Role of Transcription Factor MEF2A in Development of Coronary Artery Disease (CAD) and Myocardial Infarction (MI)

Maniragava Sai Bhagavatula
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ROLE OF TRANSCRIPTION FACTOR MEF2A
IN DEVELOPMENT OF
CORONARY ARTERY DISEASE (CAD) AND
MYOCARDIAL INFARCTION (MI)

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DEDICATION

To my parents Mr. Sambasivarao Bhagavatula and Mrs. Rajeshwari Bhagavatula, They bore me, raised me, supported me, taught me, and loved me, and my sisters Saroja Kavuri, Ramalakshmi Jammalamadaka, Gayatri Vedantam, Vasavi Kareddla and my wife Sudhatma Bhagavatula and my beloved friend Roger Goins for their constant support and encouragement.
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ROLE OF TRANSCRIPTION FACTOR MEF2A IN DEVELOPMENT OF CORONARY ARTERY DISEASE (CAD) AND MYOCARDIAL INFARCTION (MI)

MANI RAGAVA SAIKRISHNA BHAGAVATULA

ABSTRACT

Coronary artery disease (CAD) and myocardial infarction (MI) are the leading cause of death in developed countries. Genetic factors play an important role in the pathogenesis of CAD and MI. The revolutionary advances in molecular genetics have provided new insights into the genetic pathways involved in this disease. Recently, our laboratory used linkage analysis to map the first disease-causing gene for CAD and MI to chromosome 15q26 in a large family and subsequently mutation analysis identified a 7-amino acid deletion in MEF2A, a gene encoding a transcription factor. Later we identified three novel mutations N263S, P279L, G283D in four other patients or families, which has validated our initial finding of MEF2A as a gene for CAD and MI.

We recently screened another set of 200 CAD and MI patients using the single strand conformation polymorphism (SSCP) method and found another variant, T215A, in the sixth coding exon of MEF2FA in one CAD patient. The T215A mutation is clustered close to the major transcriptional activation domain of MEF2A. We then performed functional studies using the luciferase assay and found that the mutation significantly reduced the transcriptional activity of MEF2A and acted by a loss of function mechanism as the earlier mutations.
To further investigate the role of MEF2A in atherogenesis, we studied knockout mice targeted to the mouse *Mef2a* gene and created a novel *Mef2a*\(^{+/−}\) *Apoe*\(^{−/−}\) knockout mouse strain. We then analyzed diet-induced atherosclerosis and spontaneous lesion formation in the *Mef2a* and *Mef2a*\(^{+/−}\) *Apoe*\(^{−/−}\) mice. A total of 39 *Mef2a* knockout mice, including 20 males (8 wild type *Mef2a*\(^{+/+}\) and 12 heterozygous *Mef2a*\(^{+/−}\)) and 19 females (9 wild type *Mef2a*\(^{+/+}\) and 10 heterozygous *Mef2a*\(^{+/−}\)) were fed on atherogenic/paigen diet for 38 weeks. A total of 47 *Mef2a*\(^{+/+}\), *Mef2a*\(^{+/−}\) mice on *Apoe*\(^{−/−}\) knockout background, including 26 males (15 wild type and 11 heterozygous) and 21 females (9 wild type and 12 heterozygous) were maintained on a Western-type diet for 12 weeks. The mice were analyzed for quantitative atherosclerosis, platelet aggregation, and vascular permeability to determine the role of *Mef2a* in the development of atherosclerosis. Our studies showed that in the atherosclerosis-susceptible apolipoprotein E knockout mice, *Mef2a* deficiency increased the severity of atherosclerotic lesions in the proximal aorta by two-fold \((P = 0.0003)\), but not in the *Apoe*\(^{+/+}\) background. Assessment of vascular permeability in 24 *Mef2a*\(^{+/+}\), *Mef2a*\(^{+/−}\) mice on *Apoe*\(^{−/−}\) knockout background revealed that heterozygotes had high vascular leakage \((P = 0.000012)\).

We also found that lipid levels and platelet aggregation did not contribute to the formation of atherosclerotic lesions in these mice. Thus, our results support the hypothesis that *Mef2a* deficiency may promote atherosclerotic lesion formation in the proximal aorta possibly due to increased vascular permeability.

Our findings have helped us to understand the role of *Mef2a* in the development of CAD and MI. These studies will further emphasize the importance of incorporating *Mef2a* as a genetic marker to identify patients with CAD/MI. Identification of patients
with CAD/MI will assist them in improving their lifestyles to prevent and manage CAD. Future studies on MEF2A and CAD may provide additional insights into the pathogenic mechanisms of CAD, which may be important for development of new therapeutic treatments for CAD patients.
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<th>Description</th>
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<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>HTN</td>
<td>Hypertension</td>
</tr>
<tr>
<td>PTCA</td>
<td>Percutaneous coronary angioplasty</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass surgery</td>
</tr>
<tr>
<td>HL</td>
<td>Hyperlipidemia</td>
</tr>
<tr>
<td>DB</td>
<td>Diabetes</td>
</tr>
<tr>
<td>S</td>
<td>Smoking</td>
</tr>
<tr>
<td>FH</td>
<td>Family history</td>
</tr>
<tr>
<td>OB</td>
<td>Obesity</td>
</tr>
<tr>
<td>Cho</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Tri</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>RCA</td>
<td>Right coronary artery</td>
</tr>
<tr>
<td>LCA</td>
<td>Left coronary artery</td>
</tr>
<tr>
<td>SA</td>
<td>Septal artery</td>
</tr>
<tr>
<td>Ao</td>
<td>Aorta</td>
</tr>
<tr>
<td>Pu</td>
<td>Pulmonary trunk</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>RA</td>
<td>Right atrium</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrium</td>
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Coronary artery disease (CAD) and myocardial infarction (MI)

Coronary arteries are the main blood vessels that supply blood to the heart muscle. The heart is like any other organ and requires a continuous supply of oxygen and other nutrients in order to function. Coronary artery disease (CAD), the most common form of heart disease, is attributed to atherosclerotic plaque buildup in the walls of the epicardial coronary arteries (1,2). The atheroma accumulation can limit the blood flow to the myocardium, resulting in symptoms of ischemia. In some patients, inflammation at the site of atheroma leads to plaque fissure, rupture, or erosion. These events can result in sudden coronary thrombosis and myocardial infarction (MI) (1-3).

CAD/MI claims more lives each year than the other five leading causes of death combined (cancer, respiratory diseases, accidents, diabetes mellitus, and pneumonia) (4,5). It accounts for one of every 5 deaths, and overall, it is estimated to affect more than 13 million Americans. Several risk factors have been identified for CAD and MI, including a family history, male gender, high blood cholesterol and other lipids, smoking,
physical inactivity, overweight and obesity, hypertension, and diabetes (6-9). A family history of CAD is one of the most significant risk factors for the disease.

**General features of CAD**

Figure 1 shows general features of CAD in human coronary arteries. It explains plaque formation in the various branches of the arteries which include the left main artery, anterior descending artery, mid left circumflex, proximal circumflex artery, and the proximal right coronary artery; the area of the infarction of the anterior- lateral wall can also be seen in the heart which is affected by myocardial infarction.
Fig 1: Process of Atherosclerosis. (Adapted from ADAM).
Artery structure and function

The molecular mechanism of CAD can be explained by understanding the function of the arteries.

Mechanistic function of arteries (contract and relax)

When heart contracts (systole), blood is pumped into the large arteries, such as the aorta and the carotid arteries which then expand. As blood flows out of these arteries into smaller ones throughout the body the large blood vessels then retreat back to the size they were prior to the heartbeat. As the arteries relax, the heart also relaxes and fills with blood in preparation for the next heartbeat (diastole). Thus, the large blood vessels, which supply blood to the head and body, expand when the heart contracts and retracts when the heart is filling with blood for the next heartbeat.

Development of atherosclerosis

For the past several decades, the understanding of mechanism of the pathogenesis of atherosclerosis has undergone a major thought change (10). A better understanding of the biology and the pathophysiology of atherosclerosis (CAD) is critical in gaining insights into the development and cause of the disease. Recent studies have provided new insights into the understanding of this disease. The knowledge of the structure and anatomy of coronary arteries will be highly useful to develop a better understanding of this disease process.
The structure of a normal coronary artery reveals the endothelium, which is a monolayer of endothelial cells on the luminal side or the inner most, followed by the intima which consists of collagens and proteoglycans, the middle layer media is made up of a multilayer smooth muscle cells (SMC), which provide the structural integrity and the outer most layer is called the adventitia which is made up of connective tissues, fibroblasts and SMC (Fig 2).

Fig 2: Structure of normal artery and its function. (Adapted from ADAM).
The current evidence suggests that development of atherosclerosis or CAD begins with inflammation of the arteries which is initiated by infiltration of lipoproteins (fats) and monocytes into the intima region. The function of endothelial cells (ECs) as a protective sheet preventing leukocyte adhesion, is disrupted during proinflammatory stimuli, such as high fat diet, high levels of cholesterol, obesity, hyperglycemia, insulin resistance, hypertension, and smoking. This activates the expression of several adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 (VCAM-1), by the endothelial cells which help the transfer of lipid particles and blood monocytes to the endothelium through these adhesion molecules (11-13) (Fig 3).

The lipid particles (mainly LDL’s) associate with other substances of the extracellular matrix in the intima, such as proteoglycans, and gradually enlarge and reduce the size of lumen. This isolates the lipoproteins within the intima, from plasma antioxidants, as the LDL’s undergo oxidative modification (14–16). Oxidatively modified LDL particles are made up of lipid and protein moieties, which can induce a local inflammatory response (17). Oxidized LDL’s are later absorbed by monocytes. Monocytic cells interact with human ECs and increase monocyte matrix metalloproteinase 9 (MMP-9) production by several folds, thereby causing gradual infiltration of leukocytes through the endothelial layer and its associated basement membrane (18). Inflamed intima releases macrophage colony stimulating factor, which differentiates monocytes into macrophages (19, 20). This stimulation also increases expression of scavenger receptors, by macrophage, which absorbs modified lipoproteins via receptor-mediated endocytosis.

The diagram shows a cross-section through a muscular artery depicting a classic trilaminar structure. The intima of normal arteries is composed of a single layer of endothelial cells overlying a subendothelial matrix that contains occasional resident smooth muscle cells. The underlying tunica media, separated from the intima by the internal elastic lamina, contains multiple layers of vascular smooth muscle cells. The adventitia, the outermost layer of the blood vessel, separated from the media by the external elastic lamina, is not depicted in this diagram. Circulating leukocytes adhere poorly to the normal endothelium under normal conditions. When the endothelium becomes inflamed, however, it expresses adhesion molecules that bind cognate ligands on leukocytes. Selectins mediate a loose rolling interaction of leukocytes with the inflammatorily activated endothelial cells. Integrins mediate firm attachment. Chemokines expressed within atheroma provide a chemotactic stimulus to the adherent leukocytes, directing their diapedesis and migration into the intima, where they take residence and divide.
Further, macrophages are converted into foam cells by the accumulation of cholesteryl esters in the cytoplasm. Necrosis and apoptosis of foam cells lead to a necrotic core with a mass of cell debris and lipids. Macrophages and foam cells secrete cytokines, inflammatory molecules and growth factors that facilitate SMC migration and proliferation and production of extracellular matrix, forming plaques with fibrous caps. When the plaques are stable, the patient may experience stable angina. However, if plaque rupture occurs, it leads to thrombosis and the patient may experience unstable angina, acute myocardial infarction (MI), or sudden death (6,7). Thus making the process of atherosclerosis a complex disease (Fig 4).

Cholesterol hypothesize has always played a key role in understanding the pathophysiology of atherosclerosis (21). For over several years’ cholesterol and LDL concentrations have been diagnosed as a major risk factor along with age. Researchers have gained extensive knowledge in understanding the importance of modified lipoproteins in the pathogenesis of atherosclerosis. This led to the development of several treatment options to patients in reducing clinical events. The overview of the pathophysiology of CAD and MI above represents the current prevailing mechanism for the pathogenesis of CAD and MI, and suggests that CAD is a disease of lipid metabolism, smooth muscle cell proliferation and migration, and inflammation and immune responses.
However, several other risk factors have also been identified to cause the inflammation in cells involved in atherosclerosis which includes, cigarette smoking, insulin resistance/diabetes, and hypertension (22). And recent advances in the molecular genetic studies have, provided new insights into the pathogenic mechanism of CAD and MI.
Macrophages augment the expression of scavenger receptors in response to inflammatory mediators, transforming them into lipid-laden foam cells following the endocytosis of modified lipoprotein particles. Macrophage-derived foam cells drive lesion progression by secreting proinflammatory cytokines. T lymphocytes join macrophages in the intima and direct adaptive immune responses. These leukocytes, as well as endothelial cells, secrete additional cytokines and growth factors that promote the migration and proliferation of SMCs. In response to inflammatory stimulation, vascular SMCs express specialized enzymes that can degrade elastin and collagen, allowing their penetration into the expanding lesion.
**Genes causing CAD**

CAD and MI are multifactorial disorders associated with several factors such as family history, male gender, high blood cholesterol and other lipids, hypertension, diabetes etc. Genetic factors play an important role in development of CAD and MI. An effective approach for identifying a specific gene for CAD and MI appears to be a genome-wide linkage scan in large, extended families with multiple affected family members.

To date, several large-scale genome-wide scans using hundreds of sibpairs have been reported to identify chromosomal regions implicated in complex CAD or MI traits. Thus, CAD may be associated with multiple genes, making it a polygenic disorder. As CAD is a complex genetic trait, in most cases, CAD is not inherited in a clearly dominant or recessive manner. Instead, a person may have mutations in some genes that increase risk and mutations in other genes that decrease risk. The interactions among these genes and environmental factors are the key to the development of CAD.

We have recently mapped a genetic susceptibility locus for MI to chromosome 1p34-36 region by analyzing MI as a complex trait in 428 nuclear families with premature CAD and MI (23). Another susceptibility locus for MI was identified by others in the chromosome 14q region (24). Five CAD susceptibility loci have been identified on chromosome 2q21.1-22 (25), 2q36 (26), 3q13 (27), 16p13-pter (28), and Xq23-26 (25). The specific genes at all these loci, however, remain to be identified or cloned.
Table 1: List of the genes that have been identified for CAD and MI

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cause</th>
<th>Effect</th>
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<tr>
<td>ACE (Angiotensin I-Converting Enzyme)</td>
<td>Increased Risk for CAD</td>
<td>CAD</td>
</tr>
<tr>
<td>5,10-methylenetetrahydrofolate reductase (MTHFR) gene mutation</td>
<td>Val was substitute for Ala</td>
<td>CAD</td>
</tr>
<tr>
<td>angiotensinogen gene T235</td>
<td>Angiotensinogen T235 homozygotes are at significantly increased risk of CHD generally</td>
<td>CAD and myocardial infarction</td>
</tr>
<tr>
<td>LDLR (low-density lipoprotein receptor)</td>
<td>Nonfunctional receptor fails to take up plasma cholesterol</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>APOB-100 (apolipoprotein B-100)</td>
<td>Apolipoprotein B-100 fails to bind LDL receptor</td>
<td>Familial ligand-effective apolipoprotein B-100</td>
</tr>
<tr>
<td>RyR2 (ryanodine-receptor calcium-release channel)l</td>
<td>Stress-induced calcium overload in myocytes may be a mechanism</td>
<td>Catecholamine-induced ventricular tachycardia</td>
</tr>
<tr>
<td>APM1 (adiponectin gene)</td>
<td>SNP+45 T&gt;G</td>
<td>CAD</td>
</tr>
<tr>
<td>MEF2A</td>
<td>Seven-amino acid deletion, 4 missense mutations in MEF2A</td>
<td>CAD and MI</td>
</tr>
<tr>
<td>LRP6</td>
<td>Missense mutation</td>
<td>CAD</td>
</tr>
</tbody>
</table>
MEF2A, first non lipid gene for CAD and MI

Focusing on a large CAD/MI family with autosomal dominant inheritance, we recently demonstrated genetic linkage of CAD/MI with a DNA polymorphism in chromosome 15q26 region, D15S120 (we designated this locus as adCAD1) (29). This region contains ~93 genes, of which there are 43 known and 50 unknown genes (Fig 5). Of the known genes, Mef2a is a gene that encodes a transcription factor and became a strong candidate due to its mRNA expression in blood vessels during mouse early embryogenesis (30). Expression of MEF2A protein was detected as early as day 8.5 postcoitum in cells of embryonic vasculature, and the pattern is similar to vascular endothelial growth factor receptor 2 (VEGFR2) and Von Willbrand factor (both are endothelial cell markers) (31,32). Although Mef2a was initially identified as a muscle-specific gene, later it was shown to be highly expressed in the endothelium of coronary arteries (29).
Fig 5: Mapping of adCAD/MII to chromosome 15. (Adapted from Wang et al Science, 2003, 302, 1578–1581).
A systematic mutational screening of the entire Mef2a gene using direct DNA sequence analysis revealed a 21–base pair (bp) deletion in exon 11 in all ten living affected members in the large family (29) (Fig 6).

The 21-bp deletion led to a deletion of seven amino acids of MEF2A (ΔQ440P441P442Q443P444Q445P446 or Δ7aa). The deletion co-segregated with CAD in the family and absent in 119 individuals with normal angiograms, suggesting that the deletion was responsible for CAD and MI in this large family. The 7-amino acid deletion of MEF2A is a functional mutation that disrupts the transcriptional activation activity of MEF2A by a dominant-negative mechanism (29).
Fig 6: MEF2A intragenic deletion co segregates with CAD/MI in kindred QW1576. (Adapted from Wang et al Science 2003, 302, 1578–1581).
Figure 6 shows the pedigree structure of kindred QW1576. Individuals with characteristic features of CAD and MI are indicated by closed squares (males) or closed circles (females). Unaffected individuals are indicated by open symbols. Normal males under the age of 50 years or normal females under 55 years are shown with light gray color as uncertain phenotype. Deceased individuals are indicated by a slash, “/”. The proband is indicated by an arrow. Each individual's ID# is shown below each symbol. The results of genotypic analysis are shown below each symbol: "+" indicates the presence of the 21-bp deletion of MEF2A (heterozygous); "-", absence of the deletion.

The results for DNA sequence analysis of the wild type (WT) allele and the 21-bp deletion allele (D21bp) of Mef2a are shown in Figure 7. Sequence analysis of exon 11 of Mef2a in the proband (II.1) revealed the presence of a deletion. The wild type and deletion alleles were separated by a 3% agarose gel or an SSCP (single strand conformation polymorphism) gel, purified and sequenced directly. The location of D21bp is indicated.
**Fig 7:** MEF2A 21bp deletion sequence analysis. (Adapted from Wang et al Science 2003, 302, 1578–1581).
After this initial finding, we performed identification and functional characterization of three new MEF2A mutations in four families with CAD (33). We discovered that all three missense mutations are loss-of-function mutations, suggesting that MEF2A mutations can cause CAD/MI by multiple molecular mechanisms. These results strongly support the premise that MEF2A is \textit{adCAD1}. Genotype-phenotype correlation studies suggest that individuals with the dominant negative mutation may display the more severe form of CAD with a high incidence of MI than those carrying loss-of-function mutations. We also provided preliminary data that estimated the frequency of MEF2A mutations in the patient population with CAD and MI.

Later, Weng et al (34) reported the 21-bp deletion in a presumed normal individual, which may suggest that the penetrance of the deletion is not complete. On the other hand, the individual with the 21-bp deletion was not well-characterized and Weng et al. also reported the identification of a new variant S360L. The control groups were not checked for coronary angiogram results and were tested only on the resting echocardiograms (35). On the other hand, our results have been replicated in a Spanish population showing a significant association of the P279L mutation with MI patients (36).
The MEF2 family of transcription regulatory proteins

The MEF2 family of transcription factors (myocyte-specific enhancer factor-2) has been extensively studied primarily in muscle cells and has been shown to play a key role in myogenesis and morphogenesis of cardiac or skeletal muscle. MEF2 proteins are also involved in neuronal cell survival, T-cell apoptosis, and cellular responses to growth factors, cytokines and environmental stressors (37-46). Recent studies, as discussed below, suggest that MEF2 proteins also regulate vascular morphogenesis, including endothelial function and development.

The MEF2 family consists of four members, designated as MEF2A, MEF2B, MEF2C, and MEF2D (39,40). They are nuclear phosphoproteins belonging to the MADS (MCM1, agamous, deficiens, serum response factor) superfamily of DNA binding proteins. The four MEF2 proteins share approximately 50% amino acid identity overall, but have more than 95% similarity at the N-termini. MEF2 proteins have an identical structure of functional domains (Fig 8). The N-termini of the MEF2 proteins contain the MADS domain responsible for specific DNA binding and dimerization, followed by the MEF2 domain that increases DNA binding and mediates interactions of MEF2 proteins with other cofactors. The C-termini of MEF2 factors contain the transcriptional activation domains, which are also the targets of MAP kinase activation pathway and intracellular signaling cascades activated by calcium (47-50). The C-terminal ends contain the nuclear localization signals of MEF2 proteins for transporting these transcription factors into the nucleus.
The MEF2 proteins bind as homo- and heterodimers to a *cis*-element with the consensus sequence (C/T)TA(A/T)₄TA(G/A) in the promoter and regulatory regions of many cardiac and skeletal muscle-specific genes. The 3-dimensional structure of a complex between DNA and the core dimeric DNA binding domain of MEF2A (residues 1-85; containing residues 1-58 for MADS domain and residues 59-73 for MEF2 domain) has been determined. The MADS domain mediates recognition and specificity of DNA binding, and both the major and minor grooves of the DNA are involved in this interaction (38). MEF2 proteins have been shown to interact with many different cofactors, which may be a mechanism by which MEF2 factors control many different cellular processes. These factors include proteins that can bind and stimulate MEF2 activity including MyoD, GATA, NFAT, TH receptor, p300/PCAF, 14-3-3, and ERK5, and other proteins that suppress MEF2 function such as HDAC4, HDAC5, HDAC7, HDAC9, MITR, and Cabin. The interaction of MEF2 with MyoD regulates skeletal muscle differentiation, and the interaction with GATA factors may be important for cardiovascular development.
Fig 8: MEF2A structure and locations of CAD-associated mutations in MEF2A.

The N-terminal end of the MEF2A protein contains the MADS domain responsible for specific DNA binding and dimerization, followed by the MEF2 domain that increases DNA binding and mediates interactions of MEF2 proteins with other cofactors (GATA, ERK5, HDAC, etc.). The C-terminal region of MEF2A contains the transcriptional activation domains, the C-terminal end of MEF2A contains the nuclear localization signal (NLS).
MEF2B, a distinct member in the MEF2 family

Similar to other MEF2 members, MEF2B also binds to A/T-rich DNA sequences and regulates gene expression through the interactions with other co-activators and co-repressors in several tissues. MEF2B is the only member of MEF2 family that regulates smooth muscle myosin heavy chain gene expression in muscle cells (51). Whereas, in neuronal cells, MEF2B and MEF2C, together with adhesion related kinase (Ark), repress the expression of gonadotropin releasing hormone (GnRH) (52).

Among the MEF2 family of transcription factors in vertebrates, MEF2B is the most divergent member. It is significantly homologous with other MEF2 members within the MADS/MEF2 domains, whereas the C terminal region (transactivation domain) shows relatively little homology (53).

MEF2s are in an inactive state in the presence of class II histone deacetylases (HDACs). HDACs repress transcription by deacetylating core histones, which results in chromatin condensation. Whereas MEF2 activity is repressed by Cabin1 in T lymphocytes, Cabin 1 recruits class I HDAC to MEF2 target genes and thus represses MEF2 activity. Recent studies provide further information as to how MEF2 interacts with HDACs and Cabin1. Han et al solved the crystal structure of the MADS-box/MEF2 domain of human MEF2B bound to a motif of Cabin1 and DNA (54). MEF2 domain was shown to be on the surface of the MADS domain stably folded and Cabin1 binds a hydrophobic groove on the MEF2 domain. Later studies revealed the structural complexity of MADS-box and MEF2 domain of MEF2B. Furthermore, the MEF2-binding motif of class II HDAC9 was also determined which is found to be similar to the
structure of the Cabin1/MEF2/DNA complex (55). Thus, these studies provide a clear insight about the process in which MEF2 proteins recruit transcriptional co-repressor Cabin1 or HDACs to specific DNA sites through the MEF2 domain.

The functional role of MEF2B remains largely unknown. Unlike other MEF2 members, MEF2B is not phosphorylated by p38 or BMK1. Further studies need to be performed to understand how MEF2B becomes phosphorylated (55,56). MEF2B binds with reduced affinity to the other family members which have the MEF2 consensus sequence, while MEF2A, -C and –D have the same DNA specificity (55, 57).
MEF2C, another member of the MEF2 family and its significance for the development of the vascular system

MEF2C, is highly expressed in various cell types, such as skeletal, cardiac, and SMC as well as the neural crest and the vascular endothelium. It plays a major role in vascular development and is required for cardiac myogenesis and morphogenesis (58-60). MEF2C null mice exhibited profound cardiac defects including a near complete loss of the right ventricle and its outflow tract, defective aortic arch looping, and a disorganized endocardium. As a result, mouse embryos lacking MEF2C failed to form a functional vascular system and had major defects in vasculogenesis that was lethal by embryonic day E9.5. Although endothelial cells were found to develop, they failed to organize into vascular plexus, suggesting a significant role of MEF2C in endothelial cell differentiation.

A separate study on MEF2C null mice also revealed lack of vascular formation, such as arrest of yolk sac capillary network and yolk sac vessel disruption (61). A reduced number of capillaries, and enlarged vascular lumen were observed in the head and those caudal to the heart, whereas the vessels located dorsally and anterior to the heart have smaller lumens than normal control animals. The endocardium of MEF2C null embryos was malformed, and the endothelial cells in this region were rounded with erratic orientations with respect to the underlying myocardium. This suggests that a defect in endothelial cell differentiation or a defect in angiogenesis occurs. Moreover, expression of angiopoietin 1 and VEGF was reduced in the endocardial defects, which indicates that MEF2C has an essential function in the myocardium to regulate the proper
expression of these cytokines and to direct endocardial formation.

BMK1 (Big MAPK-1, also called ERK5) and MEKK3 null mice (MAPK kinase kinase-3), members of the MAPK signaling pathway and upstream targets of MEF2C also have similar embryonic abnormalities. The MAPK signaling pathways start with activation of MEKK by G-protein-coupled receptors. Activated MEKK phosphorylates a subordinate MAPK kinase (e.g. MEK5), which further activates a MAPK (e.g. BMK1 or ERK5) by phosphorylation. BMK1 can stimulate transcriptional activation of MEF2 factors by phosphorylating the transcription activation domains or by direct interactions with MEF2 factors (62-64).

Studies with BMK1 conditional knockout mice provided direct genetic evidence that a BMK1-MEF2C pathway is critical for endothelial cell survival and blood vessel integrity (65). Removal of BMK1 protein led to the death of endothelial cells partially due to the deregulation of transcriptional factor MEF2C. Studies of the MEF2C have demonstrated that MEF2C is an early marker of the vascular development that mediates endothelial cell organization and angiogenesis.
MEF2D, the other member of MEF2 family with a key role in apoptosis

Similar to MEF2A, MEF2D is also ubiquitously expressed. Studies on MEF2D reveal that it plays a significant role in T-cell receptor (TCR)-mediated apoptosis during thymic negative selection. MEF2D mediates calcium-dependent transcription of Nur77, which is also a transcription factor involved in TCR-mediated apoptosis of thymocytes (66, 67). MEF2D mediated the calcium-dependent induction of Nur77 by binding to two calcium-responsive DNA elements in the Nur77 promoter (67). Further studies are needed to investigate if there is any relationship between MEF2D and vascular development.
MEF2A model of atherosclerosis

*Fig 9: MEF2A model of atherosclerosis*

A schematic diagram of a coronary artery. It consists of the endothelium and intima, internal elastic lamina, media, and adventitia. MEF2A is expressed in the endothelium. Mutations detected in this gene lead to a dysfunctional endothelial cell layer and culminate in the generation of atherosclerosis.
Based on the previous findings that mutations in MEF2A are associated with CAD and MI, and MEF2A is highly expressed in endothelial cells in coronary endothelium, we proposed that dysfunctional or abnormally-developed endothelium is a trigger for the development of CAD (45,48). As a transcription factor, MEF2A is expected to control the expression of many genes in the coronary endothelium. Mutations/deletions in MEF2A will alter the expression of a subset of genes and thus reprogram the transcriptional profile in the endothelium, leading to the abnormally developed or malfunctional endothelium. Thus a defective endothelium, which acts as a barrier between blood and artery, is now susceptible to the infiltration of inflammatory particles such as LDLs, monocytes, and macrophages, which ultimately leads to atherosclerosis.

To test the above hypothesis and to determine the functionality of Mef2a, we studied the Mef2a knockout mice and also crossed Mef2a deficient mice (Mef2a +/-) with apolipoprotein (apo) E knockout (Apoe^-/-) mice which were previously shown to spontaneously develop atherosclerosis on a western or chow diet. We analyzed quantitative atherosclerosis, platelet aggregation, and vascular permeability in these murine models to determine the effects of the Mef2a deficiency on atherosclerosis.
CHAPTER II

METHODS

IDENTIFICATION OF MEF2A MUTATIONS ASSOCIATED WITH CAD AND MI

Study subjects and isolation of genomic DNA

The study participants were identified and enrolled at the Cleveland Clinic Foundation through the Gene Bank program at the Cleveland Clinic Heart Center, which is a registry of data in conjunction with a repository of DNA/serum/plasma samples for the individuals undergoing coronary catheterization. Family data and blood samples were also obtained when possible from family members if a positive mutation was identified in the proband. This study was approved by the Cleveland Clinic Foundation Institutional Review Board on Human Subjects.

The diagnostic criteria for CAD include: 1) any preceding or existing indication of MI which is based on the existence of at least two of the following: prolonged chest pain, ECG patterns consistent with acute MI, or significant elevation of cardiac enzymes;
2) percutaneous coronary angioplasty (PTCA); 3) coronary artery bypass surgery (CABG); or 4) coronary angiography with >70% stenosis (28). The controls were selected as those individuals with coronary angiography showing no luminal stenosis, and were ≥55 years of age. After drawing the blood from these patients, genomic DNA was isolated using the Mammalian DNA Isolation Kit (Roche Diagnostic Co., Indianapolis, IN, USA).

**Mutational analysis**

The genomic structure of the *MEF2A* gene was determined by comparing its cDNA sequence to the genomic sequence using BLAST. PCR primers were designed based on the flanking intronic sequences of each exon (Table 2). The complete coding region and the intron splice sites of the *MEF2A* gene were amplified by PCR. Amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and sequenced with forward and reverse primers by an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Mutation screening of genomic DNA was carried out using single strand conformation polymorphism analysis (SSCP) from CAD and MI patients as well as with control individuals as described (68-72).
Table 2: PCR primers for amplification of *MEF2A* exons and mutational analysis.

<table>
<thead>
<tr>
<th>Exon(s)</th>
<th>Forward Primer (5' to 3')</th>
<th>Reverse Primer (5' to 3')</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGAAGCTGCTAGATCGATGATTAG</td>
<td>ACCCAACCATTTCTGCTATGTT</td>
<td>64°C</td>
</tr>
<tr>
<td>2</td>
<td>AGATTCACCTTAGAGCTAGAATA</td>
<td>ACAAGTCATTGTGGTCTAGTTA</td>
<td>64°C</td>
</tr>
<tr>
<td>3</td>
<td>AGGTACCTCCATGCGCTGGTCTTA</td>
<td>AAGTAGAGTAAAGTAGTACCTT</td>
<td>66°C</td>
</tr>
<tr>
<td>4</td>
<td>TAAGTACTTTTATCTACCTACTC</td>
<td>GCAAAGATGTGTGCTAATCTCT</td>
<td>66°C</td>
</tr>
<tr>
<td>5</td>
<td>AGTAACCTTGGATTACCTGTGGCA</td>
<td>GAACCTGCTTTGTGAAACCATGA</td>
<td>50°C</td>
</tr>
<tr>
<td>6</td>
<td>TCTCTATTCAAGTCAGTTCAGTGT</td>
<td>TGATATTAGCTGAAAGTCCTTTAG</td>
<td>50°C</td>
</tr>
<tr>
<td>7</td>
<td>GATACCTCAACCTGAGTGGCTAG</td>
<td>GGAAGCTACAGATTTGACATGT</td>
<td>55°C</td>
</tr>
<tr>
<td>8</td>
<td>TGIGAGTACCAACGATGGTCTAG</td>
<td>GGTAGATAAACAACACGTAGAG</td>
<td>60°C</td>
</tr>
<tr>
<td>9</td>
<td>TCACATCAGATGCTTTCAGAA</td>
<td>CACAGAAAGGACAGTGATCGA</td>
<td>64°C</td>
</tr>
<tr>
<td>10</td>
<td>ATAGATTCCGTTAGGGACCTTTCA</td>
<td>AAGACAGTGTGAGCCAGAGTG</td>
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</tr>
<tr>
<td>11</td>
<td>TGCAAGGTTACCTTGCAAGCCAT</td>
<td>AGATATGTAGGAGCCAGTCCTAG</td>
<td>64°C</td>
</tr>
</tbody>
</table>
SSCP method has been proved to be a very useful way of detecting mutations for large-scale studies. We did PCR to amplify all 11 exons of MEF2A and by use of $\alpha^{32}\text{P-dCTP}$ in the PCR reactions. The PCR products were denatured, and separated by electrophoresis through a polyacrylamide gel at 4°C. The abnormal bands were detected by exposing the gel to a film. After the autoradiography, the abnormal bands were cut and dehydrated and then re-amplified using the original PCR primers, and sequenced by the Big Dye Terminator cycle sequencing reaction and an ABI PRISM 3100 Genetic Analyzer.

**Plasmid constructs and mutagenesis**

The MEF2A expression construct was created by cloning the full-length MEF2A cDNA into plasmid vector pcDNA3, and was kindly provided by Dr. Eric N. Olson at University of Texas Southwestern Medical Center. Each missense mutation of MEF2A was introduced into the wild type construct by PCR-based site-directed mutagenesis, and verified by DNA sequence analysis.

The full-length $GATA-1$ cDNA was isolated by RT-PCR, and cloned into pcDNA3 (29), resulting in the $GATA-1$ expression construct. The reporter gene for transcriptional activation assays, $ANFp-700/Luc$, has –700 bp to +1 bp upstream region from the transcription start site of the human atrial natriuretic factor ($ANF$) gene fused to the luciferase gene (29).
**Transcriptional activation (luciferase) assays**

The effect of missense mutations of MEF2A on transcriptional activation was analyzed using the *ANFp-700/Luc* reporter gene. The reporter gene was co-transfected into HeLa cells with either the wild type or mutant MEF2A expression construct alone or in combination with the *GATA-1* expression construct. Transcriptional activation activity was assayed with luciferase activity.

HeLa cells were grown to 95% confluence in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 50 ng of DNA for the expression construct, 1 µg of DNA for the reporter gene, and 50 ng of internal control plasmid pSV-galactosidase. Luciferase assays were performed using a Dual-Luciferase assay kit according to the manufacturer's instructions (Promega, Madison, MI). The β-galactosidase activity expressed from pSV-galactosidase was used to normalize the transfection efficiency. The experiments were repeated two times in triplicate. Data are expressed as mean ± S.E.

**Western blot analysis**

The efficiency of transfection was examined by Western blot analysis. Cells were transfected as described above, harvested, and lysed 24 h after transfection. Forty µg of total cellular lysates were separated by 12% SDS-PAGE and electro-transferred to a polyvinylidene fluoride membrane. The membrane was probed with goat polyclonal anti-MEF2A antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) as the primary
antibody and the rabbit anti-goat IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). ECL Western blotting detection reagents (Amersham Pharmacia Biotech) were used to visualize the protein signal.
DEVELOPMENT OF AN IN VIVO MOUSE MODEL FOR CAD

Mef2a knockout mice

We collaborated with Dr. Eric Olson who has provided us with the Mef2a knockout mice. The Mef2a +/- mice have been created by Dr. Eric Olson at University of Texas Southwestern Medical Center (Fig 10). However, these mice were found to be infected with hepatitis C. Thus, our lab had these mice rederived by Jackson Lab. We have received 42 Mef2a +/- mice from Jackson Lab which performed hepatitis C cleaning up (rederivation). These Heterozygous Mef2a +/- mice were interbred to generate Mef2a wild type (+/+) and homozygous mutant (-/-) mice.

Generation of Mef2a knockout mice

Homozygous Mef2a +/- mice are susceptible to sudden death during the first week of life (32) (Fig10). The sudden death phenotype in the Mef2a +/- mice is strain-dependent (32). In the mixed background, about 90% of Mef2a +/- mice die suddenly by 3 weeks of age, and 10% survived to 1-9 months. In the isogenic 129Sv genetic background 100% of Mef2a +/- mice die within the first week after the birth, while nearly 100% survived when mixed background 129Sv/C57B16 Mef2a +/- mice were crossed to the FVB/N background. No structural heart defects were detected before the death, but dilation of the right ventricle was detected for the homozygous Mef2a +/- mice with sudden death at necropsy.
Fig 10: Generation of Mef2a knockout mice (Adapted from Naya, F.J., et al, 2002 Nat. Med., 8,1303–1309)

a. A diagram of the MEF2A protein is shown below the mouse Mef2a locus around exon 2, which encodes amino acids 17–86. Homologous recombination resulted in deletion of coding exon 2 and insertion of a neomycin-resistance gene. B, BamHI; E, EcoRI; N, NheI; X, XhoI. b, PCR amplification of genomic DNA. Primers 1 and 2 (indicated in a) generate a 240-bp product (WT) and primers 1 and 3 produce a 380-bp product (Mutant). c, RT-PCR of total RNA from skeletal muscle of wild-type and Mef2a−/− mice at 3 wk of age. A PCR fragment (240 bp) was detected in the wild-type (WT) sample (indicated by arrowhead to the right), but no specific PCR product was detected in the sample from mutant mice. Arrowhead to the left points to a nonspecific PCR product present in all lanes. Reactions were performed in the presence and absence of reverse transcriptase (RT). ■, wild type; □, Mef2a+/−, □, Mef2a−/−
Propagation of knockout mice deficient in *Mef2a*

*Mef2a*+/− mice on the mixed background 129Sv/C57Bl6 were brother-sister mated to generate three strain-matched groups of mice, *Mef2a*+/+, *Mef2a*+/−, and *Mef2a*−/− in a 1:2:1 ratio. A total of 39 mice (17 wild type and 22 heterozygous) upon weaning were maintained on a paigen diet or an atherogenic diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid (Harlan-Teklad 88051) for 38 weeks. Aortic atherosclerotic lesions were quantified in *Mef2a*+/+, *Mef2a*+/− mice at this time point. *Mef2a*−/− mice were unable to survive until this time point.

Creation of *Apoe* and *Mef2a* deficient mice

*Mef2a* knockout mice were crossed with *Apoe*−/− mice, a widely used genetic model for atherosclerosis (Apoe knockout mice was kindly provided by Dr. Jonathan Smith from Cleveland Clinic). Although no murine model can truly simulate human situation, the Apoe–deficient mouse model has proved to be one of the best models to study the pathogenesis of atherosclerosis. In order to minimize lethality of the *Mef2a*−/− progeny, the *Mef2a*+/− mice in the 129Sv/C57Bl6 background were crossed to *Apoe*−/− mice on the FVB/N, yielding 50% *Mef2a*+/− mice that are hemizygous for *Apoe*. After mating, these progeny were crossed back to FVB/N *Apoe*−/− mice. We have selected *Mef2a*+/− mice that are homozygous for *Apoe*-deficiency (50% of the progeny). These mice were brother-sister mated to generate the three strain-matched groups of *Apoe*-deficient mice that are *Mef2a* +/+, +/−, and −/− in a 1:2:1 ratios, which were used for
analyses (Fig 11). Littermate controls were used to decrease the potential for a background gene to be responsible for the phenotype.

Both males and females were used for this study. A total of 47 mice, of which 26 were males (15 wild type and 11 heterozygous) and 21 were females (9 wild type and 12 heterozygous) upon weaning, were maintained on a western-type diet containing 21% fat, 0.2% cholesterol, and no cholic acid (Harlan-Teklad 88137) until 16 weeks of age. Aortic atherosclerotic lesions were quantified in $Mef2a^{+/+} \text{Apoe}^-$, $Mef2a^{+/} \text{Apoe}^-$ mice at this time point.
Step 1: Mef2a knockout mice were crossed to Apoe-deficient mice. In order to minimize lethality of the Mef2a^-/- progeny, the Mef2a +/- mice in the 129Sv/C57Bl6 background were crossed to Apoe-deficient mice on the FVB/N, yielding 50% Mef2a +/- mice that are hemizygous for Apoe. Step 2: After mating these progeny were crossed back to FVB/N Apoe-deficient mice, we have selected Mef2a +/- mice that are homozygous for Apoe-deficiency (50% of the progeny). Step 3: These mice that were brother sister mated to generate the three strain-matched groups of mice Apoe-deficient mice that are Mef2a +/-, +/-, and -/- in a 1:2:1 ratios, which were used for analyses.

Fig11: Schematic representation for generation of Mef2a and Apoe deficient knockout mice.
Determination of *Mef2a* animal genotype

Mice were genotyped by polymerase chain reaction (PCR) analysis. A small piece of mouse tail was digested in 0.4 ml lysis buffer (pH 7.5) containing (in mM) 50 Tris, 100 EDTA, and 100 NaCl and 1% sodium dodecyl sulfate and 0.5 mg/ml proteinase K overnight at 55°C. After the lysis, the mixture was centrifuged (10,000 rpm/min, for 10 min). The DNA (5 µl) sample was amplified by one sense primer (5'- GCT AGC CAA CAT TTC ACC TTT GAG ATC T -3' for binding to the MEF2A DNA) and two antisense primers (5'- CAA CGA TA T CCG AGT TCG TCC TTT C -3' for normal sequence and 5'- GCC TTC TAG TTG CCA GCC ATC TGT TGT TTG -3' for knockout targeting sequence, respectively). The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. The results were analyzed as shown below.
**Fig 12: Genotyping of the Mef2a knockout mice.**

A. Genotyping Results of MEF2A Wt and Mutant. PCR Primers were designed to amplify the Wt and Mutant genomic DNA. Primers 1 and 2 amplified the Wt and Primers 1 and 3 amplified Mutant genome. The bands in the +/- indicate WT, +/- indicate Homozygous, +/- indicate Heterozygous, NC indicate Negative control (Homozygous Control). B. Genotyping results of the F1 Generation of MEF2A Homozygous and Heterozygous pair reveals three Homozygous and three Heterozygous mice.
**Determination of Apoe animal phenotype**

The total cholesterol assay was performed to identify the Apoe\(^{-/-}\) mice. Briefly, plasma was isolated from whole blood and the biochemical assay using Stanbio enzymatic kit was performed. Mice with cholesterol levels of 350 mg/dl and above were considered to be Apoe\(^{-/-}\) mice.

**Dietary Restrictions**

All mice were weaned at 4 weeks of age and subdivided into Mef2a knockout and Mef2a wild type and heterozygous on Apoe knockout background. The Mef2a knockout mice (n=39) were fed with the paigen diet or an atherogenic diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid for 38 weeks after the weaning.

Mef2a wild type and heterozygous on Apoe knockout background mice were fed with the western diet containing 21% fat, 0.2% cholesterol, and no cholic acid (Harlan-Teklad 88137) for 12 weeks after the weaning. The food consumption was monitored on regular basis. No differences in the food consumption were visible among animals of these two experimental groups. The animals were housed in the Cleveland Clinic Animal Facility in a specific pathogen-free environment in rooms with a 7 a.m. to 7 p.m. light dark cycle. The Cleveland Clinic Institutional Animal Care and Use Committee approved all procedures involving mice.
Blood collection and glucose measurement

At one week before the end of the treatment period, all animals were fasted overnight but water was allowed to access. 200-300 μl of blood was collected from all the mice from the orbital sinus by using micro capillary tubes with EDTA coated appendorfs. The peri-orbital sinus of the mouse was used as a source of venous blood. These mice were anesthetized using isoflurane. The main purpose of this was to study correlation with total and HDL cholesterol levels. Blood glucose levels were also measured in these animals with a single drop of whole blood with a One Touch II monitor.

H&E staining and immunostaining

Hearts from the Mef2a+/+, Mef2a+/- and Mef2a -/- mice were fixed in 4% zinc formalin. After dehydration in a graded series of ethanol, they were paraffin embedded, cut into serial sections at 5 μm thickness (saggital sections), and subjected to Hemotoxylin and Eosin staining. After deparaffinization slides were placed in harris hematoxylin for 45 seconds followed by 1% eosin for 1 minute. Slides were then dehydrated in an ascending gradient of ethanol followed by xylene, and mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ) for examination by light microscopy. Immunostaining was also performed to determine the cell layers in the coronary arteries and other vessels as previously described (73-75). The antibodies for CD31 (PECAM-1) (Sigma) and vascular smooth muscle alpha-actin (Sigma), MOMA2 (Sigma) were used as the markers for endothelial and smooth muscle cells, and macrophages respectively.
ATHEROSCLEROSIS LESION ANALYSIS

Although there are several different assays to quantitatively assess mouse atherosclerosis at various locations, the aortic root assay is still considered the standard assay in many laboratories, as lesions start developing at this area very early this site compare to other areas and the aorta region is highly susceptible to atherosclerosis in mice. Other sites besides the aorta such as carotid, pulmonary, femoral and innominate arteries can also be used to locate lesions (76, 77).

A detailed protocol for the assessment of mouse atherosclerosis in the aortic root is as follows (78):

**Materials**

1. Ketamine/xylazine anesthesia is prepared by adding 10 ml of ketamine stock (100 mg/ml) and 1.5 ml xylazine stock (20 mg/ml) to 35.5 ml sterile phosphate buffered saline (PBS).
2. Small animal surgical tools: fine scissors, fine forceps, and coarse forceps with serrated tips.
3. PBS or normal saline in a 10 ml syringe with a 24 g needle for perfusion.
4. 10% phosphate buffered formalin (Fisher, Pittsburgh, PA).
5. Glass Staining racks with removable trays and wire handles.
6. Oil red-O stain (0.24%): This is prepared by combining 1 g oil red-O powder (Sigma Aldrich, St. Louis MO) and 250 ml 2-Propanol for 10 minutes. 150 ml of ddH₂O is added and mixed for an additional minute, allowed to stand for 6 minutes and then filtered.
through a 0.45 μm. Stericup (Millipore, Billerica, MA). Oil red-O stain should be made freshly and used within 2 hours of preparation.

7. Harris Hematoxylin (2.4%): 100 ml Harris Hematoxylin stock solution (Sigma Aldrich, St. Louis MO) is combined with 98 ml of ddH₂O and 2 ml of glacial acetic acid and filtered.

8. Bluing Solution: 5 drops of ammonium hydroxide reagent in 1 l of ddH₂O.

9. Light Green Solution (0.25%): 50 ml of a 1% stock solution (2 g Light Green SF) powder (Fisher, Pittsburgh, PA), 198 ml ddH₂O and 2 ml acetic acid) is added to 150 ml ddH₂O.

10. Glycerol gelatin (Sigma Aldrich, St. Louis MO).

11. 22 x 60 mm #1 cover slips (Fisher, Pittsburgh, PA).


13. Microscope equipped with video imaging camera, video capture card, and PC: Olympus CX41 microscope (Melville, NY), a Panasonic Industrial Color CCD Camera, Model #GP-KR222 (Secaucus, NJ), Pinnacle Systems video capture card and software in a Dell PC.
Mouse sacrifice, heart removal, and fixation

1. After the treatment period all study group mice were weighed and a 25 g mouse was anesthetized by intraperitoneal injection with 200 μl of the ketamine/xylazine stock, the volume was adjusted according to the weight of the mouse using ~8 μl of anesthesia stock per g body weight. Depth of anesthesia was tested by lack of response to a firm squeeze of the hind foot. Additional anesthesia was administered in 50 μl increments until this depth is achieved. The age of the mice under study should was kept constant ± 0.5 weeks, as lesion area is extremely age dependent.

2. The legs of the supine mouse were taped down onto several folded paper towels, and the ventral surface of the mouse was wet with water or 70% ethanol (to keep the hair matted down). A lateral skin incision was made at the base of the abdomen extending to the width of the rib cage. Some skin was wrapped from the top side of the incision around a blunt forceps with serrated tips the mouse’s rear legs were hold down, and forcefully skin was torn and pulled up over the mouse’s head exposing the peritoneum and chest. Using a fine scissors the peritoneal membrane was cut opened from the base of the abdomen to the ribcage. The sternum was lifted and quickly cut with a fine forceps, and penetrate the diaphragm with the tip of a fine scissors. Two lateral cuts were made at the top of the ribcage and the ventral ribs were removed exposing the beating heart.

3. The circulatory system was perfused with 10 ml of PBS or normal saline by placing the needle of the saline-loaded syringe into the left ventricle, using a fine scissors a cut
was made to the right atrium, (allowing drainage of the circulatory system into the chest cavity), and slowly pumping the saline into the heart. The mouse was euthanized at this point by exsanguination.

4. The heart was gently lifted out by the apex, without squeezing the top which can damage the aortic root, which is completely embedded within the heart. Any fibrous connective tissue attached to the heart was cut, and finally the aorta was cut just above the level of the heart, and the heart is put into a vial containing 5 to 10 ml of 10% phosphate buffered formalin. The vial was placed at 4°C indefinitely, until ready for embedding, but for a minimum of 24 hrs.

**Mounting the hearts on a pedestal in OCT and sectioning hearts with a cryostat**

1. The lower half of the heart was removed by cutting with a razor blade. The alignment of this cut is crucial to getting sections perpendicular to the axis of the aortic root. The cut was made ~ 1mm beneath the base of and parallel to the right and left atria.

2. The top half of the heart was then placed into a plastic tissue mold (lower chamber is 1.5x 1 x 0.5 cm, L x W x D) with the cut surface at the bottom of the mold, and then covered in OCT compound in both the lower and upper chambers. The heart was moved around carefully to fill the aorta with OCT. The mold was placed in the cryostat set at -22°C to freeze the OCT.
3. The heart was removed from the base mold and mounted with the cut side facing up on the sample pedestal, which is mounted on the cryostat freezing stage, upon a bed of not-yet solidified OCT.

4. The pedestal was placed into the chuck of the cryostat and a 30 μm thick section was cut and transferred to a microscope slide for observation to check this section was parallel to the cut surface of the heart, or oblique yielding a partial section. More sections were cut with observation every ten sections to detect the atria, appearing as appendages, which signal the approach of the aortic sinus. The aortic sinus appears as 3 bipartite valve bases with attached leaflets along with an intact intima.

5. Once the aortic sinus was visible, the section thickness was decreased to 10 μm and every other section was saved, four sections per slide covering a total distance of 80 μm. Six slides were prepared in this manner, covering a total distance of 400 μm, at which point the valve bases are shrunken, but still visible, and the valve leaflets may not all be visible. Slides were labeled, using a solvent resistant marker or a pencil, with the mouse identification number and the slide number and stored at room temp until ready to stain, or at -20°C for immunostaining.


**Staining sections for aortic root analysis**

1. 20 slides were placed back to back in the 10 slots of each staining rack. A series of staining trays were set up with the solutions listed below and the slides are immersed for the specified times.

2. ddH₂O for 2 minutes.

3. 60% 2-propanol for 30 seconds.

4. Filtered oil red-O staining solution for 18 minutes, in order to stain neutral lipids.

5. 60% 2-propanol for 30 seconds.

6. ddH₂O for 1 minute

7. ddH₂O for 1 minute

8. Harris Hematoxylin stain for 2 minutes.


10. ddH₂O rinse for 1 minute.

11. Light Green counterstain for 30 seconds.

12. ddH₂O for 1 to 15 minutes to partially destain Light Green.

13. Air dry slides until ready for cover slipping.

14. For cover slipping, glycerol gelatin was heated to 55°C in a water bath. One drop was placed over each of the 4 sections on the slide. The cover slip was placed on the slide and pressed down firmly to remove any air bubbles. After the glycerol gelatin sets for an hour, excess was removed by washing the slides in soapy water.
**Quantification of aortic root lesion area**

1. The slide was observed microscopically under bright field illumination using a 4x or 10x objective lens. Video images were captured with a video capture card and frame grabber software.

2. One section on each of the six slides was quantified. Lesions consist of oil red-O staining lipid-filled regions as well as any fibrous regions lumenal to the internal elastic lamina. Using Image Pro or other similar software, and after calibration with a stage micrometer (for each objective lens), the lesions were circled and the area of each lesion for the quantified section on the slide was exported to a spreadsheet in μm². This was repeated for each of the five remaining slides from the heart, using 80 μm intervals between the sections, if possible.

3. The sum of the lesion areas were calculated for each of the six quantified sections. Then the mean lesion area was calculated for all of the six quantified sections.

**Plasma lipid analysis**

Mice were on a 12 week western diet and were 16 weeks old. These mice were fasted overnight. 200-300 μl of blood was extracted through the retro orbital sinus and blood was immediately spun down for 10 min at 4°C and plasma was separated. Total cholesterol, HDL cholesterol, and triglycerides were measured by quantitative – enzymatic colorimetric determination Stanbio kits with the plasma isolated as described.
above. VLDL/LDL cholesterol was deduced by subtracting HDL cholesterol from total cholesterol.

**In vivo vascular permeability assay**

We performed this assay using Evans blue dye (30mg/kg in 100 µl PBS; Sigma) as described previously (79). Briefly, permeability was stimulated using mustard oil (Sigma) and after 30 minutes, mice were euthanized and ears were removed and weighed. Evans blue dye was extracted, measured spectrophotometrically at 560 nm and the leakage was expressed as nanograms of dye per milligram of tissue.

**Statistical analysis**

For each mouse, the mean aortic root lesion area in µm² was used as the primary measurement. Lesion areas varied among individual mice of the same age, gender, and genetic background. Gender had an effect on lesion area, and females of most strains had larger aortic lesions than males.

Data are expressed as mean±SE. An unpaired Student t test was used to detect significant differences when two groups were compared. P values less than 0.05 were considered statistically significant.
CHAPTER III

RESULTS

Identification of three novel MEF2A mutations associated with CAD

The genomic structure of MEF2A and the PCR primers for successfully amplifying all 11 exons of MEF2A for mutational analysis were previously reported (29). Single-strand conformation polymorphism (SSCP) and DNA sequence analyses were used to screen for mutations in MEF2A. 207 independent patients with unequivocal diagnosis of CAD based on cardiac angiography and/or development of MI, and 191 controls were screened (Table 3). Patients with onset of CAD ≤55 years of age in males, and ≤60 years of age in females were selected. The control individuals were individuals with coronary angiography documenting lack of any significant coronary arterial narrowing.
The CAD cases and controls were matched for ethnicity, age, and body mass index (p>0.05, Table 3). The CAD population has significant higher rates of males and individuals with a history of diabetes, hypertension, and smoking (p <0.01, Table 3), all of which are established risk factors for CAD.

The entire coding region of MEF2A and the intron-exon boundaries were screened for mutations in the 207 patients and 191 controls. MEF2A mutations were identified in four Caucasians among the 207 CAD patients (33, Figs. 13-15 and Table 3), accounting for 1.93% of this patient population. No mutations were identified in 191 controls. MEF2A mutations identified in this study were clustered in exon 7 which encodes portion of a transcription activation domain of this transcription factor (31). After the mutation was identified, a significant effort was made to characterize family members, albeit with limited success.
Table 3: Characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CAD Cases</th>
<th>Normal Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>207</td>
<td>191</td>
<td>-</td>
</tr>
<tr>
<td>Mean current age (SD) (years)</td>
<td>44.8 (4.8)</td>
<td>43.9 (7.8)</td>
<td>0.18¹</td>
</tr>
<tr>
<td>Mean Body mass index (SD) (kg/m²)</td>
<td>31.0 (6.8)</td>
<td>30.6 (8.4)</td>
<td>0.60¹</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>77.8%</td>
<td>74.4%</td>
<td>0.63²</td>
</tr>
<tr>
<td>African-American</td>
<td>17.9%</td>
<td>19.4%</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>2.9%</td>
<td>5.2%</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>1.5%</td>
<td>1.1%</td>
<td></td>
</tr>
<tr>
<td>% male</td>
<td>77.3</td>
<td>49.2</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>% Hypertension</td>
<td>69.6</td>
<td>38.2</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>% Diabetes</td>
<td>27.5</td>
<td>8.4</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>% current smokers</td>
<td>31.9</td>
<td>19.4</td>
<td>0.004²</td>
</tr>
<tr>
<td>Qualifying events</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiogram &gt;70% stenosis</td>
<td>77.8</td>
<td>0</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>Coronary artery bypass graft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CABG)</td>
<td>40.1</td>
<td>0</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>17.9</td>
<td>0</td>
<td>&lt;0.001²</td>
</tr>
<tr>
<td>Angioplasty (PTCA)</td>
<td>40.5</td>
<td>0</td>
<td>&lt;0.001²</td>
</tr>
</tbody>
</table>

¹An unpaired t-test was used for the continuous factors (age and body mass index). ²A chi-square test was used for the categorical factor
N263S mutation

Fig 13: Identification of MEF2A mutation N263S in CAD patients GB04146, GB05072.

(A) Results of SSCP analysis showing an aberrant SSCP conformer (indicated by an arrow). N, normal control. (B) Sequence analysis of the normal (WT) and aberrant SSCP conformers revealed an A to G substitution at codon 263 in exon 7 of MEF2A. This mutation causes substitution of amino acid residue asparagines by serine (C).
The *MEF2A* mutation N263S (change of amino acid asparagine to serine) was identified in two independent patients, GB04146 and GB05072 (Fig. 13 and Tables 4, 5). GB04146 is a 49 year old male patient who was diagnosed with CAD at the age of 41 years, and had coronary artery bypass grafting. His risk factors include history of occasional tobacco use. GB05072 is a 47 year old male who was diagnosed with CAD at the age of 45 years. A stent was placed in the right coronary artery (RCA) at that time. In 2003, the patient had a coronary procedure with a cutting balloon, brachytherapy, and 2 stents placed to the mid-RCA. Risk factors include hyperlipidemia (Table 5), hypertension with age of onset at 30 years, and history of smoking 1 pack per day for 17 years (Table 4). Family history includes a father who died of a massive MI at age 38.
Fig 14: Identification of MEF2A mutation P279L in CAD patient GB00305.

A) Results of SSCP analysis showing an aberrant SSCP conformer (indicated by an arrow). N, normal control. (B) Sequence analysis of the normal (WT) and aberrant SSCP conformers revealed a C to T substitution at codon 279 in exon 7 of MEF2A. This mutation causes substitution of amino acid residue proline by leucine (C).
Mutation P279L (change of amino acid residue proline to leucine) was identified in patient GB00305 (Fig. 14). The patient is a 39 year male with history of CAD (diagnosed at 36), and a stent was placed to the proximal LAD and balloon angioplasty was performed in the first diagonal artery in 2001. In 2002, the patient had a coronary procedure with unsuccessful attempt to cross a proximal total occlusion in the RCA. Risk factors include dyslipidemia (triglycerides level of 252 mg/dL), history of diabetes mellitus, and history of 1 pack per day smoking with cessation in 2001 (Tables 4-5). Further analysis of family members revealed that the 66 year father of the patient also carried the mutation and had a clear diagnosis of CAD. The father was diagnosed with CAD in 2001 and had coronary artery bypass grafting. Other risk factors include hyperlipidemia, hypertension, obesity, diet controlled diabetes, and a remote history of smoking.
Fig 15: Identification of MEF2A mutation G283D in CAD patient GB04583.

A) Results of SSCP analysis showing an aberrant SSCP conformer (indicated by an arrow). N, normal control. (B) Sequence analysis of the normal (WT) and aberrant SSCP conformers revealed a G to A substitution at codon 283 in exon 7 of MEF2A. This mutation causes substitution of amino acid residue glycine by aspartic acid (C).
Mutation G283D (change of amino acid residue glycine to aspartic acid) was identified in patient GB04583 (Fig. 15). This patient is a 50 year old female with history of CAD first diagnosed in 2002 after a catheterization revealed left main trunk disease with 70-80% stenosis. The patient had coronary artery bypass grafting. Risk factors include hypertension and hyperlipidemia (Tables 4-5). The patient additionally was treated for carotid artery disease with right internal carotid endarterectomy in 2000.
Table 4: Demographics of the patients

<table>
<thead>
<tr>
<th>Individual ID</th>
<th>MEF2A Mutation</th>
<th>Current Age</th>
<th>Age at Diagnosis</th>
<th>Clinical Diagnosis for CAD*</th>
<th>Risk Factors*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB04146</td>
<td>N263S</td>
<td>49</td>
<td>41</td>
<td>CABG</td>
<td>HL, SM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Angina class IV</td>
<td></td>
</tr>
<tr>
<td>GB05072</td>
<td>N263S</td>
<td>47</td>
<td>45</td>
<td>PTCA/stent</td>
<td>HL, HTN, SM, FH</td>
</tr>
<tr>
<td>GB00305</td>
<td>P279L</td>
<td>39</td>
<td>36</td>
<td>PTCA/stent</td>
<td>HL, DB, SM</td>
</tr>
<tr>
<td>CAD669</td>
<td>P279L</td>
<td>66</td>
<td>63</td>
<td>CABG</td>
<td>HL, HTN, OB, DB, SM</td>
</tr>
<tr>
<td>GB04583</td>
<td>G283D</td>
<td>50</td>
<td>48</td>
<td>CABG</td>
<td>HL, HTN, SM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Angina class IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>70-80% stenosis</td>
<td></td>
</tr>
</tbody>
</table>

*PTCA, percutaneous coronary angioplasty; CABG, coronary artery bypass surgery; HL, hyperlipidemia; HTN, hypertension; DB, diabetes; S, smoking; FH, family history; OB, obesity
Table 5: Lipid Profile of MEF2A mutation carrier

<table>
<thead>
<tr>
<th>Individual ID</th>
<th>MEF2A Mutation</th>
<th>Lipid Level* Before Medication*</th>
<th>Treatment (medication)</th>
<th>Lipid Level* After Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB04146</td>
<td>N263S</td>
<td>Cho, 177</td>
<td>Fluvastatin (Welchol)</td>
<td>Cho, 160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tri, 54</td>
<td></td>
<td>Tri, 116</td>
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<td></td>
<td>HDL, 40</td>
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<td>HDL, 33</td>
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<td></td>
<td>LDL, 126</td>
<td></td>
<td>LDL, 110</td>
</tr>
<tr>
<td>GB05072</td>
<td>N263S</td>
<td>Cho, 280</td>
<td>Atorvastatin (Lipitor)</td>
<td>Cho, 148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tri, 515</td>
<td></td>
<td>Tri, 340</td>
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<td>HDL, 39</td>
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<td>HDL, 31</td>
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<td></td>
<td>LDL, 164</td>
<td></td>
<td>LDL, 49</td>
</tr>
<tr>
<td>GB00305</td>
<td>P279L</td>
<td>Cho, 182</td>
<td>Simvastatin (Zocor)</td>
<td>Cho, 167</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tri, 252</td>
<td></td>
<td>Tri, 134</td>
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<td></td>
<td>HDL, 27</td>
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<td>HDL, 30</td>
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<td></td>
<td>LDL, 130</td>
<td></td>
<td>LDL, 110</td>
</tr>
<tr>
<td>CAD669</td>
<td>P279L</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>GB04583</td>
<td>G283D</td>
<td>Cho, 274</td>
<td>Atorvastatin (Lipitor)</td>
<td>Cho, 165</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tri, 107</td>
<td></td>
<td>Tri, 93</td>
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<td></td>
<td></td>
<td>HDL, 71</td>
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<td>HDL, 61</td>
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<tr>
<td></td>
<td></td>
<td>LDL, 182</td>
<td></td>
<td>LDL, 85</td>
</tr>
</tbody>
</table>

*Lipid levels in mg/dL; Cho, cholesterol; Tri, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Normal Cho, Tri, HDL, LDL levels are <200, <150, >45, and <130, respectively. N/A
Functional defects of MEF2A missense mutations in transcriptional activation

As all three MEF2A missense mutations identified in this study, N263S, P279L, and G283D, are located close to or within the transcription activation domain (amino acids 274-373) (31), we assessed the functional effect of these mutations on transcriptional activation using a reporter gene (ANFp700/Luc, Fig. 16A). The reporter gene has the ANF promoter (region from –700 bp to +1bp) fused to the luciferase gene (29). The ANFp700/Luc reporter gene was co-transfected with wild type or various mutant MEF2A expression constructs into HeLa cells. Western blot analysis showed that both wild type and all mutant MEF2A proteins were successfully expressed in transfected cells (Fig. 16B). Transcriptional activity was examined and expressed as relative luciferase units. As shown in Fig. 16B, expression of wild type MEF2A activated transcription of the ANF promoter. Mutations N263S, P279L, and G283D significantly reduced transcription activation by MEF2A. Transcription activation activity in cells cotransfected with wild type MEF2A plus each mutant MEF2A was indistinguishable from those transfected with wild type MEF2A alone (data not shown). These data indicate that mutant N263S, P279L, and G283D MEF2A proteins did not form functional transcriptional factors, and did not cause dominant-negative suppression of wild type MEF2A function. Thus, patients with the missense mutations are expected to express half of the normal amount of functional MEF2A.
We previously showed that MEF2A alone can activate transcription of its target genes, but in the presence of transcription factor GATA-1, synergy in transcriptional activation by these two proteins was observed (29). Accordingly, we then investigated whether the missense mutations in MEF2A could disrupt the synergistic transactivation between MEF2A and GATA-1. As shown in Fig. 16 C, either wild type MEF2A or GATA-1 alone activated expression of the ANF promoter, but co-transfection of MEF2A and GATA-1 into HeLa cells showed synergistic activation of the ANF promoter. However, mutations N263S, P279L, and G283D significantly reduced synergistic transcription activation by MEF2A and GATA-1.
Fig 16: Functional defects of MEF2A missense mutations in transcriptional activation.

(A) Structure of MEF2A protein with CAD/MI-associated mutations indicated. The MEF2A gene consists of 11 exons and encodes a 507 amino acid protein. The MADS domain and MEF2 domain at the N-terminus are responsible for DNA binding, dimerization, and interaction with other transcription factors. The transcription activation domain is located in the middle portion, and the C-terminus is responsible for nuclear localization site (NLS).

(B) The effect of missense mutations of MEF2A on transcription activation activity in the presence (+) or absence (-) of GATA-1. Transcriptional activity is shown as relative luciferase activity on the y-axis. The transcriptional activity for the vector alone was set arbitrarily to 1. WT, wild type. Inset: Western blot analysis to determine whether mutant MEF2A was successfully expressed in transfected HeLa cells. C, empty vector control; WT, lane with lysate from HeLa cells containing expressed wild type MEF2A; N263S, P279L, G283D, lanes with lysates from HeLa cells containing expressed MEF2A proteins with corresponding mutations.
Identification of T215A MEF2A mutation associated with CAD

After the initial studies of mutational screening of 207 patients, we screened another set of 200 CAD and MI patients using single stranded conformation polymorphism analysis as described earlier. This screening revealed another mutation in exon 6 of the MEF2A gene. The mutation causes a change of amino acid from threonine to alanine (T215A).
T215A mutation

Fig17: Identification of MEF2A mutation T215A in CAD patient GB04583.

A) Results of SSCP analysis showing an aberrant SSCP conformer (indicated by an arrow). N, normal control. (B) Sequence analysis of the normal (WT) and aberrant SSCP conformers revealed an A to G substitution at codon 215 in exon 6 of MEF2A. This mutation causes substitution of amino acid residue threonine by alanine (C).
Functional defect of the T215A missense mutation in transcriptional activation

MEF2A mutation T215A is also located close to or within the transcription activation domain (amino acids 274-373) (31). We assessed the functional effect of this mutation on transcriptional activation using a reporter gene (ANFp700/Luc, Fig. 18A) as described earlier. Transcriptional activity was examined and expressed as relative luciferase units. As shown in Fig. 18B, expression of wild type MEF2A activated transcription of the ANF promoter. The T215A mutation significantly reduced transcriptional activation by MEF2A. Transcription activation activity in cells co-transfected with wild type MEF2A plus the mutant MEF2A was indistinguishable from that transfected with wild type MEF2A alone (data not shown). This data indicates that mutant T215A MEF2A protein did not form a functional transcription factor, and did not cause dominant-negative suppression of wild type MEF2A function. Thus, the patient with the missense mutation is expected to express half of the normal amount of functional MEF2A. Furthermore, the T215A mutation significantly reduced synergistic transcription activation by MEF2A and GATA-1 (Fig 18).
Fig 18: Functional defect of T215A missense mutation in transcriptional activation.

(A) Structure of MEF2A protein with CAD/MI-associated mutations indicated. (B) The effect of missense mutation of MEF2A on transcription activation activity in the presence (+) or absence (-) of GATA-1. Transcriptional activity is shown as relative luciferase activity on the y-axis.
Characterization of Mef2a knockout mice for phenotype relevant to CAD and MI by atherosclerotic lesion analysis

The Mef2a mutations identified in a large family and also in the sporadic patients suggest that Mef2a can play a very significant role in the pathogenesis of CAD. To further validate this finding, we characterized Mef2a knockout mice.

We examined the atherosclerotic lesions in the Mef2a +/+ and +/- mice. A total of 39 mice, including 20 males (8 wild type and 12 heterozygous) and 19 females (9 wild type and 10 heterozygous) were fed on paigen diet for 38 weeks. 250-300 µl of blood was drawn after overnight fasting through retro orbital sinus for lipid profile analysis. Whole blood was also used before and after fasting to measure the glucose level. The mice were also weighed to study for any growth defects. The heart containing the aortic root was fixed in phosphate-buffered zinc formalin and examined for atherosclerotic lesions in cross sections of the aortic root stained with oil Red O (1.8% oil red O, wt/vol, in 60% isopropanol, filtered twice through a 0.2-µm filter) (77,78). Atherosclerotic lesions were compared among these mice per genotype per gender to study lesions, Of the 39 mice, 35 did not show any lesions. However, 3 heterozygous mice showed lesions with an average of 3095.33 µm² and 1 wild type showed a lesion size of 2005 µm² ($P > 0.05$, data not shown).
Increased atherosclerosis in \textit{Mef2a} heterozygous mice deficient in \textit{Apoe} fed on western diet

The \textit{Apoe} deficient mice have been proved to be one of the best models to study the pathogenesis of atherosclerosis and the \textit{Mef2a} and \textit{Apoe} double knockout mice helped us determine if \textit{Mef2a} defects can worsen the atherosclerosis phenotype under the \textit{Apoe} knockout background.

\textbf{Quantitative atherosclerosis and lesion morphology}

A total of 47 mice, including 26 males (15 wild type and 11 heterozygous) and 21 females (9 wild type and 12 heterozygous) upon weaning, were maintained on a Western-type diet containing 21\% fat, 0.2\% cholesterol, and no cholic acid (Harlan-Teklad 88137) until 16 weeks of age. 250-300 µl of blood was isolated after an overnight fasting through retro orbital sinus for obtaining plasma. The plasma was used to determine total and HDL cholesterol and other lipid parameters as described (80,81). Whole blood was also used before and after fasting to measure the glucose level. These mice were also weighed to study for any growth defects. These mice were then anesthetized and sacrificed, and the heart containing the aortic root was fixed in phosphate-buffered zinc formalin and examined for atherosclerotic lesions. Quantitative atherosclerosis assays were performed to these mice as mentioned above.

Total aortic lesion area was about twofold greater in \textit{Mef2a}\textsuperscript{+/−}\textit{Apoe}\textsuperscript{−/−} mice than in \textit{Mef2a}\textsuperscript{+/+}\textit{Apoe}\textsuperscript{−/−} controls (Fig. 19A and 19B). Aortic lesions in all groups were observed in the proximal aorta. The lesions were increased by two fold in the heterozygotes ($P \leq 0.0003$). The average size of lesions observed in the wild type males
and females was 46621 µm² and 61568 µm², respectively and in the heterozygous males
and females was 83408 µm² and 137822 µm², respectively. Thus there was a two fold
increase of the lesion sizes among the heterozygous mice compared to controls
($P \leq 0.0003$).

Compared with $Mef2a^{+/}\text{Apoe}^{-/-}$ controls, $Mef2a^{+/}\text{Apoe}^{-/-}$ males had 78%
larger lesions ($P = 0.0003$). Compared to the $Mef2a^{+/}\text{Apoe}^{-/-}$ controls, $Mef2a^{+/}\text{Apoe}^{-/-}$
females had 123% larger lesions ($P = 0.0003$).

In a subset of mice, we examined the morphology of the aortic root lesions. Lesions of $Mef2a^{-/-}\text{Apoe}^{-/-}$ mice consistently appeared more complex than those of
$Mef2a^{+/}\text{Apoe}^{-/-}$ controls, with more area occupied by necrotic core and cholesterol
crystals and some lesions having fibrous caps (Fig. 20).
Fig 19 A: Atherosclerotic lesion analysis in Mef2a\textsuperscript{+/+}, Mef2a\textsuperscript{+/-} male mice on Apoe knockout background.

A total of 26 Males were maintained on western diet for 12 weeks. They consisted of 15 wild type and 11 heterozygous mice. The average size of lesions observed in the wild type males were 46621 µm\textsuperscript{2} and 83408 µm\textsuperscript{2} in the heterozygous males (P = 0.00037).
Fig 19 B: Atherosclerotic lesion analysis in Mef2a\textsuperscript{+/+}, Mef2a \textsuperscript{+-} female mice on Apoe knockout background.

A total of 21 females were maintained on western diet for 12 weeks. They included 9 wild type and 12 heterozygous mice. The lesions were increased by more than two fold in the heterozygous mice compared to wild type control mice (\(P = 0.0003\)). The average size of lesions observed in the wild type were 61568 \(\mu\text{m}^2\) and 137822 \(\mu\text{m}^2\) in the heterozygous mice.
Fig 20: Morphology of aortic lesions from proximal aortic roots.

Representative section from the aortic root showing lesions in Mef2a<sup>+</sup>/Apoe<sup>−</sup> mouse at low- (X4)(A) and high- magnification detail of lower right profile in A (X20). (B) Representative section at the aortic root lesion from Mef2a<sup>+</sup>/Apoe<sup>−</sup> mouse at low-(X4) (C) and high-magnification detail of lower right profile in C (X20). (D) Lesions in Mef2a<sup>+</sup>/Apoe<sup>−</sup> mice show more complex features, which include several sharp circular lucencies indicative of cholesterol crystals and necrotic core.
The graphs in Fig 19A and B represent the lesion sizes in both male and female mice used in this study. The graphical representations of lesions of the wild type are seen on the left side and the heterozygous mice can be seen on the right. These studies show a significant two fold increase of the lesions in the heterozygous mice.
Plasma lipid levels

To establish that the differences in atherosclerotic lesion development did not result from differences in plasma levels of cholesterol or other lipid parameters, we measured total cholesterol, high density lipoprotein cholesterol (HDL-C), very low density lipoprotein cholesterol (VLDL-C), triglycerides (TG) in the plasma. Total plasma cholesterol levels were similar in mice of all genotypes (Table 6), as were HDL-C, VLDL-C levels and TG (22.7 ± 4.6 vs. 24.8 ± 6.3 mg/dl for $Mef2a^{+/+} Apoe^{-/-}$ and $Mef2a^{+/-} Apoe^{-/-}$ mice, respectively). In addition, whole blood was also used before and after fasting to measure the glucose level. These mice were also weighed to study for any growth defects. However, no significant difference was observed between the genotypes.

Thus the high lipid profile results among the wild type and heterozygous mice (Table 6) suggests, the development of atherosclerosis is due to genetic effect but not due to increase levels of lipid profile.
Table 6: Lipid profile analysis

<table>
<thead>
<tr>
<th>Lipid parameters (n=22)</th>
<th>Mef2a\textsuperscript{+/+} Apoe\textsuperscript{-/-}</th>
<th>Mef2a\textsuperscript{+/-} Apoe\textsuperscript{-/-}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>567.6 ± 158.6</td>
<td>517.4 ± 140.3</td>
<td>0.44</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>22.7 ± 4.6</td>
<td>24.8 ± 6.3</td>
<td>0.40</td>
</tr>
<tr>
<td>VLDL/LDL (mg/dl)</td>
<td>544.8 ± 156.2</td>
<td>493.01 ± 140.54</td>
<td>0.42</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>237.9 ± 24.57</td>
<td>253.4 ± 23.2</td>
<td>0.14</td>
</tr>
<tr>
<td>Glucose (mg/dl) (Whole blood)</td>
<td>Fast 104.8 ± 21.9 Fed 179.9 ± 119</td>
<td>Fast 106.25 ± 20.7 Fed 175.3 ± 82</td>
<td>0.87 0.92</td>
</tr>
</tbody>
</table>

Data is presented as mean ± SD. Plasma cholesterol, HDL, VLDL/LDL, triglycerides and glucose levels were measured one week before the mice were sacrificed for atherosclerotic lesion studies. Mice were on a 12 week western diet and were 16 weeks old. These mice were fasted overnight. 200-300 µl of blood was extracted through the retro orbital sinus and blood was immediately spun down for 10 min at 4°C and plasma was separated. Whole blood was used for glucose measurements at both fast and fed stage.
Vasculature in \textit{Mef2a}\textsuperscript{+/-} \textit{Apoe}\textsuperscript{+/-} mice is leaky

\textit{In vivo} vascular permeability assay

In order to study the mechanism of atherosclerosis in our mice, we performed \textit{in vivo} vascular permeability assay (Miles Assay) using Evan’s blue dye. This assay was done on 24 \textit{Mef2a}\textsuperscript{+/-}\textit{Apoe}\textsuperscript{-/-}, \textit{Mef2a}\textsuperscript{+/-}\textit{Apoe}\textsuperscript{-/-} mice, including 12 wild type and 12 heterozygous mice, kept on western diet after weaning for 12 weeks. All mice were age matched and gender matched. Notably, the permeability of preexisting blood vessels in response to a proinflammatory stimulus (with mustard oil) was 3.2 fold higher in the \textit{Mef2a}\textsuperscript{+/-}\textit{Apoe}\textsuperscript{-/-} mice compared to the wild type mice (\(P = 0.000012\)) (Fig.21, 22).

This suggests that the \textit{Mef2a}\textsuperscript{+/-}\textit{Apoe}\textsuperscript{-/-} endothelium junctions are leaky and are more permeable to the influx of proinflammatory particles, leading to the development of atherosclerosis.
**Fig 21: In vivo vascular permeability assay.**

A total of 24 mice (12 wild type and 12 heterozygous) were kept on 12 weeks western diet and assessed for Vascular defects. Miles assay was performed by injecting Evans blue dye. Dye leakage can be seen in the heterozygous mouse ear tissues.

*Number: 1, 3, 5, 8, 10, 11, 12, 14, 17, 18, 19, 20 – Mef2a +/- Apoe-/- Mice (heterozygous)  
Number: 2, 4, 6, 7, 9, 13, 15, 16, 21, 22, 23, 24 – Mef2a +/- Apoe-/- Mice (wild type)*

*A: Stimulated with Mustard Oil; B: Unstimulated*
Fig 22: *In vivo vascular permeability assay.*

*Plasma leakage in ear skin assessed by Evans blue extravasation after treatment with mustard oil, with no difference in control (unstimulated ears)*
CHAPTER IV

DISCUSSION AND FUTURE INVESTIGATIONS

Previously, we studied a large family with CAD and MI, and mapped a genetic locus for autosomal dominant form of CAD to chromosome 15q26 (adCAD1) (29). Further studies identified a 7-amio acid deletion (Δ7aa) in the myocyte enhancer factor 2A (MEF2A) that is responsible for the disease in the family (29). In this study, we identified three new mutations in MEF2A in four families with CAD. These mutations include N263S in two independent CAD patients, P279L in a family and G283D in a single patient. These three MEF2A mutations were not identified through SSCP in 200 control individuals with normal angiograms (lack of any significant coronary arterial narrowings). Functional studies indicate that all three mutations significantly reduce the transcription activation activity, suggesting that they are associated with CAD. We conclude that mutations in MEF2A cause CAD.
The pathophysiology of chromosome 15-linked CAD is related to the deregulation of transcription programs by MEF2A in coronary arteries. We have detected high levels of MEF2A protein expression in human coronary arteries by Western blot analysis (S Arckacki and Q Wang, data not shown). Our immunostaining experiment also demonstrated the co-localization of MEF2A protein signal with endothelial cell marker CD31 signal in coronary arteries (29). These results suggest that MEF2A protein is expressed at significant levels in the endothelium, a single layer of endothelial cells that act as a barrier between the blood and coronary vessels. The detailed molecular mechanism of the development of CAD and MI associated with chromosome 15-linked CAD is uncertain. If CAD-associated mutations in MEF2A cause dysfunctional or abnormally-developed endothelium, the coronary vessels could be prone to inflammation and thrombosis, ultimately leading to development of CAD or MI.

Chromosome 15-linked CAD/MI results from multiple molecular mechanisms that reduce the transcriptional activity of MEF2A. Coexpression of mutant and wild type MEF2A led to loss or dominant suppression of MEF2A transcription function. The Δ7aa mutation, reported previously (29), acts by a dominant-negative mechanism, whereas the three mutations identified in this study (N263S, P279L, G283D) act by a loss-of-function mechanism. Similar findings of the spectrum of functional effects by disease-causing mutations has been reported in other cardiovascular diseases, including Holt-Oram syndrome (74,75) and long QT syndrome (LQTS) (82,83). Sanguinetti et al. found that LQTS-causing mutations Δbp1261 and ΔI500-F508 of HERG act by a loss-of-function mechanism, but mutations G628S and A561V act by a dominant-negative mechanism (84).
Some LQTS-causing mutations in the LQT1 gene KCNQ1, A341V, R190Q, or G189R did not affect the function of wild type KCNQ1 (loss of function) (85). The other mutations, A177P, V254M, L272F, T311I, G306R, A341E, G345E, resulted in dominant negative suppression of KCNQ1 function (85,86). No clear correlation was identified between the degree of HERG or KCNQ1 dysfunction and the clinical outcome. Interestingly, a difference in pattern of the clinical features was observed between MEF2A gene carriers with a dominant negative mutation and those with a loss-of-function mutation. The carriers with the ∆7aa mutation appear to have a more severe form of CAD than those with the three missense mutations. Nine of the 13 carriers with the ∆7aa mutation developed MI, however, none of the five carriers with missense mutations developed MI (Table 3).

The frequency of MEF2A mutations in the CAD population is unknown. This study offers a preliminary estimate. Identification of four mutation carriers among 200 independent CAD patients suggest that as many as 2% of CAD population may carry a MEF2A mutation. These numbers need to be viewed as preliminary since the sample size is small and we limited our studies to affected male patients of <55 years and females of <60 years of age, characteristics of premature coronary artery disease. More studies with large sample sizes are now needed.

In order to further establish the association between MEF2A mutations and CAD/MI, we carried out another study with additional 200 patients. We identified one new mutation in exon 6 of the MEF2A gene in a CAD patient, which causes a change of amino acid from threonine (T) to alanine (A) at codon 215 (T215A).
The functional studies on this mutation using the luciferase assay revealed that the mutation reduced transcriptional activity of MEF2A and acted by a loss of function mechanism. The result provides further support that mutations in MEF2A are associated with CAD/MI.

Atherosclerosis is a multi factorial disorder and caused by various factors such as family history, diet, smoking, obesity, environmental factors etc., which makes it a complex disease. Progressive cellular changes are seen for decades before the acute manifestation of cardiovascular disease. The complexity and chronocity of disease process, and the difficulty to distinguish lesions in an affected patient due to the shortcomings of diagnostic tools makes it even harder to understand the underlying mechanism of atherogenesis. Therefore, an alternative approach developed to study this disease is the use of animal models, which are now extensively used to define mechanistic pathways.

Over the last decade, the mouse has become the predominant species used to create models of atherosclerosis due to the following reasons: 1) There is a great diversity of inbred strains with a clear genetic backgrounds which helps to link the genes to the development of the disease; 2) The recent advances in the genetic engineering has made it easier to modify genes in the mice by either over or under expressing specific genes, and creating knockout, conditional knockout, double knockout models has facilitated the definition of pathways in the atherogenic process.
Most of the mouse models such as apolipoprotein E (*Apoe*) and LDL receptor knockout mice are based on perturbations of lipoprotein metabolism through dietary and/or genetic manipulations.

Although hyperlipidemia is necessary for the development of atherosclerosis, mouse models have demonstrated that many non-lipid factors can influence the severity and characteristics of lesions (77). Our work highlights a novel double knockout mouse model developed by crossing Mef2a and *Apoe* knockout mice.

Our study examines the effect of *Mef2a* deficiency on atherogenesis as a single modifying factor. Our results indicate that *Mef2a* deficiency promotes lesion formation in the proximal aorta in the setting of increased susceptibility to atherosclerosis. The increase in atherosclerotic lesion area in aortas was significant (increased by 78-123% in *Mef2a*<sup>+/−</sup> *Apoe*<sup>−/−</sup>-deficient mice), and similarly, atherosclerosis has been reported in humans with Mef2a gene defects (29, 33).

This study was carried out to investigate our hypothesis that defects in Mef2a by itself or defects in *Mef2a* under a susceptible background setting such as *Apoe* can lead to the development of atherosclerosis. This study was carried out on 39 *Mef2a* knockout mice and 47 *Mef2a, Apoe* double knockout mice. These two different groups of mice were maintained on atherogenic diet for 38 weeks and on Western diet for 12 weeks. At the end of the study, several parameters were measured before the mice were used for quantitative atherosclerosis analysis.
These parameters include lipid profile analysis, glucose measurements both in the fed and fast stage, body weight measurements and also platelet aggregation assay. These were done to understand if there was any correlation between the lipid levels or the glucose levels to the lesions that were seen. We did not find any correlation between these parameters, which suggest that the development of atherosclerosis observed in our study mice was not related to abnormal lipid profile.

Of the 39 Mef2a knockout mice that were maintained under atherogenic diet, 35 did not show any lesions. However, 3 heterozygous mice showed lesions with an average of 3095.33 µm² and 1 wild type showed a lesion size of 2005 µm² ($P > 0.05$, data not shown). Whereas, Mef2a Apoe deficient knockout mice showed a significant difference in the lesion formation. The major effects of Mef2a Apoe deficiency on lesion formation were observed in the proximal aorta. The lesions were increased by two fold in the heterozygotes ($P = 0.0003$). The average size of lesions observed in the wild type males and females was 46621 µm² and 61568 µm², respectively and 83408 µm² and 137822 µm² in the heterozygous males and females, respectively. Thus, there was a two fold increase of the lesion sizes among the heterozygous mice compared to wild type controls ($P \leq 0.0003$).

To identify a mechanism of the Mef2a$^{+/−}$Apoe$^{−/−}$ effects on atherosclerosis, we speculated that Mef2a defect can cause dysfunctional or abnormally-developed endothelium. To test the hypothesis, we assessed the function of vascular permeability in the study group mice using miles assay.
Our findings showed a significant increase in the leakage of the dye from the stimulated ears of the $Mef2a^{+/−}Apoe^{−/−}$ mice compared to the wild type mice ($P = 0.000012$) (Fig. 22). This experiment was repeated thrice to confirm our findings.

At a mechanistic level, vascular leakage is linked to the infiltration of inflammatory particles responsible for causing atherosclerosis (87), hence our novel $in vivo$ model may provide insights into the cause and effects of these genetic abnormalities. These results demonstrate a previously unrecognized function for $Mef2a$ in basement membrane that is required for the maintenance of vascular permeability. Our findings are consistent with the studies on human patients who were heterozygous to the 21bp deletion (29) and also to the other four single point mutations (33).

We must acknowledge the difficulties in studying the homozygous double knockout mice ($Mef2a^{+/−}Apoe^{+/−}$). We observed 95% lethality in the $Mef2a^{+/−}Apoe^{+/−}$ double knockout mice (the 5% that survived also died before the end of the experimental period). Offspring from heterozygous intercrosses were born with the expected Mendelian distribution. However, most of the $Mef2a^{+/−}Apoe^{+/−}$ pups born were smaller in size and died at P1 stage and few of the females that survived were, however, infertile. This fertility defect presumably could be from vasculature defects. $Mef2a^{+/−}Apoe^{+/−}$ males had no obvious impairment in fertility. $Mef2a^{+/−}Apoe^{−/−}$ mice were generally healthy. Hence, all our experiments were carried with the $Mef2a^{+/−}Apoe^{+/−}$ and $Mef2a^{+/−}Apoe^{−/−}$ mice. We feel that these experiments are still representative of the intended phenotype, as reported mutations in human patients are heterozygous.
Our results have important implications for future investigations. First, the $Mef2a^{+/−} \ Apoe^{−/−}$ mice provide an exciting genetic model of atherosclerosis. In the present study, we used this model to address the role of $Mef2a$ on atherosclerosis, and this murine model will likely prove valuable for exploring the detailed molecular pathogenic mechanisms of CAD. In addition, the recognition of $Mef2a$ as a molecular determinant of vascular permeability and atherosclerosis may provide insights to determine how a basement membrane regulates the permeability property of endothelium.
FUTURE INVESTIGATIONS

The central finding of this dissertation is that Mef2a plays a key role in the development of CAD and MI in humans and also in mice. The initial linkage mapping and identification of the 21-bp deletion in the large CAD family laid the foundation for the studies in this thesis. This observation led us to the hypothesis that there could be various other mutations in Mef2a that are associated with CAD and MI. Thus, we carried out screening of MEF2A in 407 CAD patients and 191 controls and identified four novel missense functional mutations. Our transcriptional activation assays suggest that the 7 amino acid deletion in Mef2a acts by a dominant-negative mechanism and the missense mutations act by a loss-of-function mechanism.

To further establish the association between MEF2A and CAD, we studied Mef2a knockout and Mef2a/Apoe double knockout mice. These studies have provided major insights into the role of the Mef2a gene in atherosclerosis. Our finding that heterozygous Mef2a knockout mice showed a 2-fold increase of atherosclerosis than wild type mice at the Apoe-/- background indicates that Mef2a deficiency causes atherosclerosis. Furthermore, we established that increased vascular permeability may be one mechanism for development of atherosclerosis in Mef2a+/ Apoe-/- mice. However, the detailed molecular mechanism by which MEF2A deficiency causes CAD/MI remain to be further explored. These studies are briefly discussed below:
A) *In vivo* properties of mutant *Mef2a* in mouse models

The development of mouse models for CAD based on *Mef2a* is the most convincing approach to validate the finding in human patients that *Mef2a* is a disease-causing gene for CAD and to identify novel molecular mechanisms by which *Mef2a* causes CAD. This was also the only feasible approach to study the *in vivo* physiological roles of *Mef2a* in the development of CAD. This will also be one of the best ways to test potential pharmacological and gene-based therapies that may benefit CAD patients with *Mef2a* mutations. The alternative method to the use of animals is to use coronary artery tissues from human subjects. However, such tissues are not available and studies with human subjects are highly risky and limited.

Thus we proposed to study *Mef2a* deficient mice, along with its appropriate strain matched controls.
Vasculature and growth defects in knockout mice deficient in \textit{Mef2a}

The homozygous \textit{Mef2a}^{-/-} mice are susceptible to sudden death during the first week of life (32). No structural heart defects were detected before the death, but dilation of the right ventricle was detected for the homozygous \textit{Mef2a}^{-/-} mice with sudden death at necropsy (32). Mice that escaped the perinatal sudden death and reached adulthood are also susceptible to sudden death (32). The cause of the sudden death phenotype in homozygous \textit{Mef2a}^{-/-} mice remains largely unknown. Heterozygous \textit{Mef2a}^{+/-} mice exhibit an overall normal phenotype. Potential phenotypic changes in coronary arteries and other vessels were not examined in \textit{Mef2a}^{+/-} or \textit{Mef2a}^{-/-} mice. In this study we examined the coronary arteries or other vessels to determine whether these mice showed any phenotype related to human CAD and MI.

\textit{Mef2a}^{+/-} mice on the mixed background 129Sv/C57Bl6 were brother sister mated to generate three strain-matched groups of mice \textit{Mef2a}^{+/-}, +/-, and -/- in a 1:2:1 ratio. We examined the histology of the aortic root and coronary arteries in these 3 groups of mice, at P1, P2 and other stages of the pups that were alive and in those pups that have died suddenly. This study revealed interesting preliminary insights into development of \textit{Mef2a}^{-/-} mice. They were significantly smaller than the wild type mice and further studies on the heart showed distinct reduction or absence of coronary arteries. Initially we observed that the pups died at the P1 stage were very small and the pups might be born dead. The genotyping results on the dead pups showed that they were homozygous mice. Hearts from the \textit{Mef2a}^{+/-} and \textit{Mef2a}^{-/-} mice were fixed in 4% paraformaldehyde overnight. After dehydration in a graded series of ethanol, they were paraffin embedded,
cut into serial sections (both cross-sections and longitudinal-sections), de-paraffinized and subjected to hematoxylin and eosin staining. This study was designed to detect any overall defects in the heart.

Figure 23 shows growth defects in the \textit{Mef2a} homozygous mice. The homozygous mice showed extremely small size when born and died shortly after birth and a clear vasculature defects were physically seen.
Fig 23: Vasculature and growth defects in knockout mice deficient in Mef2a.

Vascular and growth defects were observed in the mutant Mef2a mice compared to the wild type mice.
The H&E staining of the hearts revealed that the mutant hearts had no blood. Coronary artery defects, and the absence of inter ventricular septum, ventricular chamber abnormalities were observed in Mef2a homozygous mice. Very few numbers of the blood vessels were observed in the homozygous mice. This is a preliminary investigation and further studies are required to confirm this finding (Fig 24-26).
A) *Mef2a* Wild Type

B) *Mef2a* Homozygous- Heart

Fig 24: Coronary artery defects in *Mef2a* knockout mice.

The morphological appearance of mouse hearts, absence/very few of coronary arteries were seen in the homozygous pup compared to wild type mice.
Fig 25: H&E staining of sagittal sections of mouse heart.

A) Wild type B) Homozygous. RA, right atrium; LA, left atrium; RV, right ventricle; IVS, Inter ventricular septum; LV, left ventricle; CA coronary arteries. Very few numbers of the blood vessels were observed in the homozygous mice.
A) *Mef2a* wild type  

B) *Mef2a* homozygous

*Fig 26: H&E staining of saggital sections of mouse hearts*
The histological studies of the coronary arteries and other vasculature of the \textit{Mef2a}^{+/+} and \textit{Mef2a}^{-/-} pups revealed a reduced number of coronary arteries, or collapse of blood vessels (Fig 26) at higher magnification (40x), which might be leading to the lack of blood supply in these mice, causing the death of these mice at a very early age. A further systematic and in depth study will provide additional insights.
B)  *In vivo* properties of *Mef2a* and *Apoe* double knockout mice

Vasculature and growth defects in *Mef2a* and *Apoe* double knockout mice

*Mef2a*<sup>+/+</sup>* Apo<sup>e−/−</sup> mice on the mixed background were brother-sister mated to generate three strain-matched groups of mice *Mef2a*<sup>+/+, +/−, and −/−</sup> in a 1:2:1 ratio. We examined the histology of the aortic root and coronary arteries in the 3 groups of mice at P1 and other stages that were alive and in those pups that died suddenly. The *Mef2a*<sup>+/−</sup>* Apo<sup>e−/−</sup> mice were significantly smaller than the wild type mice and further studies on the heart showed similar results as in the *Mef2a*<sup>+/−</sup> knockout mice (Fig 27). A detailed systematic and in depth study will provide additional insights.
A) $\text{Mef2a}^{+/+} \text{ Apoe}^{--}$  
B) $\text{Mef2a}^{--} \text{ Apoe}^{--}$

Fig 27: Vasculature and growth defects in deficient in Mef2a and Apoe double knockout mice.
The double knockout mice also showed 90% lethality and the pups that were homozygous were small when compared to the wild type counterparts and also showed the absence of vasculature (Fig 27). These mice might be born dead or might have died at P1 stage just after birth. A possible explanation could be that in these mice vascular structure might not be developed completely, which could be causing the lack of blood supply and thereby leading to the death. A further detailed study is required to understand the possible mechanism behind this phenotype.
C) To identify downstream target genes affected by the Mef2a deletion in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs)

ECs and VSMCs will be isolated from Mef2A knockout mice using the following procedure. Thoracic aortas are isolated and 1-mm rings are cut and placed between two layers of Matrigel, supplemented with 20 U/ml heparin. Microvessels grow out from the aortic rings and endothelial cells are recovered using a collagenase-containing dissociation buffer. The murine endothelial cells are cultured as for HUVECs; however, for every other passage, they are plated onto Matrigel, rather than onto fibronectin, which appears to prolong the life of the cultures. VSMCs can also be isolated from the aortas by the enzyme-dispersed method (collagenase, type V, Sigma).

The cells will be used to isolate RNA using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA will be converted to cRNA for Affymetrix microarray analysis. Duplicate arrays will be performed. The microarray data will be analyzed as previously described by us (88). The microarray data will be confirmed by reverse-transcription PCR, Northern blot analysis, in situ hybridization, (88) or Western blot analysis if the antibody is available.

These studies will lead to the identification of the genes regulated by Mef2a in ECs and VSMCs. Future studies can be designed to explore the biological functions of a selected group of genes in relation to CAD and MI. Some genes identified here will become very strong candidate genes for CAD and MI if they locate in the significant MI loci reported in the literature. Association studies can be carried out to test whether the candidate genes are associated with CAD and MI in humans.
C) Molecular and cellular properties of ECs and VSMCs from mutant Mef2a, Mef2a Apoe mice

Because the coronary arteries are composed of two main cell types, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), we hypothesize that Mef2a plays a critical role in differentiation and function of these two types of cells, and Mef2a mutations may affect proliferation, apoptosis, or cell adhesion of ECs.

Cell proliferation assay

Many cell proliferation assay kits are commercially available. We can use Amersham Biosciences [3H] Thymidine Uptake Assay kit for cell proliferation assays. The principle for this assay is based on the close association between DNA synthesis and cell doubling (divided into two daughter cells). If [3H] thymidine is added to the cell culture, cells that are undergoing cell division will incorporate the labeled nucleotide into their DNA. The amount of [3H] thymidine incorporated into the cellular DNA is quantitated. The [3H] thymidine uptake assay system from Amersham Biosciences is based on a polyvinyltoluene bead containing scintillant. The outer surface of the bead has been modified to enable only [3H] DNA to bind, which will generate a measurable light signal.
Apoptosis assay

The apoptosis assay will be carried out to examine the difference from ECs and VSMCs from wild type and mutant mice. This assay is to investigate if the Mef2a deficiency can induce apoptosis. Apoptosis can be analyzed using a flow cytometry that detects DNA breaks labeled by fluorescein anti-BrdU antibody and total cellular DNA labeled by Propidium Iodide (APO-BRDU™ Kit; Pharmingen, San Diego, CA). The CCF LRI Flow Cytometry Core has necessary equipment for this assay.
REFERENCES


