Generation of both an shRNA-resistant MEF2A over expression construct and a dominant negative construct in adenovirus for rescue and knockout experiments in muscle

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GENERATION OF BOTH AN shRNA-RESISTANT MEF2A OVER EXPRESSION CONSTRUCT AND A DOMINANT NEGATIVE CONSTRUCT IN ADENOVIRUS FOR RESCUE AND KNOCKOUT EXPERIMENTS IN MUSCLE

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GENERATION OF BOTH AN shRNA-RESISTANT MEF2A OVER EXPRESSION CONSTRUCT AND A DOMINANT NEGATIVE MEF2 CONSTRUCT IN ADENOVIRUS FOR RESCUE AND KNOCKOUT EXPERIMENTS IN MUSCLE

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ABSTRACT

The Myocyte Enhancer Factor-2, or MEF2, transcription factor family is necessary for the differentiation and regeneration of both skeletal and cardiac muscle tissue. The transcription factors in this family are responsible for the activation of many muscle specific growth factor-induced and differentiation genes. There are four individual isoforms of MEF2; MEF2A, -B, -C, and –D, and the roles of these individual transcription factors are not completely understood. Knockdowns of these individual isoforms revealed that a MEF2A knockdown mouse model displays severe myofibrillar defects in cardiac muscle. This knockdown also has shown that MEF2A is required for myogenesis in vitro, where the other 3 isoforms, -B, -C, and –D, are not necessary for this process. One method of knocking down MEF2A to study its roles further is through the use of short hairpin RNAs (shRNA). The purpose of my research was two-fold. First, in order to test the specificity of this shRNA method, an shRNA-resistant MEF2A over expression construct in an adenoviral vector was created to perform rescue experiments. Second, to compare individual MEF2 isoform knockouts to a complete knockout of the entire MEF2 family, a dominant negative construct was created in an adenoviral vector. In both cases, a pShuttle-CMV adenoviral vector was used. The
results of this experiment can be used to further investigate the roles of MEF2A in both regeneration and differentiation of skeletal and cardiac muscle tissue.
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LIST OF ABBREVIATIONS

°C: degrees Celsius

A: adenine

aa: amino acid(s)

bHLH: basic helix-loop-helix

BMP: bone morphogenic protein

C-terminal: carboxy terminal

ChIP: chromatin immunoprecipitation

DNA: deoxyribonucleic acid

dsRNA: double stranded RNA

E: embryonic day

EDTA: Ethylenediaminetetraacetic Acid

ES cells: embryonic stem cells

EtOH: ethanol

FGF: fibroblast growth factor

g: gram

h: hour

HDAC: histone deacetylase

kb: kilobase

kpb: kilobase pair

L: liter
LB: Lysogeny broth (Luria broth)
m: milli
M: molar
MADS-box: MCM1, Agamous, Deficiens, SRF box
MCK: muscle creatine kinase
MCM: mini chromosome maintenance complex component
MEF2: Myocyte Enhancer Factor-2
min: minute
mIR: micro RNA
MHC: myosin heavy chain
MRF: myogenic regulatory factor
mRNA: messenger RNA
Myf5: Myogenic factor 5
MyoD: Myogenic differentiation 1
N-terminal: amino terminal
NaOH: sodium hydroxide
NLS: nuclear localization signal
NRC: neonatal rat cardiomyocyte
Pax: paired homeobox transcription factor
RNA: ribonucleic acid
rpm: revolutions per minute
SDS: sodium dodecyl sulfate
Sec: second

SHH: sonic hedgehog

shRNA: short-hairpin RNA

SRF: serum response factor

T: thymine

Tris: Tris (Hydroxymethyl) aminomethane

μ- micro

UTR: untranslated region

UV: ultraviolet

Wnt: wingless-type MMTV
CHAPTER 1: Introduction

1.1 Introduction

This thesis focuses on the roles of the MEF2 transcription factor family within mammalian cardiac and skeletal muscle. This family is composed of four MEF2 isoforms; MEF2A, -B, -C, and –D. There is a high level of conservation between each isoform, however they all have distinct roles in the development of muscle. This work specifically focuses on the MEF2A isoform, which has been shown to be necessary for myogenesis \textit{in vitro}. The purpose of this study has two parts. The first is to create an shRNA-resistant MEF2A over expression construct in an adenoviral vector to use in rescue experiments in order to determine the specificity of the MEF2A knockdown using an shRNA method. The second is to create a dominant-negative MEF2 construct in adenovirus to compare knockouts of individual isoforms to the knockout of the entire MEF2 family.

1.2 Development and Differentiation of Striated Muscle

1.2.1 Mammalian Myogenesis

Myogenesis is the process by which mammalian muscle is formed, and it involves the differentiation of muscle precursor cells into mature myocytes. This process happens not only during the development of the embryo, but also in adult muscle regeneration. Gastrulation takes place during embryogenesis, at embryonic day 7 (E7) in mice, which forms the ectoderm, endoderm, and mesoderm (Mok and Sweetman 2010). A specific population of cells is separated from the newly formed mesoderm for the formation of both the cardiac
and skeletal muscle tissue.

Both cardiac and skeletal muscles are forms of striated tissue. They are given this name due to their striated pattern of the contractile subunits, or sarcomeres. The sarcomeres consist of actin and myosin filaments that move against each other to lengthen or shorten a muscle (Figure 1.1). The Z-disc is what links each sarcomere to its adjacent partner, which produces the aforementioned striated pattern within the larger contractile units, called myofibrils. Many myofibrils bundled together form each myofiber, which is a single multi-nucleated muscle cell (Braun and Gautel 2011).

The pathways for both cardiac and skeletal muscle myogenesis involve the same small set of signaling molecules and transcription factors. Signaling cascades involving Wnt, FGF, and BMP provide not only cues involving positioning, but also differentiation and proliferation signals to the myogenic precursor cells (Yokoyama and Asahara, 2011). Cardiac and skeletal muscle also each use MEF2 transcription factors to help in the development of mature myocytes.

1.2.2 Cardiac Muscle Development

As the embryo develops, the first mesodermal tissue to differentiate after gastrulation is the heart. The development of heart muscle begins at about E7.5 in mice, as is indicated by the expression of BMPs, Wnt inhibitors, and fibroblast growth factor 8, which work together to aid in the making of progenitor cells in the mesoderm (Marvin, Di Rocco et al., 2001). A segregated population of cells
that arises from the newly formed mesoderm migrates under the head folds to form the cardiac crescent, which initiates the formation of the heart. This activates a network of cardiac-specific transcription factors, including the MEF2A family of proteins (Harvey, Lai et al., 2002). A bit later in development, at about E8.25, the linear heart tube is formed. This is made up of an inner endothelial layer surrounded by a layer of myocardial cells. This tube goes through a complex rightward looping process, followed by the formation of the atria, ventricles, and valves, which can be seen by E10.5. The functional heart, which includes all four chambers, pulmonary arteries, and the aorta, is formed by E12.5 (Zheng, Wen et al., 2003). Cardiac malformations can occur with a loss of function of many transcription factors that are present during this process, including the MEF2A family.

1.2.3 **Skeletal Muscle Development**

Skeletal muscle is a contractile structure that is composed of multiple layers that is designed for both controlled and voluntary movement. Bundles of myofibers are combined together to make up the muscle groups of this tissue. The myofibers each have multiple nuclei just beneath the sarcolemma, and just outside of the sarcolemma lies a population of satellite cells. Satellite cells are muscle specific stem cells that are involved in both the repair and homeostasis of the muscle. All of these cells work together to regenerate whole muscle in response to injury.

A short time after gastrulation occurs, somites, which are mesodermal cells
that are positioned next to the notochord and neural tube, give rise to the dorsal
dermomyotome from which the skeletal muscle will form. The specification of
the myogenic progenitor cells in the dermomyotome begins with the expression of
paired homeobox transcription factors (Pax) 3 and 7, which are triggered by Wnt,
BMP, and SHH signals from the surrounding tissues. The cells that are positive
for Pax3 will migrate from the dermomyotome to form the myotome (Mok and
Sweetman, 2010).

Differentiation within the myotome starts with the down regulation of Pax3,
followed by an exit from the cell cycle and the expression of myogenic regulatory
factors (MRF) such as Myf5, MyoD, and myogenin (Figure 1.2) (Mok and
Sweetman, 2010). MEF2 factors are also expressed. Loss-of-function
experiments have shown that several of these transcription factors are important
for the development of skeletal muscle. Once the cells leave the cell cycle,
myogenin will kick off the differentiation of myoblasts into mature muscle. Cells
that express Pax3 and Pax7 from the dermomyotome at this stage are important
for the specification of myogenic progenitor cells that will form the adult satellite
cells (Mok and Sweetman, 2010).

1.3 Myocyte Enhancer Factor-2 Transcription Factors

1.3.1 Identification and Structure of MEF2

Myocyte enhancer factor-2 (MEF2) proteins are part of the MADS-box
superfamily of transcription factors. These proteins were originally identified as a
nuclear factor in skeletal myocytes that bound to a conserved sequence that is A/T
rich, located within the muscle creatine kinase (*mck*) promoter. MEF2 was expressed before MCK in differentiated myotubes, so this factor was designated as an early molecular marker of muscle differentiation (Gossett, Kelvin et al., 1989). Since its discovery, MEF2 binding sites have also been identified in many control regions of muscle specific genes. Because of this, MEF2 has been recognized as a regulator of muscle development. There are four known genes that belong to the MEF2 family in mammals; MEF2A, -B, -C, and –D, and they are located on four different chromosomes (Cripps and Olson, 2002). In contrast, *Drosophila* and *C. elegans* have only one MEF2 gene that encodes D-MEF2 and CeMEF2, while *Xenopus* has two MEF genes designated *Xmef2a* and *Xmef2d* (Dichoso, Brodigan et al., 2000; Wong, Pisegna et al., 1994).

The N-terminal regions of MEF2 factors are highly conserved, containing a MADS-box domain and an adjacent MEF2 domain, while the C-terminal regions have little to no homology (Figure 1.3). The MADS-box is a 57-aa motif, and this family of proteins is named after the first four members that were identified (MCM1, Agamous, Deficiens, and SRF) (Shore and Sharrocks, 2005). The MADS-box domain and the MEF2 domain work together to mediate dimerization and DNA binding, and they are necessary for co-factor interactions of some MEF2 proteins (Molkentin, Black et al., 1995). MEF2 factors can homo- and heterodimerize with each other through this domain, but they cannot form heterodimers with other MADS-box family members. They also can bind the DNA consensus sequence YTA(A/T)₄TAR (Pollock and Treisman, 1991). The...
factors in the MEF2 family share about 50% aa identity overall, and there is 95% similarity between the MADS and MEF2 domains. There is a transactivation domain located in the C-terminal region that is highly variable and is subject to alternative splicing, which produce some muscle and neuronal specific isoforms (Breitbart, Smoot et al., 1992; Breitbart, Liang et al., 1993).

1.3.2 MEF2 Gene Expression

The patterns of expression for MEF2 transcripts are distinct but overlapping in both embryonic development and adult muscle tissues. The expression of MEF2 proteins in chondrocytes, neurons, and muscle occurs together with the onset of their differentiation during embryogenesis. Throughout the development of the embryo and into adulthood, MEF2 expression is highest in the cardiac and skeletal muscle (Potthoff and Olson, 2007).

_Mef2b_ and _Mef2c_ mRNAs are the first to be detected during development at E7.5 in the cardiac mesoderm of mouse embryos, and in the skeletal dermomyotome at E8.5. _Mef2a_ and _Mef2d_ are detected in the myocardium by E8.5 and are expressed in the heart throughout development and into adulthood (Edmonson, Lyons et al., 1994; Molkentin and Olson, 2002). The expression of _Mef2b_ and _–c_ begins to decline around E10.5 and they are undetectable post E13.5. The expression pattern of MEF2 transcripts is similar in skeletal muscle, where _Mef2c_ is detected at around E8.5, and the expression of _Mef2b_ follows at E9.0 (Figure 1.4). Beginning at E9.5, _Mef2a_ and _Mef2d_ are detectable and are expressed throughout the development of skeletal muscle and into adulthood. In
contrast to cardiac muscle development, *Mef2b* and –*c* continue to be expressed in skeletal muscle through development and after birth (Edmonson, Lyons et al., 1994; Molkentin and Olson, 2002). There are several skeletal muscle lineage markers and structural genes that are expressed prior to *Mef2*, and this suggests that these markers may initiate the expression of *Mef2* in skeletal muscle. Several studies suggesting that MEF2A activates *myogenin* have also shown that MEF2A, -B, and –D are expressed in myoblasts before the expression of this gene (Breitbart, Liang et al., 1993). MEF2 transcripts are also detected in smooth muscle, the central nervous system, and cartilaginous tissue, starting in the late stages of development and continuing into adulthood (Edmonson, Lyons et al., 1994).

1.3.3 MEF2 Regulation

1.3.3.1 Transcriptional Control

MEF2 factors are responsible for regulating a large array of developmental programs such as differentiation, cell division, and cell death (Potthoff and Olson, 2007). Mutually exclusive cues are necessary for a cell to make the decision to divide or die, and this is highly regulated. Because of their strong regulating roles, MEF2 factors are subject to strict and complex regulation at the translational and post-translational levels through interactions with both activators and repressors (McKinsey, Zhang et al., 2002). In mammals, the *Mef2* genes have long 5’ untranslated regions (UTR), which undergo alternative splicing. This makes it hard to study the control of these genes at the transcriptional level. *Mef2*
enhancers have been identified and characterized in *Drosophila*. In this model, at least 12 separate enhancers exist in the 12 kbp upstream of *D-mef2*, and the direct transcription of *D-mef2* is in a temporospatial pattern (Black and Olson, 1998). The regulatory regions upstream of *Mef2a* and *Mef2c* in mammals have only been partially characterized. To regulate transcription, the 5’ UTR regions of mammalian *Mef2* genes have regulatory regions that will bind to myogenic bHLH and proteins belonging to the MEF2 family. In addition, *Mef2A* is a transcriptional target of nuclear respiratory factor 1 (NRF1) (Ramachandram, Yu et al., 2008). Because of the complexity of the regulation patterns, no regulatory regions have been identified for *Mef2b* or –*d*. These findings support the idea that the regulation of the expression of MEF2 occurs at the transcriptional level, and therefore it can be said that transcriptional regulation can account for the overlapping but distinct temporal expression patterns of mammalian MEF2 transcripts.

1.3.3.2 Translational Control

With the exception of *Mef2c*, whose expression is limited to the brain, spleen and skeletal muscle, the expression of MEF2 transcripts is ubiquitous (Pollock and Treisman, 1991). The protein expression of MEF2 transcripts, however, is restricted to striated muscle and the brain due to translational control (Breitbart, Liang et al., 1993). Studies have shown that microRNAs regulate the expression of transcription factors that are important for the development of both cardiac and skeletal muscle (Small and Olson, 2011). In the 3’ UTR region of *Mef2a*, there is
an evolutionarily conserved sequence, which inhibits the expression of any MEF2A proteins in anything but muscle cells. The binding of miR-155 to the 3’ UTR of MEF2A prevents translation, and therefore inhibits the differentiation of skeletal myoblasts.

1.3.3.3 Post-translational Control

Repressive complexes at MEF2-dependent gene regulatory regions are formed by histone deacetylases (HDACs) to inhibit the transcriptional activity. Class II HDACs 4, 5, 7, and 9 are associated with DNA-bound MEF2 proteins and deacetylate histones to repress the expression of the genes. The phosphorylation of HDACs is caused by an increase of intracellular calcium, and this creates docking site for 14-3-3, which will disrupt the interactions between the HDACs and MEF2 proteins, and it masks the HDAC nuclear localization signal (NLS). After this, HDAC is removed from the nucleus so that the transcription of MEF2-dependent genes can occur. The activity of MEF2D can also be stimulated by the direct interaction of 14-3-3 with the MADS/MEF2 domains (McKinsey, Zhang et al., 2002). It is assumed that the post-translational control of MEF2 occurs through mechanisms other than this HDAC path due to the fact that Class II HDACs are mainly expressed in the brain, heart, and skeletal muscle.

Both the p38 and ERK/MAPK pathways phosphorylate MEF2, which leads to an increase in the transcriptional activity of MEF2. This in turn promotes differentiation and cell growth. MEF2D is activated by p38 by the direct phosphorylation of the MADS/MEF2 domain. This activated MEF2D will
interact with MyoD and activate the expression of late myogenic markers. MEF2 proteins regulate a large amount of developmental programs by interacting with many transcription factors. This allows the MEF2 binding partners to modulate the MEF2 activity (Black and Olson, 1998).

1.3.4 Loss-of-Function and Gain-of-Function Models for MEF2 Factors

1.3.4.1 Invertebrate MEF2 Loss-of-Function

Two mutations were created to determine the function of CeMef2 in C. elegans. The first mutation deleted the MADS box and MEF2 domain and it was considered a null allele. The second mutation deleted a sequence in the 3’ region of the gene, and this gene made a product but it was missing 167 amino acids in the C-terminal protein. The results from these experiments showed that CeMef2 is not required for the development of muscle in C. elegans and the mutants do not have a strong visible phenotype or defects during myogenesis. As adults, the CeMef2 mutants are shorter than the wild type, which suggests that MEF2 has a different pathway other than muscle development in C. elegans (Shore and Sharrocks, 2005). These results suggest that CeMef2 has developed an evolutionarily divergent role in respect to myogenesis, or its ability to regulate the differentiation of muscle has been replaced by another transcription factor.

D-mef2, the MEF2 gene in Drosophila, is primarily expressed in the mesoderm and muscle cell types. A null mutation of D-mef2 in Drosophila produces flies that do not develop differentiated muscle and die during embryogenesis (Bour, O’Brien et al., 1995). This indicates a crucial role for this MEF2 protein in
myogenesis and muscle differentiation.

1.3.4.2 MEF2A

Mice that lacked both *mef2a* alleles (*mef2a*−/−) were created using homologous recombination embryonic stem (ES) cells. These knockout mice suffer from perinatal lethality and sudden death from severe cardiac cyto-architectural defects that include myofibrillar disorganization, right ventricular chamber dilation, and random mitochondria distribution. Microarray analyses using cardiac RNA show that structural, mitochondrial, and stress-responsive genes are dysregulated in these *mef2a*−/− mutants, and their gene programs resemble one that is characteristic of cardiac failure. These mice show no skeletal muscle defects at the histological level (Naya, Black et al., 2002). ChIP-chip analyses did show that MEF2A associates with a variety of promoters whose genes are known to be important in the process of muscle differentiation and function (Paris, Virtanen et al., 2004). Skeletal muscle regeneration, however, is impaired in adult mice without MEF2A due to abnormal Wnt signaling (Snyder, Rice et al., 2012). Differentiation is not impaired in MEF2A knockouts targeted to the adult satellite cells in the context of muscle injury, however it is perturbed when MEF2A, -C, and –D are deleted simultaneously (Liu, Nelson et al., 2013). A dominant-negative form of MEF2A that lacks a transcriptional activation domain inhibits the formation of myotubes and also the expression of important muscle regulatory genes. The expression of this dominant-negative MEF2A in C3H10T1/2 fibroblasts impairs MyoD mediated myogenic conversion. This result suggests that MEF2A is necessary for
muscle specific gene expression and myogenesis (Ornatsky, Andreucci et al., 1997).

Overexpression studies of mef2a in the heart have shown that careful modulation of the expression level of this gene is necessary for this muscle to function properly. Transgenic mice overexpressing mef2a displayed a thinning of ventricular walls, ventricular chamber dilation, and a MEF2A dose-dependent activation of the fetal gene program (van Oort, Rooij et al., 2006). The overexpression of MEF2A in cultured neonatal rat cardiomyocytes (NRCs) is sufficient to dysregulate expression of α-myosin heavy chain (α-MHC) and α-actinin, two structural proteins associated with the sarcomere. Other proteins related to ion channels, adhesion, and the extracellular matrix, were also dysregulated (Xu, Gong et al., 2006).

1.3.4.3 MEF2B

The expression of mef2b is in line with the expression of mef2c in cardiac muscle cells and lags slightly behind mef2c in skeletal muscle lineages of the developing embryo (Molkentin, Lu et al., 1998). MEF2B is known to bind to the MEF2 consensus sequence along with forming a heterodimeric complex with E12 and myogenin to bind to and activate regulatory regions of target genes (Molkentin and Olson, 1996). According to research demonstrated by Black et al. (1998), MEF2B null mice are viable with no obvious phenotype. As myogenesis proceeds beyond the early differentiation stages, MEF2B is expressed at its highest levels in the ends of myofibers and myotubes. This indicates a possible
role for MEF2B in the fusion of the myotubes with the ends of the myofibers (Molkentin, Lu et al., 1998).

1.3.4.4 MEF2C

MEF2C is the earliest factor expressed during development of the mouse embryo (Molkentin and Olson, 1996). A MEF2C knockout caused mice to die prenatally due to defects during the heart looping process, and this results in a failure to form the future right ventricle (Lin, Schwarz et al., 1997). Genes such as α-MHC and α-actinin fail to be expressed, which impairs circulation, worsening the morphogenic heart defects that are observed (Lin, Schwarz et al., 1997). MEF2C null embryos fail to form vascular systems and have disorganization in the assembly of endothelial cells (Lin, Lu et al., 1998). A conditional skeletal muscle MEF2C knockout was used to show that MEF2C null skeletal muscle quickly degenerates perinatally, the integrity of the sarcomeres is compromised, and its function is impaired (Potthoff and Olson, 2007).

Overexpression of MEF2C in the p19 cardiac cell line supports the cardiac phenotype that was seen in MEF2C null mice. In this model, MEF2C is sufficient to induce cardiomyogenesis. This overexpression upregulates the expression of Bmp4, Nkx2-5, Gata4, cardiac α-actinin, and MHC, and this is sufficient to direct the early stages of differentiation in cardiomyocytes (Skerjanc, Petropoulos et al., 1998).

Together, the aforementioned studies suggest a possible conserved role for MEF2C in the maintenance and assembly of the heart and vascular systems, along
with being required for the proper assembly and maintenance of sarcomeres.

Additionally, studies have shown that MEF2C could be active in the precardiac mesoderm and could act redundantly to ensure proper differentiation of striated muscle.

1.3.4.5 MEF2D

The expression of *mef2d* in the developing mouse is expressed in the same temporal pattern as *mef2a* in both cardiac myocytes and the developing somite myotome, and these are the predominant MEF2 isoforms that are expressed in adult cardiomyocytes. This suggests a dual role for MEF2D in both the development and maintenance of mature muscle (Edmonson, Lyons et al., 1994; Naya, Black et al., 2002). Loss- and gain-of-function studies have been done with MEF2D in both cardiac and skeletal muscle to investigate its function further.

*Mef2d*−/− knockout mice are viable with no histological abnormalities in a range of tissue types, including cardiac and skeletal muscle. These mice do, however, display a blunted response to cardiac stress due to pressure overload, which included a resistance to cardiac remodeling and fibrosis when compared to the wild type mice (Kim, Phan et al., 2008). Overexpression of MEF2D in transgenic mice resulted in hearts that were sufficient in activating both fibrosis and hypertrophy (Kim, Phan et al., 2008). MEF2D overexpression also results in a distinct cardiac phenotype since the overexpression of MEF2A of MEF2C results in dilated cardiomyopathy with little to no hypertrophy (Xu, Gong et al., 2006). This suggests a unique set of genes that MEF2D may target.
Analysis of skeletal muscle MEF2D null mice showed no obvious histological abnormalities (Kim, Phan et al., 2008). The conditional deletion of \textit{mef2d} in skeletal muscle did, however, indicate a role for MEF2D in the activation of slow, oxidative-fiber type muscle genes. This is because the activity-dependent ability of muscle cells to make a fast- to slow-fiber type transformation is lost when \textit{mef2c} or \textit{mef2d} is deleted in skeletal muscle (Potthoff and Olson, 2007). A study that used a morpholino knockdown of MEF2C and MEF2D in zebrafish skeletal muscle showed a need for these two factors in the expression of thick filament contractile proteins that are necessary for the proper assembly of sarcomeres (Hinits and Hughes, 2007). The studies mentioned above suggest a role for MEF2D in the activation of specific muscle contractile proteins in the adult striated muscle in response to pathological or physiological cell stress. Further investigation is needed to determine the precise role of MEF2D in striated muscle.

1.4 Short Hairpin RNA

RNA interference (RNAi) is a biological response to exogenous double-stranded RNA (dsRNA) that was first discovered in \textit{C. elegans}, and this response induces a sequence-specific form of gene silencing. The regulatory motif of RNAi is conserved across a wide range of eukaryotic organisms, and endogenously encoded triggers of gene silencing can work through elements of this machinery to in turn regulate the expression of genes that code for specific proteins. Small temporal RNAs are transcribed as short hairpin precursors that are 17 nucleotides long, and they are processed into active, 21 nucleotide long
strands of RNA by Dicer (Figure 1.5). These strands then recognize and target specific mRNAs based on interactions between base pairs (Paddison, Caudy et al., 2002). Paddison et al. (2002), among others, showed that short hairpin RNAs (shRNAs) can be created to knock down the expression of certain desired genes in mammalian cells by using this specific base-pairing interaction method. By using shRNAs to suppress the expression of genes, we can further understand gene programs. In the case of this thesis, shRNA can be used to acutely knock down each MEF2 member to study the phenotypes created by these mutants and therefore learn more about the function of each MEF2 member.

1.5 Statement of Thesis Rationale

The focus of my thesis is two-fold. The first goal is to generate an shRNA resistant MEF2A construct in an adenoviral vector to use for rescue experiments in both cardiac and skeletal muscle. Although the shRNA method of suppressing genes is sequence specific, we do not know if these strands of RNA have other off-site targets that could create the phenotypes observed. The generation of this construct would allow us to over express the shRNA resistant form to demonstrate that we can rescue the phenotype in cardiac and skeletal muscles caused by the knockdown of MEF2A to show the shRNA method’s specificity. If there is a strong rescue, we can say that the shRNA used to knockdown MEF2A is specific for that gene and not targeting any others. If there is no rescue at all, it implies that the shRNA is targeting a gene other than MEF2A that is causing the observed phenotype. If there is an incomplete rescue with the overexpression of the
shRNA resistant form of MEF2A, it could suggest that the shRNA is targeting both MEF2A and other off target genes to produce the observed phenotypes.

The second focus of this thesis is to create a dominant negative MEF2 construct in an adenoviral vector. This can be used to knock out all members of the MEF2 transcription factor family at once as opposed to knocking down each factor separately. This could allow us to observe what happens to the specific gene programs that involve all members of the MEF2 family.
Figure 1.1 The contractile unit of striated muscle: the sarcomere. Schematic showing the cross-striations of a skeletal myocyte (left) and a cardiomyocyte (right). The sarcomere is composed of antiparallel filaments of actin and myosin. This image shows the pattern created by the Z-disc regions in each sarcomere that line up end to end in the myofibrils (Adapted from Braun and Gautel, 2011).
Figure 1.2 Schematic of satellite cell myogenesis. Satellite cells are quiescent in normal muscle but can be activated by things such as muscle damage. When activated, they divide to produce myoblasts that will proliferate and fuse to form myotubes, which will then form myofibers. The activation of satellite cells is marked by expression of myogenic regulatory factors such as Myf5, MyoD, and myogenin (Zammit et al., 2006).
Figure 1.3 The sequences of the Myocyte Enhancer Factor 2 transcription factor family are conserved. The MEF2 transcription factor family is evolutionarily conserved. The proteins in this family share approximately 95% similarity within the MADS box and MEF2 domains (Potthoff and Olson, 2007).
Figure 1.4 MEF2 Temporal expression during muscle development. The pattern of cardiac muscle expression is shown in black, and a similar expression in skeletal muscle is shown in red, approximately one day later. MEF2 is detected as early as E7.5 in the heart, and E8.5 in skeletal muscle (Edmondson et al., 1994).
**Figure 1.5 shRNA processing schematic.** The path by which shRNA is processed in order to be used to bind to the target mRNA sequence so that the desired strand of mRNA can be cleaved. This process is used in knockdown experiments (De Rienzo, et al., 2012).
CHAPTER 2: Materials and Methods

2.1 Preparation of the MEF2A-FLAG Insert

A double digestion of the shRNA-resistant MEF2A plasmid in a pCDNA3 vector was performed using KpnI and NotI. 1 μL (1 μg) of the shRNA-resistant MEF2A plasmid (stored at -20°C), 0.5 μL of Kpn1, 0.5 μL of Not1, 2 μL of Buffer 2.1, and 16 μL of dH₂O were added to a 1.5 mL microfuge tube to reach a final volume of 20 μL. The tube was Parafilmed and placed in a 37°C water bath for 1 h. Once the incubation was complete, the digested plasmid was stored at -20°C. The restriction enzymes and buffer were purchased from New England Biolabs (NEB) (Ipswich, MA) and were stored at -20°C.

2.2 Preparation of the pShuttle-CMV Vector

A double digestion of the pShuttle-CMV vector was performed using KpnI and NotI. 1 μL (1 μg) of the pShuttle-CMV vector (stored at -20°C), 0.5 μL of Kpn1, 0.5 μL of Not1, 2 μL of Buffer 2.1, and 16 μL of dH₂O were added to a 1.5 mL microfuge tube to reach a final volume of 20 μL. The tube was Parafilmed and placed in a 37°C water bath for 1 h. Once the incubation was complete, the digested plasmid was stored at -20°C.

2.3 Gel Purification and Extraction

The digested pShuttle-CMV vector and shRNA-resistant MEF2A insert, along with the undigested vector and insert, were electrophoresed on a 0.8% agarose gel containing ethidium bromide and visualized with UV. The digested products were excised and gel extracted with the QIAquick gel extraction kit (QIAGEN)
according to manufacturer’s instruction. The extracted products were stored at -20°C.

2.4 Ligation Reaction and Transformation

15 µL of the extracted insert were added to 10 µL of the extracted vector, along with 1 µL of T4 DNA ligase and 4 µL of 10X T4 buffer (New England Biolabs). The solution was incubated at 16°C for 4 h and then stored at 4°C.

20 µL of thawed DH5α cells were combined with 10 µL of the ligation reaction and the solution was incubated over night at 37°C. The cells were plated on pre-warmed LB plates with ampicillin (100 µg/mL), sealed with Parafilm, and incubated upside-down for 16-18 h at 37°C.

2.5 Mini-preps of Selected Colonies

Three solutions were made for this procedure. Solution 1, a resuspension buffer, contained 50 mM glucose, 10 mM EDTA (pH 8.0), and 25 mM of Tris (pH 8.0). Solution 2, a lysis solution, contained 0.2 M NaOH and 1% SDS. Solution 3, a neutralization buffer, contained 29.4 g Kac (3 M final concentration), 11.5 mL Acetic Acid, and brought up to 100 mL final volume with dH2O.

25 colonies were selected for mini-preps and inoculated in 3 mL of LB-ampicillin (100 µg/mL) overnight. 1.5 mL were transferred to a microfuge tube and spun at 14000 rpm for 30 sec. The supernatant was aspirated and the pellet was resuspended in 100 µL of solution 1. 200 µL of solution 2 was added and mixed by inversion and then incubated for 4 min at room temperature. 150 µL of solution 3 was added and mixed by inversion and 400 µL of phenol/chloroform
was added and the solution was vortexed and spun at 14000 rpm for 1 min. 400 μL of the upper phase was transferred to a new tube containing 1 mL of cold 100% EtOH. The solution was spun at 14000 rpm for 5 min and the supernatant was aspirated and the pellet was left to air dry at room temperature for 15 min. Once dry, the pellet was resuspended in 20 μL of RNase water (100 μg/mL) and stored at -20°C.

This procedure was completed for both the shRNA-resistant MEF2A construct (MEF2A-FLAG) as well as the dominant negative form of MEF2 (MEF2-En). A double digestion of the mini-preps for the dominant negative MEF2 was performed with BamH1 and Not1 using buffer 3.1 (New England Biolabs) to see if the insertion was successful.

CHAPTER 3: Results and Discussion

3.1 Preparation of the shRNA-resistant MEF2A Insert and pShuttle-CMV Vector

In order to achieve high level expression of the shRNA resistant form of MEF2A, one approach is to clone this expression construct in adenovirus. A number of cells lines are efficiently transduced by adenoviruses and high levels of expression can be achieved. To begin, shRNA-resistant MEF2A and the pShuttle-CMV plasmids that were used to create the overexpression construct in this experiment were digested with the restriction enzymes NotI and KpnI. The products from the digestion were run on a 0.8% agarose gel to make sure that the plasmids were appropriately digested in preparation for the ligation reaction. The
first three times that the restriction enzyme digestion and subsequent agarose gels were completed and visualized with UV, the bands shown for the digested insert and vector were no different than the control bands of the undigested vector and insert. After the first two unsuccessful times of incubating the digestion reaction for one hour, the incubation time was increased to overnight. Once the third digestion reaction was also unsuccessful, a new stock of the enzyme Not1 was ordered so as to make sure the enzyme had not lost its function. The fourth time the experiment was performed, the gel displayed both the insert and the vector having the appropriate bands, indicating the desired digestion had occurred.

3.2 Ligation Reaction and Transformation

After the restriction enzyme digestion, the desired fragments of the digested plasmids are combined to create the desired adenoviral vector. A ligation reaction between the shRNA-resistant MEF2A and the pShuttle-CMV insert was performed and combined with thawed DH5α cells. The cells were plated on plates containing LB agar and Ampicillin. This procedure was completed twice. The first was unsuccessful, as no colonies grew on the plate, so new plates were prepared to ensure that there were no issues with the medium on which the cells were grown on. The second trial was also unsuccessful, indicating that an error had most likely been made in previous steps of the experiment. The experiment was performed again up until the preparation and gel purification of the insert and vector.

3.3 Mini-preps From Colonies for MEF2-En Insert and pShuttle-CMV Vector

In order to achieve high expression of the dominant negative MEF2, this
construct also has to be cloned in an adenovirus. The restriction enzyme digestion, ligation, and transformation was performed for this adenoviral vector, and from the transformation 25 colonies were selected for mini-preps used. Colonies 4-9 were selected and mini-preps were made for each. The mini-preps were double digested with HindIII and EcoRV and the products were run on a 0.8% agarose gel to ensure that the insertion was successful. Three bands were expected; ~7.5 kb, ~0.9 kb, and ~0.5 kb. The mini-preps and digestion results for colonies 4 and 5 were inconclusive because the gel was not run for long enough. The digestion of the mini-preps created from colonies 6 and 7 were unsuccessful due to a non-functioning NotI enzyme. The mini-preps and restriction enzyme digestion of colonies 8 and 9 appeared to display bands that indicated a successful insertion of the MEF2-En insert into the pShuttle-CMV vector.

### 3.4 Future Directions

More steps must be taken in order to create the desired shRNA resistant MEF2A overexpression vector as well as the dominant negative MEF2. For the shRNA resistant over expression vector, the gel purified products from the successful restriction enzyme digestions of the insert and vector will be used in a ligation reaction and transformed into DH5α cells and plated on LB agar containing Ampicillin. Some of the resulting colonies will be selected to create mini-preps of the desired plasmid, and an agarose gel will be run to make sure that the insertion was successful. Once the appropriate mini-preps for both the dominant negative form of MEF2 and shRNA resistant form of MEF2A are
created, the plasmids will be transformed into cells so as to amplify and generate a stock of the adenoviruses.

The shRNA resistant over expression vector will be used in rescue experiments in order to demonstrate the specificity of the shRNA method used to knockdown the MEF2A in previous experiments and to ensure that the shRNA used does not have any targets other than the desired MEF2A that would create the observed phenotypes. The dominant negative form of MEF2 can be used to transform into cells so as to knock out the entire MEF2 family of transcription factors in cells to determine the resulting phenotype, which can be used as a comparison to methods that knock down each isoform individually.
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Work Experience:

Researcher (Sept 2013-Present)  
Boston University, Boston, MA:  
I currently work in the laboratory of Dr. Frank Naya. My Master’s thesis is based on research involving the creation of an adenovirus from an shRNA resistant form of the transcription factor MEF2A. My experience includes mouse dissection, cell culture, transfection, transduction, restriction enzyme digestion, DNA extraction and purification, agarose gels, and other applicable laboratory techniques.

Teaching Fellow (Sept 2013-Present)  
Boston University, Boston, MA:  
I teach lab sections for the following classes: Introduction to Biology, Systems Physiology, and Animal Behavior. I am responsible for teaching a background on the laboratory procedures, guiding students through labs, designing exams, and grading laboratory work.

Lab Assistant (Jan 2012-May 2013)  
University of New Hampshire, Durham, NH:  
I perform basic laboratory maintenance such as equipment care and inventory. I have also worked on research projects that include working with laboratory animals: socializing the rats, meeting animal care guidelines and standards, euthanizing, and proper dissection technique. I have sectioned tissue, stained immune cells, evaluated blood smears for differential cell counts, captured microscopic images, and documented interpreted results.

Academic Tutor/Mentor (Aug 2011-May 2013)  
University of New Hampshire (CFAR/SSS), Durham, NH:  
I work as an academic tutor for students attending the University of New Hampshire. Subject areas include: organic chemistry, biochemistry, and genetics. I help students with subject content, note taking strategies, study techniques, to improve grades and comprehension.

Substitute Teacher (May 2010- Jan 2012)  
Nashua School District, Nashua NH:
As a substitute teacher, I worked with children in grades K-8. I taught all subject fields including gym and music classes. I also helped other faculty such as teachers and principals with paper work and grading.

The Atrium Salon and Spa, Nashua, NH:
As a part-time receptionist for almost five years, my principal responsibility was customer assistance. I also operated their computer systems for both front and back of store. Specifically, I was responsible for making and confirming appointments, inventory, cashing out, and opening and closing the salon. When necessary, I helped to train new receptionists in these same procedures.

**Veterinary Intern (Dec 2011-Jan 2012)**
Lowell Road Veterinary Center, Hudson, NH:
As an intern, I was able to sit in on many veterinary appointments. I also was able to observe certain lab procedures such as blood work.

**Education:**
Bachelor of Science
University of New Hampshire, Durham, NH (Aug. 2009 – May 2013)
GPA: 3.9
Major: Molecular, Cellular, and Developmental Biology
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Boston University, Boston, MA (Sept. 2013 – Sept. 2015)
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**Achievements:**
Annual Presidential Merit Scholarship recipient
National Honors Society
Induction into the Golden Key International Honors Society
Dean’s List and High Honors every semester in college
Teaching Fellowship from Boston University

**Skills:**
General lab techniques – lab animal work, immunohistochemistry, blood smears, blood counts, cryotome use for tissue sectioning, lab maintenance, capturing microscopic images, interpreting and presenting data, cell culture, transfection, DNA digestion, western blots, agarose gels, etc.
Computer work – Excel, PPT, Word, Mac, PC