HISTONE DEACETYLASE 7 IN CARDIOVASCULAR DEVELOPMENT

AND TRANSCRIPTIONAL REGULATORY NETWORKS

OF THE VASCULAR ENDOTHELIUM

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Dedicated to my family,

For showing me the way;

And to Melissa,

For walking it with me.

HISTONE DEACETYLASE 7 IN CARDIOVASCULAR DEVELOPMENT AND TRANSCRIPTIONAL REGULATORY NETWORKS OF THE VASCULAR ENDOTHELIUM

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2008

Acknowledgements

I wish to thank my mentor, Dr. Eric Olson, for his constant support and guidance. I must also acknowledge all past and present members of the Olson lab, especially Chris Davis, whose intensive instruction instilled in me an appreciation for the finer points of the trade. It was also a pleasure to work with Shurong Chang, whose work on HDACs laid the groundwork for my own. Whether working with miRs or mice, my collaboration with Mark Hatley was both enjoyable and productive. Viviani Moresi was a terrific benchmate and miR collaborator. Without Cheryl Nolen's assistance and expert advice, I could not have maintained the enormous mouse colony necessary for this project. I would like to thank my committee members, Dr. Michelle Tallquist, Dr. Raymond MacDonald, and Dr. Joseph Hill for the valuable advice they have provided throughout my training. I am very grateful to Dr. Rhonda Bassel-Duby, whose attention and patience help graduate students such as myself remain grounded in the often dizzying pace of the Olson lab environment. I would particularly like to thank Rusty Montgomery and Yuri Kim for advice, assistance, and endlessly entertaining interaction, both scientific and otherwise. Finally, I would like to thank my family for all the encouragement through the years.

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Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2008

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Cells respond to stimuli in part through the modulation of gene expression. Signal transduction from the environment to the nucleus culminates in the activation of factors that modify chromatin structure to either facilitate or inhibit gene transcription. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two such classes of enzymes that regulate the epigenetic code. Their opposing actions – to activate transcription by histone acetylation and to inhibit transcription by deacetylation – are tightly regulated to coordinate the vast gene programs required for cellular growth and differentiation.

The class II HDACs are restricted in their expression patterns, and each have unique developmental and physiological functions. The studies described here focus on

HDAC7, a class II HDAC that is expressed in vascular endothelial cells and whose function is essential for the maintenance of vascular integrity during embryogenesis. Mice lacking HDAC7 die by e11.5 with complex cardiovascular malformations including endothelial, vascular smooth muscle, and myocardial defects. By generating HDAC7 conditional knockout mice, it was observed that all of these defects are recapitulated in mice bearing an endothelial-specific deletion of HDAC7, but no defects are observed upon deletion of HDAC7 in the other cell types that were affected in the HDAC7 nulls. This *in vivo* evidence demonstrated that HDAC7 acts cell autonomously to maintain normal vascular development, and lead to the identification of the genetic abnormalities and mechanism leading to cardiovascular failure in the HDAC7 knockout.

Further, this work begins the investigation of HDAC7 in adult vascular physiology, the findings of which will reveal new mechanisms whereby the vasculature responds to stress signals or disease. To this end, methods have been developed for the deletion of HDAC7 in the adult mouse using an inducible cre recombinase system together with the HDAC7 conditional allele. Additionally, these studies present progress toward the identification of the enhancer elements driving the endothelial-specific expression pattern of HDAC7. Detailed characterization of this enhancer is likely to implicate new signaling pathways as being involved in the genetic regulation of vascular development and maintenance. Finally, this work investigates the role of microRNA-mediated gene silencing in the vascular system by identifying microRNAs involved in MEF2-dependent signaling in endothelial cells.

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aKO	**adult knockout**
α-MHC	**alpha myosin heavy chain**
ANF	**atrial natriuretic factor**
bFGF	**basic fibroblast growth factor**
b-MHC	**beta myosin heavy chain**
BNP	**brain natriuretic peptide**
CaMK	**calcium/calmodulin-dependent protein kinase**
cAMP	**cyclic adenosine monophosphate**
CBP	**CREB binding protein**
cDNA	**complimentary DNA**
ChIP	**chromatin immunoprecipitation**
cKO	**conditional knockout**
CREB	**cAMP responsive element binding protein**
dKO	**double knockout**
DMEM	**Dulbecco's Modified Eagle's Medium**
DNA	**deoxyribonucleic acid**
EDTA	**ethylenediaminetetraacetic acid**
EMSA	**electrophoretic mobility shift assay**
FGF	**fibroblast growth factor**
GAPDH	**glyceraldehydes-3-phosphate dehydrogenase**
H&E	**hematoxylin and eosin**
HAEC	**human aortic endothelial cells**
HAT	**histone acetyltransferase**
HDAC	**histone deacetylase**
HUVEC	**human umbilical vein endothelial cells**
MAPK	**mitogen-activated protein kinase**
MARK	**microtubule affinity-regulating kinase**
MEF2	**myocyte enhancer factor 2**
miR	**micro RNA**
MMP10	**matrix metalloproteinase 10**
N-CoR	**nuclear receptor co-repressor**
NRSF	**neuron-restrictive silencer factor**
NFAT	**nuclear factor of activated T cells**
NLS	**nuclear localization signal**
PCR	**polymerase chain reaction**
PKA	**protein kinase A**
PKC	**protein kinase C**
PKD	**protein kinase D**
RACE	**rapid amplification of cDNA ends**
RAR	**retinoic acid receptor**
RNA	**ribonucleic acid**
RT-PCR	**reverse transcriptase-polymerase chain reaction**

RXR	**retinoid X receptor**
SIK1	**salt inducible kinase 1**
siRNA	**small interfering RNA**
SMRT	**silencing mediator for retinoic acid and thyroid hormone receptors**
SRF	**serum response factor**
TIMP1	**tissue inhibitor of matrix metalloproteinase 1**
TSA	**trichostatin A**
UTR	**untranslated region**
VEGF	**vascular endothelial growth factor**
VDR	**vitamin D receptor**
WT	**wild type**

Chapter One

Histone Deacetylases in Cardiovascular Development and Disease

Chromatin modifying enzymes are central regulators of the gene programs that drive developmental processes, including cell growth, cell differentiation, and organogenesis. Two classes of these enzymes, the histone acetyl transferases (HATs) and histone deacetylases (HDACs), act at the interface between cellular signaling pathways and DNA-bound transcriptional coactivators. The modulation of these signaling pathways represents many possibilities for future therapeutics, as many of the gene regulatory networks essential for normal development and physiology are dysregulated in conditions of stress and disease.

HATs and HDACs

Both the development and stress-induced growth of the heart are controlled by combinatorial interactions of transcription factors, including myocyte enhancer factor 2 (MEF2), serum response factor (SRF), nuclear factor of activated T-cells (NFAT), and GATA family zinc finger proteins (Zhang, Azhar et al. 2001; Braz, Bueno et al. 2003; Pikkarainen, Tokola et al. 2004). The ability of these factors to activate gene expression is due in part to their ability to recruit HATs (Fig. 1.1). HATs provide these coactivators the ability to modulate the "histone code" – a diverse array of post-translational modifications which together determine the higher-order structure of chromatin (Strahl and Allis 2000). Specifically, HATs transfer acetyl groups from acetyl coenzyme A to conserved lysine residues on histone tails. This modification results in a neutralization of the positive charge of the residue, leading to the local relaxation of intranucleosome and internucleosome interactions. In this less condensed state, chromatin is more accessible to transcriptional machinery, favoring gene expression. The most extensively studied HATs in muscle are the closely related coactivators p300 and CREB-binding protein (CBP). The activity of p300 and CBP is increased in response to hypertrophic signaling pathways in the cardiac myocyte (Gusterson, Brar et al. 2002; Miyamoto, Kawamura et

al. 2006). Also in the cardiac myocyte, a p300-responsive element in the *skeletal* α-*actin* promoter was mapped to a MEF2-binding site, and a ternary complex containing this DNA element, MEF2, and p300 was demonstrated (Slepak, Webster et al. 2001). Moreover, ectopic overexpression of p300 and CBP stimulates, while dominant negative mutants of p300 block agonist-mediated cardiac growth in adult mice (Gusterson, Brar et al. 2002; Gusterson, Jazrawi et al. 2003; Yanazume, Hasegawa et al. 2003). The importance of p300 in cardiac development is illustrated by the phenotype of p300 knockout mice, which die between days 9 and 11.5 of gestation. These mice show reduced expression of muscle structural proteins such as α-MHC and α-actinin, as well as cardiac structural defects (Yao, Oh et al. 1998).

The stimulatory effect of HATs on gene expression is countered by the HDACs. The HDACs promote chromatin compaction and thereby repress gene expression. In the myocyte, HATs and HDACs coordinately act as links between signal transduction pathways and the DNA-binding transcription factors that ultimately drive the gene programs of myogenic growth and differentiation. Eighteen human HDACs have been identified to date (Figure 1.2). They fall into three classes based on their homology with three structurally and biochemically distinct yeast HDACs. Class I HDACs (1, 2, 3, 8 and 11) are related to yeast RPD3, class II HDACs (4, 5, 6, 7, 9 and 10) to yeast HDA1, and class III HDACs (Sirt1–7) to yeast Sir2. Class II HDACs are further divided into two subclasses, IIa (HDACs 4, 5, 7 and 9) and IIb (HDACs 6 and 10). Herein, the term "*class II HDACs*" will refer specifically to the class IIa HDACs. Members of this subset (HDACs 4,5,7, and 9) have been shown to repress MEF2-mediated gene expression and myocyte growth (McKinsey, Zhang et al. 2002; Zhang, McKinsey et al. 2002). In contrast, increasing evidence suggests class I HDACs may be involved in promoting cellular growth. (Antos, McKinsey et al. 2003; Kook, Lepore et al. 2003). The class III HDACs, which act as nicotinamide adenine dinucleotide (NAD)-dependent deacetylases,

have been implicated in the inhibition of cardiac hypertrophy and enhanced cardiomyocyte survival (Alcendor, Kirshenbaum et al. 2004), as well as in nutrient sensing and aging (Guarente 2008).

Class I HDACs are expressed ubiquitously and are composed mainly of a catalytic domain (Grozinger and Schreiber 2002). In contrast, class II HDACs show more restricted expression patterns, being enriched in heart, skeletal muscle, and brain. The class II HDACs contain an approximately 500 amino acid N-terminal extension that mediates interactions with other transcriptional cofactors and confers responsiveness to calcium-dependent signaling pathways through conserved serine phosphorylation sites (Grozinger, Hassig et al. 1999; Miska, Karlsson et al. 1999; Lu, McKinsey et al. 2000).

HDACs as Repressors of MEF2-Mediated Transcription

A series of studies demonstrated that the class II HDACs control muscle growth and differentiation specifically through their associations with MEF2 and MyoD. The MEF2:HDAC interaction was initially identified by yeast two-hybrid screens for MEF2-interacting proteins (Sparrow, Miska et al. 1999; Lu, McKinsey et al. 2000). It is now clear that class II HDACs associate with MEF2 and act as potent inhibitors of MEF2-dependent transcription (Miska, Karlsson et al. 1999; Wang, Bertos et al. 1999; Lemercier, Verdel et al. 2000; Haberland, Arnold et al. 2007). Binding of these HDACs to MEF2 is mediated by 18 conserved amino acids in their amino-terminal extensions, whereas class I HDACs lack this domain and thus do not directly associate with MEF2. The Class II HDACs bind sequences in MEF2 at the junction of the MADS/MEF2 domains, which mediate DNA binding and dimerization (Lu, McKinsey et al. 2000). However, association of HDAC with MEF2 does not appear to significantly alter these properties (Lu, McKinsey et al. 2000), and the crystal structure of MEF2 bound to DNA is consistent with the formation of a ternary complex of MEF2, HDAC, and MEF2-target

genes (Santelli and Richmond 2000). Further, the interaction of MEF2 with HATs and HDACs is mutually exclusive (Sartorelli, Huang et al. 1997; Zhang, McKinsey et al. 2001). Thus, MEF2 acts as platform to respond to positive or negative transcriptional signals by exchanging HATs and class II HDACs.

The class II HDACs possess at least two separable repression domains, located at the amino- and carboxy-termini of the protein. The HDAC catalytic domain mediates the repressive activity of the carboxy-terminal region (Kao, Downes et al. 2000; Lu, McKinsey et al. 2000). There is evidence to suggest that full-length class II HDACs lack intrinsic catalytic activity, instead deriving their deacetylase activity from the recruitment of class I HDACs (Fischle, Dequiedt et al. 2002).

The amino-terminal domain of class II HDACs does not possess catalytic activity but instead represses transcription by recruiting other HDACs and corepressors (Grozinger, Hassig et al. 1999; Sparrow, Miska et al. 1999; Dressel, Bailey et al. 2001; Zhang, McKinsey et al. 2001). While the amino- and carboxy-terminal regions function independently as transcriptional repressors in transient promoter-reporter assays, the repressive activity of both domains appears to be required to inhibit endogenous MEF2 target genes (Lu, McKinsey et al. 2000). MEF2-interacting transcription repressor (MITR), an HDAC9 splice variant, lacks a catalytic domain. Yet, it is able to repress MEF2-dependent transcription and cardiac hypertrophy through the recruitment of other corepressors (Sparrow, Miska et al. 1999; Zhou, Richon et al. 2000; Zhang, McKinsey et al. 2001; Zhang, McKinsey et al. 2002).

As the class II HDACs do not bind DNA, they adopt target gene specificity by recruitment through MEF2 factors to MEF2 recognition sites. However, MEF2 is also capable of interacting with GATA and NFAT factors (Blaeser, Ho et al. 2000; Morin, Charron et al. 2000). Thus, class II HDACs may also repress elements of the

developmental and hypertrophic cardiac gene program via recruitment by MEF2 to complexes assembled at GATA and NFAT response elements.

Abnormal cardiac growth in HDAC-knockout animals correlates with super-activation of the MEF2 transcription factor, (Zhang, McKinsey et al. 2002) suggesting a relationship between the MEF2-HDAC interaction and the control of the hypertrophic response. These knockout animals are discussed in further detail later in this chapter.

MEF2-independent Functions for Class II HDACs

Class II HDACs are capable of associating with nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT), suggesting they have MEF2-independent roles (Huang, Zhang et al. 2000; Kao, Downes et al. 2000). Indeed, SMRT and N-CoR repress transcription by association with a vast array of transcription factors. The binding site for SMRT/N-CoR on class II HDACs overlaps with their nuclear export sequence (Huang, Zhang et al. 2000; Kao, Downes et al. 2000; McKinsey, Zhang et al. 2001). It has been shown that SMRT can drive HDAC4 from the cytoplasm to the nucleus, further suggesting a role for this interaction in the control of HDAC subcellular localization (Wu, Li et al. 2001). Also, SMRT has been shown to undergo nuclear export in response to MEK-1 signaling (Hong and Privalsky 2000), suggesting that CaMK-independent pathways may control class II HDAC activity indirectly via SMRT. In this regard, it has also been found that the ERK1/2 MAP kinases phosphorylate HDAC4, similarly promoting nuclear export (Zhou, Richon et al. 2000). Thus, it is likely that class II HDACs are regulated both directly and indirectly by multiple signaling networks.

Other MEF2-independent functions for class II HDACs include the control of cardiac gene expression through indirect interactions with stress-responsive transcription

factors. These indirect interactions include HDAC5 and Nkx2.5 via calmodulin binding transcription activator 2 (CAMTA2) (Song, Backs et al. 2006), HDAC5 and SRF via myocardin (Cao, Wang et al. 2005; Xing, Zhang et al. 2006), and HDAC4 and NFAT via mammalian relative of DnaJ (Mrj) (Dai, Xu et al. 2005).

Class II HDACs as Regulators of Cardiac Remodeling

Much of the characterization of the functions of the class II HDACs has focused on their role in the heart. Because their well-studied role in hypertrophy elegantly demonstrates how they act as molecular switches in response to stress stimuli, these findings are used here as a starting point for understanding how they may function in stress signaling in other systems.

In response to stress signals that arise from a variety of cardiovascular disorders, including myocardial infarction and hypertension, the adult heart typically becomes enlarged due to cardiomyocyte hypertrophy. During this hypertrophic response, the individual myocytes increase in size without dividing and assemble additional sarcomeres to maximize force generation. While this response may provide initial compensatory advantages, such as the normalization of wall tension, prolonged hypertrophy in response to pathological signals is associated with increased morbidity and mortality due to both systolic and diastolic dysfunction (Levy, Garrison et al. 1990). These observations are mirrored in animals models, in which prevention of the hypertrophic response ultimately maintains cardiac performance and enhances survival in conditions of hemodynamic stress (Frey and Olson 2003).

The Development-Hypertrophy Connection

At the cell surface, humoral factors such as isoproterenol, angiotensin II, and endothelin-1 trigger cardiac hypertrophy and remodeling by activating diverse

downstream signaling pathways. These include pathways involving the calcium/calmodulin-dependent phosphatase calcineurin, CaMK, and MAPKs (Zou, Takano et al. 2002; Molkentin 2004). Many of these pathways converge upon members of the MEF2, GATA, and NFAT families of transcription factors that together control fetal cardiac gene expression. Through these pathways, stress signaling in the myocardium results in upregulation of genes encoding embryonic isoforms of proteins that govern contractility, calcium handling, and energetics, with a concomitant downregulation of adult isoforms. For example, stress signals enhance the expression of embryonic beta-myosin heavy chain (β-MHC) and reduce expression of adult alpha-MHC (α-MHC). This isoform switch results in diminished myofibrillar ATPase activity and impaired cardiac contractility (Mercadier, Lompre et al. 1981; Whalen, Sell et al. 1981). This thick filament isoform switch is best characterized in rodent models, but there is strong evidence that it also contributes to the progression of heart disease in humans. Specifically, phenotypic improvement in patients receiving beta-adrenergic antagonists (β-blockers) directly correlates with an increase in adult α-MHC and a decrease in fetal β-MHC (Abraham, Gilbert et al. 2002).

The potential involvement of chromatin remodeling in the regulation of α- and β-MHC expression was first suggested by the demonstration that nuclease hypersensitive sites appear in the *α-* and *β-MHC* promoters in a spatio-temporal pattern that correlates with expression of each isoform (Huang and Liew 1998). Enhancer mapping of the *β-MHC* gene revealed muscle-specific regulatory elements including recognition sites for MEF2 and MyoD (Huang, Chen et al. 1997), which further suggests a potential involvement of HATs and HDACs in the regulation of contractile protein isoforms.

The genes encoding atrial and brain natriuretic peptides (ANP and BNP) are also components of the fetal gene program that are upregulated in hypertrophic and failing

hearts (Cameron and Ellmers 2003). ANP and BNP bind to the natriuretic peptide receptor-A (NPR-A), which possesses intrinsic guanylyl cyclase activity and produces the second messenger cyclic guanosine monophosphate (cGMP) following peptide binding. NPR-A knockout mice spontaneously develop cardiac hypertrophy (Knowles, Esposito et al. 2001; Holtwick, van Eickels et al. 2003), suggesting that natriuretic peptides might serve counterregulatory functions to negatively control pathological cardiac signaling, perhaps through the activation of cGMP-dependent protein kinase (PKG) (Fiedler, Lohmann et al. 2002). ANP and BNP gene expression is repressed by neuron-restrictive silencer factor (NRSF), a repressor that recruits both class I and class II HDACs to repress the fetal cardiac gene program (Nakagawa, Kuwahara et al. 2006). This study further demonstrated decreased interaction between NRSF and class II HDACs in both *in vitro* and *in vivo* cardiac hypertrophy models.

Signal-Dependent Regulation of Class II HDACs

Class II HDACs levels do not appear to change in the stressed myocardium (Zhang, McKinsey et al. 2002; Chang, McKinsey et al. 2004). Instead, these HDACs are regulated by shuttling from the nucleus to the cytoplasm in response to stress signals, providing a posttranslational mechanism to override HDAC-mediated repression of cardiac growth (Bush, Fielitz et al. 2004; Harrison, Roberts et al. 2004; Vega, Harrison et al. 2004). This redistribution of HDACs enables MEF2 and other transcriptional activators and coactivators to associate with HATs resulting in increased local histone acetylation and activation of downstream genes that promote cellular growth (Youn, Grozinger et al. 2000; Han, Pan et al. 2003). The nuclear-cytoplasmic translocation of class II HDACs is induced by phosphorylation of two conserved, serine-containing motifs found in the amino-terminal extensions of HDACs 5 and 9, and three conserved serines in the amino-terminal extensions of HDACs 4 and 7 (Grozinger and Schreiber

2000; McKinsey, Zhang et al. 2000; McKinsey, Zhang et al. 2000). When phosphorylated, these motifs associate with a chaperone protein, 14-3-3, which results in masking of the nuclear localization sequence located between the phosphorylation sites, and induces a conformational change that unmasks a nuclear export sequence at the C terminus of the HDAC (Figure 1.3) (McKinsey, Zhang et al. 2001; Wang and Yang 2001). The nuclear export sequence is subsequently bound by the CRM1 nuclear export receptor, which facilitates translocation from the nucleus to the cytoplasm (Harrison, Roberts et al. 2004). Induction of cardiac hypertrophy includes the posttranslational activation of MEF2, which occurs in part as a consequence of the dissociation and nuclear export of class II HDACs (Lu, McKinsey et al. 2000). Thus, signal-dependent HDAC export couples cellular signaling pathways to the MEF2-dependent activation of the fetal cardiac gene program (Figure 1.4).

Further insight was provided by the generation of signal-resistant class II HDACs. By mutating the conserved serines to alanines, HDAC phosphorylation is blocked, thereby inhibiting 14-3-3 docking and nuclear export. Such a signal-responsive, constitutively nuclear HDAC5 was shown to block cardiomyocyte hypertrophy induced by known hypertrophic agents (Zhang, McKinsey et al. 2002).

HDAC Kinases

Investigations thus far have identified four families of HDAC kinases, three of which belong to the Ca2+/calmodulin-dependent protein kinase (CaMK) superfamily. The families include CaMKII, protein kinase D (PKD), and microtubule affinity-regulating kinase (MARK). The fourth HDAC kinase, salt inducible kinase 1 (SIK1), is part of a novel, three member subfamily of the cAMP-dependent protein kinase (PKA) family.

Protein Kinase D

The PKD family includes three highly homologous PKD isoforms, all of which are able to phosphorylate all four class II HDACs (Chang, Bezprozvannaya et al. 2005; Dequiedt, Van Lint et al. 2005; Parra, Kasler et al. 2005), indicating that the PKD family may exert redundant control over the class II HDACs. PKD is activated and translocates to the nucleus in response to G-protein coupled receptor agonists, which include the hypertrophic agonists phenylephrine, angiotensin II, and endothelin-1 (Rey, Young et al. 2001; Auer, von Blume et al. 2005; Harrison, Kim et al. 2006). Some of these agonists, such as phenylephrine, activate protein kinase C (PKC) which directly phosphorylates PKD. Others, such as endothelin-1, activate PKD through PKC-independent mechanisms (Wood, Marklund et al. 2005; Harrison, Kim et al. 2006).

In the heart, PKD activation occurs not only in response to the agonists previously mentioned, but also in response to chronic hypertension and pressure overload, such as that caused by aortic constriction. Activation of PKD in cultured cardiomyocytes causes the activation of the fetal cardiac genes, including ANF, BNP, and α-skeletal actin (Vega, Harrison et al. 2004).

CaMKII

CaMKII is the most highly expressed CaMK family member in the heart (Edman and Schulman 1994). CaMKII activity is low at basal states and is activated by Ca^{+2}/calmodulin complexes. Overexpression of calmodulin in mouse hearts causes pathological remodeling (Gruver, DeMayo et al. 1993), implicating CaMKII in the transduction of cardiac stress signals. *In vitro* assays based on the expression of constitutively active CaMKI and IV in fibroblasts clearly identify this kinase family as strong class II HDAC kinases (McKinsey, Zhang et al. 2000). However, studies in cultured cardiac myocytes fail to clearly establish a connection between endogenous

CaMKI and IV activity and HDAC export. Moreover, pressure overload hypertrophy selectively upregulates CaMKII, further implicating this CaMK family member in cardiac stress signaling (Colomer, Mao et al. 2003).

Suprisingly, overexpression of a constitutively active CaMKII is unable to efficiently drive HDAC5 nuclear export (Backs, Song et al. 2006), and calcium signaling in myocytes that leads to CaMKII activation drives HDAC4, but not HDAC5, nuclear export (Liu, Randall et al. 2005). The inability of CaMKII to induce HDAC5 export is explained by the finding that HDAC4 contains a unique CaMKII docking site that is absent in HDACs 5 and 9 (Backs, Song et al. 2006). This domain confers HDAC4 responsiveness to Ca^{+2} signaling relayed through CaMKII. In support of this, adrenergic agonist-dependent export of HDAC4, but not HDAC5, is sensitive to CaMK inhibitors. Together, these findings indicate that the class II HDACs can be differentially exported in response to different signals and may regulate different sets of genes (McKinsey 2007).

MARK Kinases

Of the MARK kinase family members (MARK1, 2, 3, and 4), MARK1 and 2 are most abundant in the heart (Drewes 2004; Tassan and Le Goff 2004). MARK2 phosphorylates the same sites on HDAC5 that are targeted by PKD, and these sites are conserved among the other class II HDACs (Drewes, Ebneth et al. 1997). MARK3 is able to phosphorylate HDAC7 (Dequiedt et al., 2006), and appears to preferentially phosphorylate the amino-terminal 14-3-3 binding site. Despite these findings, there is not yet any evidence that the MARK kinases mediate HDAC phosphorylation and export in cardiomyocytes (Vega, Harrison et al. 2004) (McKinsey, Zhang et al. 2000). Thus, more studies are needed to determine if MARK kinases have a role in cardiac development or remodeling.

SIK1

The SIK kinases are serine/threonine kinases expressed in the heart, adipocytes, nervous system, and other tissues (Ruiz, Conlon et al. 1994; Wang, Takemori et al. 1999; van der Linden, Nolan et al. 2007). SIK1 is a direct target of the CREB family of transcriptional coactivators (Berdeaux, Goebel et al. 2007). Like MEF2, CREB is induced in response to calcium signaling and activates the genes programs responsible for myogenic growth and differentiation (Wu, Huang et al. 2006) (Chen, Ginty et al. 2005). Berdeaux *et al.* found that transgenic mice expressing a dominant-negative CREB die prematurely due to severe muscle wasting, and SIK1 is downregulated in skeletal muscle of these mice. SIK1 was shown to phosphorylate HDAC5 and thereby increase MEF2 activity. In the dominant-negative CREB transgenic mice, muscle necrosis was shown to be substantially abrogated by replacement of SIK1 in skeletal muscles. Thus, SIK1 acts on the class II HDACs to mediate cooperative crosstalk between CREB- and MEF2-mediated myogenic signaling.

HDAC Knockout Mice

HDAC9 and HDAC5 Knockout Mice

HDAC9 is the most abundantly expressed HDAC in the myocardium, and the primary product of the HDAC9 locus, MITR, is a highly effective suppressor of hypertrophy *in vitro* (Zhang, McKinsey et al. 2001). The HDAC9 gene was inactivated in mice by homologous recombination in ES cells (Zhang, McKinsey et al. 2002). Mice homozygous for the null allele are obtained in predicted Mendelian ratios and show no pathology in early life. However, they display a dramatically increased hypertrophic response to thoracic aortic banding, a model for cardiac pressure overload. HDAC9 nulls

also show an exaggerated hypertrophic response to a known inducer of cardiac growth, the heart-specific calcineurin transgene. This increased hypertrophy in response to calcineurin includes an exaggerated upregulation of ANF, BNP, and β-MHC. Finally, even in the absence of such cardiovascular stresses, HDAC9 nulls develop cardiac hypertrophy by eight months of age. A transgenic mouse bearing a MEF2 consensus binding site-driven *lacZ* cassette (Naya, Wu et al. 1999) revealed that *HDAC9* nulls also displayed an exaggerated elevation in MEF2 activity in response to the calcineurin transgene. Together, these findings identify HDAC9 as a participant in hypertrophic signaling pathways *in vivo.*

Targeted deletion of HDAC5 resulted in similar findings (Chang, McKinsey et al. 2004). HDAC5 nulls are viable, fertile, and show no abnormalities early in life. Expression of *lacZ* from the targeted allele revealed that HDAC5 is strongly expressed in the looping heart tube at embryonic day 9.5 (E9.5). Expression becomes broader during later embryogenesis, appearing in the spinal cord and skeletal muscle. The adult expression pattern is broader, with staining present in the heart, lung, brain, skeletal muscle, liver, and other tissues. They show similar age-dependent cardiac hypertrophy and exaggerated responses to calcineurin signaling and pressure overload due to aortic constriction. Mice lacking both HDAC5 and HDAC9 show some embryonic or early perinatal lethality. Double nulls that survive show cardiac hypertrophy by one month of age. Analysis of double null embryos at E15.5 reveals ventricular septal defects, multifocal hemorrhages, and thinning of the ventricular myocardium. Similar defects were also seen in a subset of the mice that survived to birth. Thus, HDAC5 and 9 appear share a redundant function in normal cardiovascular development.

HDAC4 Knockout Mouse

HDAC4 is expressed in prehypertrophic chondrocytes of the developing skeleton and determines the timing and extent of endochondral bone formation (Vega, Matsuda et al. 2004). Mice homozygous for an HDAC4 mutation exhibit lethal ossification of endochondral cartilage due to ectopic hypertrophy of chondrocytes, whereas ectopic expression of *HDAC4* in chondrocytes inhibits hypertrophic growth and differentiation. HDAC4 directly interacts with and represses runt related transcription factor 2 (Runx2), a factor known to drive chondrocyte hypertrophy.

Hypertrophic growth of different cell types depends upon different cellular stimuli and different sets of transcription factors. The HDAC4 knockout phenotype suggests that even within these diverse cellular contexts, class II HDACs maintain their role as repressors of pathological or developmental hypertrophic gene programs.

HDAC7 Knockout Mouse

Targeted deletion of HDAC7 results embryonic lethality due to vascular dilation and rupture (Chang, Young et al. 2006). This phenotype is totally penetrant, with all embryos dying by E11.5 and showing the same defects. HDAC7 was deleted by replacing the first three coding exons with a β-galactosidase protein-coding cassette fused in frame with the initiating codon of HDAC7, facilitating the characterization of the gene expression pattern. Analysis of mice heterozygous for the targeted allele revealed that HDAC7 is expressed specifically in vascular and endocardial endothelial cells. By gross examination, *lacZ* staining is present in many organs, especially in heart, skeletal muscle, major vessels, and lungs. Histological examination reveals this broad expression to be highly specific for the vascular endothelial cells within all of these tissues, being excluded in vascular smooth muscle, cardiomyocytes, and other organ parenchyma. Notably, expression is absent in the yolk sac vascular endothelium.

In wild type embryos, electron microscopy reveals normal tight junctions between adjacent endothelial cells of major vessels in wild type embryos. However, such junctions are often missing in mutant embryos just prior to death. Endothelial cells appear to extend processes that fail to establish tight endothelial cell-cell interactions, which are essential for maintaining vascular integrity.

In addition to the endothelial cell defects, mutants have a reduced number of smooth muscle cells surrounding their major vessels. The dorsal aortae show extreme dilation, and the myocardium is significantly thinner in both the atria and ventricles. Thus, the HDAC7 knockout (HDAC7 KO) phenotype includes non-endothelial cell developmental abnormalities. Likely explanations for the phenotypic features that are not restricted to the HDAC7-expressing cell type include aberrant paracrine signaling between endothelial cells and adjacent cell layers, or the misregulation of some other secreted factor or factors that influence adjacent cells or the surrounding matrix.

The necessity of HDAC7 gene expression specifically in the endothelium is further confirmed by targeted, conditional gene deletion. The absence of HDAC7 in the endothelium recapitulates with complete penetrance the same phenotypic features as seen in the null mice. These finding are discussed in detail in Chapter Two.

Subsequent analysis of the HDAC7 KO reveals that loss of HDAC7 leads to a MEF2-dependent upregulation in matrix metalloproteinase 10 (MMP10) and downregulation of tissue inhibitor of metalloproteinase 1 (TIMP1) *in vivo* and *in vitro.* These findings suggest that the misregulation of secreted matrix remodeling enzymes is contributing to the loss of normal vascular development and maintenance of vascular integrity.

In sum, HDAC7 is essential for normal cardiovascular development. Its critical role, at least in part, is to modulate MEF2 activity in the endocardium and in the major

vessels. Further, these findings introduce the possibility that HDAC7 may have roles in physiological maintenance or pathological processes in the adult vasculature.

HDAC1 Knockout Mouse

Deletion of the class I HDAC, HDAC1 results in embryonic lethality by E10.5 due to severe developmental defects involving the head and allantois (Lagger, O'Carroll et al. 2002). These morphological defects appear to result from impaired cellular proliferation. Analysis of ES cells from mutant embryos reveals increased levels of the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ secondary to the hyperacetylation of their promoters. These findings are consistent with the observation that the activation of tumor suppressors by HDAC inhibitors is integral to their anti-tumorgenic activity (Kramer, Gottlicher et al. 2001).

HDAC1 is not expressed in the heart prior to E10.5, but is expressed in the adult heart (Yang, Yao et al. 1997). Interestingly, the cyclin-dependent kinase inhibitor p21 is upregulated in human cells treated with HDAC inhibitors (Sowa, Orita et al. 1999; Richon, Sandhoff et al. 2000), and several studies have implicated p21 as a repressor of pressure- and angiotensin-induced cardiac hypertrophy (Li and Brooks 1997; Nozato, Ito et al. 2000). Therefore, HDAC1 inhibition in cardiac myocytes may upregulate p21 and thereby block agonist-induced hypertrophy (Backs and Olson 2006). These findings may in part explain the observation that HDAC inhibitors to prevent stress-induced cardiac hypertrophy.

HDAC2 Knockout Mouse

HDAC2 KO mice are cyanotic, bradycardic, and die within 24 hours of birth (Montgomery, Davis et al. 2007). They have severe cardiac malformations including an

enlarged interventricular septum and nearly complete loss of the right ventricular lumen. Further analysis revealed increases in apoptosis and cell proliferation in the myocardium.

Conditional deletion of HDAC2 in the heart resulted in viable animals with no gross abnormalities. Thus, the lethality and defects in HDAC2 KO mice are not due to a cardiac myocyte autonomous function of HDAC2. Subsequent deletion of HDAC2 in the endothelium, neural crest, and secondary heart field likewise failed to reproduce the phenotype of the HDAC2 KO. Together, these findings suggest HDAC2 acts in multiple cell types in the developing heart.

Conditional deletion of both HDAC1 and HDAC2 in the heart leads to lethal cardiac dilation and arrhythmias during the second week after birth (Montgomery, Davis et al. 2007). Hearts from these double knockout (dKO) mice show upregulation of a T-type calcium channel, CaV3.2. This channel is normally expressed in the embryonic heart and downregulated after birth (Yasui, Niwa et al. 2005), and it has been shown to cause susceptibility to cardiac arrhythmias and sudden death in mice (Kuwahara, Saito et al. 2003). In addition, hearts from dKOs show dramatic upregulation of skeletal muscle troponin isoforms Tnni1 and Tnni2, which regulate the response of contractile apparatus to calcium fluxes. Because these proteins are not typically induced in cardiac hypertrophy (Bodor, Oakeley et al. 1997), their upregulation may represent a primary defect leading to cardiac failure in these animals. Finally, as the phenotypic defects and gene misregulation in the heart-specific dKOs is not observed in the HDAC1 or HDAC2 single cardiac deletions, these HDACs have partially redundant roles in cardiac morphogenesis and contractility, specifically with regard to their cardiac myocyte-autonomous functions.

Toward a General Model for HDACs as Mediators of Stress Signaling

The mechanism of action and function of the HDACs, especially the class II HDACs, has been studied primarily in the context of cardiac hypertrophy. However, HDACs are expressed in all cell types and are certain to have many functions that have not yet been identified. The function of the class II HDACs in hypertrophy provides a starting point for developing a general model that will inform the investigation of HDACs in other aspects of development and physiology.

In general terms, stress signals cause cells to respond by altering their gene expression profile. In the heart, the stress responsive gene program is highly similar to the gene program active in the developing heart. Their expression both in development and in hypertrophic stress signaling is dependent on MEF2 activity. In other cell types, stresses may similarly induce members of the MEF2 family or other transcription factors, and this induction may include the signal-dependent relief from HDAC-mediated repression. Future investigation will reveal whether this general model of class II HDACs acting as molecular switches in stress signaling is applicable in other cell types.

HDAC Inhibitors and Therapeutics

HDAC Inhibitors and Cardiac Remodeling

Given the role of class II HDACs as repressors of the hypertrophic gene program, it would follow that pharmacological inhibitors of HDACs should stimulate cardiac hypertrophy. Paradoxically, treatment of cardiomyocytes with general antagonists of both class I and class II HDACs, such as trichostatin A (TSA) and sodium butyrate, represses the increases in cell size and fetal gene expression that are normally evoked by hypertrophic agonists (Antos, McKinsey et al. 2003; McKinsey and Olson 2004). It was also demonstrated that TSA upregulates α-MHC expression in cultured cardiac

myocytes, as well as in an *in vivo* model of hypothyroid rats, while it downregulates the expression of α- and β-tubulins and prevents their induction in response to angiotensin II (Davis, Pillai et al. 2005). HDAC inhibitors, therefore, have the capacity to antagonize pathological gene expression that leads to the impairment of contractility (Kong, Tannous et al. 2006).

There are several possible explanations for the apparent conflict between the anti-growth effects of HDAC inhibitors and the role of class II HDACs in repressing pro-hypertrophy factors such as MEF2. First, MITR, the splicing variant of HDAC9 that lacks the catalytic domain, can repress MEF2 as efficiently as the full-length HDAC9 protein (Zhang, McKinsey et al. 2002). This indicates that the deacetylase domain is dispensable for the antigrowth effect of class II HDACs and therefore might not be affected by HDAC inhibitors. Also, class II HDACs appear to lack intrinsic catalytic activity (Fischle, Dequiedt et al. 2002) and the general HDAC inhibitors described here do not affect NAD-dependent class III HDACs (Imai, Armstrong et al. 2000). Thus, it has been proposed that distinct HDACs play positive or negative roles in the control of cardiac growth by regulating opposing sets of target genes via interactions with different sets of transcription factors, and it is the class I HDACs that are required for the repression of pro-hypertrophic genes (McKinsey et al., 2004). The simplest interpretation may be that class I HDAC activity is necessary to repress genes that encode anti-hypertrophic factors. Alternatively, class I HDACs may stimulate expression of pro-growth genes. While HDACs are typically associated with gene repression, there are several studies in which HDACs have been linked to gene induction (Chang, McKinsey et al. 2004; Zupkovitz, Tischler et al. 2006). Finally, class I HDACs may act through the deacetylation of non-histone targets (Westermann and Weber 2003; Yanazume, Hasegawa et al. 2003), altering signal transduction or the cytoskeleton. HDAC inhibitors

might then oppose hypertrophic signaling in part by affecting the acetylation state of these non-histone targets.

Many of the same transcription factors that are crucial for embryonic cardiac gene expression and development, including MEF2, SRF, and GATA, act as endpoints for stress-responsive hypertrophic signaling pathways. Activation of the fetal gene program with repression of corresponding adult cardiac genes ultimately leads to cardiac failure (Lowes, Gilbert et al. 2002). Thus, tremendous effort has been devoted toward the identification of the mechanisms coupling cardiac stress signaling pathways to the fetal gene program. Studies have demonstrated that both genetic and pharmacological blockade of these signaling pathways in the context of chronic cardiac stress preserves cardiac function (Koch, Rockman et al. 1995; Rothermel, McKinsey et al. 2001; Antos, McKinsey et al. 2002).

HDAC Inhibitors and Cancer

HDAC inhibitors (HDACi) have multiple mechanisms of action that contribute to their efficacy as anti-cancer agents, and are now showing success in clinical trials for the treatment of both hematologic and solid tumors (Bolden, Peart et al. 2006) (Marks and Breslow 2007) (Garcia-Manero and Issa 2005). HDACi have dramatic effects on transcription, with varying reports of 2-10% of genes being regulated more than 2-fold on microarrays using cancer cell lines (Chambers, Banerjee et al. 2003; Glaser, Staver et al. 2003). Not surprisingly, the broad influence of the HDACs across the genome corresponds with the modulation of numerous signaling pathways. In cancer cells, HDACi have been shown to induce numerous pro-apoptotic pathways, block cell cycle progression, block tumor invasion, inhibit tumor angiogeneisis, and influence many other cellular processes (Xu, Parmigiani et al. 2007).

Because HDACs have both redundant and nonredundant functions in various cell types, having the ability to modulate multiple or single family members to selectively affect the HDAC targets of interest would conceivably increase the clinical utility of HDAC inhibition. Although achieving specificity with HDAC inhibitors has been challenging due to the high similarity of the catalytic domains, compounds showing varying specificities have been characterized.

Targeting the class II HDACs for clinical benefit may be best advanced by strategies other than catalytic inhibition, based on studies that suggest the class II HDACs lack intrinsic catalytic activity, and instead repress transcription through the recruitment of class I HDACs(Fischle, Dequiedt et al. 2001; Lahm, Paolini et al. 2007). If HDACi are only able to modulate the activity mediated by the ubiquitous class I HDACs, alternative molecular strategies will be necessary to specifically access the unique and restricted biological functions regulated by the class II HDACs. Thus, a detailed understanding of their signal-responsive regulation as has been characterized in the setting of cardiac hypertrophy, as well as the characterization of each member's unique biological function in other tissues, will be essential to developing novel therapeutics targeting the class II HDACs.

Conclusions and Future Perspectives on Therapeutics

General inhibitors of HDACs are tolerated well *in vivo* and are currently being tested as anti-cancer drugs. Gain- and loss-of-function experiments in cancer cell lines, cardiomyocytes, and mouse models are aiding in the development of effective isoform-specific HDAC inhibitors. New high-throughput screens and medicinal chemistry strategies are beginning to identify small molecules with superior activity and more narrow target specificities (Arts, de Schepper et al. 2003; Haggarty, Koeller et al. 2003; Hu, Dul et al. 2003). Such discoveries should lead to novel therapeutic strategies that

enable the treatment of cancer, pathological cardiac remodeling, and potentially other diseases while minimizing unpredicted clinical side effects.

The identification of signaling pathways that control cardiac gene transcription has provided new opportunities for drug discovery (Bush, Fielitz et al. 2004; Wu, Ding et al. 2004). Understanding the mechanisms and patterns of gene regulation in the developing heart has enabled the identification of molecular signals that cause pathological remodeling in the stressed heart. Furthermore, applying the model of HDACs in cardiac stress signaling to other biological systems has the potential to advance our understanding of other diseases and stress signaling. Studies in mice have identified roles for the HDACs in processes such as bone development and vascular development – roles that remain largely unexplored with regard to therapeutic possibilities.

By acting at the interface between diverse signaling pathways and the genome, the HDACs occupy a unique position in the control of both pathological and developmental gene expression. In this capacity, the HDACs control diverse biological processes in both development and disease. Thus, further insight into the biological roles of the HDACs will continue to reveal new possibilities for therapeutic intervention.

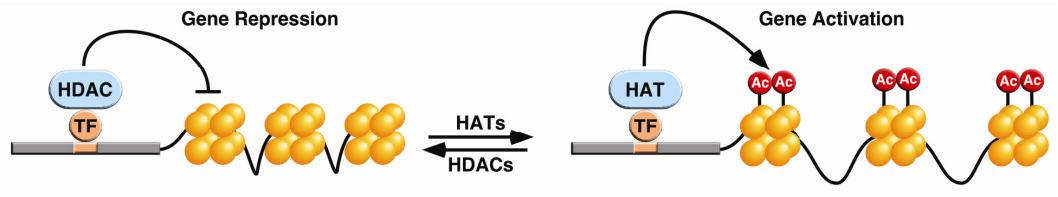

Figure 1.1. Model of histone acetylation and deacetylation. HATs and HDACs are recruited to DNA by association with transcription factors (TFs). Acetylation of histone tails promotes chromatin relaxation and transcriptional activation. Deacetylation promotes chromatin condensation and transcriptional repression.

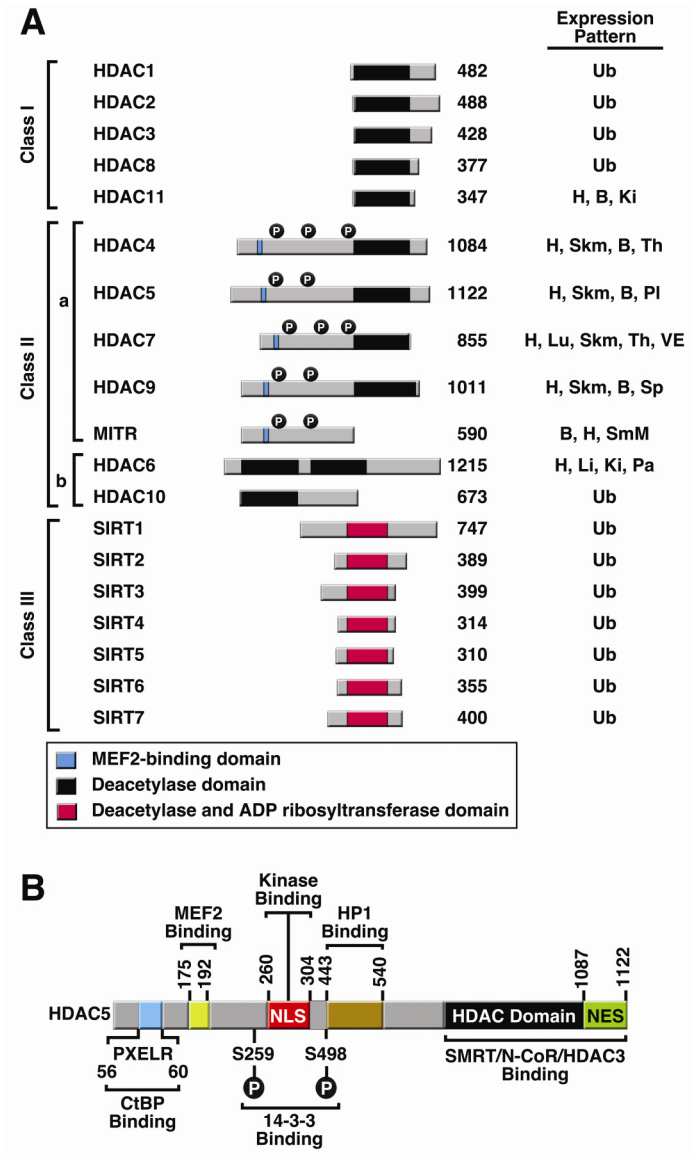

Figure 1.2. Schematic representations of histone deacetylases. (a) HDACs are grouped primarily into three classes — I, II, and III — on the basis of their homology with three structurally and biochemically distinct yeast HDACs, Rpd3p, Hda1p and Sir2, respectively. The Class IV HDAC, HDAC11, is most closely related to the Class I HDACs. The Class III HDACs are also called the Sirtuins. Class IIa HDACs are expressed in a tissue-restricted manner (H, heart; B, brain; VE, vascular endothelium and endocardium; Ki, kidney; Skm, skeletal muscle; SmM, smooth muscle; Th, thymus; Pl, placenta; Lu, lung; Sp, spleen; Pa, pancreas; Ub, ubiquitous). (b) Schematic showing functional domains, phosphorylation sites, and cofactor binding regions of human HDAC5 (NLS, nuclear localization signal; NES, nuclear export signal; HP1, heterchromatin protein 1; CtBP, C-terminal binding protein 1). Annotated features and domains are conserved among class II HDACs. Adapted from (McKinsey et al., 2002).

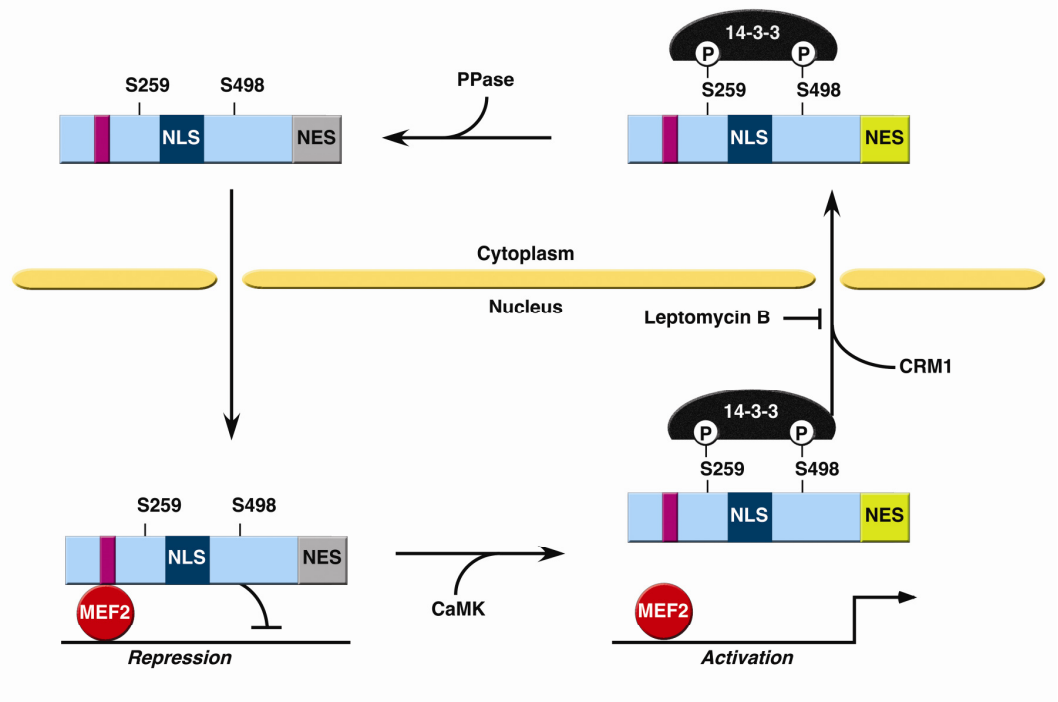

Figure 1.3. Model of the regulated nuclear-cytoplasmic shuttling of class II HDACs In the unphosphorylated state, HDAC5 is localized to the nucleus and associates with MEF2, resulting in repression of MEF2 target genes. Phosphorylation of conserved serines that flank the NLS of HDAC5 results in recruitment of 14-3-3 and dissociation from MEF2. Binding of 14-3-3 masks the NLS and activates a cryptic NES at the carboxyl terminus of HDAC5, resulting in nuclear export. This process is blocked by leptomycin B, which inhibits the CRM1 exportin protein. Re-entry of HDAC5 into the nucleus requires the action of a protein phosphatase (PPase). Adapted from (McKinsey et al., 2001).

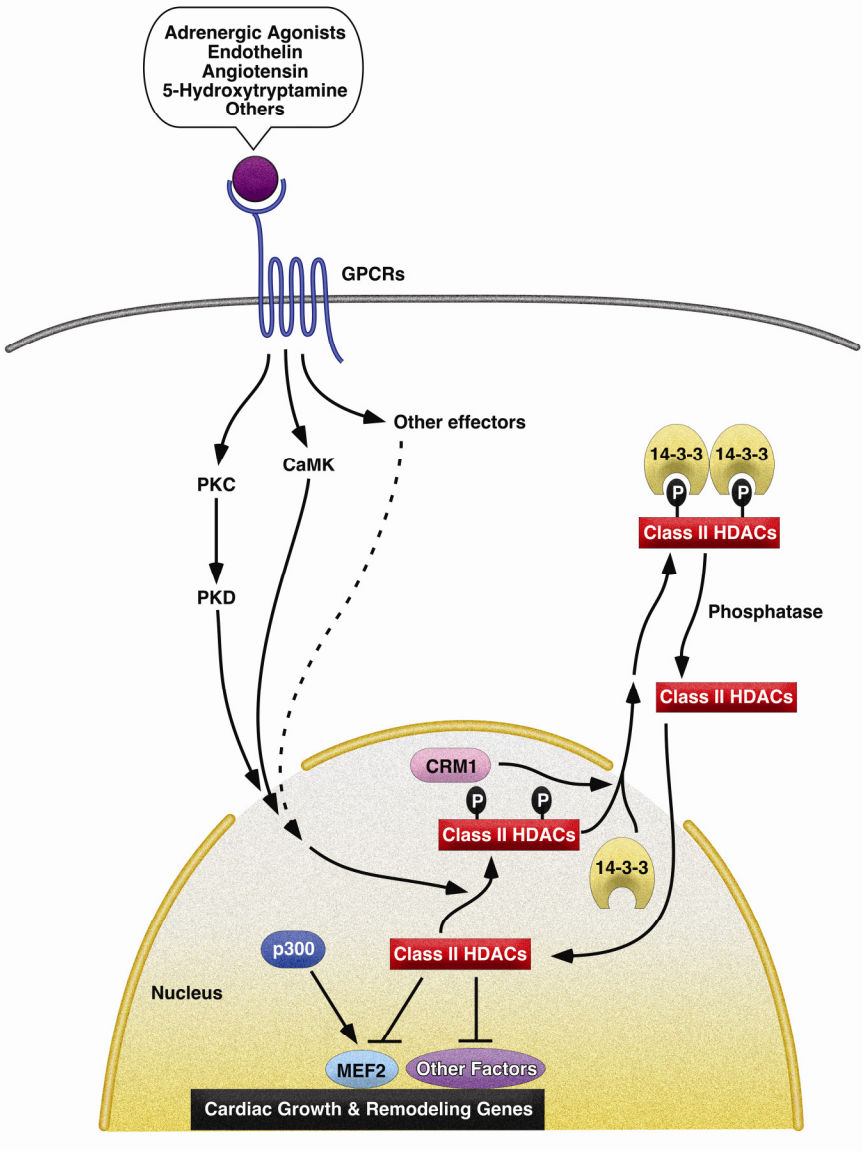

Figure 1.4. Model for stress signaling pathways in cardiac hypertrophy. Hypertrophic agonists activate kinases that phosphorylate class II HDACs. Nuclear export of the class II HDACs allows the activation of growth and remodeling genes by MEF2 and other transcription factors. (Adapted from Backs and Olson, 2006)

Chapter Two

Requirement for HDAC7 in Vascular Development

Introduction

Targeted deletion of HDAC7 in mice results in complex vascular defects and embryonic lethality by e11.5 (Chang, Young et al. 2006). The HDAC7 knockout (HDAC7 KO) provided two essential observations. First, loss of HDAC7 resulted in abnormalities in multiple cell types, including cardiac myocytes, vascular smooth muscle cells, and vascular endothelial cells. Second, the expression of *lacZ* confirmed the results of *in situ* analysis of HDAC7 mRNA expression, which is that HDAC7 is expressed specifically in endothelial cells around the time of embryonic lethality (Figure 4.1). Expression was also seen in the cells of the endocardium, which is continuous with the vascular endothelium. After birth, HDAC7 is expressed in the alveolar cells of the lung and in some neurons of the central nervous system. Notably, no expression is observed in cardiac myocytes or vascular smooth muscle cells.

The thinning and disorganization observed in the vascular smooth muscle and myocardium might be explained by one of several hypotheses. First, HDAC7 could be expressed and necessary in these cell types, and the global deletion of HDAC7 caused changes in gene regulation that directly led to the defects observed in these cells. This possibility is not supported by the *lacZ* knockin, which shows no staining within smooth muscle cells or cardiac myocytes. However, consideration of the *lacZ* data cannot exclude the possibility that the recombination of the allele for the *lacZ* knockin disrupted the endogenous gene expression pattern, or that HDAC7 is expressed at a very low but functionally significant level in other cell types that is not readily detectable in the *lacZ* staining and histology. Thus, further evidence was sought to definitively identify the cell type or types that experienced changes in gene regulation that in turn caused the various cardiovascular defects.

An alternative hypothesis is that the direct changes in gene regulation caused by the loss of HDAC7 occur only in the endothelial cells, and subsequent intercellular

interaction would then lead to the smooth muscle and myocardial defects. These intercellular interactions could include secreted factors that act on cell surface receptors. Signaling between the endocardium and myocardium is known to involve ET-1, retinoic acid, VEGF, neuregulin, and other signaling pathways (Wagner and Siddiqui 2007) (Eisenberg and Markwald 1995). Similarly, signals from the vascular endothelial cells are known to be essential in the recruitment and organization/patterning of the vascular smooth muscle (Hellstrom, Kalen et al. 1999). Endothelial cells also secrete factors that are involved in the remodeling of the intercellular stroma. Thus, the disruption of gene expression within the endothelial cells could lead to effects in other cell types by intracellular signaling mechanisms or secreted factors that remodel the surrounding matrix.

Endothelial cells are known to send and receive paracrine, autocrine, and endocrine signals that are essential to the development of many cells types and organs (Cleaver and Melton 2003), (Hungerford and Little 1999). The role of endothelial signaling to other cells has been extensively characterized as a requirement for vascular smooth muscle cell layer formation (Lindahl, Johansson et al. 1997) and reviewed in (Tallquist, Soriano et al. 1999). Such signaling is also required for the induction of endocrine pancreatic differentiation by blood vessels. In the embryo, a series of reciprocal interactions occurs between the prepancreatic endoderm and the developing vascular plexus (Lammert, Cleaver et al. 2001). These interactions include a signal from vascular endothelial cells that induces the specification and differentiation of insulin-secreting pancreatic islet cells.

Such intercellular cues from endothelial cells are also essential in hepatic morphogenesis. Embryos lacking the VEGF receptor *flk-1* fail to form endothelial cells (Shalaby, Rossant et al. 1995). Lacking endothelial cells, endoderm that has undergone hepatic specification fails to proliferate and expand into the surrounding mesenchyme,

two of the early and essential steps in liver organogenesis (Matsumoto, Yoshitomi et al. 2001).

Another example of such developmental signaling from the endothelium is the induction of myocytes by the adjacent endothelial cells into Purkinje fibers, highly specialized cells of the cardiac conduction system (Takebayashi-Suzuki, Yanagisawa et al. 2000). The endothelial cells of developing arteries within the myocardium and the endothelial cells of the endocardium express endothelin converting enzyme 1 (ECE-1). This enzyme converts myocyte-expressed precursors of the signaling pepetide endothelin 1 (ET-1) to its mature form. This creates a domain in which mature ET-1 induces expression of conduction cell markers in a subset of myocytes adjacent to the endothelial cells. Thus, the timing and location of Purkinje fiber development is directed in part by the endothelium, as it provides intercellular spatial cues that direct the cell fate determination of a subpopulation of developing myocytes.

The *HDAC7* gene expression data, together with previously described mechanisms whereby endothelial cells direct the specification, differentiation, and growth, of many diverse cells types and structures, support the hypothesis that the complex defects seen in the cardiovascular system of the HDAC7 knockout are secondary to defects within the endothelial cells. Specifically, the loss of HDAC7 results in gene regulatory changes whose effects on other cell types adjacent to the endothelium are mediated by some secreted factor that is disruptive to the development of these cells or to their assembly into organized multicellular structures.

In order confirm the hypothesis that HDAC7 acts cell autonomously within endothelial cells to regulate vascular development, and to further characterize the role of HDAC7 in the endothelium and other tissues, a conditional allele of HDAC7 (HDAC7 *loxP)* was generated using the cre recombinase-*loxP* system. This approach allows the tissue-specific analysis of the HDAC7 knockout phenotype as well as the study of the

role of HDAC7 at time points beyond the point of developmental arrest. The utility of the conditional allele for these studies is dependent on several factors that were considered in the design of the targeting construct. First, the targeted allele must function as wild type, with the *loxP* sites not disrupting the production of full length HDAC7 transcript. Second, cre-mediated excision of the region between the *loxP* sites must result in the complete loss of HDAC7 transcript from that allele. Finally, the targeting construct should be designed to facilitate a convenient strategy for the detection and differentiation of the wild type, knockout (*lacZ* knockin), *loxP*, *loxP*ΔNeo, and Cre-excised alleles.

Methods

Targeting of HDAC7 Conditional Allele

The murine *HDAC7* gene consists of 23 coding exons that span approximately 18 kB, with a 5'-UTR that is variably comprised of one or more distant upstream exons. Here, the first coding exon is designated exon 2. To generate a conditional allele, *loxP* sites were inserted upstream of exon 2, and downstream of exon 11 (Figure 2.1). Deletion of exons 2-5 by *lacZ* knockin was confirmed to be a transcript null, so it was inferred that cre-mediated excision of exons 2-11 would likewise result in complete loss of transcript. The 5'-*loxP* site was inserted in a region of low conservation 190 bp upstream of exon 2 to reduce the possibility of disrupting any gene regulatory elements. Introns 5-10 were rejected as potential 3'-*loxP* site locations due to concern that modification of these small introns might disrupt normal gene expression.

Regions of homology were amplified by high-fidelity PCR from 129SvEv genomic DNA. Restriction sites were engineered into the construct to facilitate the

identification of targeted embryonic stem cells by Southern blotting. The vector was linearized and electroporated into embryonic stems cells. Stem cell colonies were picked, amplified, and screened by Southern blot. Targeted cells were identified, amplified, and infected with adenoviral cre construct to verify the functionality of the *loxP* sites Southern blotting. The targeted ES cells were then injected into blastocysts. Chimeric males were bred with wild type C57BL6 females, and germline transmission was confirmed by Southern blot and PCR. FLPe transgenic mice (Rodriguez, Buchholz et al. 2000) were bred to mice with the HDAC7 *loxP* allele to remove the *FRT*-flanked neomycin-resistance cassette from the allele. Mice homozygous for the conditional allele with the intact neomycin-resistance cassette were not obtained, presumably due to failure of this allele to express functioning HDAC7 and subsequent embryonic lethality. However, following removal of the neomycin cassette, mice homozygous for the conditional allele were found to be born in Mendelian ratios, fertile, and phenotypically indistinguishable from wild type mice. The FLPe transgene was subsequently segregated out of the HDAC7 *loxP/loxP* line.

Genotyping of HDAC7 Alleles

DNA was extracted from tail biopsies, or in the case of embryos, yolk sacs. Primer sequences for genotyping are: (*fwd*) 5' -cca gtg gac gag cat tct gga gaa agg c- 3'; (*rev*) 5'-gtt gca ggg tca gca gcg cag gct ctg- 3'; (*lacZ*) 5' -gcc agt ttg agg gga cga cga cag tat cg- 3'. Primers are combined in a single reaction and enable the identification of any of the three HDAC7 alleles (+/KO/*loxP*). Product for WT allele is 400 bp; loxP is 500 bp; KO is 640 bp; cre-excised loxP allele produces no product with this primer set. Reverse primer for detecting cre excised allele is (*short arm rev*) 5' -gag agc cag ctg cag cga gaa gtg t- 3' (used with *fwd*). Product for loxPΔneo is 4.8 kb; WT 4.6 kb; cre-excised (floxed) is 267 bp.

Generation of Tissue-Specific HDAC7 Knockout Mice

The HDAC7 *loxP* allele and the HDAC7 *KO* allele were utilized together with Cre recombinase transgenic lines to generate tissue-specific HDAC7 knockout animals (Figure 2.2). For these studies, cre was expressed in cardiac myocytes by the α-myosin heavy chain cre transgene (Agah, Frenkel et al. 1997). Endothelial cre expression was achieved using the *Tie-2* cre trasngene (Kisanuki, Hammer et al. 2001). For smooth muscle cre expression, a transgenic line was used in which cre is driven by the minimal cardiac and smooth muscle enhancer of *myocardin* (Creemers, Sutherland et al. 2006). Neural crest deletion was achieved using the *Wnt-1* cre line (Chai, Jiang et al. 2000). Skeletal muscle deletion was performed using the *Myo-cre* transgenic line (Li, Czubryt et al. 2005), in which cre is under control of the *myogenin* promoter (Cheng, Wallace et al. 1993) and *MEF2C* skeletal muscle enhancer (Wang, Valdez et al. 2001).

Undesired cre expression in the female germline has been reported for some cre lines, resulting global gene deletion in offspring for which the cre transgene has been maternally transmitted (unpublished observations). Therefore, the cre lines were carried on the male animals until the final conditional knockout generation was acquired. Additionally, a PCR-based genotyping strategy was designed to differentiate between conditional alleles in which cre-mediated recombination had or had not occurred. Thus, this strategy was used to monitor for unwanted global deletion of the conditional allele.

Results

Conditional Knockout of HDAC7 in Endothelial Cells

The endothelial-specific deletion of HDAC7 resulted in embryonic lethality by e11.5 (Chang, Young et al. 2006). Embryos were collected from multiple females around

e11.0, just prior to or at the time of death. PCR genotyping of the yolk sacs revealed that HDAC7 conditional knockout (cKO) mice were represented near the Mendelian ratios at e11.0. The endothelial knockouts showed a consistent and completely penetrant phenotype nearly identical to that of the HDAC7 KO (Figure 2.3). The myocardium was thin and failed to develop into an organized, compact layer. The vascular smooth muscle cell layer around most major arteries was thin due to reduced smooth muscle cell numbers. Vascular dilation and rupture, interstitial edema, and pericardial effusion were present in cKO embryos, though the vascular dilation and effusions were typically less severe than observed in the HDAC7 KO embryos. These differences could be attributed to the deletion occurring later in endothelial cells in the cKO as compared to the standard knockout, as Tie-2 cre does not cause recombination in all endothelial cells until around e9.5 (Kisanuki, Hammer et al. 2001). Alternatively, differences of a few hours in the time of embryo collection may have resulted in the observed differences in the severity of some of the phenotypic features.

The PCR strategy designed to detect only an HDAC7 conditional allele that had not undergone cre-mediated recombination was applied to the cKO embryo yolk sac DNA. This PCR product was generated with approximately the same efficiency in all cre-postive and cre-negative embryos. Unless all cKO samples were contaminated with maternal tissue, this confirmed that the *Tie2*-cre transgene had not caused the undesired global deletion in these litters. This was the expected result, as we had experienced no precedent for germline cre expression by the *Tie2*-cre transgene in the male germline.

Conditional Knockouts of HDAC7 in Other Cell Types are Viable

No abnormalities were observed upon deletion of HDAC7 in the myocardium, smooth muscle, skeletal muscle, or neural crest. These animals were born in Mendelian

ratios, were viable, and indistinguishable from their wild type littermates by gross analysis or standard H&E histology of the tissues of interest (data not shown).

Conclusions and Future Directions

Together, the results obtained from the conditional deletion of HDAC7 support the hypothesis that HDAC7 acts within endothelial cells to maintain vascular integrity during development. Similar defects in cardiac myocytes and vascular smooth muscle cells are found when HDAC7 is deleted globally or in endothelial cells alone. The defects in these cell types are secondary to the loss of HDAC7 within the endothelial cells.

These findings indicated that in order to identify the molecular mechanism leading to the HDAC7 knockout phenotype, the identification of genetic dysregulation within the endothelial cells would be the appropriate starting point. By analyzing gene expression in cultured endothelial cells following siRNA-mediated knockdown of HDAC7, candidate genes were identified (Chang, Young et al. 2006). Among these, matrix metalloproteinase 10 (MMP10), also referred to as stromelysin 2, was found to be upregulated 6.5 fold, and tissue inhibitor of metalloproteinase 1 (TIMP1) was found to be downregulated 8.6 fold (Table 2.1). These two enzymes were of particular interest because of their ability to act outside the cell in which they originated and thus potentially cause the non-cell autonomous features of the phenotype. Subsequent experiments demonstrated that a loss of HDAC7 inhibition of MEF2 was responsible for the *MMP10* gene upregulation, and that this led to excess MMP10 protein *in vivo.* It was thus concluded that the loss of vascular integrity was secondary to pathological matrix remodeling and a loss of appropriate intercellular interactions and adhesion (Figure 2.4).

Future studies are required to determine the significance of other genes showing differential regulation in endothelial cells upon HDAC7 knockdown. The array data

included multiple genes involved in FGF or retinoic acid signaling (Table 2.2). These observations warrant further investigation, as these pathways are known to be involved in cardiovascular development and vascular remodeling (Yasuda, Nishi et al. 1992; Bohnsack, Lai et al. 2004). Moreover, class II HDACs, including HDAC7, are recruited to transcriptional repressor complexes associated with the nuclear receptor family members retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Kao, Downes et al. 2000). Thus, HDAC7 may regulate multiple transcription factors and gene programs that are important in vascular development and maintenance. Many recent studies regarding the modulation of HDACs as a means of cancer therapy have implicated the effects on tumor angiogenesis as an important mechanistic component of the inhibitors' therapeutic efficacy (Qian, Kato et al. 2006; Mehnert and Kelly 2007). As an HDAC whose functions appear specific to the vascular system, HDAC7 is a unique member of the HDAC family. Thus, further characterization of the molecular pathways surrounding HDAC7 is likely to yield new avenues for targeted therapeutic intervention for vascular disease and tumor angiogenesis.

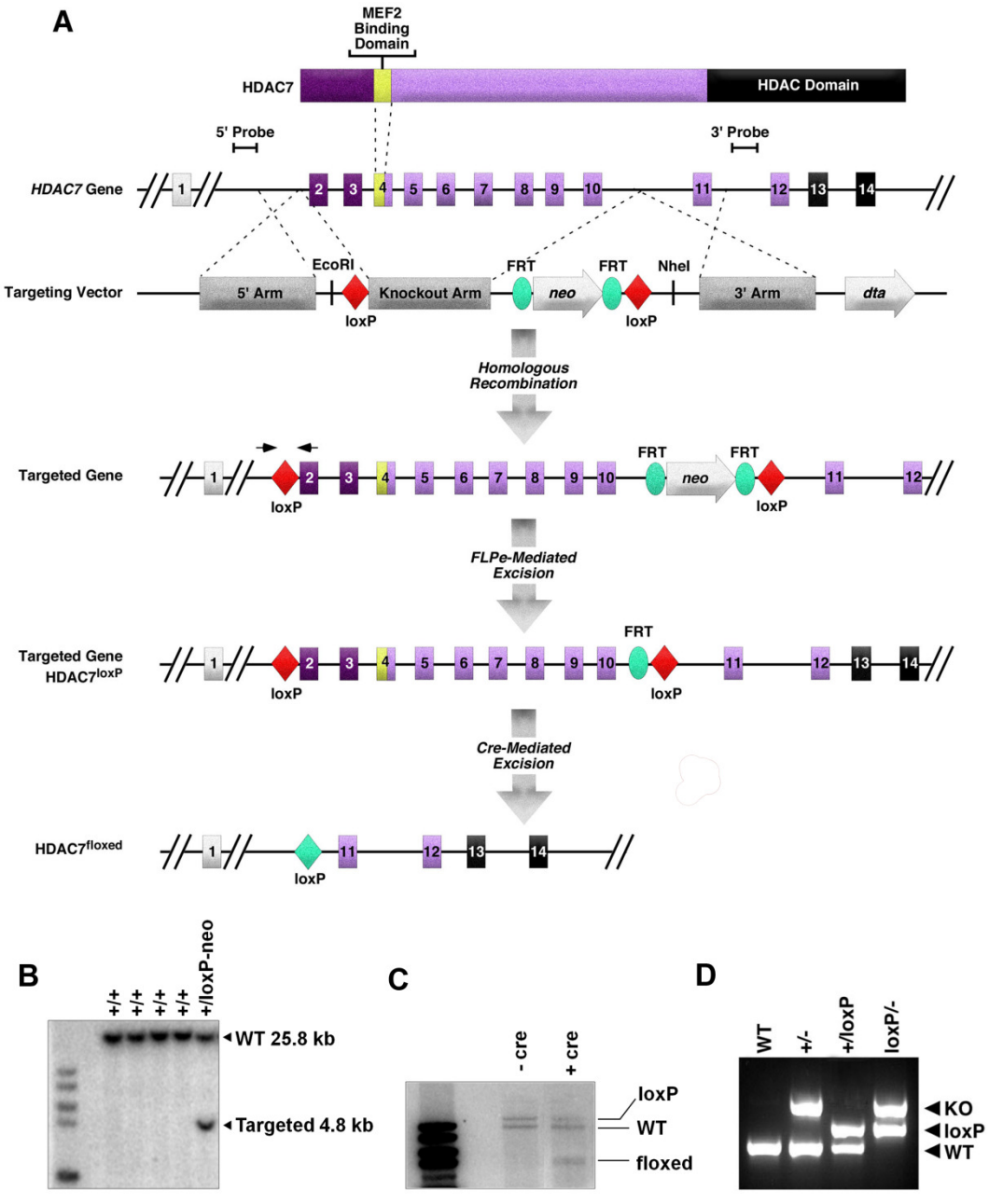

Figure 2.1. Strategy for HDAC7 conditional targeting and recombination. (A) Targeting and recombination strategy to generate a conditional HDAC7 mutant allele. loxP sites were inserted into introns 1 and 10. The structures of the genomic locus, the targeting vector, and the targeted allele are shown. The neomycin resistance cassette, flanked by FRT sites, was removed in the mouse germline by breeding heterozygous mice to hACTB::FLPe transgenic mice. Each conditional deletion of HDAC7 was achieved by breeding to a transgenic mice line harboring a cre transgene. Positions of

probes and restriction sites used for Southern analysis and primers used for PCR (arrowheads) are shown.
(B) Southern blot analysis for targeted ES cells. ES cell DNA was digested with *EcoRI*, and the corresponding wild-type (25.8 kb) and targeted (4.8 kb) bands are indicated for the 5' probe. (C) Southern analysis showing *loxP*-mediated recombination of targeted allele. ES cells were electroporated with PGK-cre plasmid or control. DNA was digested with *XbaI,* and WT (9.6 kb), *loxP* (11.5 kb), and *floxed* (5.1 kb) bands are indicated. (D) Genotyping of HDAC7 mutant mice. Primers flanking the 5'- *loxP* site as well as the 5' end of the *lacZ* insertion generate PCR products indicating WT (400 bp), loxP (500 bp), and KO (640 bp). Lane 1 shows wild-type, lane 2 shows HDAC7$^{+/KO}$, lane 3 shows HDAC7^{loxP}, and lane 4 shows HDAC7$^{loxP/KO}$.

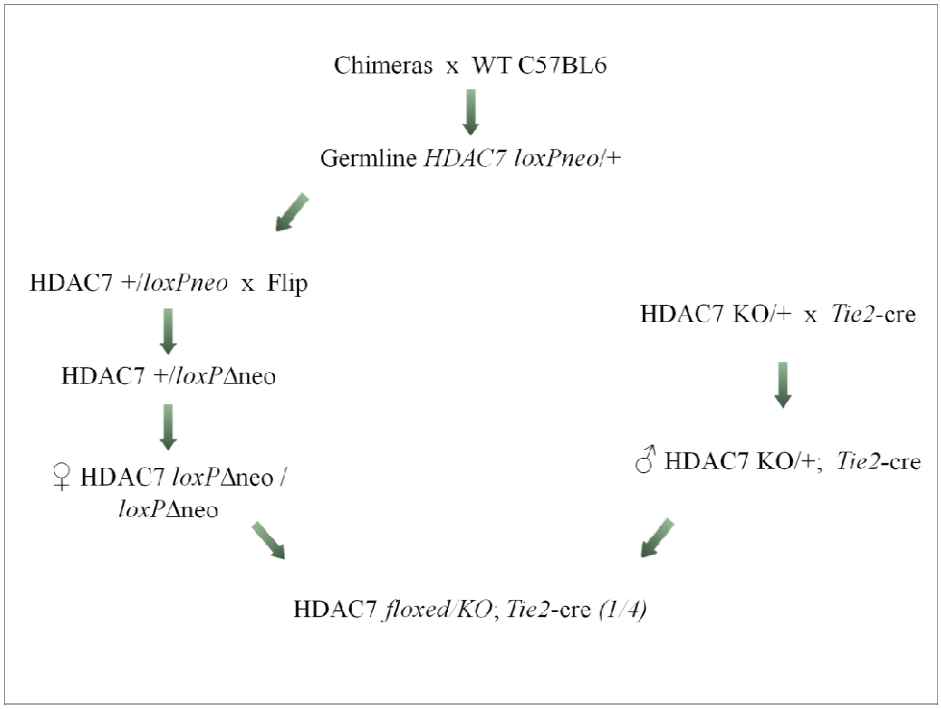

Figure 2.2. Breeding strategy for tissue-specific HDAC7 knockout mice. Endothelial-specific deletion by *Tie2*-cre is shown. The same strategy was used for all cre lines shown in Table 1.1.

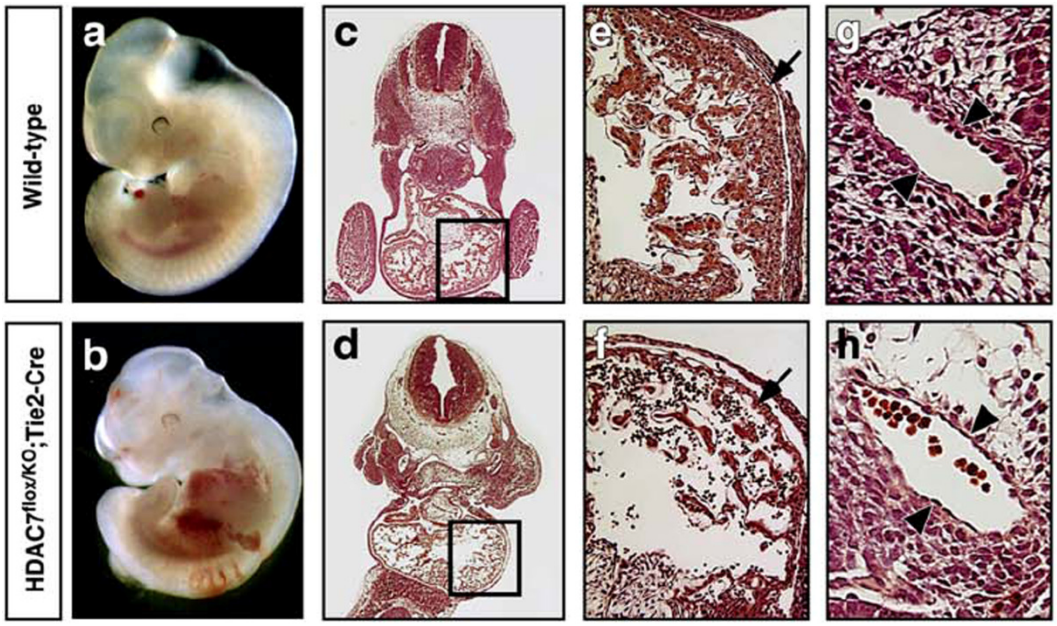

Figure 2.3 HDAC7 endothelial-specific knockout phenotype. Wild-type (top panels) and HDAC7flox/KO; Tie2-cre mutant (bottom panels) embryos at E11.5. Panels (a) and (b) show whole embryos. Diffuse edema around the neural tube and hepatic vascular congestion can be seen in the mutant. Panels (c) and (d) show transverse sections. Boxes show regions of the heart expanded in panels (e) and (f). Arrows point to the myocardial cell layer, which is hypocellular in the mutant. Panels (g) and (h) show dorsal aortae. Discontinuity of the smooth muscle cell layer and endothelial elongation are present in the mutant (arrowheads).

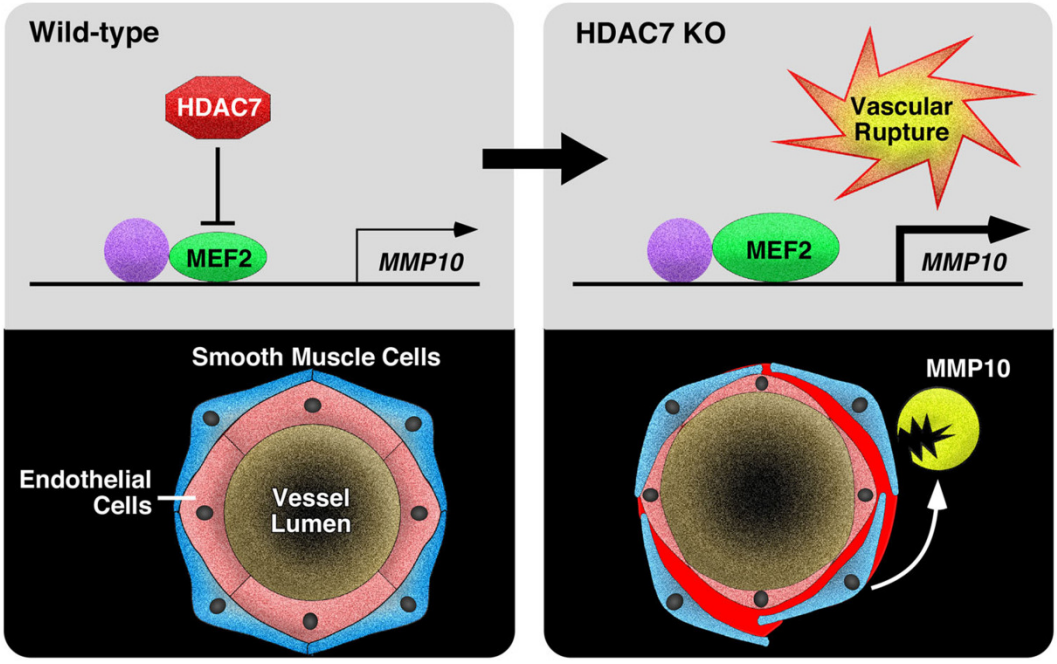

Figure 2.4. Model for modulation of MMPs by HDAC7 through inhibition of MEF2 activity. In endothelial cells, MMP10 expression is controlled by MEF2, which is antagonized by HDAC7. In the absence of HDAC7, MMP10 is overexpressed. Combined with downregulation of TIMP1, the extracellular matrix is greatly disrupted, which leads to dilatation and rupture of blood vessels.

Cre Driver	Expression Pattern	Result
Tie2	**Endothelial Cells**	**Lethal at e11.5**
α-MHC	**Cardiac Myocytes**	**Viable**
Myocardin	**Smooth Muscle**	**Viable**
Wnt1	**Neural Crest**	**Viable**
Myogenin	**Skeletal Muscle**	**Viable**

Table 2.1. Cre recombinase drivers used to delete HDAC7. *Tie2* cre is the only cre driver to result in lethality. All others were found to be viable and indistinguishable from wild type littermates.

Fold change	Gene
48.5	calcineurin-binding protein calsarcin-1
7.0	growth arrest-specific 1 (GAS1)
6.5	matrix metalloproteinase 10 (stromelysin 2) (MMP10)
5.2	histone deacetylase 9 (HDAC9)
4.6	retinol dehydrogenase homolog isoform-1 (RDH)
4.3	retinol dehydrogenase homolog isoform-2 (RDH2)
4.0	sprouty (Drosophila) homolog 1 (antagonist of FGF signaling)
2.8	matrix metalloproteinase 1 (MMP1)
1.9	endothelin receptor type A (ETA)
−22.6	DBL proto-oncogene splicing variant 1 (Ras GEF)
−13.9	5-hydroxytryptamine (serotonin) receptor 2B (HTR2B)
−11.3	retinol-binding protein 4, interstitial (RBP4)
-9.2	retinoic acid receptor responder (RARRES1)
−8.6	tissue inhibitor of metalloproteinase 1 (TIMP1)
-8.6	vitamin D (1,25-dihydroxy vitamin D) receptor (VDR)
−7	claudin 14 (CLDN14)
−6.5	hairy and enhancer of split (Drosophila) homolog 2 (HES2)
−5.7	angiopoietin-related protein (ANGPTL2)
−5.3	fibroblast growth factor 1 (acidic) (FGF1)
−4	retinoic acid receptor, gamma (RARg)

Table 2.2. Genes differentially regulated by HDAC7 knockdown. Representative results from array data are shown. Genes involved in intercellular signaling pathways are highly represented among HDAC7 targets identified by microarray.

Chapter Three

Deletion of HDAC7 in the Adult Mouse

Introduction

The MEF2-dependent reactivation of fetal cardiac genes in response to stress signals leads to cardiac remodeling (Passier, Zeng et al. 2000). The loss of HDAC5 or HDAC9, signal-dependent repressors of MEF2, results in an increase in this hypertrophic response (McKinsey, Zhang et al. 2000) (Zhang, McKinsey et al. 2002). These specific observations in the heart may be taken to suggest a general hypothesis, that class II HDACs are general repressors of stress signals that reactivate fetal gene programs. In the adult organism, this signal responsive activation of developmental genes results in tissue remodeling that may be adaptive or pathological depending on the cell type and the nature of the stress.

While HDAC7 modulates MEF2-dependent gene expression in the developing vascular system, a role for HDAC7 in the physiology of the adult animal remained unidentified. The identification of such roles for HDAC7 *in vivo* required a system whereby the HDAC7 function could be ablated beyond the point of embryonic lethality. The finding that HDAC7 is essential to cardiovascular development led to the hypothesis that the lack of HDAC7 in the adult animal would cause gene dysregulation, and that this would contribute to spontaneous or stress-induced cardiovascular disease.

Methods, Significant Problems Encountered, and Results

A globally-expressed transgene encoding a cre recombinase fused to a modified estrogen receptor has been described (Hayashi and McMahon 2002). This transgene provides the ability to induce recombination in *loxP* targeted gene loci upon the

administration of the estrogen analog, tamoxifen. The cre-estrogen receptor fusion protein is constitutively expressed by a strong promoter, but lacking its ligand, the modified estrogen receptor domain causes the recombinase to remain in the cytoplasm. Thus, unlike *tet*-on and *tet*-off systems previously utilized for inducible cre expression, the tamoxifen-inducible transgene does not depend on the control of transgene expression (Bockamp, Sprengel et al. 2008). This system is designed to provide strong cre activity upon tamoxifen administration while avoiding "leaky" cre activity prior to induction.

To generate HDAC7 adult knockout mice (HDAC7 aKO), HDAC7 loxP/loxP females were bred with HDAC7 loxP/loxP males who were heterozygous for the tamoxifen-inducible cre transgene. Additionally, a smaller number of animals were bred according to the strategy used with the other cre lines described in chapter 2 (Figure 2.2). This set had the benefit of the HDAC7 endothelial lacZ expression, should such staining become useful in later analysis. HDAC7 loxP/loxP and HDAC7 loxP/KO mice with and without the tamoxifen inducible transgenes were allowed to reach at least six weeks of age. During the study, it was found that survival through the tamoxifen administration was better at eight weeks than at six weeks, as initially attempted. Tamoxifen (Sigma) was administered at first by intraperitoneal injection. The protocol was later modified such that tamoxifen was administered by gavage, as this method is believed to achieve superior gene deletion (personal communication from multiple investigators). For both methods of delivery, dose efficacies and tolerances were evaluated for tamoxifen at 250 to 500 mg/kg body weight, dissolved in 100 to 200 μL of 95% sesame oil, 5% ethanol. The mixture was forced through a 0.45um syringe filter, stored at 4C, heated to 55C to resuspend the tamoxifen the day of use, and cooled to approximately room temperature

before injection. Doses were administered daily over five consecutive days or every 48 hours for a total of five injections (Figure 3.1).

Hypertension was induced in HDAC7 adult knockout (aKO) mice by feeding them a high salt (8.0%) diet (Harlan Teklad) as previously described (Yu, Larson et al. 2004). The high salt diet began one week following the last tamoxifen injection in these animals. Animals were maintained on high salt diet for 12 weeks.

Significant morbidity and mortality during the procedure was associated with large subcutaneous axillary masses in both aKO and control animals. Histology of these masses identified them as bacterial abscesses, so organism identification and antibiotic sensitivity testing was performed. Subsequently, animals were given 1 mg/mL doxycycline *ad libitum* via drinking water during the full course of tamoxifen treatment and for one week following the final dose. This procedure eliminated the abscess formation and significantly reduced lethality during the procedure.

In addition to the adult knockout induction at 6 to 8 weeks of age, we attempted to delete HDAC7 in late stage embryos or immediately after birth. Previous studies suggest that gene deletion efficiency with the tamoxifen-inducible cre is higher when induced at earlier timepoints such as in embryos or in nursing pups (Hayashi and McMahon 2002). We administered tamoxifen to pregnant females starting from e13.5 and beyond using protocols previously described (Hayashi and McMahon 2002). Additionally, we delivered tamoxifen to nursing mothers starting at P1 in accordance with established procedures (laboratory of T. Carroll, personal communications). Both of these approaches resulted in loss of all pups due to premature delivery or to failure of the mother to maintain the litter.

For the administration of tamoxifen in adult animals, we empirically identified the optimal protocol with consideration for both tamoxifen tolerance and for HDAC7 deletion efficiency, while being guided by published methods. This was found to be 5 doses of 5 mg of tamoxifen dissolved in 95% sesame oil, 5% ethanol, administered every 48 hours with concurrent administration of 1.0 mg/mL doxycycline in the drinking water. Survival was significantly higher in animals treated at 8 weeks than in animals treated at 6 weeks. The reason for this age-dependent difference in treatment tolerance is unclear, as the tamoxifen dose is adjusted to body weight.

Two weeks following the final tamoxifen injection, gene deletion in genomic DNA from tail biopsies was evaluated by Southern blotting, and deletion of HDAC7 mRNA in a variety of organs was evaluated by rtPCR (Figure 3.2). Cases of lethality were evaluated by necropsy and, in some cases, sectioning and H&E staining of select organs. This protocol consistently achieved approximately 80-90% gene deletion in most tissues, while causing treatment-related lethality at an approximate rate of 10%. Complete deletion of HDAC7 was never observed with any method.

To address the potential for genetic redundancy among the class II HDACs, we generated HDAC7 aKOs on HDAC5 -/- or HDAC9 -/- backgrounds. No phenotype was observed in these animals. However, these mice were only obtained in small numbers due to the number of alleles required, and were not part of the high salt diet group.

In sum, no phenotype was observed in HDAC7 aKOs, either grossly or by histological examination (Figure 3.3). The animals receiving a hypertension-inducing diet were likewise phenotypically unaffected by the loss of HDAC7 (data not shown). In addition, the HDAC7 aKOs are fertile and have normal life spans.

Discussion and Recommendations

There are several possible explanations for the absence of an observed phenotype in the HDAC7 aKO. First, because complete gene deletion was never observed, the remaining HDAC7 transcript may be sufficient for normal gene regulation and to maintain vascular integrity. To address this possibility, future experiments should be directed toward inducing gene deletion at earlier timepoints, as such protocols have been reported to achieve greater recombination efficiency (personal communications from Carroll laboratory, UTSouthwestern; also, (Hayashi and McMahon 2002)). In this study, single-dose tamoxifen administration to pregnant or nursing females resulted in the loss of entire litters, possibly due to the anti-estrogenic activity of tamoxifen, as has been described (Fang, Wong et al. 1996). Previous investigations indicate that further titration of doses and timepoint adjustment may be able to alleviate these effects to a degree that aKOs may be obtained in sufficient numbers (Danielian, Muccino et al. 1998).

An alternative explanation for the apparent lack of defects in HDAC7 aKOs is that other class II HDACs may become functionally redundant with HDAC7 late in embryogenesis and after birth as the specificity of their expression patterns diminish. The expression of HDAC9 was increased 5-fold upon siRNA-mediated knockdown of HDAC7 in HUVECs, suggestive of a compensatory mechanism. Further supporting this hypothesis, HDAC9 is a direct transcriptional target of MEF2 *in vivo* (Haberland, Arnold et al. 2007). If HDAC7 functions primarily through the repression of MEF2 to maintain vascular integrity, the MEF2-mediated activation of HDAC9 could act as a direct mechanism for providing redundant protection against a detrimental excess of MEF2

activity. Studies of cardiac stress signaling provide precedent for functional redundancy among the class II HDACs. Prior studies of HDAC5 and HDAC9 included *in situ* hybridizations at late embryonic timepoints (e13.5 and later) that reveal modest HDAC5 and HDAC9 expression in blood vessels including the endothelial cell layer (unpublished data). Further supporting this possible explanation, the redundancy among class II HDACs has been described in other cell types. HDAC5 and HDAC9 expression overlaps in the myocardium, where they play redundant roles in regulating cardiac development and stress-induced hypertrophy (Chang, McKinsey et al. 2004), and class II HDACs redundantly regulate myofiber isotype switching in skeletal muscle (Potthoff, Wu et al. 2007).

If correct, this class II HDAC redundancy hypothesis may be applied to predict when the loss of a given HDAC will cause developmental defects or disease. Specifically, the deletion of a single class II HDAC will only result in phenotypic changes within developmental windows in which it is the only class II HDAC expressed within a given tissue. For example, HDAC7 KO may be embryonic lethal because there is a time period in which it is the only class II HDAC expressed in the endothelial cells. An experimental approach for characterizing redundant functions of class II HDACs in the endothelium would be to combine multiple KO or cKO HDAC mouse lines using the inducible cre system. Such an approach was successfully undertaken in the previously mentioned study addressing the role of class II HDACs in skeletal muscle fiber type switching.

A third, and non-mutually exclusive explanation for the lack of a phenotype in HDAC7 aKOs is that the adult vasculature no longer exhibits the same changes in gene

regulation in response to HDAC7 loss, or that these genetic changes still occur and fail to cause an observable change in physiology or structure of the vasculature. Several experimental approaches would be able to test these possibilities. Immunohistochemistry of tissue sections or rtPCR analysis of isolated endothelial cells from aKOs could be used to identify the presence of MMP10 or other gene dysregulation at the protein or transcript level. The presence of elevated MMP10 levels, for example, would rule out the hypothesis that genetic redundancy is preventing the loss of vascular integrity. The absence of genetic regulation would neither prove nor rule out the genetic redundancy hypothesis, as signals or activating factors necessary for MMP10 activation may not be present at later timepoints. That is, even in the absence of the repression provided by HDAC7, the molecular conditions may not be present to drive the same genes that were identified as contributing to embryonic demise at e11.5.

Finally, it may be that a phenotype in HDAC7 aKOs will only be observed if the vascular system is stressed or tested in such a way that subtle defects in cell behavior can be identified. Therefore, aKOs will be tested for *in vivo* angiogenesis using the Matrigel (BD Biosciences) implantation model (Senger, Claffey et al. 1997; Watanabe, Hasegawa et al. 2004). In this model, Matrigel infused with heparin and with or without growth factors such as VEGF or FGF is subcutaneously implanted in male aKO mice or control littermates. After 6-7 days, the matrigel plug is removed, sectioned, stained for an endothelial marker, and scored for endothelial cell invasion and tube formation. In this way, the neoangiogenic capabilities of the aKOs vascular network can be assessed. For this assay, tamoxifen must be allowed to clear prior to the assay to avoid the known effects of tamoxifen on angiogenesis (McNamara, Harmey et al. 2001). In the mouse,

orally administered tamoxifen has an elimination ($t_{1/2}$) in the serum of approximately 12 hours, and detailed pharmacodynamic data is available to estimate the elimination times needed depending on dose and method of administration (Robinson *et al.* 1990). While the protocol should be designed to avoid effects of tamoxifen on the assay, treatment of wild type littermates will provide experimental control for this complication.

In conclusion, the hypothesis that HDAC7 is involved in vascular maintenance or disease in adult animals warrants further investigation, as the negative findings of this study remain complicated by inefficiency of gene deletion. Further improvements in the tamoxifen administration protocol are needed to address this issue. Also, further analysis of the vasculature of these mice should be undertaken to identify more subtle defects that are not apparent by the basic examination previously undertaken. This should include Matrigel implantation to assess angiogenesis, as well as sectioning and staining with established vascular markers that have higher sensitivity for vascular defects than the basic histologic analysis used thus far in this study. Finally, the addition of new genetic backgrounds such as the deletion of other class II HDACs may show that genetic redundancy is protecting the mice from the vascular disruption seen in KO embryos. While HDAC7 aKO/HDAC5 KO mice and HDAC7 aKO/HDAC9 KO mice have been generated in small numbers and show no phenotype, further analysis of these animals as well as additional potentially redundant combinations is warranted by other studies demonstrating the complexity of class II HDAC redundancy in other systems.

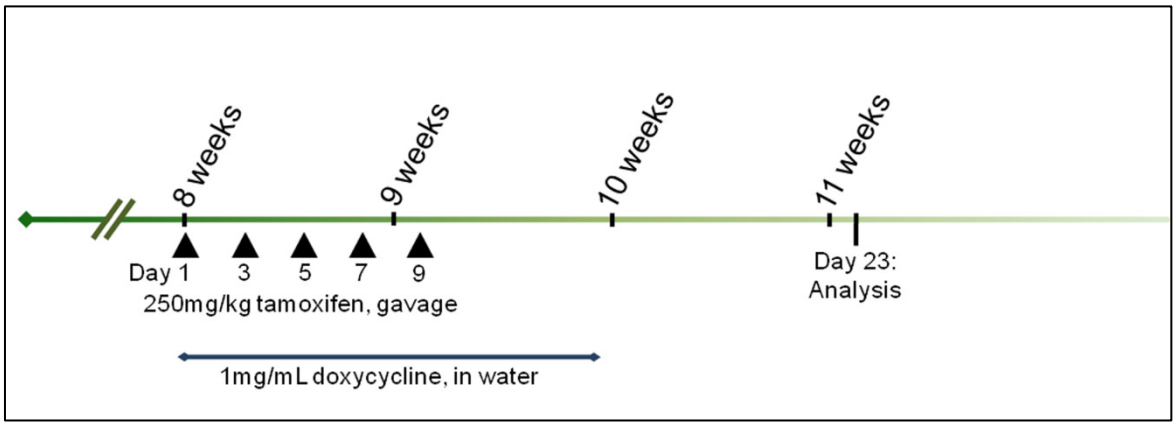

Figure 3.1 Schematic of tamoxifen administration protocol. For some mice, analysis for takeout efficiency and histology was performed on day 23 as shown. The high salt diet group was analyzed at the end of the twelve week diet. Histology was performed for a small group six months after injection. Some animals were not sacrificed, including some of the HDAC5/7 and HDAC7/9 dKOs. All aKO experiment animals surviving the treatment period exhibit normal life spans.

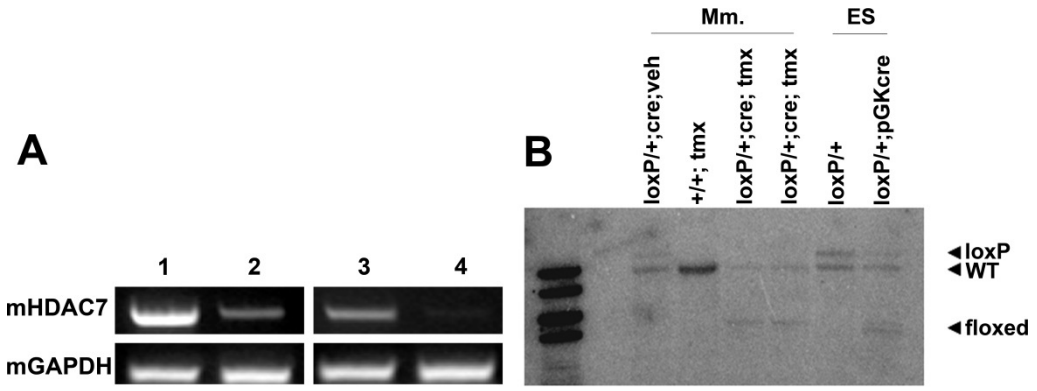

Figure 3.2. HDAC7 deletion efficiency in adult mice. (A) rtPCR showing gene deletion in aKO lungs. RNA was extracted from lung tissue two weeks following the final tamoxifen injection. Mice shown here were administered tamoxifen by gavage starting at six weeks of age. Lanes 1 and 3, loxP/+,tamoxifen; lanes 2 and 4, loxP/-,cre,tamoxifen. HDAC7, 35 cycles; GAPDH, 27 cycles. (B) DNA was extracted from tail biopsies (lane 1-4) or from targeted ES cell clones (lane 5-6) and analyzed by Southern blotting.

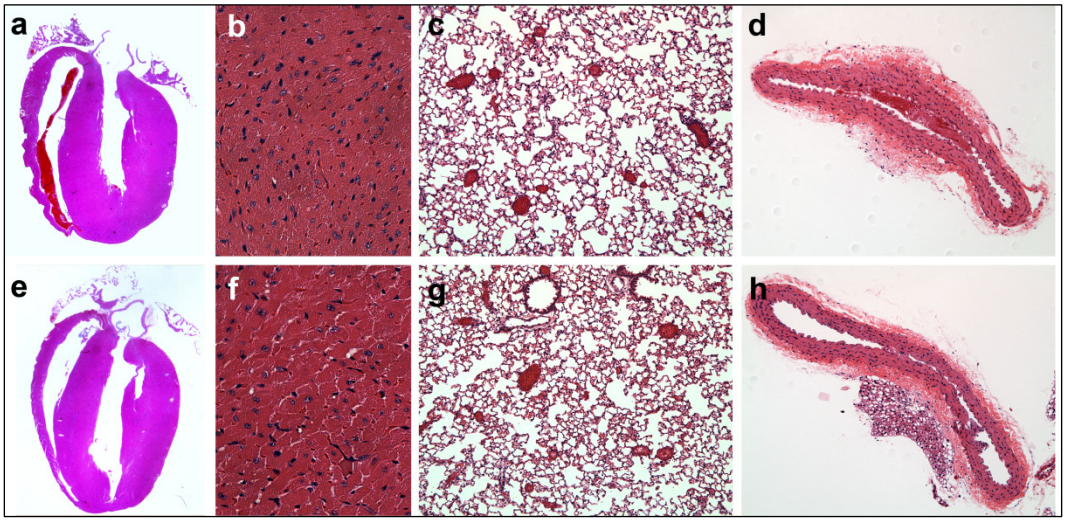

Figure 3.3. Histology in organs of adult knockout mice. (a) Heart, low magnification; (b) heart, high magnification; (c) lung; (d) aorta of wild type littermate control treated with tamoxifen. (e,f,g,h) Same tissues from adult knockout mouse. No differences between WT and aKO were identified. HDAC5/7 and 9/7 dKO, high salt diet mice, and vehicle control mice (sesame oil only) were similarly indistinguishable from WT (data not shown).

Chapter Four

Regulatory Elements Driving Expression of HDAC7 in the Endothelium

Introduction

HDAC7 exhibits one of the most restricted and unique expression patterns of the HDAC family. While the necessity of HDAC7 in endothelial cells has now been demonstrated, the gene regulatory elements responsible for this endothelial-specific expression throughout development and adult life have not been identified. Finding the minimal genomic regions necessary to drive this expression can lead to the identification of the *trans*-acting factors responsible. Knowing these factors is likely to implicate new pathways in which HDAC7 is involved, and will provide new insight into the transcriptional networks regulating vascular development.

HDAC7 Locus Structure

HDAC7 is detected in endothelial cells as early as e9.5, and is present throughout development and the life of the adult mouse (Figure 4.1) (Chang, Young et al. 2006). HDAC7 is expressed in the endothelium of all major vessels and in the microvasculature of the embryo and adult organs. Expression is absent in the yolk sac vasculature. Another report indicates that HDAC7 is also highly expressed in $CD4^+/CD8^+$ T-cells in the thymus (Dequiedt, Kasler et al. 2003). The enhancer elements driving thymocyte expression have not been identified.

The mouse *HDAC7* gene is composed of 23 coding exons spanning 18kb (Figure 4.2). The *HDAC7* locus is flanked by genes that together bound an 89 Kb region. The 3' end of the vitamin D receptor gene (VDR) ends approximately 43 Kb upstream of *HDAC7* exon 2. The cloning and genomic mapping of expressed sequence tags and full length mRNAs from a variety of tissues support the existence of three noncoding exons

within this 43 Kb upstream region. While many reportedly full length mRNAs have been cloned from a variety of tissue sources and deposited into public databases, the characterization of the alternative splicing of the *HDAC7* 5'-UTR has not been investigated. Moreover, the identification of the promoter of *HDAC7* is complicated by the ambiguous location of the first exon. It is conceivable that the alternative 5'-UTRs are transcribed in different cell types in response to different proximal promoters. Thus, the identification of a bias in endothelial cells for a particular alternative exon 1 could provide insight to the enhancer elements responsible for endothelial expression.

Ets Sites in Known Endothelial Enhancers

Ets-family transcription factors are known to directly activate the endothelial expression of many of the early genes critical in endothelial cell development, notably including four endothelial cell-specific tyrosine kinase receptor genes. These four genes include that of the VEGF receptors *Flt1* and *Flk1,* and the angiopoetin receptors *Tie1* and *Tie2* (Dube, Akbarali et al. 1999) (Iljin, Dube et al. 1999) (Kappel, Schlaeger et al. 2000) (Wakiya, Begue et al. 1996). In addition, *MEF2C* expression is activated in endothelial cells *in vivo* by a phylogenetically conserved enhancer containing Ets binding sites (De Val, Anderson et al. 2004). The expression and necessary function of HDAC7 in endothelial cells led to the hypothesis that one of several regions near the *HDAC7* locus that were observed to have conserved Ets binding sites was the endothelial enhancer.

Resources for Enhancer Prediction

Many studies have sought to develop methods of predicting the conserved genomic elements for any particular gene of interest that are *cis*-acting regulatory elements governing expression (Michelson and Kopan 2002). Recently, Hallikas *et al.* developed a computational algorithm, Enhancer Element Locator (EEL), by which comparative genomics and known combinatorial interactions of DNA-binding transcription factors are used to score regions of conservation according to the identity and arrangement of transcription factor binding sites within the region (Hallikas, Palin et al. 2006). Used to supplement a candidate-based approach toward enhancer identification, such software may identify candidate regions or contribute to the prioritization of already recognized enhancer candidates.

Methods

Computational Identification of Putative Enhancers

Enhancer candidate regions were first identified by manually assessing the transcription factor binding sites within regions conserved across multiple species using data from the rVISTA and Evolutionary Conserved Regions (ECR) public database (Loots and Ovcharenko 2004; Ovcharenko, Nobrega et al. 2004). The sequence conservation plots and transcription factor binding site annotation provided by the ECR browser utility guided the final selection of primer location for cloning into *lacZ* constructs.

To further inform the search, 100 Kb regions containing the mouse and human *HDAC7* loci were supplied to the EEL application. Sequence comparison and

transcription factor binding site alignment were performed according to default settings, and binding sites definitions were acquired from the curated JASPAR CORE database (Sandelin, Alkema et al. 2004). Regions of conserved transcription factor binding site clusters are described, scored, and ranked in the report. The locations of the highest scoring regions were added to maps of the *HDAC7* locus so the findings could be integrated with previously identified candidate regions.

5'-RACE

Human aortic endothelial cells (Clonetics) were grown in Endothelial Growth Medium 2 (Lonza), and RNA was harvested prior to their tenth passage using Trizol (Invitrogen) according to the manufacturer's protocol. 5' adapters were ligated to full length mRNAs using the FirstChoice RLM-RACE system according to manufacturer's protocols (Ambion). 5'-RACE was performed using primers within exon 2. More than 40 products were analyzed for size by agarose gel electrophoresis. Ten clones were selected for sequencing, representing all product sizes observed.

Generation *LacZ* Transgenics

Five small regions of interest were initially identified, four of which were supported by high scores from the EEL algorithm. All five regions were separately amplified from 129SvEv genomic DNA from tail biopsies using Expand High Fidelity DNA polymerase (Roche) and each cloned into expression vector containing a *lacZ* cassette driven by the Hsp68 minimal promoter (pGH-Hsp68TATA-*lacZ*). Primers are listed in Table 4.1. Constructs were confirmed by DNA sequencing, linearized with *SalI*

to remove the vector backbone, and gel purified. Transgenic mice were generated by injecting the linearized constructs into the pronuclei of fertilized oocytes as previously described (Cheng, Wallace et al. 1993). F_0 embryos were collected for staining between e11.0 and e11.5.

LacZ Staining

Embryos harvested between e11.0 and e11.5, fixed with 4% paraformaldehyde, 0.2% glutaraldehyde for 60 minutes at 4C. They were then washed three times with cold PBS and then stained with x-gal (1mg/mL) overnight at room temperature.

Results

5'-UTR of HDAC7 in Endothelial Transcripts

5'-RACE using HUVEC-derived cDNA revealed that two variants of the 5'-UTR are present in these cells. Other alternative exons supported by EST evidence were not found in HUVEC transcripts. Approximately half of all traces reading 3' to 5' ended with the first nucleotide of exon 2, the first coding exon. This indicated that the TATA box- and MEF2 site-containing region immediately upstream of this exon was likely the proximal promoter for some of the endothelial HDAC7 transcripts. The other half of the 5'-RACE traces ended within an exon at -33 Kb relative to exon 2 (Figure 4.2).

Putative Enhancer Regions

Regions of interest were identified based on conservation, transcription factor binding sites, and scoring by EEL software. All of the regions are located between *HDAC7* exon 2 and the 3'-UTR of *VDR*. One of the regions is located near the distant 5'-exon identified as part of *HDAC7* transcripts in endothelial cells. Specific locations of the regions tested are annotated in Figure 4.2 and Table 4.1.

β-galactosidase Expression

Yolk sac genotyping confirmed that at least six *lacZ* positive embryos were obtained for each construct injected. Staining was observed in several embryos in varying patterns (data not shown). No single pattern was observed in more than one embryo, and these were attributed to positional effects from the transgene integration sites. No endothelial staining was ever observed.

Discussion and Recommendations

The enhancer candidates tested were unable to drive *lacZ* expression in mouse embryos. One explanation is that the *HDAC7* endothelial enhancer lies in a region not represented in the tested constructs. Alternatively, some combination of regions, from the candidates or otherwise, may be required to drive endothelial expression. Using currently available bioinformatics, it appears unlikely that further pursuit of this candidate approach will be successful for locating *HDAC7* regulatory elements. To continue to address this aim, bacterial artificial chromosome (BAC) clones containing the *HDAC7* locus and large amounts of the surrounding chromosome should be used to create *lacZ* knockin constructs. This approach utilizes homologous recombination in

bacteria to introduce β-galactosidase protein-coding regions at the desired location in the BAC (Copeland, Jenkins et al. 2001). Once a BAC clone knockin is found that drives endothelial *lacZ* expression, the clone is divided into progressively smaller pieces until a minimal enhancer region is identified. In contrast to the candidate region approach, BAC knockins would more easily allow for the location of the enhancer if a combination of relatively distant sites is necessarily for expression, as have been identified in other studies (Zhou and Sigmund 2008). Moreover, this approach can identify cryptic enhancers not suggested by predictive software. Finally, the large size of the BAC clones (>150 Kb) facilitates the more rapid testing of larger areas surrounding the locus.

The necessity of HDAC7 in embryogenesis, as well as studies implicating MEF2C in endothelial transcriptional networks, suggest that identifying the *HDAC7* endothelial enhancer will provide insight into the pathways controlling vasculogenesis. Thus, the pursuit of alternative methods for this enhancer analysis are warranted.

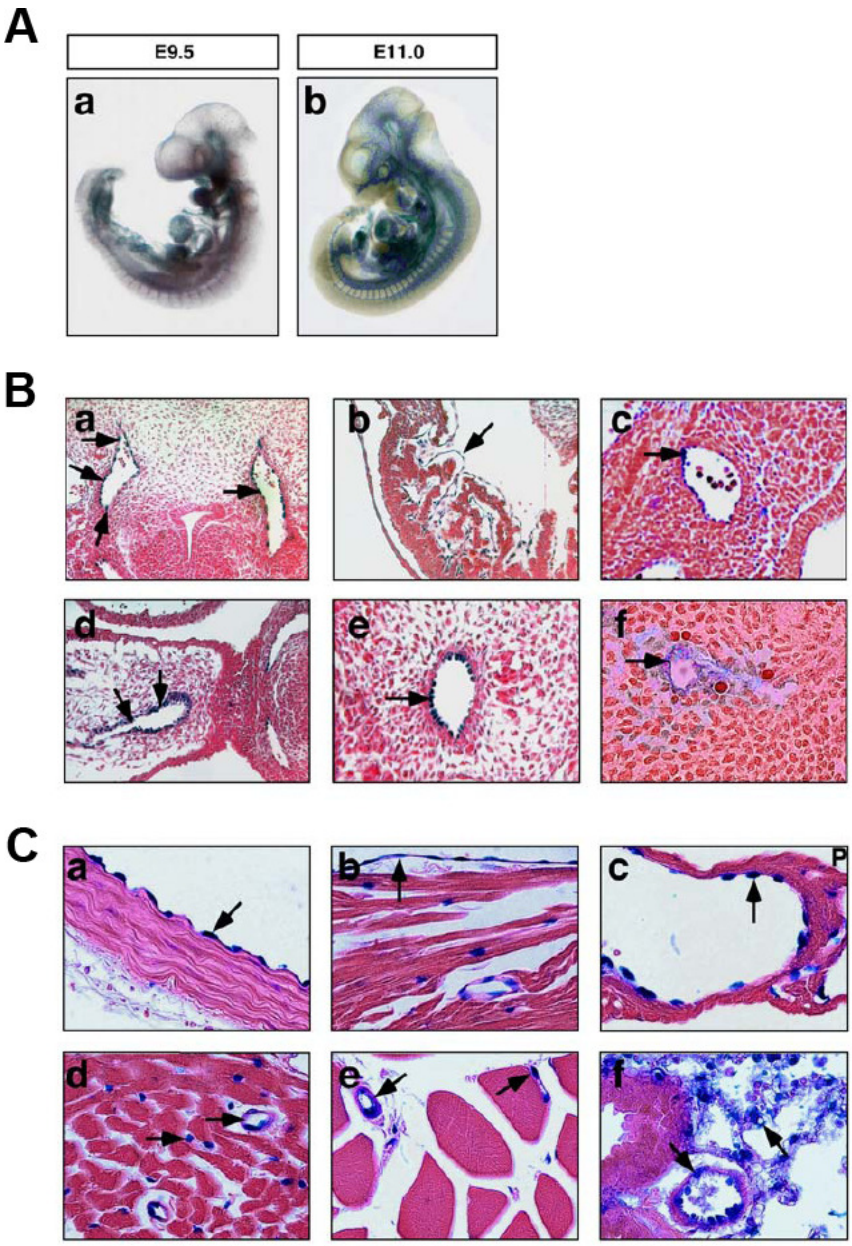

Figure 4.1. Expression pattern of HDAC7 during embryogenesis and adulthood.
(A) Expression of *lacZ* from targeted HDAC7 KO allele. (a), E9.5 and (b), E11.0.
(B) Light eosin staining of histological sections of HDAC7$^{+/KO}$ embryos at E11.0 prestained for β-galactosidase. Arrows point to ECs stained for β-galactosidase activity. Shown are (a) communication between dorsal aorta and branchial arch arteries, (b) the endocardium, (c) the third branchial arch artery, (d) the outflow tract, (e) a small vessel in the head, and (f) an intersomitic vessel.
(C) Expression of *lacZ* from the HDAC7 KO allele in adult tissues. Arrows point to ECs stained for β-galactosidase activity. Shown are (a) the ascending dorsal aorta, (b) the endocardium of ventricle, (c) endocardium of atria, (d) the small vessels and capillaries within the myocardium, (e) small vessels and capillaries within skeletal muscle, and (f) an artery and capillaries of the lung. (Adapted from (Chang, Young et al. 2006))

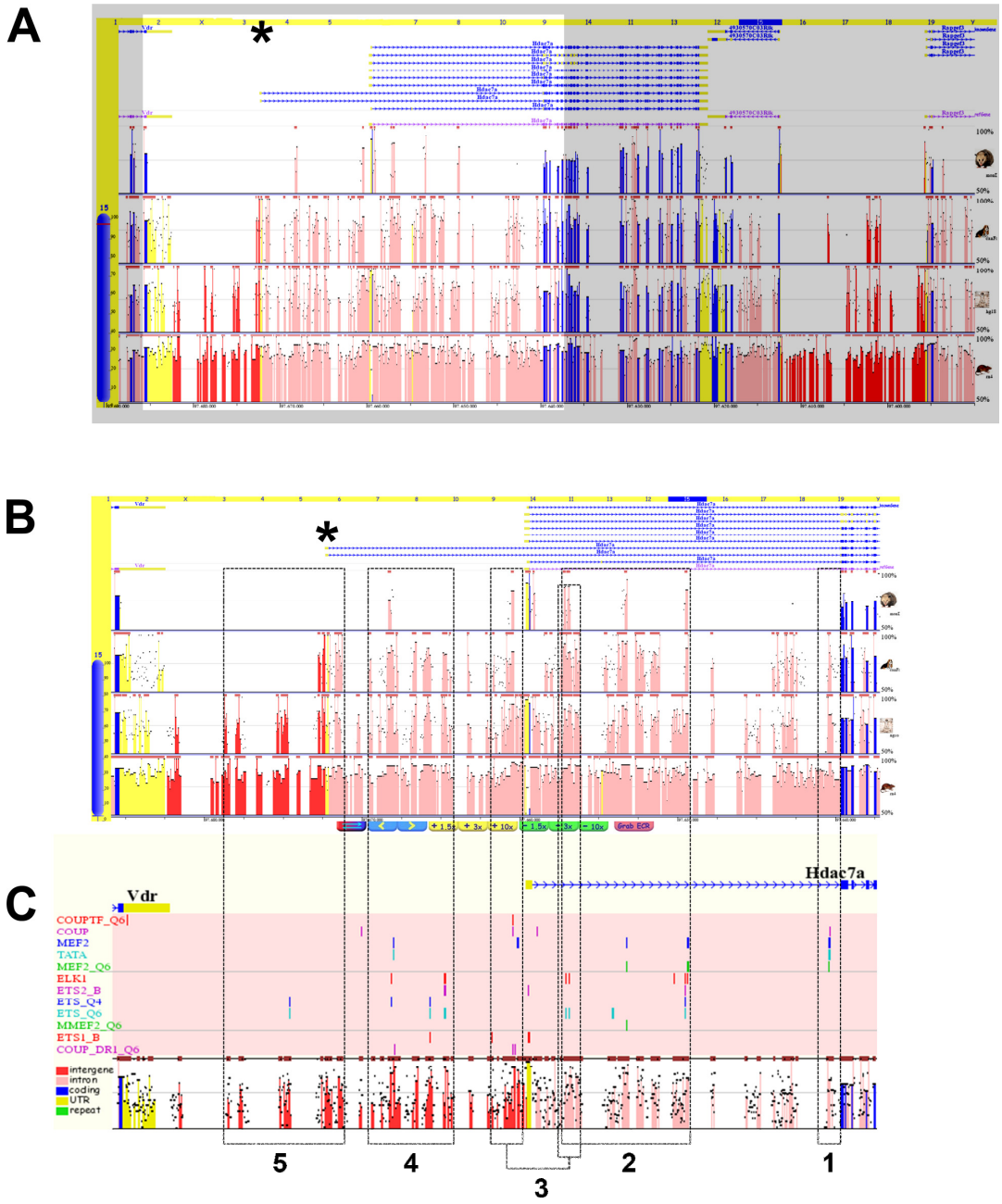

Figure 4.2. 5'RACE results and conservation map of the HDAC7 locus. Maps were generated by ECR browser using mHDAC7a annotation from RefSeq (NM_019572) as the base sequence. Conservation threshold was set at 70% for display. Blue regions are exons, red peaks correspond to conserved intergenic regions, pink to intronic regions, and yellow to UTRs. The asterisks mark the upstream exon detected in endothelial cell transcripts by 5'-RACE. (A) 100 kb map showing *HDAC7* locus in context of nearby genes. Bright box indicates 48 kb area shown in (B) and (C). Species shown (from top to bottom) are possum, dog, human, rat. (B) Boxes indicate regions tested for endothelial *lacZ* expression. Constructs 2 and 3 contained the three highest scoring regions in the EEL analysis. (C) Conserved (mouse to human) transcription factor binding sites predicted by rVISTA.

Construct #	5' end	3' end	5' primer 3' primer
1	-1353	-3	AGTGCTTAGTAACTGGAGGGCCACAG GGAGACACAGCAGAGTCAGGTGGAAG
2	-18156	-10661	CCTGACCTCTGACCTCAGGGCGCTATAG AGGTGGCTCAGCAGTTGGGAGCACTG
3	-21567	-20195	CTTCCGGCTGAGGCTACAGAAATCGTG CTGAGTGCACGCACCCGTGCATACACAG
(3)	-17858	-16743	GTTCCTAGTGGCTCAGTTGTGATG GGCTCACACTAAACAGATGCTAGCCTG
4	-28933	-24505	CTCCAAATTCACGACTTGCATCTTAG GCTCCGAGTCAGATATATTAGGATCAG
5	-41266	-32650	GTCCATGGTCCATGCTGCCACTGAG CAGAAGCTAGAGGGTAGAACAG

Table 4.1. Primers for generating putative enhancers. Each PCR product was cloned into pGH-Hsp68TATA-*lacZ*. Positions of the ends of each tested regions relative to the beginning of the first coding exon of HDAC7, exon 2, are indicated. The primer pair used to clone each region is shown; all primers are written 5' to 3'. As shown in Figure 4.2, construct 3 was a fusion of two separate regions. The area between the two regions contains a repetitive, low-complexity, CG-rich region that could not be amplified by PCR and thus omitted from the construct.

Chapter Five

MEF2-Regulated miRs in the Endothelium

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression by targeted mRNA degredation or inhibition of translation (Bartel 2004) (Ambros 2001). miRNAs may be derived from the introns of protein coding genes, from individual miRNA genes, or from polycistronic transcripts encoding multiple miRNAs (Figure 5.1). They are transcribed by RNA polymerase II, and are processed by Drosha and Dicer into a mature double-stranded miRNA. This duplex then enters the multiprotein RNA-induced silencing complex. As part of this complex, the complimentarity of the miRNA directs either the degredation or translational repression of target mRNAs. Bases 2-8 near the 5' end of the mature miRNA comprise the miRNA "seed". Complimentarity between the seed sequence and conserved sequences within 3'-UTRs is the most critical determinant when predicting miRNA targets (Lewis, Shih et al. 2003).

miRNAs that are generated as bicistronic or polycistronic miRNA transcripts, whether from a dedicated miRNA gene or as an intron of a protein-coding mRNA, are termed a cluster. miRNAs within a single cluster are thus driven by the same *cis*-acting elements or are driven by the regulatory elements of their protein-coding host gene. Thus, members of a cluster often have the same expression profiles (Lau, Lim et al. 2001). miRNAs that share the same number, such as miR-27a and miR-27b, have identical seed sequences and thus share target genes (Lewis, Burge et al. 2005), but are encoded by unique genes and are thus likely to have different expression patterns.

miRNAs perform gene regulatory functions in numerous cell types, including endothelial cells (Kuehbacher, Urbich et al. 2007). miRNAs contribute to endothelial cell activation (Harris, Yamakuchi et al. 2008), response to hypoxia (Fasanaro, D'Alessandra et al. 2008), tumor angiogenesis (Mertens-Talcott, Chintharlapalli et al. 2007), and other processes in the endothelium.

Based on findings that miRNAs regulate changes in expression of cardiac myosin isoforms during cardiac hypertrophy (van Rooij, Sutherland et al. 2007), we hypothesized that miRNAs act in other cell types in MEF2-dependent signaling pathways. Specifically, we envisioned a model in which MEF2 drives the expression of some miRNAs, and that these miRNAs in turn provide negative feedback to help govern MEF2-mediated gene expression (Figure 5.2)

Methods

RNA Isolation and Microarray

HUVECs (ATCC) were grown in 100mm dishes to approximately 50% confluency in Endothelial Growth Medium 2 (Lonza). Media was replaced, and they were then infected with adenoviruses (MOI 100) expressing MEF2-VP16, MEF2-engrailed, or *lacZ* as a control. After 48 hours, cells were washed twice with PBS, and RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions, with the addition of an isopropanol precipitation performed overnight at -80C, which reportedly increases small RNA recovery. Total RNA was sent for miRNA array (LC Sciences, Houston, TX). 503 array spots of approximately 3000 on the miRNA chips detected transcripts. These results were analyzed by pairwise sample comparison (control vs. MEF2VP16; control vs. MEF2ENGR; MEF2VP16 vs. MEF2ENGR).

Quantitative Real Time PCR

Microarray findings were verified by real time PCR using TaqMan probes (Applied Biosystems) for the miRNAs of interest. RNA from replicate HUVEC

adenoviral infections were used as template. Reactions were performed in triplicate. Samples were normalized using the control RNA probe RNU6B.

Luciferase Assay

Genomic regions containing human miR-27b and the miR-23b/24-1/27b cluster were amplified by PCR and ligated into a pCMV6 expression vector. The reporter constructs were generated by ligating the WT or seed sequence mutant form of the entire 3'-UTR of hMEF2C into the firefly luciferase (*f-luc*) reporter construct pMIR-REPORT (Ambion). The constructs were transfected into COS cells using Fugene 6. Luciferase activity was assayed according to standard protocols.

Electrophoretic Mobility Shift Assay (EMSA)

Gel shift assy was performed as previously described (Gossett, Kelvin et al. 1989). Probes corresponding to the putative MEF2 binding sites of the *APO* gene alternative start sites were tested for MEF2 binding. The MEF2 site from the *MCK* enhancer was used as a positive control for MEF2 binding (Cserjesi, Lilly et al. 1994). For the upstream *APO* promoter, probe sequences were: 5'- AGTTCTCTTTCCCT TTAAAATTAAGCAGCTCAGAATAA -3'; with MEF2 site mutated, 5'-AAGTTCTCT TTCCCTTTAcgcgTAAGCAGCTCAGAA TAA -3'. For the downstream *APO* promoter (more proximal to the miR-27b cluster), probes sequences were: 5'- GGGGTGAGG TGATG GCTATTAATATTTAATTTCATATAC -3'; with MEF2 site mutated, 5'- GGGTGAG GTGATGGCTAcgcgTATTTAATTTCATATAC -3'.

Results

MEF2-Regulated miRNAs Identified in Transfected Endothelial Cells by miRNA Microarray

A representative portion of the results of the miRNA array are shown in Table 5.1. The reliable data points on the array did not show any miRNAs to be regulated more than 2 fold. Despite the unremarkable amplitude of the expression changes, the array data was considered reliable for several reasons. First, the results were highly consistent between array chip spots representing identical miRNA sequences, such as miRNAs completely conserved across species (Table 5.2). Second, it was found that the absolute detection values for miRNAs on the control array correlated with a previous report that identified the most highly expressed miRNAs in endothelial cells (Kuehbacher, Urbich et al. 2007).

Realtime PCR Verification of Microarray Results

Realtime PCR confirmed that miR-16, miR-23a, miR-23a, miR-27a, and miR-27b were upregulated by MEF2-VP16 (Figure 5.3). Upregulation varied from 1.5 fold for miR-16, to 2.7 fold for miR-27b. The fold changes corresponded to those seen in the array. Also as seen in the results of the array, MEF2-engrailed failed to induce significant changes in expression.

MEF2 Binds and Activates a Promoter of the Gene Coexpressed with miR-23b and miR-27b

MEF2 binding sites were identified upstream of two of the transcription start sites of the C9ORF3 (aminopeptidase O) locus. MEF2 binding of these putative

promoter sequences was demonstrated by EMSA, and MEF2 activates this promoter to drive a luciferase reporter (Figure 5.5).

miR-27 Suppression of MEF2C Expression by Targeting MEF2C 3'-UTR is Weak and Not Dependent on miR27 Seed Recognition Site

A miR-27b expression construct repressed the MEF2C-3'-UTR-luciferase reporter in transfected COS cells (Figure 5.6). However, mutations in the seed recognition sequence in the 3'-UTR failed to abrogate the repression. As the mutations completely disrupt the complimentarity between the seed sequence and the recognition sequence, miR-27b is not repressing the reporter through direct targeting of the UTR. It is unclear what indirect or possibly nonspecific mechanism is responsible for the observed reporter repression.

Discussion and Future Directions

The microarray results, quantitative PCR results, and luciferase data showing the MEF2-dependent regulation of miRs appears to be reliable, but the magnitude of the observed regulation was unremarkable. The MEF2-VP16 fusion protein is a potent activator expected to cause more substantial upregulation of targets. In addition, the repression of the MEF2C 3'-UTR reporter construct was found to be nonspecific.

Preliminary work was done to more conclusively demonstrate the regulation of the miR-23/27 clusters, as well as to acquire another RNA source for more definitive microarray data. Experiments were considered to address the possibility that the changes in miRNAs were muted due to low cell infection efficiency or the relatively high stability of miRNAs over short timeframes such as the 48 hour conditions of the HUVEC array experiment. We hypothesized that an *in vivo* model could circumvent these issues by

expressing the transgenes over a longer timeframe and eliminating the technical issue of cultured cell transfection efficiency. Two transgenic mice expressing a cardiac-specific MEF2D transgene were available, providing a means of identifying MEF2-regulated miRNAs in another cell type. While this approach was complicated by transgenes that either failed to express or failed to activate known MEF2 target genes, future efforts should focus on developing such an approach. While this approach does not directly address endothelial cell biology, it is an immediately accessible means of advancing the understanding of the interaction between miRNAs and MEF2. Further, experiments could be done to find MEF2-regulated endothelial miRNAs *in vivo,* requiring only the additional step of endothelial cell isolation, such as by cell sorting.

The preliminary finding that the miR-23a/-27a and miR-23b/27b clusters are regulated by MEF2 has significant implications. The importance of MEF2C in vascular development has been described (Lin, Lu et al. 1998), and is further confirmed in this study by the analysis of the HDAC7 knockout mice. miR-27a is expressed in tumor cell lines and targets repressors of pro-growth and pro-angiogenic factors (Figure 5.4) (Mertens-Talcott, Chintharlapalli et al. 2007). By this mechanism, the expression of miR-27a increases the expression of vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGR1), and *survivin* in these cell lines. The identification of crosstalk between miRNA- and MEF2-dependent pathways would likely reveal new mechanisms for the regulation of vasculogenesis and tumor angiogenesis.

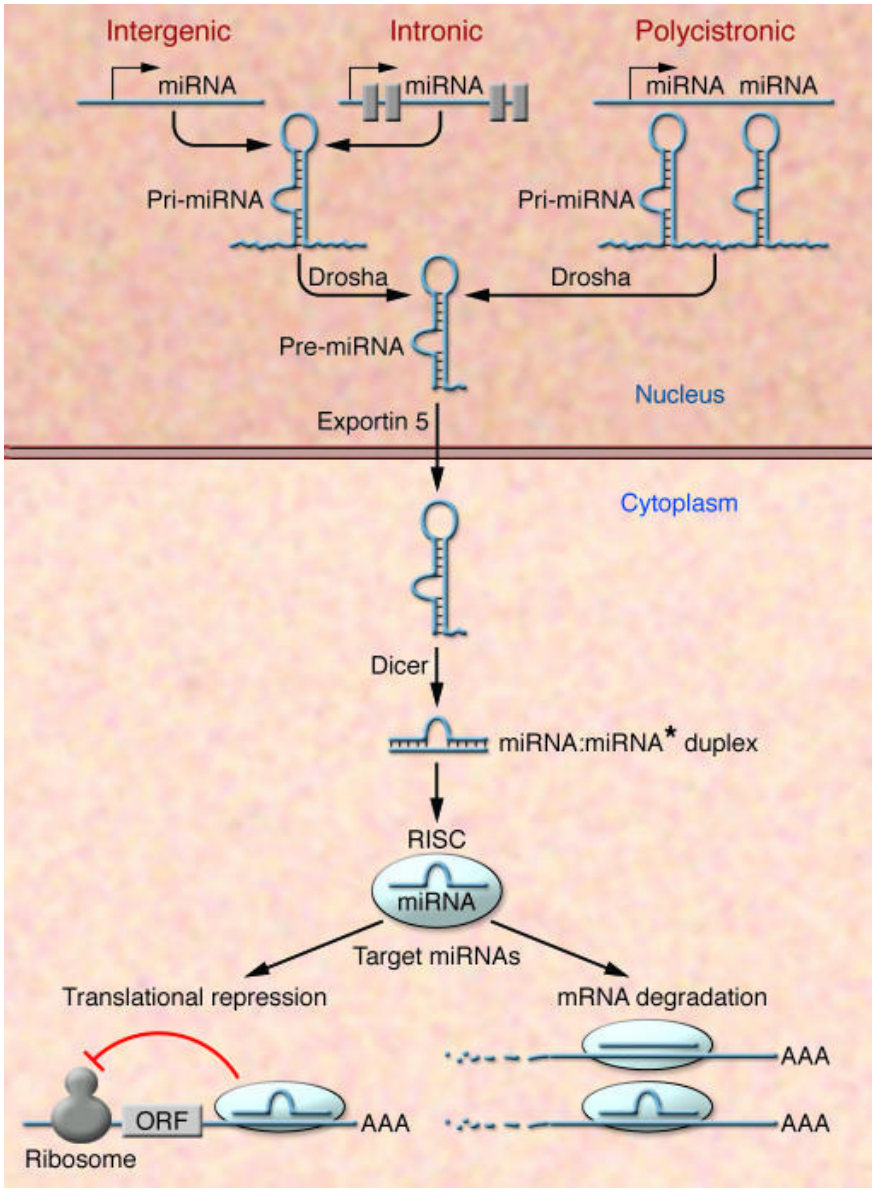

Figure 5.1. Biogenesis and activity of miRNAs. miRNAs are derived from single miRNAs genes, from introns of protein-coding mRNAs, or from polycistronic miRNA genes. From (van Rooij and Olson 2007). Used with permission.

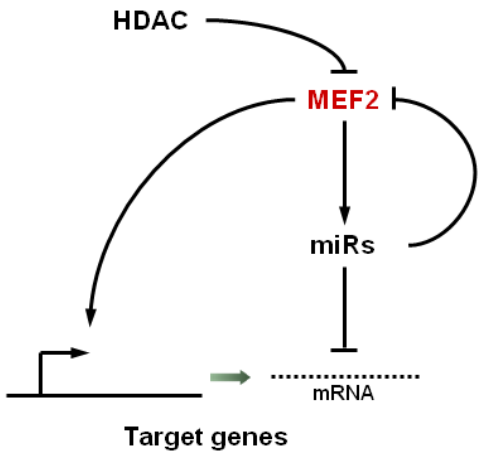

Figure 5.2. Hypothetical model for MEF2/miRNA gene regulatory networks. Members of the myocytes enhancer factor 2 (MEF2) family of transcriptional activators drive the expression of miRNAs (miRs). These miRNAs cause the compliment-mediated degradation or translational inhibition of mRNAs from MEF2 target genes, thus providing direct negative feedback. These miRNAs may also provide negative feedback by targeting the 3'-UTR of MEF2 mRNAs. The negative regulation of MEF2 by HDACs is also depicted.

A

Reporter Name	p-value	Ctrl Mean	StDev	VP16 Mean	StDev	Log2	Fold Change
ppa-miR-23b	2.20E-03	21,008	347	24,670	560	0.23	1.17
dre-miR-23b	7.99E-03	18,532	467	22,107	910	0.25	1.19
dre-miR-27b	8.65E-03	6,711	69	9,501	533	0.5	1.42
hsa-miR-27b	7.69E-03	5,360	99	7,879	432	0.56	1.47
hsa-let-7f	5.34E-03	16,157	155	20,118	525	0.32	1.25
mmu-let-7f	3.52E-03	13,472	420	18,669	232	0.47	1.39
bta-let-7g	8.80E-03	6,243	294	7,991	410	0.36	1.28
hsa-let-7g	5.13E-03	5,277	79	7,010	227	0.41	1.33
lca-miR-16	3.12E-03	927	44	1,433	104	0.63	1.55
xtr-miR-16b	4.47E-03	904	64	1,355	74	0.58	1.50

B

Reporter Name	p-value	Ctrl Mean	StDev	ENGR Mean	StDev	Log2	Fold Change
ppa-miR-23b	7.88E-04	21,008	347	16,143	458	-0.38	-1.30
dre-miR-23b	5.80E-03	18,532	467	15,577	533	-0.25	-1.19
hsa-miR-31	1.43E-03	15,588	344	11,808	423	-0.4	-1.32
mmu-miR-31	3.65E-03	15,887	934	11,496	379	-0.47	-1.38
gga-miR-31	5.47E-04	14,338	322	10,959	205	-0.39	-1.31
hsa-miR-27a	7.86E-03	13,427	313	9,376	469	-0.52	-1.43
dre-miR-27a	1.20E-03	16,444	407	10,518	109	-0.64	-1.56
bta-miR-27a	4.56E-03	15,965	249	11,769	377	-0.44	-1.36
bta-miR-16	3.72E-03	5,363	185	4,433	86	-0.27	-1.21
hsa-miR-16	2.14E-03	6,120	196	4,733	147	-0.37	-1.29

Table 5.1. Representative results demonstrating consistency within miRNA microarray data. (A) Data from comparison of MEF2-VP16 to control. (B) Data from comparison of MEF2-Engrailed to control. Orthologous miRs are highly similar in sequence. This representative data demonstrates the consistency in the array signals for a given miR across species.

		Group A	Group B	Group C
Reporter Name	p-value	Mean	Mean	Mean
hsa-miR-654	3.17E-10	174	975	15,794
mmu-miR-689	2.60E-08	4,131	1,626	5,531
mmu-miR-705	4.87E-08	9,262	4,438	8,424
hsa-miR-612	8.32E-08	344	403	87
hsa-miR-154*	1.16E-07	134	311	91
dre-miR-739	1.45E-07	13,152	3,985	11,933
mmu-miR-714	1.60E-07	740	156	285
ggo-miR-27a	1.85E-07	13,457	14,527	8,277
hsa-miR-768-5p	2.20E-07	603	549	1,436
dre-miR-27b	2.54E-07	6,711	9,501	4,110
mmu-miR-762	2.78E-07	32,054	18,033	43,599
bta-miR-139	4.16E-07	3,992	2,461	4,433
hsa-miR-638	4.32E-07	66,324	34,955	77,653
hsa-miR-19b	4.50E-07	1,427	1,258	957
dre-miR-21	5.90E-07	28,024	43,415	64,388
hsa-miR-132	6.43E-07	164	399	247
hsa-miR-29b	6.79E-07	362	688	101
gga-miR-199*	8.86E-07	396	428	797
bta-miR-21	9.08E-07	39,988	45,283	83,398
rno-miR-422b	1.12E-06	260	118	366
ggo-miR-26a	1.15E-06	11,540	12,704	6,585
hsa-miR-103	1.17E-06	9,502	5,967	6,935
bta-miR-107	1.36E-06	10,624	7,342	7,963
hsa-miR-22	1.44E-06	7,714	6,008	4,395
ppa-miR-23b	1.47E-06	21,008	24,670	16,143
bta-miR-151*	2.23E-06	9,057	8,274	13,151
mmu-let-7a	2.40E-06	18,658	23,023	16,624
bta-miR-186	2.73E-06	485	574	213
mmu-let-7f	2.79E-06	13,472	18,669	14,852
mmu-miR-127	2.86E-06	4,728	3,895	5,163
hsa-miR-663	2.92E-06	27,567	16,531	35,165
gga-miR-31	3.28E-06	14,338	15,702	10,959
mmu-miR-134	3.76E-06	2,244	1,829	3,390
bta-miR-30d	4.01E-06	3,907	3,308	2,197
bta-miR-214	4.25E-06	439	392	767
dre-miR-27e	4.26E-06	5,745	8,546	3,967
hsa-miR-768-3p	4.68E-06	313	444	1,131
dre-miR-16b	4.77E-06	595	1,009	351
rno-miR-151*	4.97E-06	8,524	6,856	12,165
hsa-miR-365	5.57E-06	1,488	3,060	1,567
mmu-miR-744	6.08E-06	17,071	9,565	10,861
hsa-miR-155	6.79E-06	2,423	2,435	4,368
dre-miR-27d	6.84E-06	4,839	7,214	3,057
bta-miR-423	7.11E-06	1,197	563	304
hsa-miR-27b	7.52E-06	5,360	7,879	3,796

Table 5.2. miRNAs showing most significant fold changes on microarray. Data was sorted by p-value. Group A is control; Group B is MEF-VP16; Group C is MEF2-Engrailed. Asterisks indicate miRNA "star" sequences that are derived from the same hairpin structure but differ in sequence from the mature miRNA of the same number.

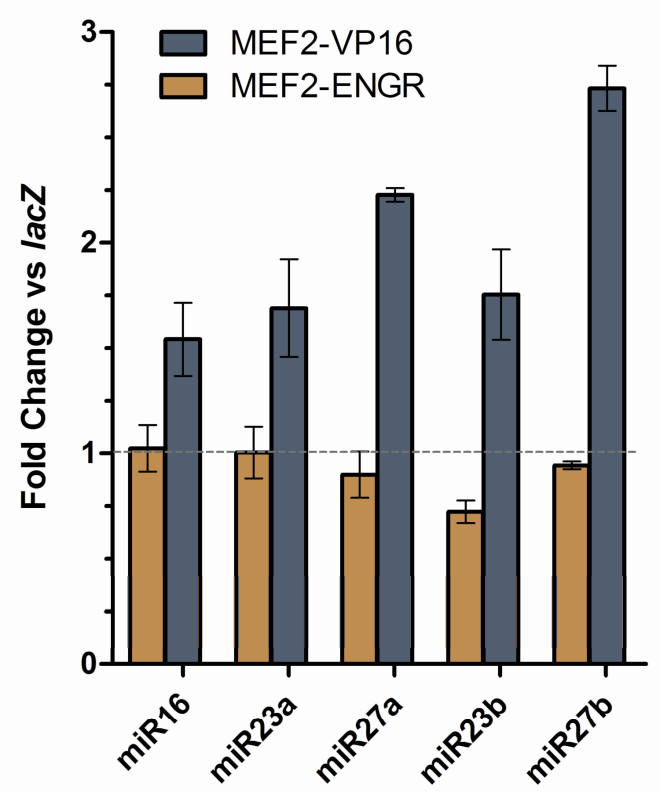

Figure 5.3. Realtime PCR confirms regulation of miRNAs by MEF2. HUVECs were infected adenovirus carrying expression vectors for MEF2-VP16 fusion protein, MEF2-engrailed fusion protein, or β-galactosidase protein as a control. RNA input was normalized to hRNU6B. Fold change shown relative to control-infected cells. Error bars represent standard deviation. As was observed in the array, MEF2-engrailed failed to achieve statistically significant changes relative to control.

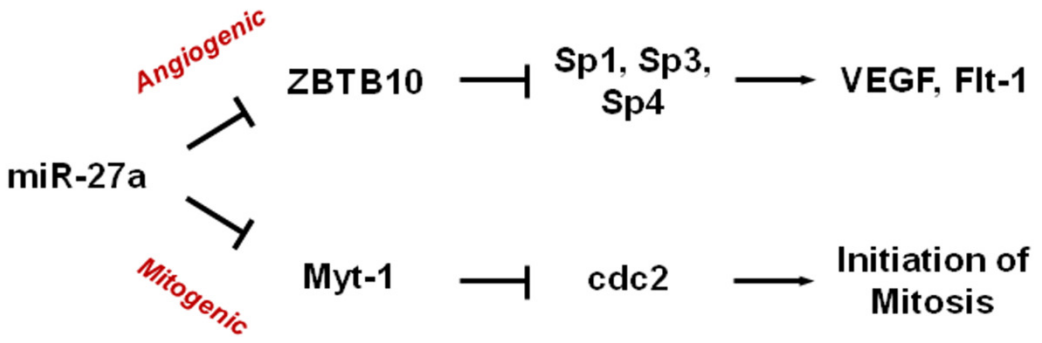

Figure 5.4. Model for pro-growth and pro-angiogenic activity of miR-27a. ZBTB10 and Myt-1 are targeted by miR-27a. ZBTB10 suppresses Sp1, Sp3, and Sp4, which promote the expression of the pro-angiogenic VEGF and the VEGF receptor 1 (VEGFR-1), or Flt-1. miR-27a also targets Myt-1, which represses cdc2 by inducing its phosphorylation. By mediating cdc2 release from Myt-1 repression, miR-27a thus promotes progression past the G2-M checkpoint.

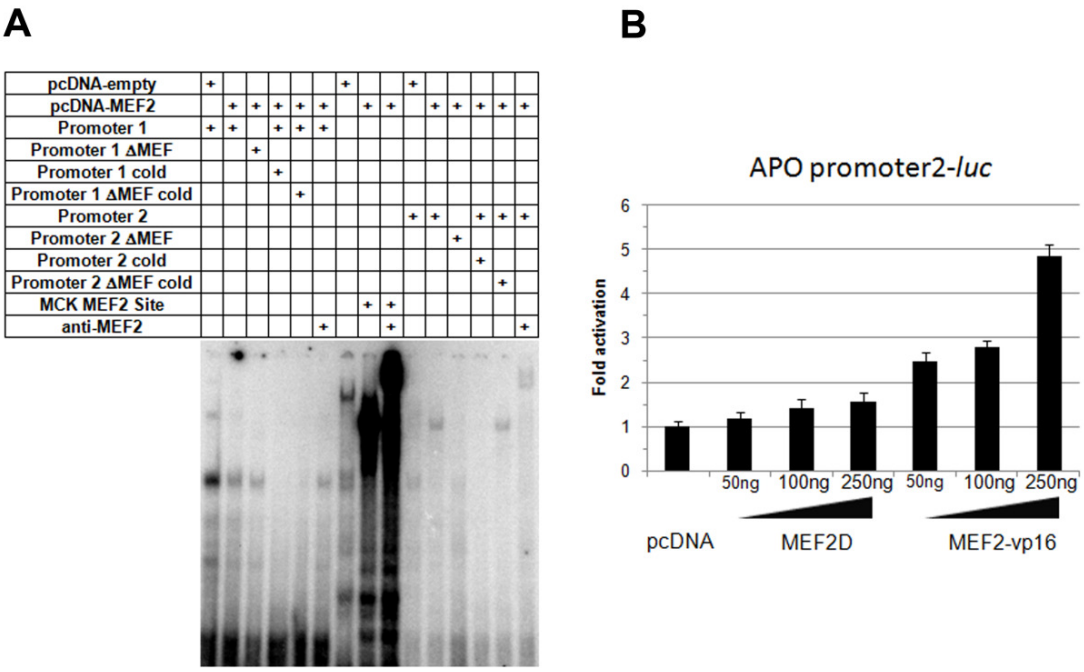

Figure 5.5. MEF2 can bind and activate a promoter driving miR-27b. The miR-23b/24-1/27b cluster is located in an intron and is coexpressed with the gene *C9ORF3* (data not shown). *C9ORF3* encodes aminopeptidase O (APO). APO genes have putative proximal promoters 83kb ("Promoter 1") and 25kb ("Promoter 2") upstream of the miRNA cluster. These putative promoters have conserved MEF2 biding sites. (A) MEF2 can bind to the Promoter 2 MEF2 site. "Cold" indicates a non-radiolabeled probe. ΔMEF indicates a MEF2 site mutation that disrupts MEF2 binding. MCK is a gene with a well-characterized MEF2-responsive promoter. (B) MEF2D activates a Promoter 2 luciferase reporter in COS cells.

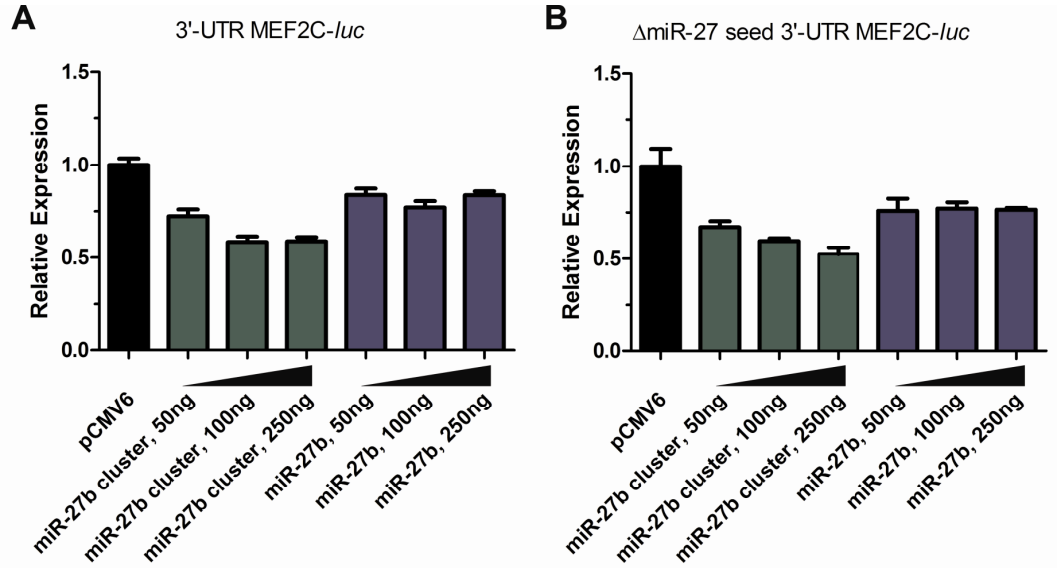

Figure 5.6. Repression of MEF2C by miR-27b cluster in cell transfection experiment. (A) miR-27b and the miR-27b/23b/24-1 cluster modestly represses MEF2C 3'-UTR-luciferase reporter expression in COS cells. (B) Mutation of the miR-27 seed sequence did not affect the repression, suggesting a nonspecific or indirect effect.

Chapter Six

Conclusions and Future Perspectives

Chromatin modifying enzymes hold great promise as therapeutic targets. HDAC inhibitors have already been approved for the treatment of some cancers, and clinical trials are ongoing. However, there is still great demand for further advances in understanding the function and regulation of the HDACs. Even as these first drugs enter the clinical arena, we are just beginning to uncover the unique biological functions of the individual members of the HDAC family.

HDACs are recruited to numerous transcription factor complexes to directly and indirectly regulate thousands of genes in response to both known and still unidentified cellular signals. Target specificity for HDAC inhibitors remains a challenge, as the eleven members of the non-sirtuin HDAC subfamilies share highly similar catalytic domains. In addition, the class I HDACs are expressed ubiquitously, allowing them potential roles in an endless array of cell types.

While HDAC inhibitors to date appear to act primarily through the more catalytically active class I HDACs, the signaling pathways involving the class II HDACs may be targets of future drug design. The class II HDACs have limited expression patterns, and each appears to have more limited and unique biological functions; HDACs 5 and 9 act together to control cardiac hypertrophy, HDAC4 regulates bone development, and HDAC7 regulates vascular development. If there is a common theme among their biological roles, it may be that they all repress a stress-induced recapitulation of developmental gene expression that centers around the MEF2 transcription factors. Just as MEF2 drives pathological hypertrophic gene expression in the heart, so too might MEF2 and other HDAC targets drive undesirable gene expression in other tissues and

diseases. The ability to modulate such stress-dependent gene expression thus depends upon our understanding of the signaling pathways that govern the expression and post-translational control of the class II HDACs.

HDAC7 is an endothelial-specific factor that regulates vascular integrity

HDAC inhibitors are known to block signaling that is required for tumor angiogenesis. Interestingly, HDAC7 has yet to be implicated as a target mediating these effects. Being expressed specifically in the vascular endothelium, HDAC7 holds promise as a new highly specific target for anti-angiogenic strategies or for the treatment of other vascular disease.

Endothelial cell-specific HDAC7 knockout embryos experience a multifaceted failure of cardiovascular development. The phenotype includes a thin myocardium, thin vascular smooth muscle layer, vascular dilation and rupture, and pericardial effusion. Endothelial deletion recapitulated every feature of the phenotype of the global knockout, while deletion in cardiac myocytes, smooth muscle cells, and other tissues failed to show any defects. Thus, HDAC7 acts within endothelial cells to allow normal cardiovascular development. This finding facilitated, and should continue to facilitate, investigation of the molecular mechanisms whereby HDAC7 affects vascular development and physiology.

The function of HDAC7 in the adult organism

Work was undertaken to extend the knowledge of HDAC7 in the vasculature beyond the point of embryonic demise. HDAC7 regulates a MEF2-dependent pathway to maintain the integrity of the vessels. Previous studies of class II HDACs and MEF2 focused on the heart, where HDACs similarly repress a MEF2-dependent gene program. A loss of this repression results in cardiac pathology. Thus, we hypothesized that in an adult mouse, loss of HDAC7 may result in vascular pathology.

Deletion of HDAC7 in the adult was performed using the conditional allele and a globally expressed tamoxifen-inducible cre transgene. These studies are ongoing, but no pathology has yet been identified in these knockouts. This may be due to failure to achieve total deletion, to genetic redundancy among the class II HDACs during adulthood, to a different molecular or physiological environment in the adult that no longer requires the function of HDAC7, or to a lack of appropriate stress necessary to elicit a phenotype. Further experiments are needed to address each of these possibilities. An HDAC7 adult knockout mouse would greatly facilitate the investigation of HDAC7 in the physiology and pathology of the adult organism. This study contains significant advances in the methods necessary to develop such a model.

The HDAC7 endothelial enhancer

Characterizing the regulatory elements of a particular gene provides new insights into the nature molecular pathways in which the gene plays a part. HDAC7 is expressed in an intriguingly restricted pattern throughout development and adulthood. This specificity is in contrast to the ubiquitous expression of class I HDACs, and is even more

dramatic than the restricted expression profiles of other class II HDACs. The regulatory elements responsible for the endothelial-specific expression of HDAC7 are unknown. This study identified highly conserved clusters of transcription factor binding sites near the HDAC7 locus that were reminiscent of the enhancers of other endothelial-expressed genes. These regions, however, were unable to drive endothelial expression of β-galactosidase *in vivo.* These studies serve as a starting point for future searches for the HDAC7 enhancer, and alternative approaches that enable high-throughput evaluation of the genomic landscape surrounding the HDAC7 locus should be considered.

MEF2-regulated miRs in endothelial cells

MicroRNAs have recently emerged as important regulators of gene expression by degrading mRNAs or inhibiting their translation. In endothelial cells, miRNAs were identified whose expression is influenced by MEF2. Some of these miRs target the mRNAs of previously identified MEF2 target genes, thus providing a negative feedback mechanism. Interestingly, one of these miRNAs, miR-27, targets the 3'-UTR of MEF2C. This finding awaits further verification, but if true, adds a new level of complexity to the negative feedback interplay between MEF2 and miRNAs.

As the mechanism of interaction between MEF2 and miRNAs is better understood, the connection between miRs and class II HDACs will likewise become more clear. miRNAs have been implicated in numerous aspects of cancer biology, as have HDACs. Further research into the functions of HDACs and miRNAs is likely to uncover common ground between these two very different classes of genetic regulators.

Class II HDACs hold great promise as future targets of therapeutic intervention. The conditional knockout of HDAC7 has unequivocally identified this enzyme as a regulator of vascular biology via gene regulation in the endothelium. The ability to modulate blood vessel growth, remodeling, and maintenance is critical to the treatment of cancer, heart disease, diabetes, atherosclerosis, and other conditions. Hopefully, advancing our understanding of the unique functions of HDAC7 will reveal new strategies for improving the treatment of these diseases.

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