, top panel). We tracked the numbers of transfected cells (green cells) inside tubule-like structures at the end of a 24-hour angiogenesis assay (Figure 2.11B, bottom panel). For both controls, transfected cells (green cells) were readily incorporated into the tubule-like structures, at the ratios close to transfection efficiency (Figure 2.11B, bottom panels, left and middle). In contrast, no green cells were observed in the tubule-like structures for HUVECs transfected with the *EGFP-HspA12B* (Figure 2.11B, bottom panels, right). Thus, tagging an EGFP group to the N-terminus, but not the C-terminus, of the HspA12B inhibited angiogenesis, suggesting an unblocked N-terminus of HspA12B is needed for angiogenesis.

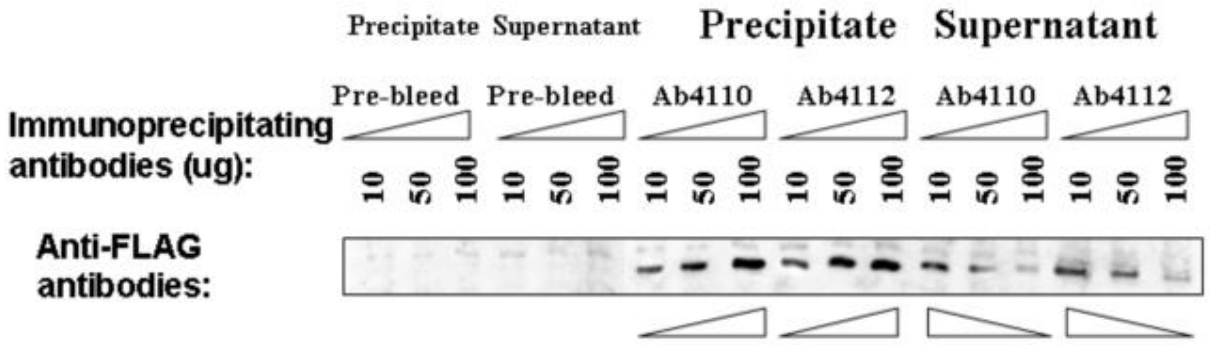


Figure 2.10 Ab4110 and Ab4112 quantitatively immunoprecipitated Flag-tagged HspA12B overexpressed in 293 cells. 293 cells overexpressing Flag-tagged mHSPA12B were lysed and the extracts were immunoprecipitated with increasing concentration of pre-immune sera, Ab4110, and Ab4112, The precipitates and supernatants were probed with anti-Flag M2 antibody

(StrataGene, Cedar Creek, TX) in a Western blot. The increasing concentrations of Ab4110 and Ab4112 resulted in increased precipitated Flag-HspA12B and minimal soluble Flag-HspA12B.

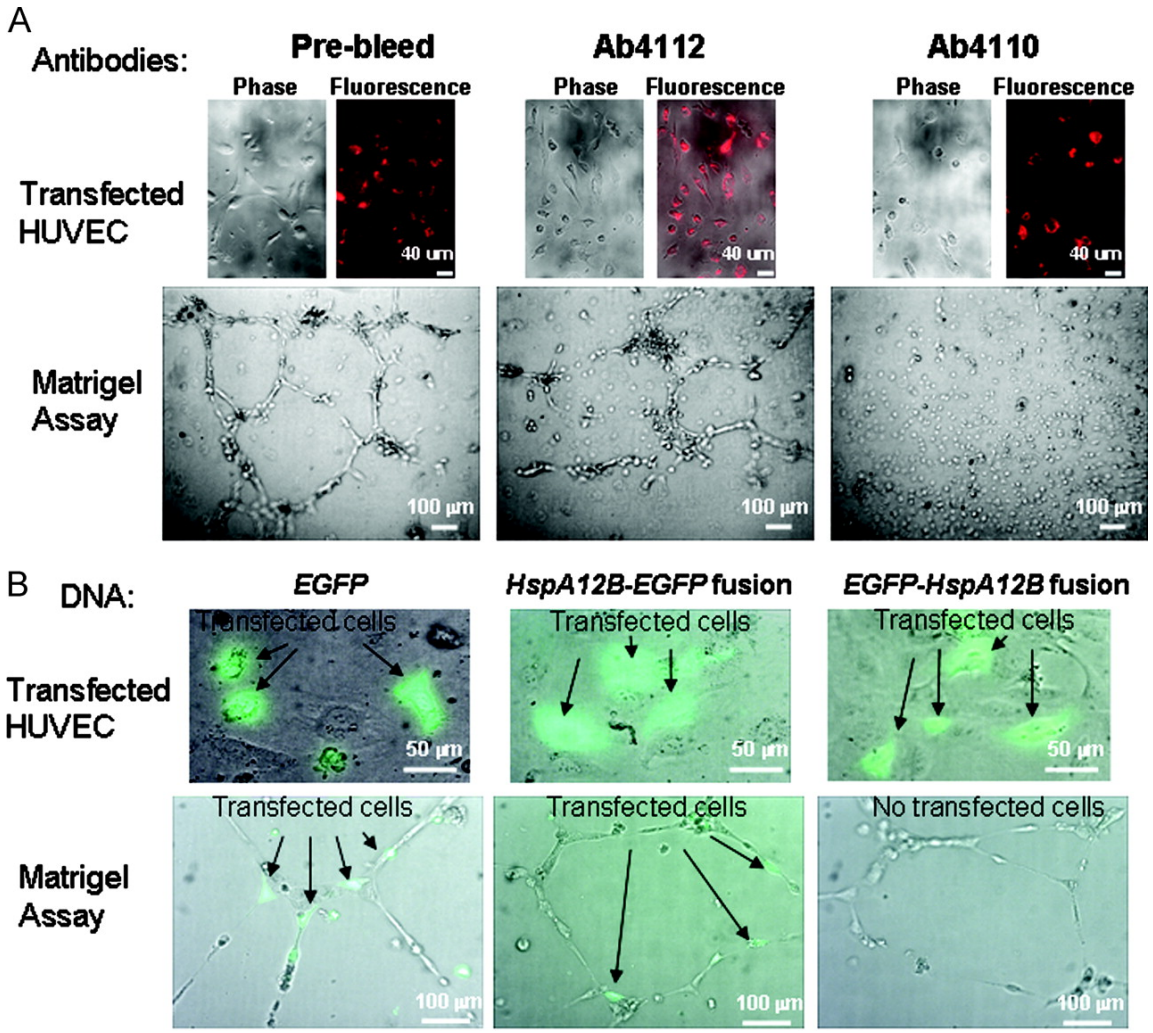


Figure 2.11 HspA12B was required for angiogenesis *in vitro*. A, the N-terminus-specific antibody Ab4110 inhibited *in vitro* angiogenesis. Top, phase contrast and fluorescent pictures of HUVECs, 4 hours after transfection of 10 µg/well pre-immune sera (left), Ab4112 (middle), and Ab4110 (right), by the protein delivery reagent BioPORTER (Sigma), spiked with 1 µg/well of AlexaFluor-555-labeled antibody (red). Bottom**,** Matrigel assays, the above HUVECs were plated onto Matrigel in 24-well plate (30 000 cells/well), and incubated in 5% CO_2, 37°C for 24 hours. B, overexpressing the N-terminus-tethered *EGFP-HspA12B* fusion, but not the C-terminus-tethered *HspA12B-EGFP* fusion, inhibited *in vitro* angiogenesis. Top, HUVECs were transfected with the *pEGFP-N1* (left), *pHspA12B-EGFP* fusion (middle), and *pEGFP-HspA12B* fusion (right); 24 hours after transfection, UV fluorescent pictures were taken to assess the expression of EGFP (left), HspA12B-EGFP fusion (middle), and EGFP-fusion (right) proteins.

Note the EGFP were distributed uniformly inside whole cells (left), whereas the HspA12B-EGFP and EGFP-HspA12B fusion proteins were located in cytoplasm (middle and right). Bottom, Matrigel assays, the above transfected cells were trypsinized and plated onto Matrigel for *in vitro* angiogenesis assay for 24 hours. Phase contrast and UV fluorescent pictures were taken for the same field to track the destination of the EGFP-labeled cells. A and B, representative photomicrographs of three independent experiments are shown.

N Terminus-Specific Anti-HspA12B Antibodies Reduce Migration

Migration is an essential early event of angiogenesis, thus we examined whether neutralizing HspA12B would interfere with migration using a Boyden chamber assay. As shown in Figure 2.12, migration was stimulated by the growth medium containing endothelial cell growth factors in HUVEC cells (2.7 ± 0.4 fold over background). In contrast, HUVEC cells transfected with Ab4110 showed a significant reduction ($P<0.01$) in the number of cells transmigrating toward chemoattractant medium (1.5 ± 0.3 fold over background). The difference between control and Ab4112 was not significant. Thus, HspA12B is involved in the HUVECs chemotactic activities toward angiogenic factors as measured in this assay.

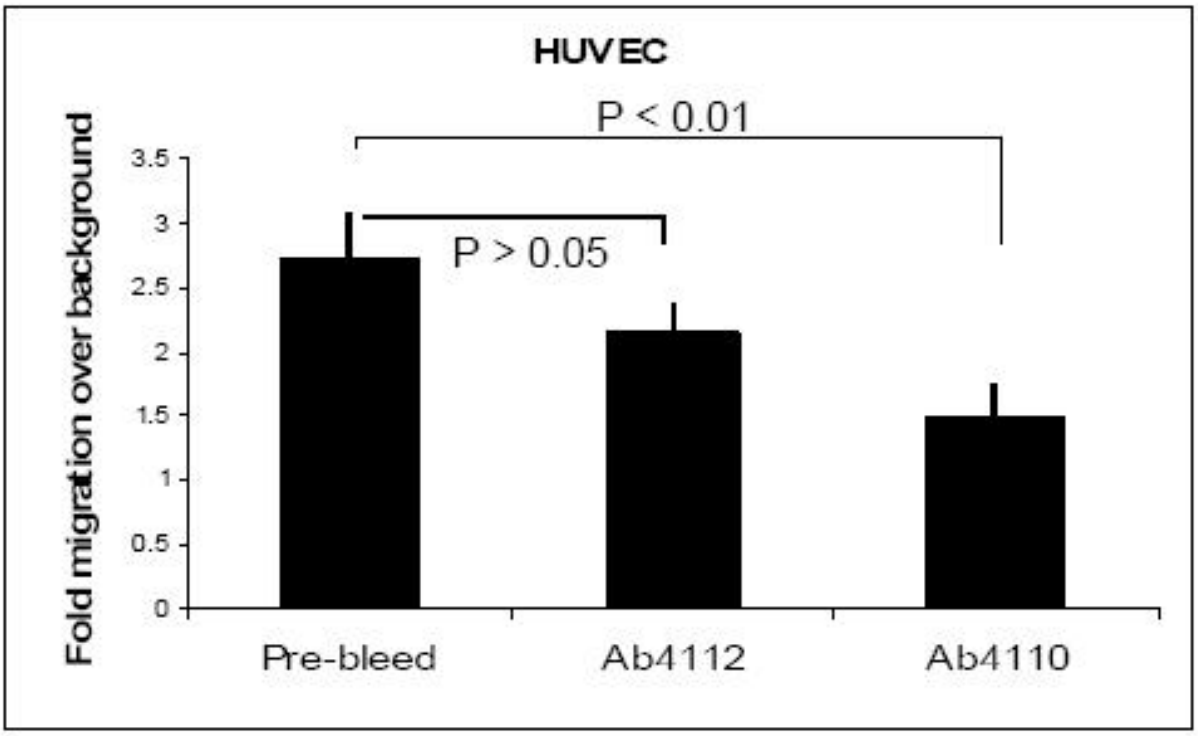


Figure 2.12 HspA12B was required for migration. HUVECs were transfected with pre-bleed sera, Ab4112 and Ab4110, and underwent migration assays. Cells were placed on the upper chambers of 8-µm cell culture inserts. Cells that migrated through inserts were fixed and counted in 10 randomly selected fields (original Magnification 10×). The number of cells that had migrated through the inserts towards M199 medium with 1% serum was considered as background and was arbitrarily set at 1. The values are the mean ± SD of three independent transfection experiments.

Discussion

As the newest member of Hsp70 family, little was known about the normal expression and function of HspA12B. In this study, we report that HspA12B is predominantly expressed in endothelial cells, as assayed by *in situ* hybridization, immunostaining, and *EGFP* expression in mice under the control of *HspA12B* native regulatory elements. We also report that HspA12B is required for angiogenesis and an unblocked N-terminus is needed for these angiogenic activities. We provide evidence that HspA12B may exert its effects during cellular adhesion and cell migration. Our data suggest that HspA12B may be an EC-specific chaperone.

A common perception is that Hsp70 proteins are expressed ubiquitously. Here, we showed that the HspA12 subfamily is highly tissue-specific. HspA12B is predominantly expressed in endothelial cells, and HspA12A is highly expressed in neuronal cells (15). Further studying of their roles in development and pathology should yield important insights into those processes. The HspA12 subfamily is the least conserved subfamily of the Hsp70 family, judging by primary sequence homology. Still, 3-dimensional modeling shows that the signature ATPase domain that covers approximately two thirds of the Hsp70 is well-conserved in HspA12 (FUGUE program, Z-score of 22.18 for HspA12B, and 28.39 for HspA12A, when higher Z-score means higher confidence and a Z-score >6 means the prediction is almost certain).

In the vascular endothelia, HspA12B expression is prevalent but not universal, demonstrating vessel heterogeneity. HspA12B is found in adipose tissue, brain, heart, kidney, and lung, but not in liver sinusoidal endothelial cells, which are fenestrated and where blood pressure is low. In brain and heart, vessels of all calibers expresses HspA12B, but in adipose tissue and lung, HspA12B is present primarily in capillaries. The latter is consistent with the expression levels observed in cultured primary cells (microvessel ≥ vein > aorta).

Ab4110, once delivered into HUVEC cells, inhibited angiogenesis in Matrigel assay. This could be the result of either complete removal/precipitation of HspA12B protein, or masking the HspA12B N-terminus region, where the conserved ATPase domain resides. The

second possibility is more likely because the C-terminus-specific Ab4112 did not block angiogenesis even though Ab4112 possessed similar levels of affinity toward HspA12B in immunoprecipitation. Accordingly, overexpressing the N-terminus-masked *HspA12B* (*EGFP-HspA12B*), but not the C-terminus-masked *HspA12B* (*HspA12B-EGFP*), prevented otherwise normal cells from participating in angiogenesis. Thus, interfering with the N-terminus region of HspA12B abrogated angiogenesis. The dominant-negative fashion of the latter results was consistent with the hypothesis that HspA12B has multiple domains and involves protein-protein interactions for its proper function. Hsp70 proteins consist of two functional domains: the highly conserved ATPase domain of 44-kDa in the N-terminus and the substrate binding region of 25-kDa in the C-terminus (16). Also, it is thought that chaperones cooperate extensively, sometimes forming multi-chaperone systems that work sequentially or simultaneously to ensure the efficient biogenesis of cellular proteins in their respective cellular compartments (17). For example, HSC70 interacts with Hsp40 and Hop through the C-terminal domain, and interacts with Hip through N-terminal ATPase domain, and BAG-1 competes with Hip in binding to the ATPase domain (18). This network of cooperating and competing cofactors regulates the chaperone activity of HSC70. It is conceivable that the presence of the EGFP group in the N-terminus might either block HspA12B activity or disrupt its normal interactions.

Recently, a zebrafish *HspA12B* homologue has been characterized and found to be specifically expressed in zebrafish blood vessels. Knocking-down of *HspA12B* by RNAi disrupted zebrafish blood vessel normal development and inhibited *in vitro* angiogenesis and migration in HUVECs (19). These results are consistent with and complementary to our findings.

In this study, we have demonstrated that the *HspA12B-BAC-EGFP* transgenic mice expressed EGFP strongly and specifically in endothelial cells. These mice will be a valuable tool for marking endothelial cells and tracking blood vessels during normal development or under pathological conditions.

The mechanism by which HspA12B influence angiogenesis remains unclear, but our data provide evidence that HspA12B may regulate endothelial cell adhesion and migration, critical steps during angiogenesis. Firstly, HspA12B is induced in active, confluent endothelial cells and during the early stage of *in vitro* angiogenesis when cells make contact and migrate. Secondly, inhibiting HspA12B reduces HUVEC migration in a migration assay. HspA12B knockout mice should ultimately help to confirm the role of HspA12B in angiogenesis and shed light on its mechanism. The characterization of HspA12B provides new insight on angiogenesis and may lead to new target for disease intervention.

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CHAPTER 3

HSPA12B, A NEWLY IDENTIFIED ANGIOGENESIS REGULATOR, PROMOTES ANGIOGENESIS BY SUPPRESSING AKAP12 THUS UP-REGULATING THE VEGF PATHWAY

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Abstract

HspA12B, a member of Hsp70 sub-family 12, is predominantly expressed in endothelial cells (ECs) and required for angiogenesis. We set out to uncover the pathway through which HspA12B exerts its pro-angiogenic effect. We identified AKAP12 (A-kinase-anchoring protein 12, also known as Gravin or SSeCKS) as an HspA12B-interacting protein through a yeast two-hybrid (Y2H) screening and confirmed the interaction by subcellular co-localization and co-immunoprecipitation. shRNA mediated knockdown of HspA12B significantly increased AKAP12 levels, whereas overexpression of HspA12B significantly decreased AKAP12 levels in primary human umbilical vein endothelial cells (HUVECs), indicating that HspA12B negatively regulates the level of AKAP12, which in turn has been reported to down-regulate vascular endothelial growth factor (VEGF) and other angiogenesis-related genes. Correspondingly, knockdown of HspA12B significantly reduced the levels of VEGF, Akt, pAkt, and Angiopioetin-1 (Ang-1), both in normoxia and hypoxia conditions, whereas overexpression of HspA12B up-regulated VEGF in HUVECs. The VEGF up-regulation by HspA12B is mediated through suppression of AKAP12, since concomitant silencing of both HspA12B and AKAP12 reversed the loss of VEGF expression observed by silencing HspA12B alone. We further identified a 32-AA domain in AKAP12 that was capable of interacting with HspA12B, and overexpressing this 32-AA in HUVECs disrupted the HspA12B-AKAP12 interaction, up-regulated AKAP12, and down-regulated VEGF. HspA12B was induced by hypoxia and HspA12B knockdown abrogated hypoxia-induced tubule formation in a Matrigel assay. HspA12B promoted angiogenesis in an *in vivo* assay. Finally, the proteasome inhibitor, MG132 reversed the reduction of AKAP12 levels caused by HspA12B overexpression, indicating that HspA12B facilitates the turnover of AKAP12 through the ubiquitin-proteasome pathway. We provide the first evidence that HspA12B promotes angiogenesis through regulating VEGF by way of repressing AKAP12, most likely by facilitating AKAp12 turnover. Thus, we provide the first example of an EC-specific molecular chaperone acting as the regulator of angiogenesis. Our findings also have significant

implication in linking G-protein coupled receptors signaling to angiogenesis and add insight to hypoxia-induced VEGF induction and angiogenesis.

Introduction

Blood vessel development and formation are essential for organ growth and repair, wound healing, and reproduction cycle, and an imbalance of functional vessels contributes to diseases such cancer and ischemia (*1*). Angiogenesis is the process whereby existing endothelial cells (ECs) form functional vessels through remodeling. Angiogenesis is a complex process involving the interplay of multiple genes and gene family members, the most prominent of which is vascular endothelial growth factor (VEGF)/VEGF receptors (VEGFR) and is critically dependent upon the precise and delicate coordination, combination, and collaboration of many molecular players in the right time, space, and dose. A molecular chaperone is a natural candidate to perform this organizational role.

We have recently characterized a potential candidate, HspA12B, for an endothelia-specific molecular chaperone. HspA12A and HspA12B make up a new sub-family of Hsp70. HspA12A is a candidate gene for atherosclerosis susceptibility, based on evidence of genetic linkage and expression profiling. HspA12B shares sequence homology (60% identical) to HspA12A and is also enriched in atherosclerotic lesions (*2*). Subsequently, we showed that HspA12B is predominantly expressed in ECs and is required for angiogenesis (*3*). This was later confirmed by others who demonstrated that the zebrafish HspA12B homolog is expressed specifically in ECs and is indispensable for zebrafish vessel development (*4*). There is some indication that Akt activation is impaired in ECs following the reduction of HspA12B level (*4*), but otherwise the mechanism by which HspA12B functions is unknown. Based upon its structural similarity to Hsp70s, we hypothesized that HspA12B acts as an EC-specific molecular chaperone that mediates the production, localization, turnover, and quality control of its cognate set of protein targets, which in turn defines the angiogenic process in ECs.

Heat shock proteins (Hsp) are a group of proteins that are abundant in cells, highly conserved among species, and associated with stress responses. Hsp70 is the largest and most conserved family of heat shock proteins (*5*). Hsp70s function as molecular chaperones, assisting

in protein synthesis, folding, assembly, trafficking between cellular compartments, and degradation (*6*). Hsp70s take part in the cellular stress response by binding and refolding or removing misfolded proteins through the ubiquitin-proteasome system by interacting with CHIP (carboxyl terminus of Hsp70-interacting protein), an Hsp70-associated ubiquitin ligase (*6, 7*). In this capacity, chaperones of Hsp70/Hsp90/CHIP mediate the "triage decision" between stabilization and degradation, and were shown to control the steady-state level of critical signaling molecules (*7*).

In this study we aimed to find the mechanisms by which HspA12B regulates angiogenesis through the characterization of its targets. We reasoned that molecular chaperones routinely bind to their substrates to effect change; therefore, we screened for HspA12B interacting proteins using a yeast two-hybrid screening. The screening identified several HspA12B-interacting proteins that have previously been shown to be relevant to angiogenesis (Table 3.1). AKAP12 (A-kinase anchor protein 12) also called SSeCKS for src-suppressed C-kinase substrate (its mouse ortholog) or gravin), was the top-ranked interacting protein from this screening.

AKAP12 was originally characterized as a cancer repressor (*8, 9*) and has recently been linked to angiogenesis regulation. A recent study demonstrated that induced expression of SSeCKS (AKAP12) suppressed metastases of prostate cancer cells in both spontaneous and experimental models of *in vivo* metastasis and decreased angiogenesis *in vitro* and *in vivo* by suppressing VEGF and other proangiogenic genes including angiopoietin and hypoxia-inducible factor-1α (HIF-1α) (*8*). Another study indicated that AKAP12 attenuates expression of angiogenic genes including HIF-1α, thus suppressing Src-induced oncogenesis (*9*). It has also been reported that AKAP12 promoted tight junction formation in blood-brain barrier by decreasing VEGF and augmenting angiopoietin-1 (ang-1) and tight junction proteins (*10*), and that AKAP12 promoted human blood-retinal barrier formation by down-regulation of HIF-1α (*11*).

AKAP12 is a scaffolding protein that regulates mitogenesis, development, and differentiation through selective binding of signaling molecules such as G-protein-coupled receptors (GPCR), PKC, PKA, calmodulin, and cyclins (*12-14*). It is hypothesized that AKAP12 provides an organizing center about which various protein kinases and phosphatases can be assembled to create a signaling complex that can be modulated and trafficked within the cell, thus compartmentalizing various enzymes that are regulated by second messengers (*15*).

In this study we set out to determine the interaction between HspA12B and AKAP12 and determine the effects of HspA12B on AKAP12 and the VEGF pathway. Because chaperones are associated with the stress response, we investigated HspA12B's induction by angiogenesis related stresses and found it to be induced by hypoxia and shear stress. Hypoxia also induces angiogenesis so we determined whether siRNA mediated knockdown of HspA12B reduced hypoxia-induced tubule formation in an *in vivo* angiogenesis assay.

Materials and Methods

Yeast Two-Hybrid (Y2H) Screening

Bait cloning and Y2H screening were performed by Hybrigenics (Paris, France) (www.hybrigenics.com). *HspA12B* was PCR-amplified and cloned into an Y2H vector optimized by Hybrigenics. The bait construct was checked by sequencing the entire insert and was subsequently transformed in the L40ΔGAL4 yeast strain (*16*). A human bone marrow endothelial cell random-primed cDNA library, transformed into the Y187 yeast strain and containing ten million independent fragments, was used for mating. The screen was first performed on a small scale to adapt the selective pressure to the intrinsic property of the bait. Neither toxicity nor auto-activation of the bait was observed. Then, the full-scale screen was performed in which 134 millions of interactions were actually tested with HspA12B (13 times of coverage of the primary complexity of the yeast-transformed cDNA library). After selection on medium lacking leucine, tryptophane, and histidine, 232 positive clones were picked, representing 22 unique clones. A ranking system, which took account of prey fragments' redundancy, independency, frequency, distributions of reading frames and stop codons, as well as appearance in all prior screens, was used to assess the reliability of each interaction (*17*).

Co-Immunoprecipitation (co-IP)

The co-IP was performed according to Harlow and Lane (*18*). 3×10^5 HUVECs were lysed in 50 µl T-per buffer (Pierce, Rockford, IL), incubated on ice for 1 hour with 2 µl normal rabbit serum, followed with addition of 2 µl fixed S. aureus Cowan I (SAC) and 30 minutes. After a 4°C centrifugation (15 minutes at 10 000 g) 1 µl of precipitating antibodies (1:50) or pre-bleed were added into the supernatant and incubated on ice for 1 hour. 10 µl protein A/G plus beads were added, followed by 1 hour incubation on ice with rocking. The beads were collected by centrifugation, washed three times with lysis buffer (150 mM NaCl, 1% NP-40, and 50 mM

Tris, pH 8.0), and analyzed by Western blot. The sheep AKAP12 antibody (S1562) was purchased from Sigma (St. Louis, MO).

Immunostaining

HUVECs or HEK 293 cells were fixed with 4% paraformaldehyde for 10 minutes, rinsed with 1× PBS, blocked in 1% triton /10% BSA in 1x PBS for 30 minutes, before incubation with the primary antibodies (1:200) in 10% BSA at 4°C overnight. The rabbit anti-HspA12B Ab4111 was made as previously described (*3*). After rinsing 4 times with 1×PBS for 15 minutes and 1 time with 10% BSA blocking buffer, the cells were incubated with 1:200 dilution of AlexaFluor-488 goat-anti-rabbit IgG (H+L) (green) and AlexaFluor-555 goat-anti-sheep IgG (H+L) (red) from Molecular Probes (Carlsbad, CA) in 10% BSA blocking buffer for 1 hour, and washed with 1×PBS four times. The plates were mounted and pictures were taken under a Nikon Diaphot fluorescent microscope and a Leica TCS SPII confocal microscope.

Cell Cultures- Normoxia, Hypoxia and Shear Stress Systems

Primary Human Coronary Artery Endothelial Cells (HCAECs) were obtained from Lonza (Rockland, ME) and were propagated through passage 6 in Endothelial Cell Basal Medium-2 supplemented with 10% FBS from Gibco (Invitrogen, Carlsbad, CA), human epidermal growth factor (hEGF), hydrocortisone, gentamicin sulfate (GA-1000), VEGF, human fibroblast growth factor-basic (hFGF-B), R3 insulin-like growth factor-1 (R3-IGF-1) and Ascorbic acid. Primary Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Lonza and were propagated through passage 6 in F-12K medium (Mediatech, Herndon, VA) supplemented with 10% FBS, 0.1 mg/ml porcine heparin and 0.05 mg/ml bovine endothelial cell growth medium (Sigma, St. Louis, MO). The H9c2 embryonal rat heart derived cells (*19*) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in monolayers in Dulbecco's modified Eagle's medium (DMEM)-supplemented with 10% fetal bovine serum (FBS), sodium

pyruvate (1 mmol/l), penicillin (100 IU/ml) and streptomycin (100 μg/ml) in normoxic condition (21% O_2, 5% CO_2, and 74% N_2) in a humidified incubator at 37°C. When the cells reached ~70–80% confluence, Ad5-HspA12B EGFP or Ad5-GFP (1×10^7 pfu/ml) was added into the cells. 24 hours after transfection the hypoxia treatments were performed in a temperature- and humidity-controlled anaerobic environmental chamber controlled by ProOx oxygen sensor (BioSperix, Redfield, NY) in an atmosphere containing <0.5% O_2, 5% CO_2, balance N_2 for 8, 24 or 48 hours. Simulated ischemia was achieved by culturing the cells in serum-deficient, low glucose DMEM in addition to the anaerobic environment. Alternatively, the hypoxia transition metal, $CoCl_2$ (100 μM) was also used to induce hypoxia for 6 or 24 hours (*20*). After hypoxia, the hypoxia/reoxygenation (H/R) cells were recovered with reoxygenation at normoxic conditions.

HCAECs were also cultured under steady flow as a ‘‘static’’ control (not exposed to shear) for 24 hrs or subjected to a low shear stress of 2 dynes/cm^2 and a high shear stress of 15 dynes/cm^2 using the parallel plate apparatus method of Sakariassen et al. (*21*). Briefly, control HCAECs were cultured statically for 4 days on collagen I slides. Shear samples were cultured statically for 2 days on collagen I slides until confluent, slides were then assembled into a parallel plate flow chamber to impose high or low shear stress for 2 days.

MG132 (Calbiochem, Gibbstown, NJ) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. For proteasomal inhibition studies cells were treated with 20 μM MG132 or an equivalent volume of DMSO for 24 hours.

The Human Embryonic Kidney 293 (HEK 293) cell line was obtained from ATCC (Manassas, VA) and the HEK 293A cell line was obtained from Quantum Biotechnologies (Montreal, Canada). Both were cultured in monolayers in Dulbecco’s modified Eagle’s medium (DMEM)-supplemented with 10% fetal bovine serum (FBS), sodium pyruvate (1 mmol/l), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in normoxic condition (21% O_2, 5% CO_2, and 74% N_2) in a humidified incubator at 37°C.

Small Interfering RNA and DNA Construct Transfection of Endothelial Cells

The small interfering RNAs (siRNA) against the human HspA12B were: (si1), CCACGGAUCUCACCUUGAAUU; (si3), GGGACUGGCUCUACUUCGAUU; Negative control (NC), GCGCAUCGACUUUCUCUUU. Transfection ready siRNA duplexes were purchased from Dharmacon (Chicago, IL). Transfection experiments were carried out using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) essentially following manufacturer instructions at a final concentration of 100 pmol in 6-well plates.

For plasmid transfection, exponentially growing HUVECs were seeded at a density of 2×10^5 cells in gelatin coated 6-well plates for 18-24 hours until ~40-60% confluence, and 0.4 μg of plasmids plus 10 μl Effectene (QIAGEN, Valencia, CA) were added for transfection. 24 hours later, cells were analyzed for protein expression, treated with hypoxia, used for co-IP or plated for Matrigel assays.

Western Blot (WB) Analysis

The western blots were performed using standard procedures. Briefly, total protein was electrophoresed on SDS-PAGE and transferred to polyvinylidene difuoride (PVDF) membranes (Invitrogen) with the Invitrogen Novex Mini-Cell system. The blotted membranes were incubated with antibodies and treated with the enhanced chemiluminescence reagent Western Dura (Pierce, Rockford, IL). For membranes probed with the HspA12B antibody, Ab4112 (ProSci Inc, Poway, CA) (*3*) a goat-anti-rabbit IgG/horseradish peroxidase conjugate (Pierce, Rockford, IL) was used as the secondary antibody. As a control for loading and normalization, membranes were reprobed with an anti-GAPDH monoclonal antibody (MMS-580S; Covance, Berkeley, CA). Additional primary antibodies included the rabbit polyclonal to GFP (2555) from Cell Signaling (Danvers, MA), the goat polyclonal to Ang-1 (sc-6320), the mouse monoclonal to HIF-1α (sc-13515), the mouse monoclonal to VEGF (sc-7269), the sheep polyclonal to

AKAP12/SSeCKS (S1562) from Sigma (Saint Louis, MO) and the rabbit monoclonal to Akt-[pS473] (44-621G) from BioSource (Carlsbad, CA).

Matrigel-Based *In Vitro* Angiogenesis

Wells of a 24-well plate were coated with 250 µl Matrigel from BD Biosciences (San Jose, CA) and incubated at 37°C for 30 minutes 5×10^4 HCAECs in complete EC Basal Medium were added to Matrigel-coated wells and incubated at 37°C with 5% CO_2 for 24 hours. Photographs were taken (magnification 40×) of three random microscopic fields. A tubule was defined as a complete ring of endothelial cells forming a vascular-like structure. To quantitate, the tubules were manually outlined with the lasso tool of Photoshop and the number of pixels selected was determined using the histogram tool.

Adenovirus Construction

Double-deleted adenovirus type 5 (Ad5) expressing HspA12B-EGFP fusion protein (Ad5-HspA12B-EGFP) (Appendix A, Figure A.1.8) was generated using the AdEasy system from Quantum Biotechnologies (Montreal, Canada). The C-terminus EGFP-tagged HspA12B (HspA12B-EGFP) expression construct (Appendix A, Figure A.1.3) was generated according to Steagall *et al*. (*3*). This construct was then digested with BglII and XbaI to digest out the HspA12B-EGFP coding sequence. The gel purified DNA fragment was inserted into the pShuttle-CMV vector (Appendix A Figure A.1.6) using the same enzymes. This was followed by recombination with the Ad5 vector. The double-deleted adenovirus expressing HspA12B-EGFP was packaged in HEK293A cells and purified by ultracentrifugation. The plaque forming units per milliliter (pfu/ml) of Ad-HspA12B-EGFP were determined by the Optical Particle Unit method according to the manufacture's protocol. Similarly, Ad5-HspA12B was generated by digesting with SalI and NotI the mHspA12B full length coding region that was previously RT-PCR amplified and subcloned into pCMV·Sport6 (Invitrogen) (Appendix A Figure A.1.5). This

fragment was subsequently cloned into the pShuttle-CMV vector (Appendix A Figure A.1.7) using the same enzymes followed by recombination with the Ad5 vector and packaging as described above. For control, we propagated Ad5-green fluorescent protein (Ad5-GFP) from Quantum Biotechnologies that expressed GFP from the same type of adenovirus vector.

sh-HspA12B and sh-AKAP12 Construction

We generated the shHspA12B through a DNA vector-based strategy that synthesize short hairpin RNA (shRNA) from U6 promoter, which has been found to be much more stable than siRNA in *in vivo* systems (*22*). The target sequence (gcagtttggagatactgagatt 1968-89) was designed empirically and contains no homology with other genes as analyzed by Blast search. The pBS/U6-sh-HspA12B (Appendix A Figure A.1.9) vector that expressed HspA12B-shRNA was generated by a triple-ligation of three DNA fragments: 1, the PCR fragment containing U6 promoter and amplified from primers gccagggttttcccagtcacgacg (P1) and cagcaagcttgaatatctcagtatctccaaactgcaaacaaggcttttctccaagggata (1968-H3L) and template pBS/U6, and subsequently digested with KpnI and HindIII; 2, the fragment annealed from agcttaatctcagtatctccaaactgcttttg (1968-2a) and aattcaaaaagcagtttggagatactgagatta (1968-2b); and 3, pBluescript (SK) digested with EcoRI and KpnI. In effect, this method outlined in (*22, 23*) with minor modification, recreated the pSilencer 1.0-U6 system but with the added advantages of creating a shRNA that has clean ends unencumbered by any restriction site and has a mismatch that favors the loading of antisense strand onto RISC over the sense strand. A similar process was adopted to generate pBS/U6-sh-AKAP12 (Appendix A Figure A.1.10), by targeting gttcaagaggttctgtcactga (3639-3660). The sequences of the three primers are 3639-3L cagcaagcttgaaacagtgacagaacctcttgaacaaacaaggcttttctccaagggata, 3639-2a agctttcagtgacagaacctcttgaacttttg, and 3639-2b aattcaaaaagttcaagaggttctgtcactgaa.

AKAP12 Interacting Domain Construction

To make pDsRed AKAP12-829-60 (Appendix A Figure A.1.11), the 32AA region of AKAP12 identified by the Y2H screening to interact with HspA12B was PCR amplified and subcloned into the pDSRed-Monomer-C1 vector from Clontech (Mountain View, CA) by HindIII and BamHI. The primers are gcttttaagcttcggacgcagggccaacaggggccaac and cgcggatcccattttctccctttctacagcatc. The template is BC022814.

Experimental Animals

All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care and were in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals published by National Institute of Health (Publication Number NIH 85-23, revised 1985).

Directed *In Vivo* Angiogenesis Assay (DIVAA)

In DIVAA (Cultrex®, Trevigen, Inc, Gaithersburg, MD), 20 µl of basement membrane extracts (Matrigel, BD Biosciences), along with 62 ng/ml vascular endothelial growth factor (VEGF) and 185 ng/ml fibroblast growth factor (FGF)-2, forms a 3D matrix within a small silicone cylinder (angioreactor) that permits EC migration and blood vessel formation. Either 1µl Ad5-HspA12B or Ad5-GFP control adenovirus [1×10^{10} plaque forming units (pfu)/ml] were included in the Matrigel. The cylinders were implanted subcutaneously in mice for 10 days before recovery, and vessel formation within the cylinders was quantified by measuring fluorescent signal FITC-dextran inside the cylinder. Briefly, mice were anesthetized and two 1 cm incisions were made on the dorsal-lateral surface above the hip-socket of each mouse. Four filled angioreactors were implanted into one mouse with the open end opposite the incision and the incisions were closed. Before collection of the angioreactors, mice received a 100 µl

injection of 25 mg/ml of FITC-dextran in PBS via the tail vein, and the amount of FITC signal recovered from the angioreactors represented the volume of vessels formed within. Quantification was performed by removal of the Matrigel and digestion in 200 µl of Dispase solution (Collaborative Research Inc, Bedford, MA) for 1 hour at 37°C. After digestion, the incubation mix was cleared by room temperature centrifugation for 5 minutes at 15 000 g in a benchtop centrifuge (Eppendorf/Brinkman, Westbury, NY). Fluorescence of the supernatant aliquots was measured in 96-well plates using a Molecular Devices GeminiXS spectrofluorimeter (Sunnyvale, CA) at excitation 485 nm and emission 510 nm.

Statistical Analysis

Results are expressed as the mean ± standard deviation (± SD). Differences between groups were tested for statistical significance by one-way analysis of variance (ANOVA) and Student's t-test ($P < 0.05$).

Results

HspA12B Binds to AKAP12 Specifically

HspA12B's distant homology to Hsp70 led us to examine the hypothesis that HspA12B is an endothelia-specific molecular chaperone that performs its function by interacting with other proteins. A Y2H system screened for HspA12B-interacting proteins identifying 22 unique DNA prey fragments from 232 positive yeast clones after screening 134 millions of interactions, using a human bone marrow endothelial cell random-primed cDNA library. After analyzing and taking account of prey fragments' redundancy, independence, frequency, distributions of reading frames and stop codons, as well as their appearance in all previous unrelated screens, we were able to generate a ranking of the reliability of each interaction. Four angiogenesis regulators were identified to interact with HspA12B (Table 3.1). The top-ranked clone was AKAP12 (A-kinase-anchoring protein 12, also known as Gravin or SSeCKS).

TABLE 3.1 HspA12B Interacting Proteins from the Yeast Two-Hybrid System

Accession #	Interacting Proteins	Functions	References
BCO22814	**AKAP12** (A-kinase anchoring protein 12)	Promotes tight junction formation Angiogenesis inhibitor	(*9, 10*)
NP 001018121	**PODXL** (Podocalyxin like)	Mediates cell adhesion Angiogenesis regulator	(*24, 25*)
AACO3365	**ARNT** (Aryl hydrocarbon receptor nuclear translocator)	Angiogenesis regulator	(*26*)
NP 842565	**SPTBN1** (Spectrin)	Forms submembrane cytoskeletal network with actin Angiogenesis inhibitor	(*27*)

To confirm the interaction between HspA12B and AKAP12, we tested whether these two proteins were co-localized by double-immunostaining HUVECs with anti-HpsA12B and anti-AKAP12 antibodies. As shown in Figure 3.1A, HspA12B and AKAP12 showed an almost

complete co-localization, confirming the interaction. We further confirmed the interaction by co-IP. HspA12B was pulled down by antibodies specific against AKAP12, and AKAP12 was pulled down by antibodies specific against HspA12B (Figure 3.1B), indicating the interaction between HspA12B and AKAP12.

Because HspA12B subcellular localization was hitherto unpublished, we further examined its subcellular localization. Immunostaining of HspA12B showed that HspA12B is preferentially distributed in cytoplasm and enriched in the perinuclear region in particular (Figure 3.1E). This distribution pattern is consistent with that of molecular chaperones. The HspA12B-EGFP fusion protein (HspA12B tagged with EGFP in the C-terminus, Appendix A Figure A.1.3) is a functional form of HspA12B (*3*) and displayed similar localization (Figure 3.1F). The factors that determine HspA12B subcellular location are likely to be present within the HspA12B protein itself and not requisite of endothelial cell, because FLAG-tagged HspA12B (Figure 3.1G) and EGFP-tagged HspA12B (Figure 3.1H) showed similar distribution in HEK 293 cells, which do not express HspA12B under normal conditions. Not surprisingly, the distribution is similar to the published AKAP12 subcellular localization.

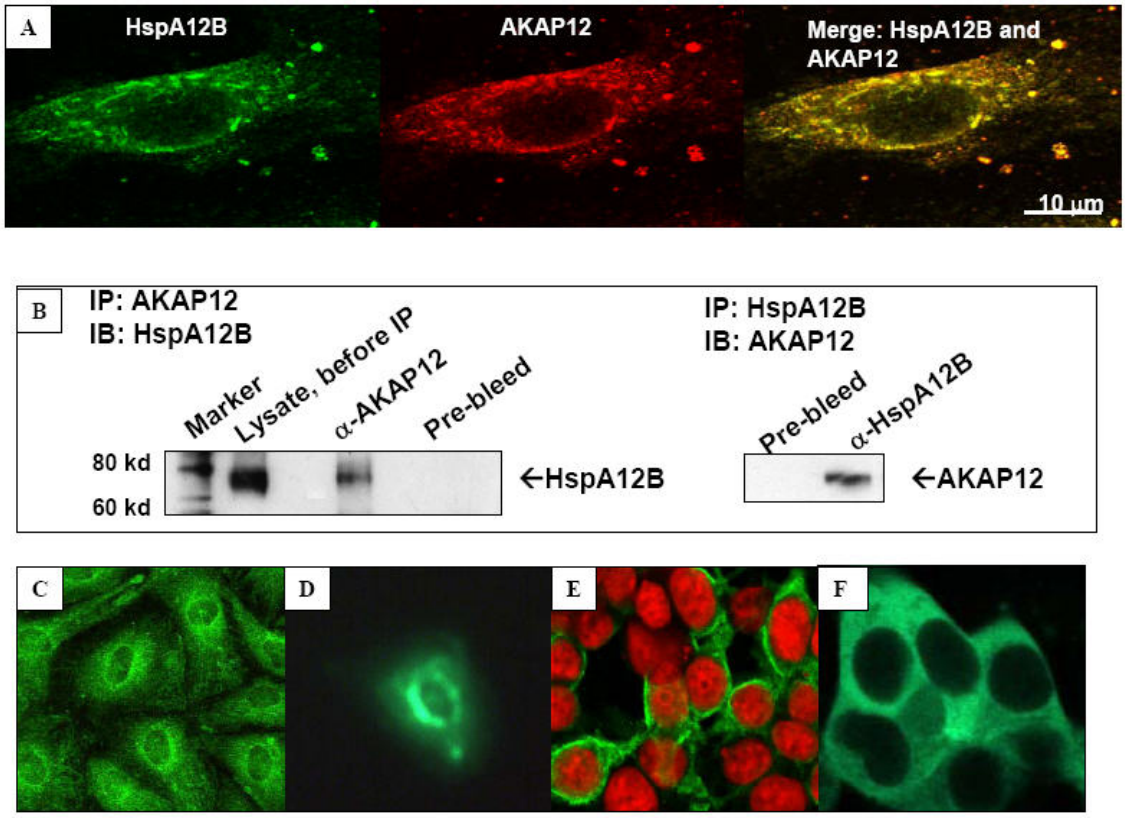


Figure 3.1 HspA12B interacts with AKAP12 and is preferentially localized in cytoplasm and perinuclear regions. A, HspA12B co-localized with AKAP12 in HUVECs. Fluorescent confocal microscopy of HUVECs double-stained with anti-HspA12B (green) and anti-AKAP12 antibodies (red), demonstrating co-localization. B, Co-immunoprecipitation of AKAP12 and HspA12B. C, Fluorescent confocal microscopy of HUVECs immunostained with anti-HspA12B antibodies Ab4111 and goat-anti-rabbit Alexa-Fluor-488 conjugate. D, Fluorescent microscopy of HUVECs transduced with adenovirus expressing HspA12B-EGFP fusion proteins. E, Fluorescent confocal microscopy of HEK 293 cells expressing Flag-tagged mHspA12B, after immunostaining with anti-Flag antibody-FITC conjugate. Nuclei were labeled red. F, Fluorescent confocal microscopy of HEK 293 cells expressing HspA12B-EGFP fusion proteins.

HspA12B Down-Regulates the Levels of AKAP12

To test whether the HspA12B-AKAP12 interaction has any biological significance, and mindful of the role of molecular chaperones in protein turnover, we examined the effect of HspA12B on AKAP12 levels. Reduction of HspA12B levels through either neutralizing antibodies (*3*) (Figure 3.2A) or siRNA (Figure 3.2B) resulted in increased levels of AKAP12 in

HUVECs. Conversely, overexpression of HspA12B (figure 3.2C) or HspA12B-EGFP (Figure 3.2D) decreased AKAP12 levels. These data indicated that HspA12B suppressed AKAP12 levels.

To test whether HspA12B suppressed AKAP12 levels by degradation through the ubiquitin-proteasome pathway, we examined the effect of an ubiquitin-proteasome pathway inhibitor on HspA12B-induced suppression of AKAP12. As shown in Figure 3.2E, the presence of the proteasome inhibitor MG132 lead to increased AKAP12 levels (lane 2). Overexpression of HspA12B reduced AKAP12 levels (lane 3), but the addition of MG132 attenuated the suppression (lane 4). Taken in whole, these data suggested that HspA12B down-regulates AKAP12 by binding to AKAP12 and facilitating its turnover through the ubiquitin/proteasome pathway.

HspA12B Regulates the VEGF Signaling Pathway and Ang-1

We have shown that HspA12B promotes angiogenesis but the mechanism is unknown. Because HspA12B suppresses AKAP12 levels, and AKAP12 has been reported to suppress proangiogenic genes (*8, 10*), we predicted that HspA12B promotes angiogenic gene expression. As expected, overexpression of HspA12B (Figure 3.2C) and HspA12B-EGFP (Figure 3.2D) in HUVECs resulted in a significant increase in VEGF. Conversely, silencing of HspA12B with shRNA significantly reduced the levels of VEGF, both under normal and hypoxia conditions (Figure 3.3). The levels of Ang-1, Akt, and phosphorylated Akt were also significantly reduced (Figure 3.3). These data indicate that HspA12B is a significant regulator of VEGF and requisite for its normal expression as well as its induction by hypoxia.

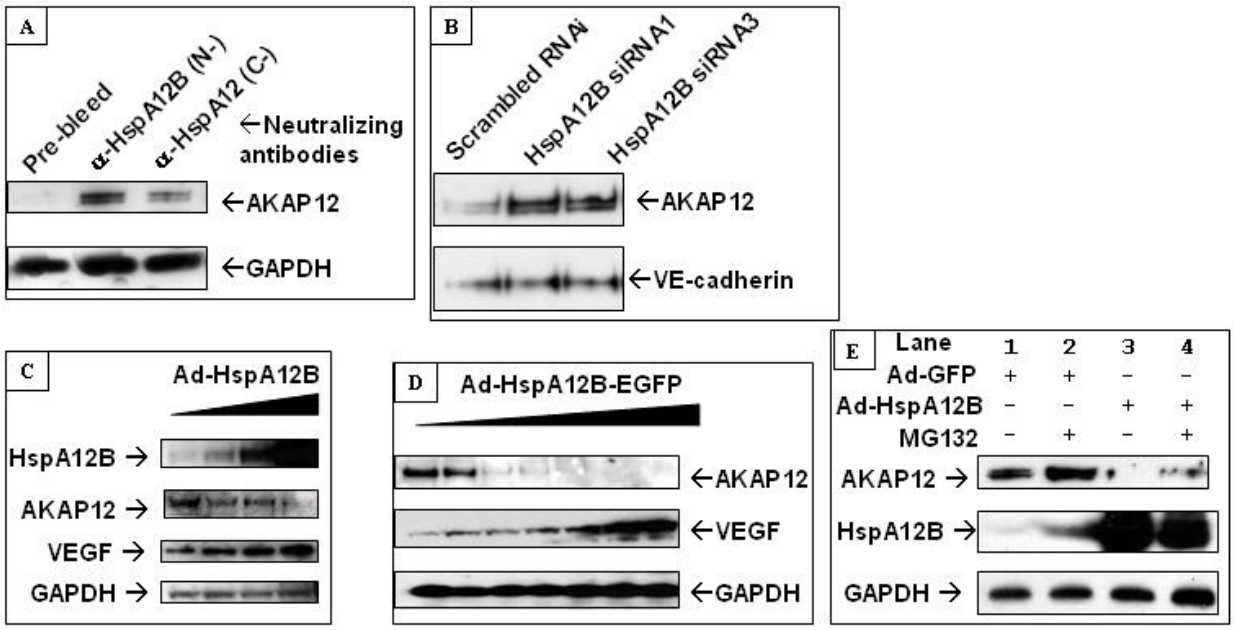


Figure 3.2 HspA12B negatively regulates the AKAP12 level. A, Neutralizing HspA12B by α-HspA12B antibodies significantly increased the AKAP12 level in HUVECs. Antibody transfection and western blots (WB) were done as previously described (*3*). 24 hours after neutralizing antibodies were transfected into HUVECs, cells were harvested for WB. α-HspA12B (N-): Ab4110 is specific for the N-terminus. α-HspA12B (C-): Ab4112 is specific for the C-terminus. Ab4110 had a stronger neutralizing effect on inhibiting HspA12B's function compared to the C-terminus-specific Ab4112 antibodies (*3*), and a consistently higher level of AKAP12 was observed with Ab4110 compared to Ab4112. One representative result out of three WBs is shown, unless stated otherwise. B, Knockdown of HspA12B by siRNA resulted in significant increase of AKAP12 level in HUVECs. HUVECs plated in 6-well plate were transfected with siRNA by Effectene following the manufacturer suggested protocol. 48 hours after transfection of siRNA WBs were performed from lysates. C, Overexpression of HspA12B reduced AKAP12 levels and increased VEGF levels. HUVECs in 6-well plate were transduced with increasing amount of Ad-HspA12B (10^4, 10^5, 10^6 10^7). After 72 hours WBs were performed from lysates. D, Overexpression of HspA12B-EGFP fusion reduced AKAP12 levels and increased VEGF levels. HUVECs in 6-well plate were transduced with increasing amount of Ad-HspA12B-EGFP that expressed HspA12B-EGFP under a CMV promoter. 72 hours later cells were harvested and WB performed. E, HspA12B down-regulated AKAP12 by ubiquitin/proteasome pathway. HUVECS were transduced with control (Ad-EGFP) or Ad-HspA12B for 48 hours, followed by a 24 hour treatment of 20 μM MG132. Cells were harvested and WB were performed. The presence of inhibitor MG132 (lane 2) increased AKAP12 level (lane 1). Ad-HspA12B reduced AKAP12 level (lane 3), but the addition of MG132 led to the increased AKAP12 level (lane 4).

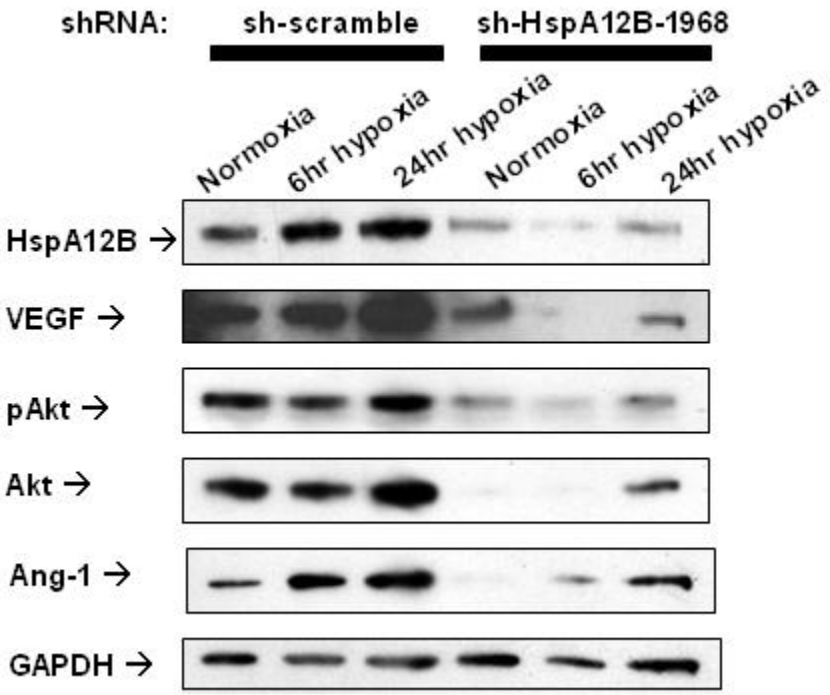


Figure 3.3 HspA12B is requisite for VEGF pathway and Ang-1. HUVECs were transfected with shRNA via Effectene (Qiagen). 48 hours later, cells underwent either 24 hours normoxia (normoxia control), or 18 hours normoxia followed by 6 hour hypoxias (6 hr hypoxia), or 24 hours hypoxia (24 hr hypoxia) as described in material and method. One representative picture out of three WBs is shown.

HspA12B Promotes VEGF Pathway by Suppressing AKAP12 Levels

Our data indicated that HspA12B down-regulates AKAP12 and up-regulates VEGF. To definitively prove HspA12B regulates VEGF pathway through AKAP12, we generated a shRNA (psh-AKAP12-3639) that was effective in silencing AKAP12 (Figure 3.4A), and compared VEGF levels in HUVECs of HspA12B/AKAP12 double knockdown to those of single knockdowns (Figure 3.4B). Compared to scrambled control (lane 1), HspA12B knockdown reduced VEGF level, as expected (lane 2). This reduction was reversed by the concomitant knockdown of AKAP12 (lane 4), indicating that HspA12B regulates VEGF levels by suppressing AKAP12. We also confirmed that AKAP12 suppressed VEGF by showing that silencing AKAP12 led to increased levels of VEGF (lane 3).

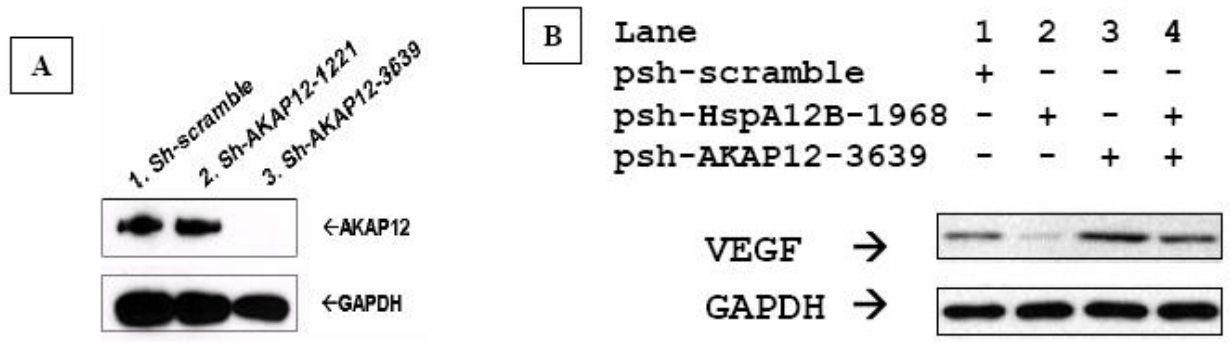


Figure 3.4 HspA12B regulates the levels of VEGF by suppressing AKAP12. A, psh-AKAP12-3639 effectively silenced AKAP12. HUVECs were transfected with shRNA via Effectene. 48 hours later cell lysates were harvested and WBs were performed. One representative WB out of three is shown. B, VEGF reduction by the HspA12B knockdown was reversed by silencing AKAP12. HUVECs were transfected with shRNA via Effectene. 48 hours later cell lysates were harvested and WBs were performed. One representative result out of three WBs is shown. Lane 1: psh-scramble control; lane 2: psh-HspA12B-1968 silencing HspA12B, resulting in loss of VEGF expression; lane 3: psh-AKAP12-3639 silencing AKAP12, leading to elevated VEGF levels; lane 4: both psh-HspA12B-1968 and psh-AKAP12-3639. Silencing of AKAP12 superceded the effects of silencing HspA12B, and restored VEGF level.

AKAP12 interacts with HspA12B through Amino Acid 829 to 860 (AA 829-60)

In our Y2H screening we used random primed cDNA libraries. Therefore, each protein partner (prey) can be identified through many fragments. The alignment of all these fragments allows the identification of the common sequence shared by all prey fragments belonging to the same prey protein (Shared Interaction Domain or SID), which had been found in numerous cases to correspond to structural domains (*28*). By comparing six independent positive clones from the Y2H screening, we were able to narrow the SID in AKAP12 to a peptide spanning amino acid 829 to 860 (AA829-60, HspA12B-Binding Domain - HBD) (Appendix A Figure A.1.11 A). To confirm that HBD interacted with HspA12B, we tested whether HBD co-localized with HspA12B, by tracking the localization of AA829-60-DsRed fusion protein and HspA12B-EGFP fusion protein in HUVECs. As shown in Figure 3.5A, AA829-60-DsRed fusion co-localized with HspA12B-EGFP. This confirmed the Y2H screening results and indicated that the HBD is

capable of binding HspA12B. Furthermore, overexpression of the HBD-DsRed in HUVECs interrupted immuno pull-down between HspA12B and AKAP12B (Figure 3.5B). These data indicated that AKAP12 interacts with HspA12B through AA829-60.

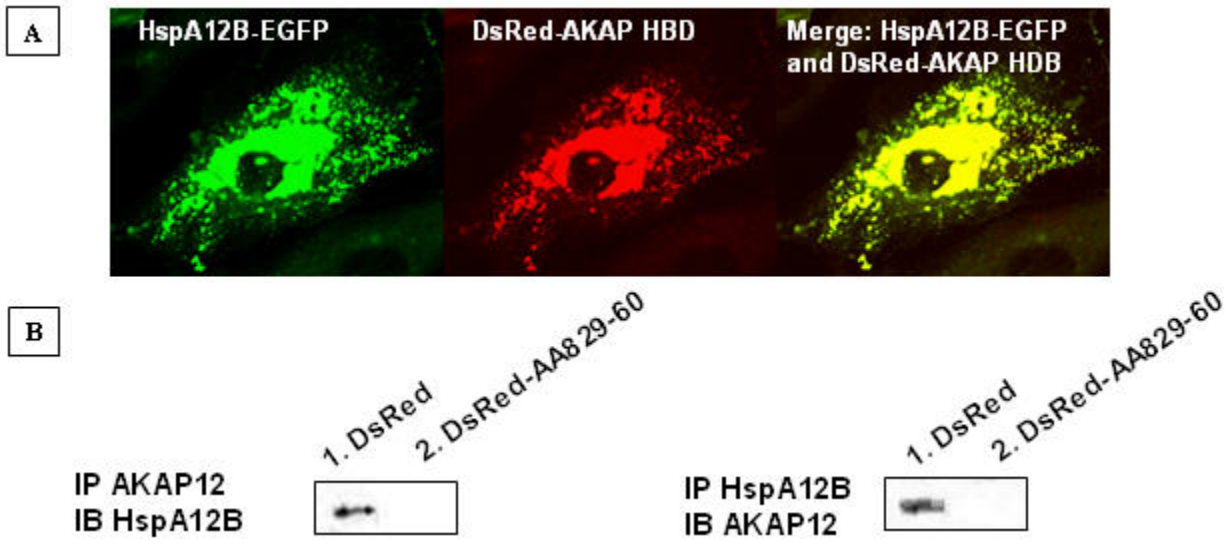


Figure 3.5 AKAP12 interacts with HspA12B by AA 829-60. A, Co-localization of AKAP12-AA829-60 with HspA12B. Left, HspA12B-EGFP; middle, DsRed-HBD; right, merge. pHspA12B-EGFP and pDsRed-AKAP HBD were co-transfected into HUVECs with Effectene. 48 hours later pictures were taken with a fluorescent confocal microscopy. Co-localization was observed. B, The AKAP12-HBD (AA829-60) domain disrupted the HspA12B-AKAP12 interaction. HUVECs were transfected with pDsRed control or pDsRed-AKAP HBD. 48 hours later co-immunoprecipitation was performed. The overexpression of HBD (AA829-60) disrupted the interaction between HspA12B and AKAP12.

The HBD Domain Overexpression Up-Regulates AKAP12 and Down-Regulates VEGF

Our data suggest that HspA12B regulates the VEGF pathway by binding to AKAP12 through the AA 829-60 (HBD) and removing AKAP12. A decisive proof and a logical conclusion of our hypothesis is that interrupting the HspA12B-AKAP12 interaction should recapitulate the same phenotypes as HspA12B knockdown. Therefore, we tested the effects of

overexpressing a HBD fused to DsRed (pDsRed-HBD), on the AKAP12 and VEGF levels. As expected, HBD overexpression led to increased AKAP12 and decreased VEGF (Figure 3.6, lane 2 compared to lane 1), indicating that disrupting the HspA12B-AKAP12 interaction lead to increased AKAP12 and decreased VEGF. In addition, the ability of ectopic HspA12B to reduce AKAP12 and increase VEGF (lane 3), was ablated by HBD overexpression (lane 4). Thus, overexpression of HBD acts in a dominant-negative fashion, and supports our conclusion that HspA12B regulates the VEGF pathway by binding and inactivating AKAP12.

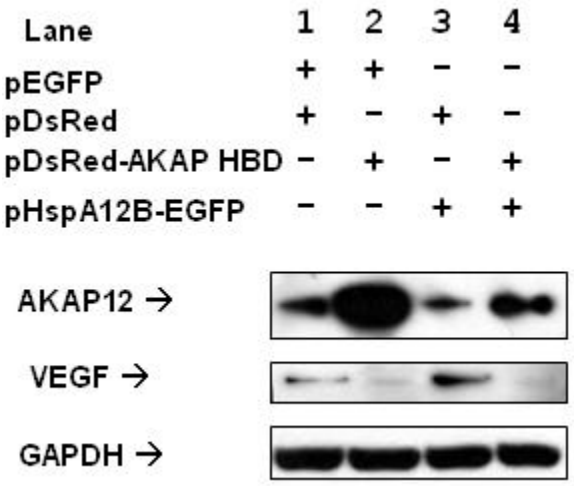


Figure 3.6 Overexpression of the AKAP12 829-60 HspA12B Binding Domain (HBD) elevated AKAP12 and reduced VEGF. HUVECs were transfected with empty vectors as a control (lane 1); the HBD domain tagged to DsRed (pDsRed-AKAP HBD) (lane 2); HspA12B tagged with EGFP on its C-terminus (lane 3); and both AKAP HBD and HspA12B (lane 4). Three days later WBs were performed to assess the levels of AKAP12 and VEGF, and one representative WB out of three is shown. Over-expressing the 32 AA HBD led to elevated levels of AKAP12 and reduced VEGF (lane 2), indicating that interrupting the AKAP12-HspA12B interaction interfered with the degradation of AKAP12. Overexpression of HspA12B reduced the level of AKAP12 and increased the level of VEGF (lane 3) compared to a control (lane 1). Overexpression of the HBD partially restored the AKAP12 level that was reduced by HspA12B (compare lane 4 to 3). Thus, HspA12B reduced the level of AKAP12 by interacting with AKAP12 and mediating its removal, and promoted the VEGF level through its regulation of AKAP12.

HspA12B is Induced by Shear Stress, by Hypoxia and is Required for Hypoxia-Mediated Tubule Formation

The finding that HspA12B regulates the VEGF pathway led us to examine whether HspA12B is induced by shear stress and hypoxia, conditions that induce angiogenesis via VEGF signaling (*29, 30*). High shear induced HspA12B expression in HCAECs compared to static or low shear stress (Figure 3.7A). Compared to control, the HspA12B protein levels were increased more than 2 fold ($P<0.05$) in HCAECs exposed to hypoxia (Figure 3.7B). A more pronounced increase in HspA12B is induced when hypoxia is followed by reoxygenation.

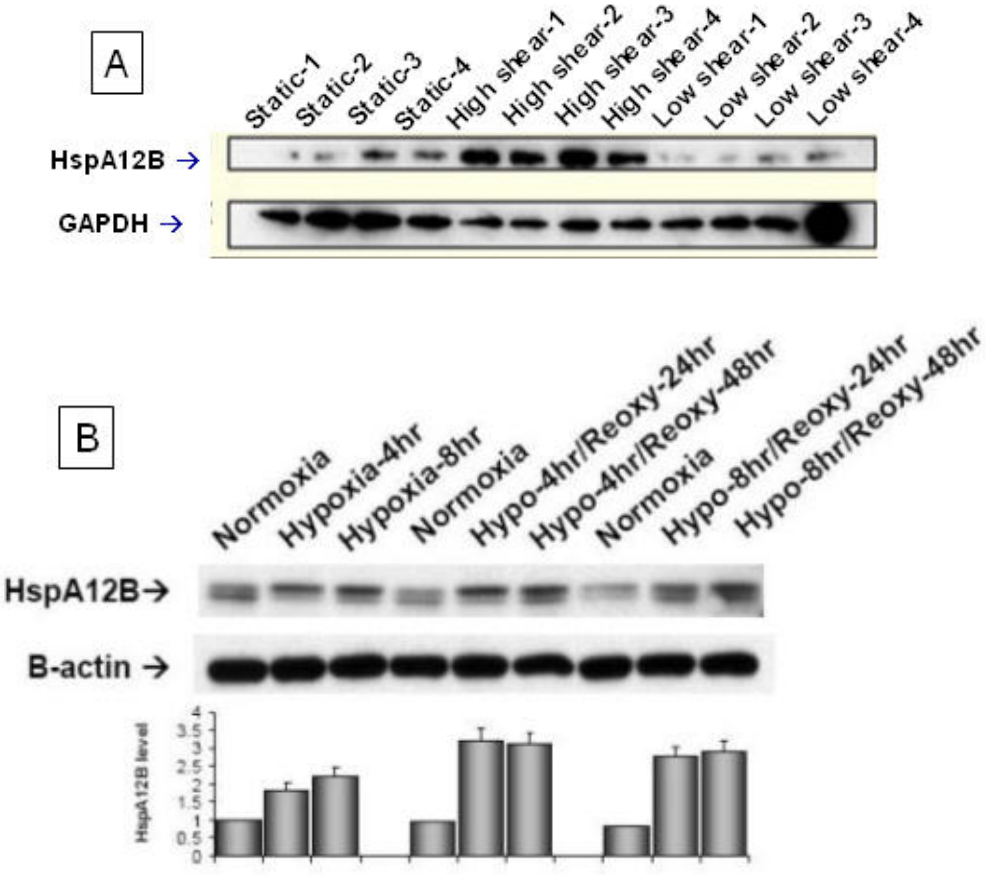


Figure 3.7 HspA12B is induced by shear stress and hypoxia. A, Control HCAECs were cultured statically for 4 days on collagen I slides. Shear samples were cultured statically for 2 days on collagen I slides until confluent, slides were then assembled into a parallel plate flow chamber to

impose high or low shear stress for 2 days. B, HCAECs in 6-well plates underwent hypoxia or hypoxia/reoxygenation at the times indicated. Lysates were collected and WBs performed to assess the levels of HspA12B. One representative WB out of three is shown. Shear stress and hypoxia was performed as described in the materials and methods.

To examine functional consequences of HspA12B induction by hypoxia, we examined whether silencing HspA12B can interfere with hypoxia-induced tubule formation in a Matrigel assay. Silencing of HspA12B abolished hypoxia/reoxygenation-induced tubule formation in HCAEC and HUVEC cells (Figure 3.8B and Figure 3.8C, respectively). These results demonstrate that HspA12B is required for hypoxia-induced tubule formation.

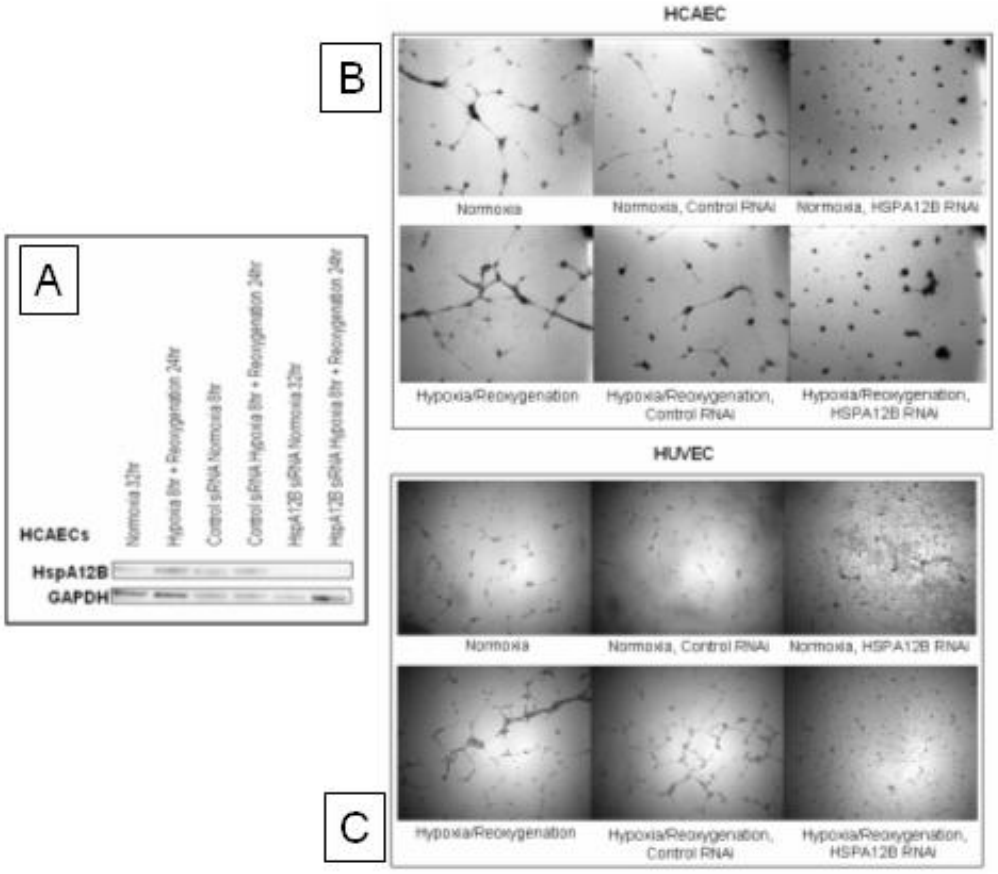


Figure 3.8 HspA12B is required for tubule formation in Matrigel assay. A, siRNA successfully knocked down HspA12B in HCAECs. B, HCAECs and C, HUVECs were transfected with

control siRNA or HspA12B siRNA for 2 days before undergoing Matrigel assays, as described previously by (*3*). The reduction of HspA12B expression was verified by WB prior to plating cells for the Matrigel assay. 24 hours after plating the cells pictures were taken under a bright-field microscope at 20× magnification.

HspA12B Promotes Angiogenesis *In Vivo*

We have shown previously that HspA12B was specifically expressed in endothelial cells and required for angiogenesis *in vitro*, but it has yet to be determined if HspA12B can promote angiogenesis in mice *in vivo*. Therefore, we examined whether HspA12B can promote vascularization in Directed *in vivo* Angiogenesis Assay (DIVAA), a robust *in vivo* assay. We have generated an adenovirus expressing HspA12B (Ad-HspA12B) and one with GFP tethered to the C-terminus of HspA12B (Ad-HspA12B-EGFP). Western blots indicate that these adenovirus express very high levels of HspA12B and HspA12B-EGFP fusion (Figure 3.9A), representing >30 fold increase in expression compared to untransfected HUVECs. Our adenovirus also transfected HUVECs at high efficiency (Figure 3.9B). To examine transfectionefficiency *in vivo* in the myocardium, we transfected rat hearts with Ad-GFP at 1×10^{10} pfu/ml. Three days after transfection, the hearts were harvested and sectioned. GFP expression was observed by fluorescence microscopy. As shown in Figure 3.9C, GFP expression was observed in most cardiac cells after transfection. Subcutaneously implanted angioreactors filled with Matrigel containing our adenovirus overexpressing HspA12B (Ad-HspA12B) displayed significantly more vessel formation after 10 days than control angioreactors filled with Matrigel containing Ad-GFP (1292 ± 241 *vs*. 873 ± 178) (Figure 3.10). Thus HspA12B promotes angiogenesis *in vivo*.

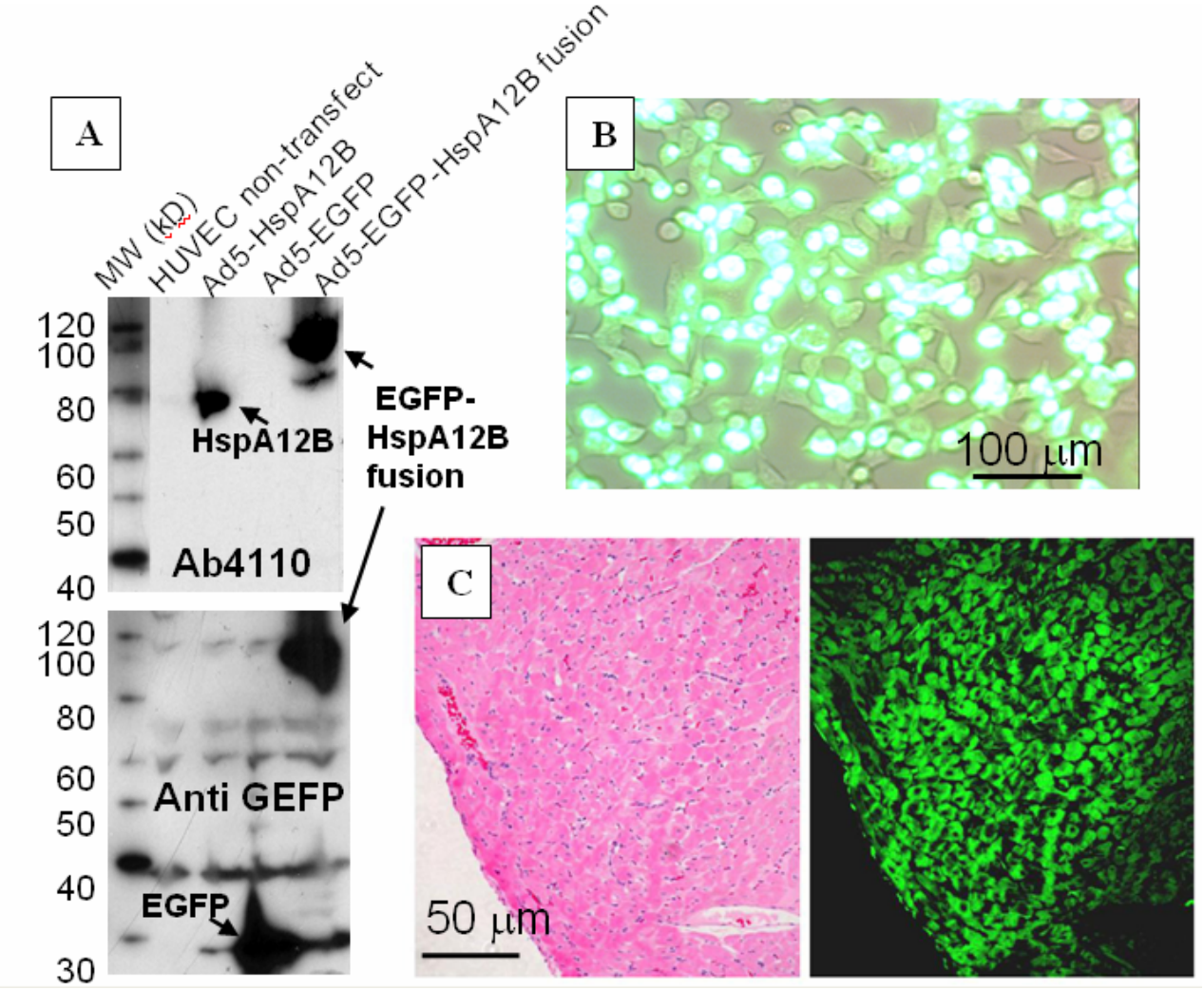


Figure 3.9 Adenovirus overexpresses HspA12B. A, Western blots with anti-HspA12B, Ab4110 (top) and anti-EGFP (bottom) as primary antibodies. 5ug lysates of HUVECs transfected with adenovirus were loaded in each well. HspA12B is 76 kD, EGFP is 27 kD, HspA12B-EGFP fusion is ~103 kD. B, Ad-HspA12B-EGFP infected HUVECs with high efficiency. **C,** Expression of GFP in the myocardium. Rat hearts were directly transfected with Ad-GFP (1×10^{10} pfu/ml). Two days after transfection of Ad-GFP, the hearts were harvested, sectioned, stained with hematoxylin and eosin (H&E), and visualized with light (left) and fluorescent (right) microscopy.

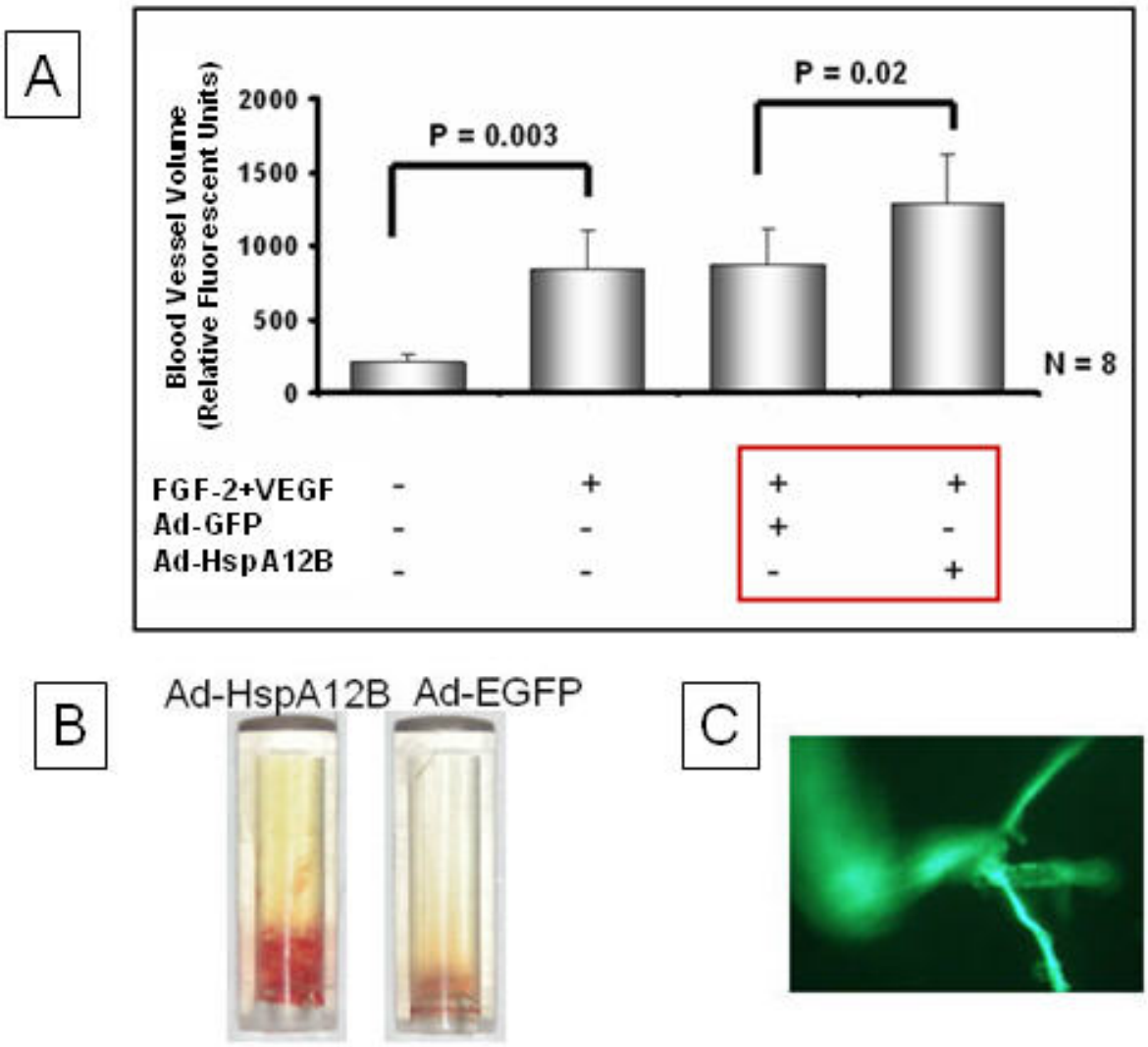


Figure 3.10 HspA12B promotes angiogenesis *in vivo*. A, FITC-Lectin DIVAA showed that the HspA12B adenovirus induced *in vivo* angiogenesis. Bar graphs are showing mean ± S.E.M. of 8 animals/group. B, Vascularization of angioreactors is significantly increased in the presence of Ad-HspA12B compared to Ad-GFP control after a 10 day implantation. C, Fluorescent imaging of FITC-dextran permeated Matrigel inside angioreactors. DIVAA assay were performed as described in the material and methods.

Discussion

In this study we searched for HspA12B-interacting proteins through a Y2H screening. We identified AKAP12 as a major interacting protein and confirmed the interaction by co-localization and co-immunoprecipitation. Not surprisingly, the HspA12B distribution is similar to the published AKAP12 subcellular localization (*31*). We provided evidence that HspA12B up-regulated the VEGF pathway by mediating degradation of AKAP12 through the ubiquitin-proteosome pathway. We also showed that HspA12B was induced by hypoxia and required for hypoxia-induced tubule-formation in an *in vitro* assay. Prior functional assays of HspA12B have been limited to cultured cells and zebrafish. Here we provided the first functional evidence of HspA12B regulating angiogenesis in mice. We also identified a 32 AA region in AKAP12 that is capable of interacting with HspA12B. Overexpression of this 32-AA domain interrupted the HpsA12B-AKAP12 interaction and down-regulated VEGF. Thus, our study characterized one pathway by which HspA12B promoted angiogenesis, and further confirmed the involvement of AKAP12 in angiogenesis.

In our current working model (Figure 3.11), HspA12B directly binds to AKAP12 and promotes its turnover through the ubiquitin-proteasome pathway. HspA12B might be part of a larger complex that includes Hsc70 and CHIP, because HspA12B binds to Hsc70 in co-immunoprecipitation assay (Han, unpublished data). Also in our model, reduction of AKAP12 levels leads to elevated HIF-1. At least two publications indicated that AKAP12 down-regulates HIF-1α (*9, 11*), and it is worth noting that HIF-1β (ARNT) interacts with HspA12B in a Y2H system (Table 3.1). Further studies are necessary to test this model.

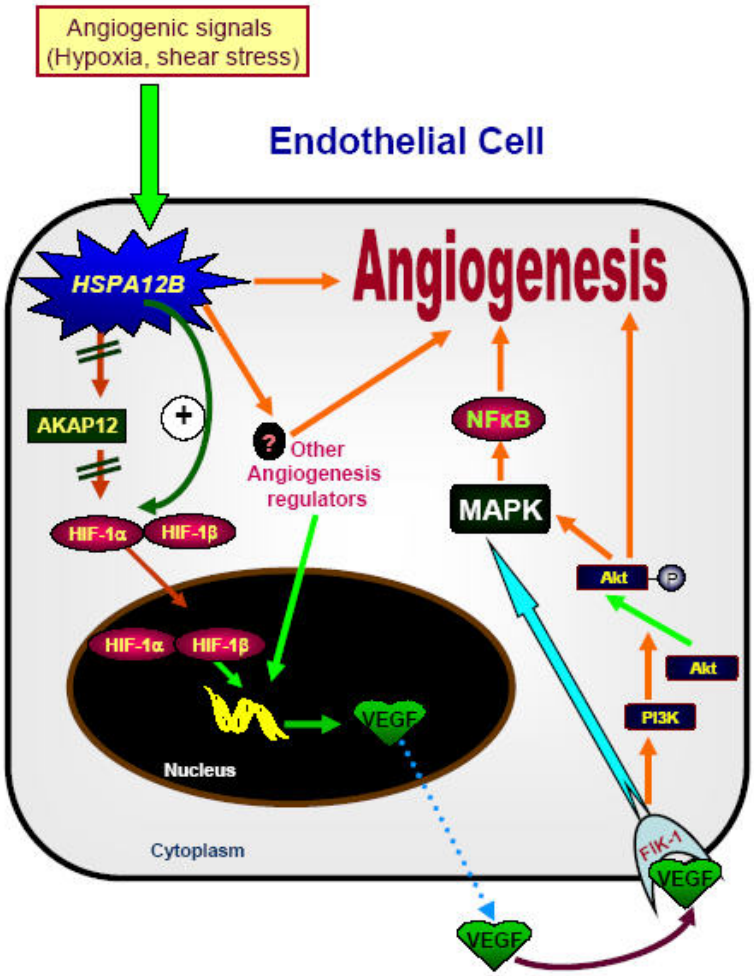


Figure 3.11 Working model of HspA12B regulation of angiogenesis: VEGF pathway.

AKAP12 has been implicated in regulating angiogenesis. One group reported that it negatively regulates the expression of angiogenesis regulators including VEGF and Ang-1 in MLL tumor cells as well as in stromal cells (*8*). Another group reported that it promoted ECs tight junction formation in the brain blood barrier (BBB) (*10*) and blood retinal barrier (BRB) (*11*) by inhibiting VEGF and promoting Ang-1 and tight junction proteins. Thus these studies concur on AKAP12's effect on VEGF but differ on Ang-1, a molecule that regulates vessel maturation. Our results confirm that AKAP12 inhibits VEGF in endothelial cells and also

suggest that HspA12B up-regulates both VEGF and Ang-1. Thus, HspA12B functions as a master regulator of angiogenesis.

AKAP12 belongs to the group of scaffold proteins called A-kinase anchor proteins (AKAPs) that display a signature binding site for the regulatory subunit of protein kinase A (PKA) (*15*) and confine the PKA holoenzyme to discrete locations within the cell. AKAP12 binds to the β_2-adrenergic receptor, a prototypical G-protein-coupled receptor (GPCR), through a domain that spans the three conserved AKAP motifs located in AKAP12 and regulates GPCR resensitization and recycling (Figure 3.12) (*12, 32*). In addition to PKA and GPCR, AKAP12 also binds to PKC (*33, 34*), phosphatase 2B (*35*), cyclin (*36*), calmodulin (*37*), Src (*14*) and is proposed to serve as a movable scaffold achieving compartmentalization of cyclic AMP signaling in cells (*15*).

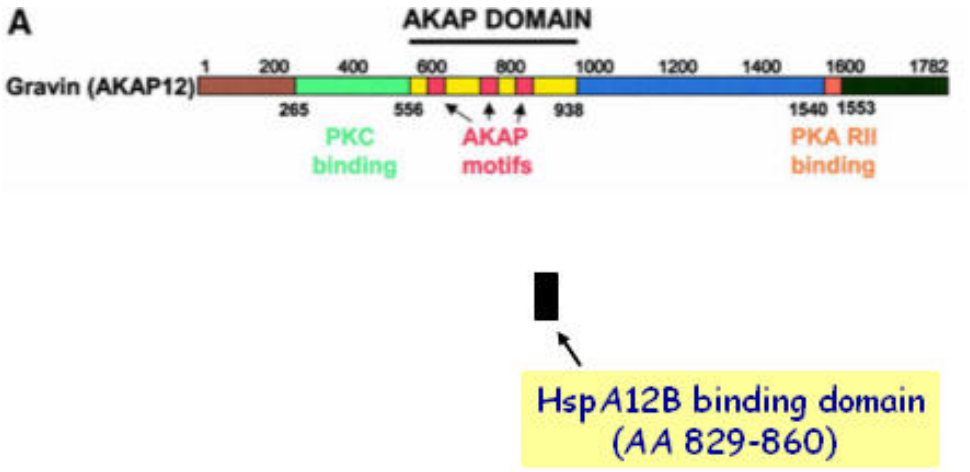


Figure 3.12 AKAP12 binding domains. The conserved AKAP motifs are located 603–633, 753–783 and 797–827. The minimum interacting domain deduced from Y2H screening is located in 829-860. It is located proximal to the AKAP12 domain. The AKAP12 domain mediates binding between the scaffold and the β2-adrenergic receptor. (Adapted from Tao et al., EMBO Journal (2003) 22, 6419-29).

Given the role of AKAP12 in modulating GPCR, our study suggests a potential mechanism by which HspA12B regulates the angiogenic signaling transmitted through GPCR. While VEGF and its cognate tyrosine kinase receptors (VEGF-R1, VEGF-R2) are central regulators of angiogenesis, a number of GPCRs and their associated proteins have also been shown to play a part in angiogenesis. For example, the GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity (*38*). Also, the disruption of $G\alpha_{13}$ impaired the ability of endothelial cells to develop into an organized vascular system (*39*). This involves thrombin as the agonist and protease-activated receptor (PAR) as the GPCR, with thrombin activating aspects of angiogenesis including sprouting, growth, migration, and remodeling of ECs (*40*). In addition, thrombin and Angiotensin II, respectively, via PAR-1 and AT-1R (both are GPCRs) can induce VEGF expression. It has been proposed that activation of GPCR by agonists leads to the activation of the small G-protein Rac and the NADPH oxidase system, increasing intracellular ROS level (*40*). This increase of ROS specifically decreases HIF-1α degradation by the proteasome pathway permitting the accumulation of HIF-1α and the formation of HIF-1 complex (*40*). HIF-1 in turn increases expression of VEGF. It will be logical to examine whether or not AKAP12 recognizes PAR-1. It is conceivable that HspA12B and AKAP12 form a large protein complex involving GPCR and HIF-1 and thus regulate angiogenesis. Because HspA12B regulates AKAP12, which in turn facilitates receptors resensitization and recycling, HspA12B may provide another control level for modification of angiogenic signaling.

Our identification of the interacting domain may provide insight into general AKAP biology. Because AKAP12 is 90% unfolded and an extended molecule (*41*), it is perhaps not surprising that a member of Hsp70, albeit a distant member, would bind to it either to stabilize or facilitate its turnover. It is worth noting that the AKAP domain in AKAP12 essential for AKAP12-GPCR interactions (AA 536-938) (*12*) closely abuts the HBD domain (AA 939-960) we characterized. Such close proximity raises the interesting prospect of mutual exclusivity of

HspA12B and GPCR binding to AKAP12. One attractive hypothesis deriving from this observation is that of a general mechanism to patrol against the accumulation of free AKAP12 unassociated with GPCR, i.e., AKAP12 dissociated from GPCR will be free to bind to HspA12B, which in turn escorts AKAP12 to undergo proteasomal degradation. A long-extended unfolded AKAP12 unassociated with GPCR would likely be deleterious to cells. On the other hand, HspA12B might contribute to AKAP12 recycling and trafficking. AKAP12 association with the cell membrane is a critical aspect of its cellular localization control and GPCR, an intrinsic membrane protein, may help anchor AKAP12 to membranes (*41*). Alternatively, HspA12B might directly regulate the binding between GPCR and AKAP12 and influence signal transmission.

It has also been reported that AKAP12 induces apoptotic cell death in human fibrosarcoma cells by regulating CDK1-cyclin D1 and caspase-3 activity (*42*), and re-expression of AKAP12 caused apoptosis in cancer cells (*43*). It is likely that as part of its pro-angiogenic effects, HspA12B protects against EC apoptosis, partly by down-regulating the AKAP12 levels.

Our prior studies identified a novel angiogenesis regulator, HspA12B, which belongs to Hsp70 family of proteins and is predominantly expressed in ECs. In this study, we showed for the first time that HspA12B is required for VEGF expression, induced by hypoxia, and promotes angiogenesis by down-regulation of AKAP12. Thus, our findings define a new pathway in which angiogenesis is regulated and we highlight the important role of chaperone and scaffolding proteins in this process. Our study points to potentially significant applications for modification of HspA12B in novel therapies for cardiovascular disease and tumorgenesis.

Acknowledgements

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CHAPTER 4

CONCLUSION

Molecular chaperones are highly specialized in expression and function. Many *hsp70* genes have been reported that encode a diversified family of proteins. This diversity is achieved at genetic, transcriptional, and post-transcriptional levels that generate a multiplicity of structures, functions, and anatomic locations (Berruti and Martegani 2002; Brocchieri et al. 2008). It has been recently reported that 130 human Hsp70 proteins alone exist (Brocchieri et al. 2008), this does not take into account the Hsp40, Hsp60, Hsp90, and Hsp100 proteins. Because of this specialization, mutations that effect chaperone function can have serious consequences.

Molecular chaperones are important in several forms of human diseases including cardiovascular disease, Alzheimer's disease and retinal degeneration disease (Matsuno et al. 1998; Chapple et al. 2001; Martin-Ventura et al. 2006; Ghayour-Mobarhan et al. 2007; Cerpa et al. 2008). Hsp20 serves a significant role in preventing platelet aggregation which may provide a defensive system to thrombus formation (Matsuno et al. 1998). siRNA knockdown of Hsp27 increased apoptosis in vascular smooth muscle cells (Martin-Ventura et al. 2006) and a direct relationship between plasma cholesterol and the levels of Hsp-60, 65, and -70 was reported in animal models (Ghayour-Mobarhan et al. 2007). Chapple *et al.* (Chapple et al. 2001) reported a mutation in molecular chaperones that causes inherited retinal dysfunction and degeneration. In Alzheimer's disease molecular chaperones modulate amyloid formation in neurons. Interestingly, tumor cells differ from normal tissues in their capacity to present Hsp70 on their plasma membrane (Gehrmann et al. 2008); however, the impact this has on tumor progression remains to be elucidated. Manipulation of chaperone levels or chaperone function might offer potential novel therapies. Chaperonomics has been developed to make available a comprehensive tool for understanding the composition of chaperone families in all cell compartments, their functional relations, and, ultimately, their role in pathogenesis (Brocchieri et al. 2007).

Intriguingly, a study done on the other member of our Hsp70 sub-family 12, HspA12A, found its mRNA expression to be neuron-specific in the human brain and significantly reduced in subjects with schizophrenia (Pongrac et al. 2004). It could be speculated that HspA12A also acts as a tissue-specific chaperone that regulates neuron homeostasis by regulating the levels of key proteins and protecting against apoptosis.

Our experiments probed the endothelial specific expression of HspA12B and that its N-terminus region where the conserved ATPase domain reside, is required for angiogenesis (Steagall et al. 2006). To more definitively classify HspA12B as a molecular chaperone, future studies will involve Thermally Inactivated Luciferase Protection Assays and ATPase activity assays. The Thermally Inactivated Luciferase Protection Assay tests a protein's ability to protect other proteins from irreversible aggregation after heat denaturation. This assay was adapted from Minami (Minami et al. 1996; Minami and Minami 1999) and it will test HspA12B's ability to refold thermally denatured luciferase by measuring restored luciferase activity. The mechanism by which chemical energy in the ATPase domain is used to open and close the peptide binding domain of Hsp70 proteins remains unresolved. However, coordination between the peptide-binding domain activity and the ATPase activity must be fundamental for the chaperone activity of Hsp70 proteins. As mentioned previously, the putative ATPase domain of HspA12B is atypical in that it is split into two segments by spacer AA's 175-189. A typical ATPase assay that measures the fraction of ATP hydrolyzed to ADP requires ~15 µg of purified protein. A monoclonal antibody for HspA12B will be made in order to affinity purify protein for ATPase activity assays.

We characterized HspA12B's role in the regulation of angiogenesis by defining the mechanisms by which HspA12B regulates angiogenesis through the identification of its targets. The top ranked interacting clone in a Y2H, AKAP12, was originally reported by Grove et al. to demonstrate endothelial cell-specific expression similar to HspA12B (Grove et al. 1994; Grove and Bruchey 2001). However, its expression has since been extended to several adherent cell

lines but not in nonadherent cells such as neutrophils, lymphocytes, and leukemia cell lines. Moreover, human erythroleukemia cells, which are normally nonadherent and do not express AKAP12, display strong up-regulation of AKAP12 expression when induced to adhere and spread by phorbol ester treatment (Gordon et al. 1992). This characteristic implicates AKAP12 in cancer biology. It is well known that cancer cells are non-adhesive. The nonadherent nature of malignant cancer cells allows these cells to metastasize. Our identified HBD may be an important binding domain for AKAP12 in cell types other than endothelial cells. It would be interesting to determine if our HBD peptide regulates AKAP12 levels in various cell types that express AKAP12 including metastasized cancer cells.

In summary, this dissertation provides evidence of an endothelial-specific chaperone. HspA12B promotes angiogenesis by facilitating the turnover of angiogenic proteins, specifically AKAP12, through the ubiquitin-proteasome pathway. The proteasome is a barrel-shaped multi component protease that recognizes proteins decorated with ≥ 4 ubiquitin residues. Future experiments that could conclusively label AKAP12 as a target for proteasome degradation would consist of immunoprecipitation of AKAP12 from HUVECs followed by anti-ubiquitin western blots. We predict that overexpression of HspA12B in the HUVECs prior to IP would result in an increase in ubiquitin signal at the site of AKAP12. This signal would decrease with the shRNA mediated knockdown of HspA12B or with the overexpression of our HBD. As an angiogenic stress induced heat shock protein, HspA12B promotes angiogenesis through regulating VEGF by moderating AKAP12. Our study provides insight into the basic mechanisms of angiogenesis.

The characterization of HspA12B as an early angiogenesis regulator will likely have significant potential applications in cardiovascular diseases and cancer. In an *in vivo* study using Dr. Nilanjana Maulik's ischemia/reperfusion rat model, the activation of the VEGF pathway by HspA12B has been confirmed by demonstrating that Ad-HspA12B activated the VEGF pathway. In addition, Ad-HspA12B gene therapy protected hearts against damages caused by

ischemia/reperfusion in a rat model (Figure 4.1), likely by promoting angiogenesis and perhaps also through anti-apoptosis (manuscript submitted).

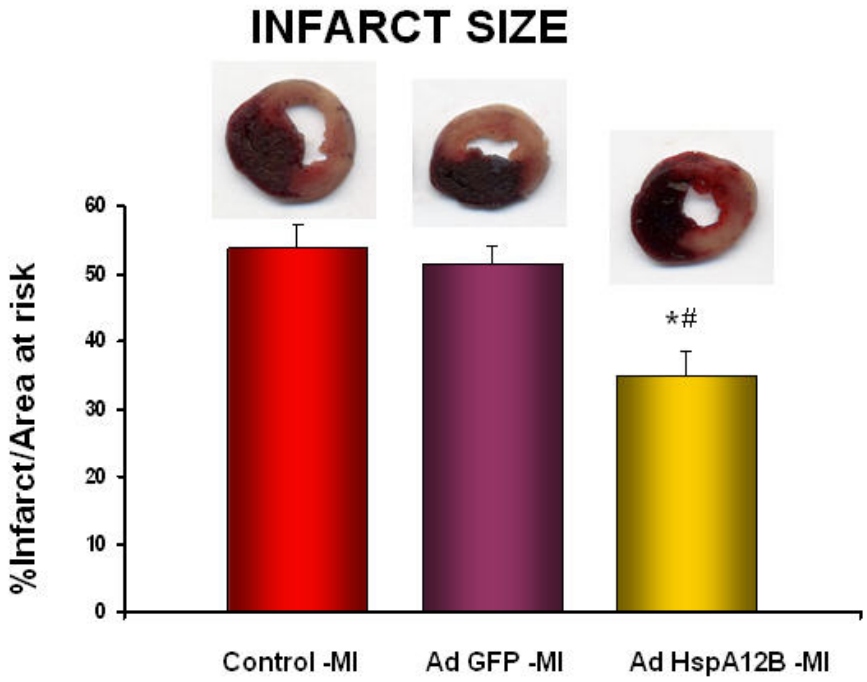


Figure 4.1 Ad-HspA12B gene therapy decreases infarct size in rat myocardium. Control-Myocardial Infarction (MI) represents a surgical procedure without ischemia. The Ad GFP- MI and Ad-HspA12B- MI groups are rats transfected with Adenovirus into the myocardium two days prior to the ischemia/reperfusion procedure. Injection of Ad-HspA12B resulted in significant decrease in infarct size (34% *vs* 46%) as compared to Ad-LacZ injected group. There was no significant difference in the Ad-LacZ MI group and the Control –MI group. Representative pictures are shown above the bar graph. Bar graphs are showing mean ± S.E.M. of 6 animals/group.

On the other side of the coin, HspA12B's role in promoting vessel formation and its suppression of AKAP12 also begs the examination of its role in tumorgenesis. Initial results from transgenic mice overexpressing HspA12B has shown increased tumorgenesis 9 days after B16 cell transplantation in HspA12B tg/+ mice compared to wild type (Han, unpublished data).

If our HBD peptide inhibits angiogenesis assays *in vitro* it could be a new target of tumor angiogenesis for cancer therapy. There has been initial success using liposomes to encapsulate and deliver drugs to angiogeneic vasculature in tumor-bearing animals (Maeda et al. 2004; Asai et al. 2008; Katanasaka et al. 2008). One study has identified an angiogenic vessel-homing peptide, Ala-Pro-Arg-Pro-Gly (APRPG), which was used to design an APRPG-PEG-modified liposomal angiogenic inhibitor carrying the VEGF inhibitor, SU1498. Administration of this liposome significantly decreased tumor microvessel density in Colon26 NL-17 cell-bearing mice and prolonged the survival time of the mice (Katanasaka et al. 2008). It is plausible that our HBD peptide, a recombinant adenovirus expressing our HBD peptide sequence, or our Ad-HspA12B-shRNA could be delivered via this liposome to angiogenic endothelial cells in tumors and thus inhibits tumor-induced angiogenesis.

As indicated by the Y2H data and our signaling data, HspA12B may work to regulate angiogenesis via pathways that are independent of VEGF (such as Ang-1, PODXL, and Spectrin). A set of functional experiments that would help to elucidate whether HspA12B also works independently of VEGF would be matrigel assays of HUVECs that have shRNA mediated knockdown of VEGF. If overexpression of HspA12B induces angiogenesis at the same level as control it is possible that HspA12B is acting through VEGF independent pathway. We have defined HspA12B as a master regulator that coordinates the expression of a number of angiogenic genes. Characterization of and eventually the knowledge to control HspA12B induced angiogenesis will be invaluable for novel angiogenic disease intervention.

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APPENDICES

APPENDIX A

Expression Constructs

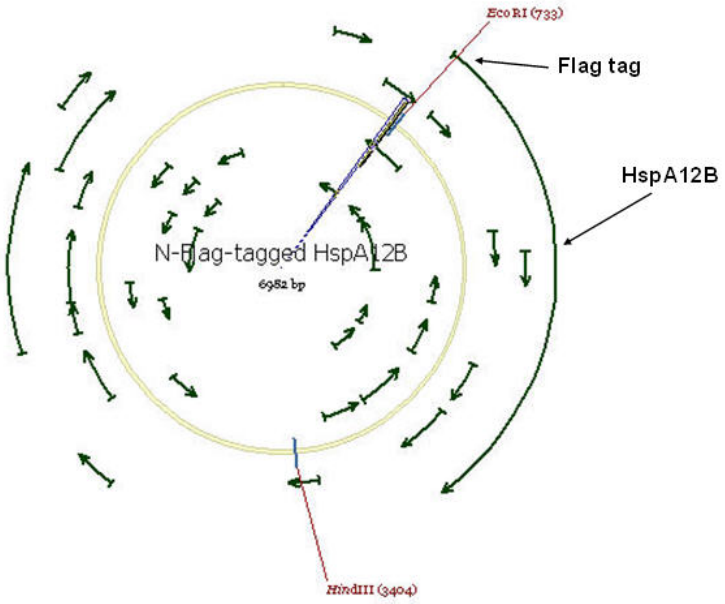


Figure A.1.1 N-Flag-tagged HspA12B construct

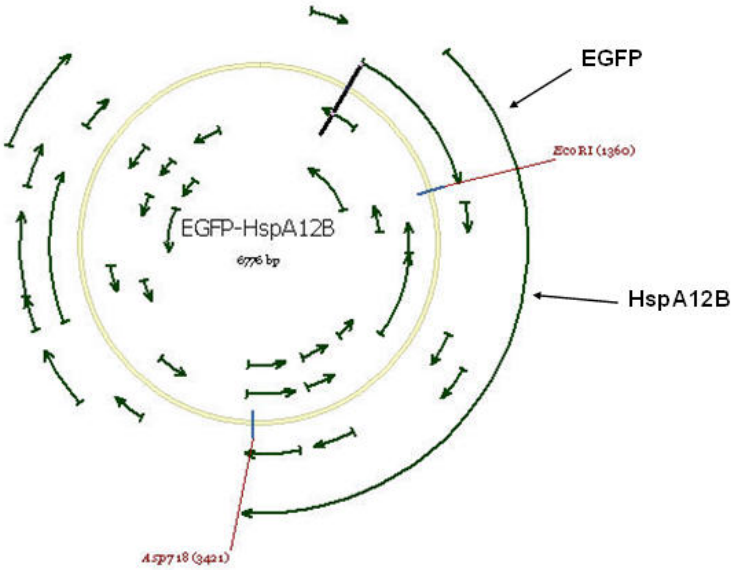


Figure A.1.2 N-EGFP-tagged HspA12B (EGFP-HspA12B) construct

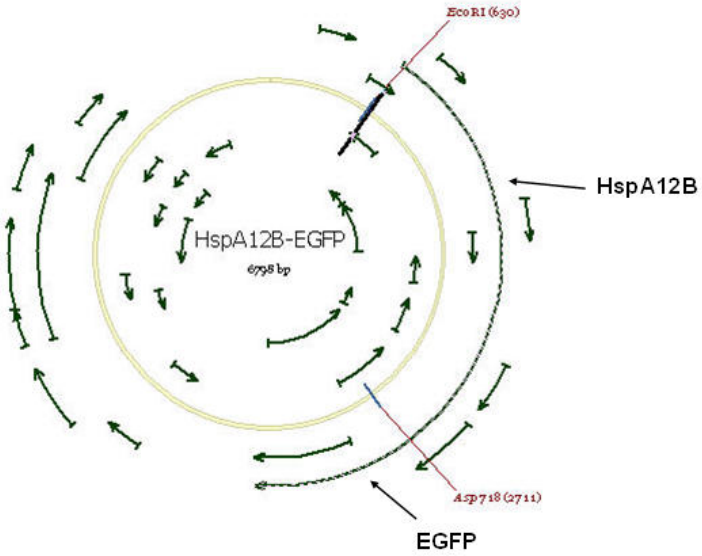


Figure A.1.3 C-EGFP-tagged HspA12B (HspA12B-EGFP) construct

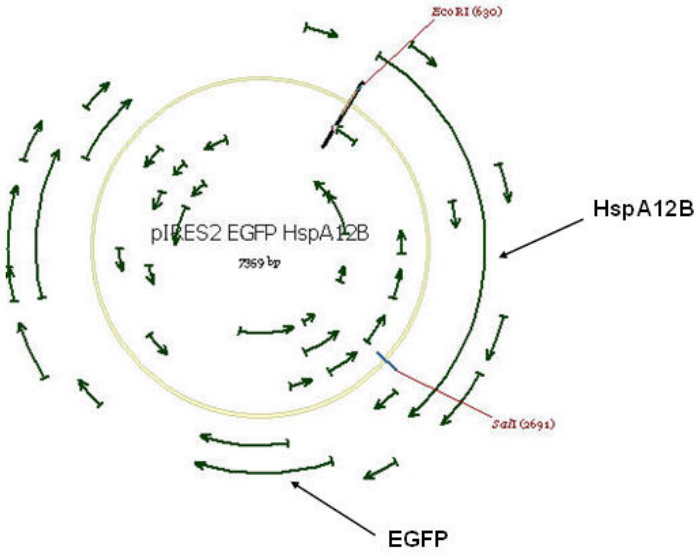


Figure A.1.4 Bi-cistronic wild type HspA12B construct

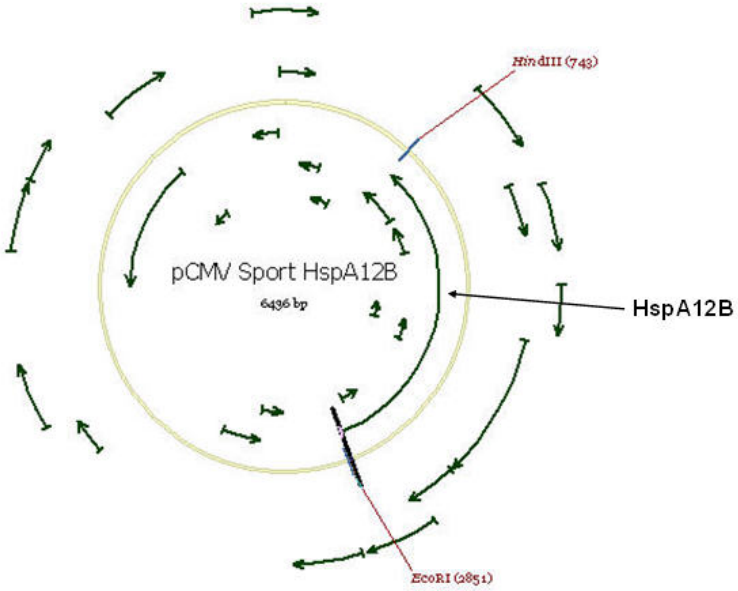


Figure A.1.5 pCMV•Sport 6 HspA12B construct

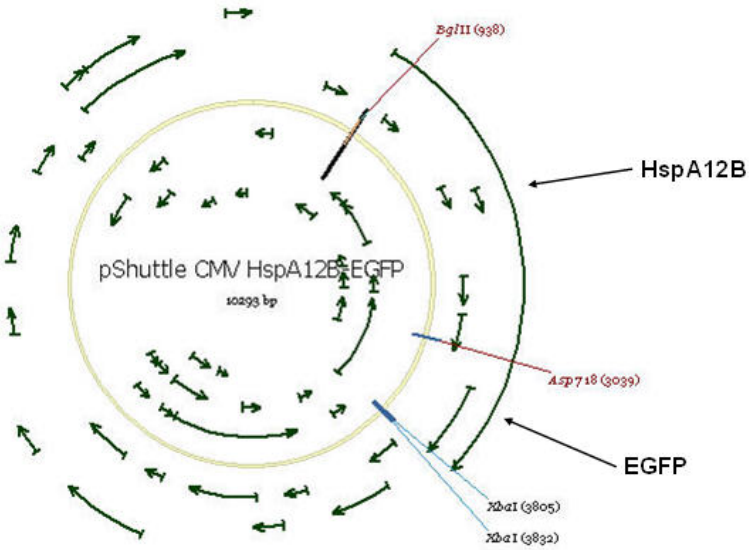


Figure A.1.6 pShuttle-CMV HspA12B-EGFP construct

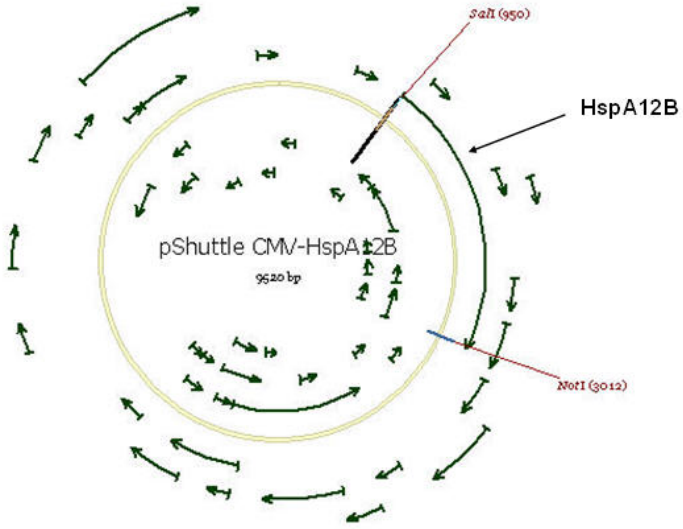


Figure A.1.7 pShuttle-CMV wild type HspA12B construct

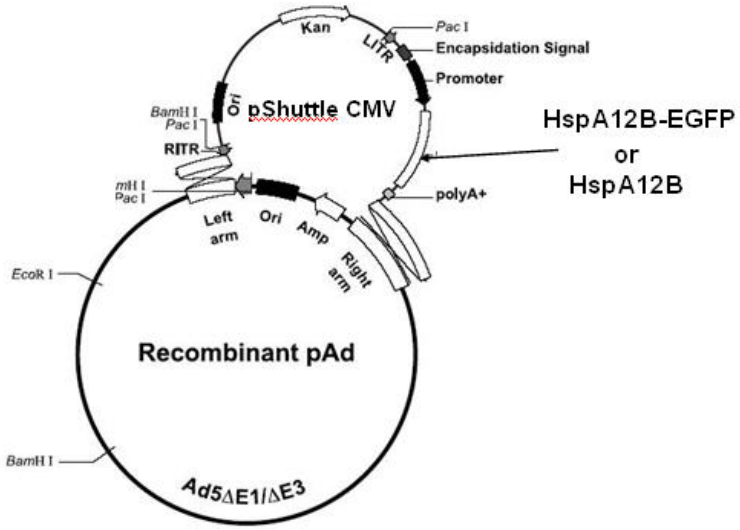


Figure A.1.8 Adenovirus HspA12B recombination figure (adapted from pAdEasy manual from Quantum Biotechnologies (Montreal, Canada))

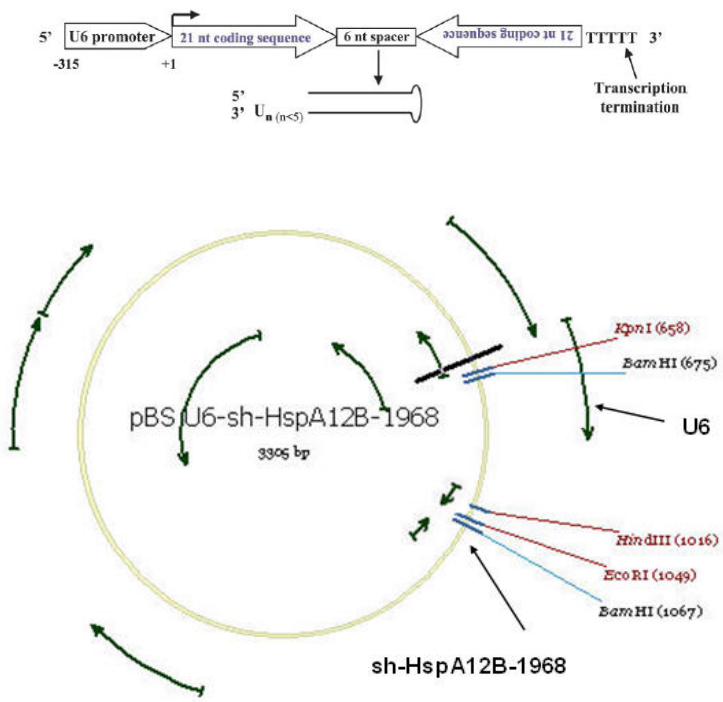


Figure A.1.9 pBS/U6-sh HspA12B construct

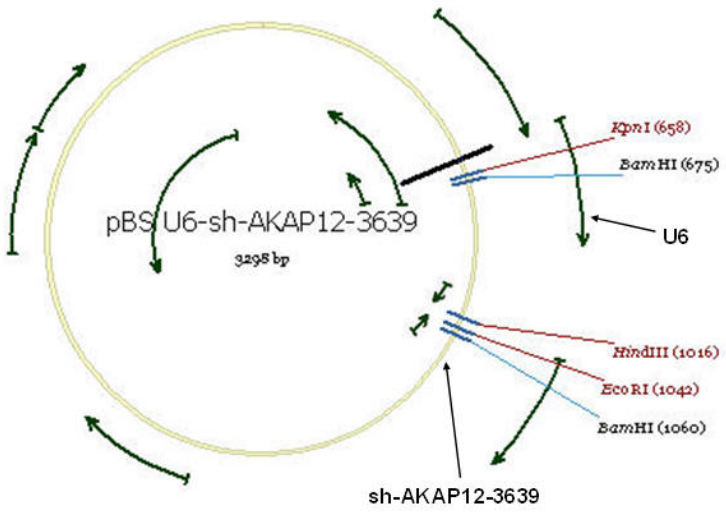


Figure A.1.10 pBS/U6-sh AKAP12 construct

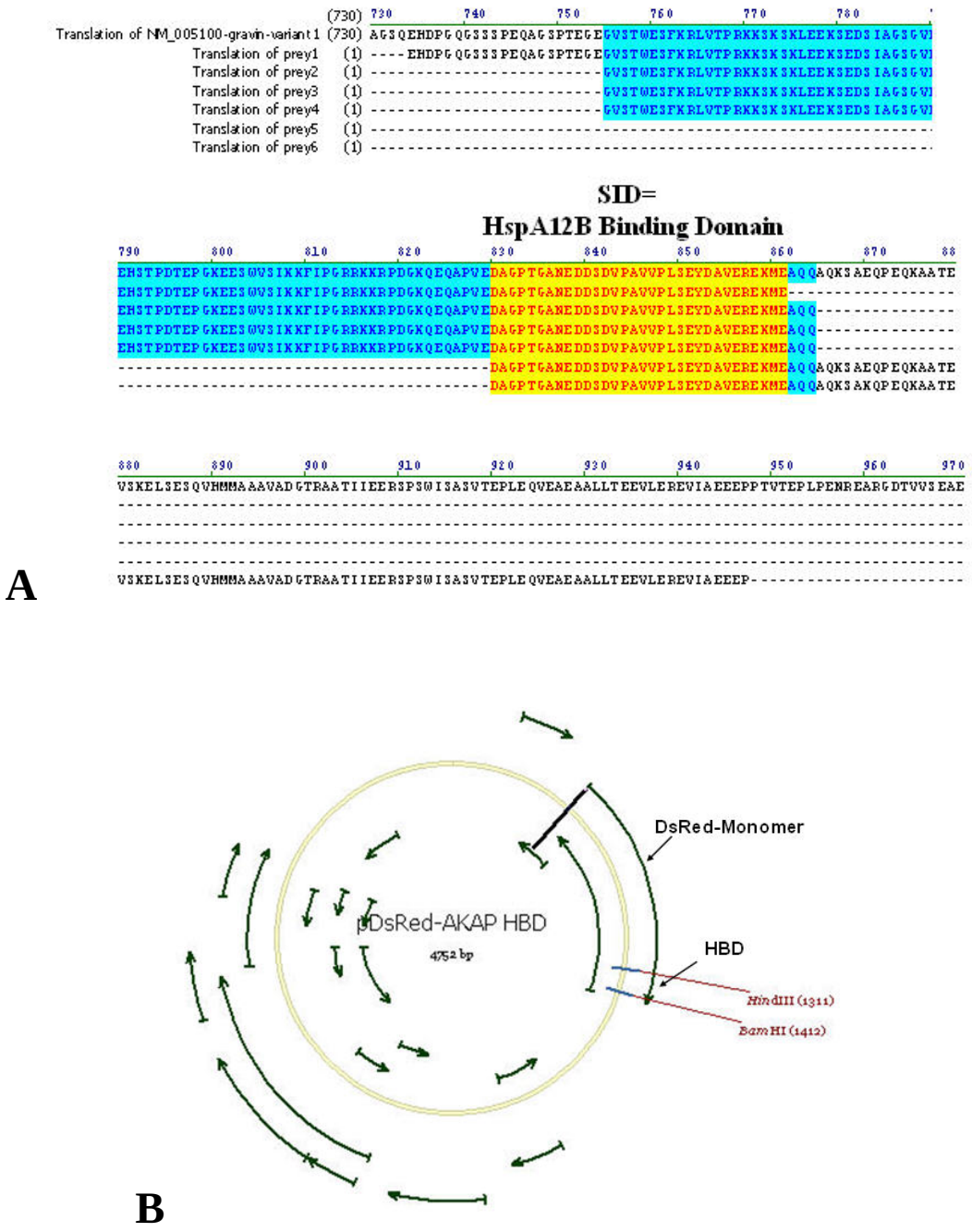


Figure A.1.11 pDsRed AKAP12-829-60 HspA12B binding domain (HBD) construct. A, shows the AKAP12 Shared Interaction Domain (SID) determined from the Y2H. HspA12B interacted with human AKAP12 through 6 fragments which allowed us to determine the SID start at amino acid 829 and the SID stop at amino acid 860. B, is the Vector NTI figure of the pDsRed AKAP HBD.

APPENDIX B

Abbreviations

bp	-Base pairs
BSA	-Bovine serum albumin
DNA	-Deoxyribonucleic acid
ECs	-Endothelial cells
EDTA	-Disodium ethylenediamine tetraacetate
EtOH	-Ethanol
FBS	-Fetal bovine serum
gm	-Gram
Hsp70	-Heat shock protein 70
kD	-Kilodalton
mg	-Milligram
ml	-Milliliter
mm	-Millimeter
mM	-Millimolar
M	-Molar
MOPS	-3-(N-Morpholino)-propanesulfonic acid
OD	-Optical density
PBS	-Phosphate buffered saline
PCR	-Polymerase chain reaction
Pfu	-Plaque forming units
PVDF	-Polyvinylidene difluoride
RFU	-Relative fluorescent units
SDS-PAGE	-Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

siRNA	-Small interfering ribonucleic acid
TBST	-Tris buffered saline tween 20
µg	-Microgram
µl	-Microliter.

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2. Thirunavukkarasu M, Han Z, Zhan L, Koneru S, **Steagall RJ**, Maulik N. (2008). Ad-HspA12B-EGFP gene therapy leads to VEGF/NFκB activation followed by down-regulation of ventricular remodeling in the rat infarcted myocardium through the inhibition of AKAP12, a negative regulator of angiogenesis. (Manuscript in preparation)

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11. Smith TJ, **Kottke RJ**, Lum H, Andersen TT. (1993). Human orbital fibroblasts in culture bind and respond to endothelin. *AM J Physiol,* Jul; 265 (1 Pt 1), C138-142.

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