# Regulation and function of MEF2A in cardiomyocytes

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## Abstract

Regular formation of the mammalian heart needs precise spatial and temporal transcriptional regulation of gene programs in cardiomyocytes. Cardiac transcription factors are defined, in this context, as essential transcriptional activators that are expressed predominantly in the myocardium which regulate the expression of the cardiac genes encoding structural proteins of cardiomyocytes. Unsurprisingly, disruptions in this elaborate transcriptional machinery can lead to severe cardiac abnormalities including hypertension, cardiomyopathy, and congenital heart disease. In this field, Myocyte Enhancer Factor 2 (MEF2) transcription factor is considered one of only a few core cardiac transcription factors that play important roles in cardiac development, survival, contractility, and in postnatal adaption to a wide array of physiological and pathological signals.

MEF2 functions as a transcriptional switch by potently activating or repressing transcription through interaction with a variety of co-factors which serve as positive and negative regulators of transcription. The interaction of MEF2 with its co-factors is controlled by a multitude of signaling pathways that result in post-translational modification of MEF2 and in the subsequent MEF2-dependent repression or activation of target gene transcription. Our project studied regulation and function of MEF2A in cardiomyocytes. We hypothesized that the combinatorial interactions between transcription factors and promoter elements that are required for the regulation of cardiac gene expression may operate in pathological cardiac remodeling and hypertrophy. Therefore, studying and characterizing the regulation of proteins which bind to MEF2A in cardiomyocytes may unravel the underlying dysregulation of the cardiac

transcriptome in the pathogenesis of cardiovascular disease and heart failure. In this project, HL-1 cardiomyocytes have been chosen as a model of study. An agonist (Isoproterenol) was used to mimic cardiomyocytes hypertrophy in HL-1 cells. Isoproterenol activates adrenergic signaling which can trigger many mechanisms in the heart contributing to the hypertrophic phenotype. We developed two different methods to capture MEF2A interacting partners (interactome), including immunoprecipitation (IP) of endogenous MEF2A and IP of Flag-MEF2A proteins in normal and hypertrophy conditions. Our optimization will allow characterization of MEF2A interactome partners through state of the art quantitative proteomics approaches.

In previous research, transcriptome analysis (RNA-seq) from left ventricular RNA samples and MEF2A depleted cardiomyocytes identified some genes, including *kf2, junb, alas2* and *rarres2* which may have implications in cardiac hypertrophy. Our ChIP-qPCR data indicated that MEF2A is recruited to the *rarres2* promoter in primary cardiomyocytes. Thus, *rarres2* is a novel MEF2A target gene and further, it will be interesting to uncover functions of MEF2A interactome partners on *rarres2* gene regulation in cardiac diseases. A study has indicated that *klf2*, *junB*, *alas2*, and *rarres2* may have a role in promoting cardiomyocyte hypertrophy in cultured HL-1 cells and primary neonatal rat cardiomyocytes. Taken together, this project developed the methods to study characterization of MEF2A interactome in cardiomyocytes. Additionally, we showed the capacity of some MEF2 target genes, including *rarres2* to promote cardiomyocyte hypertrophy.

## ACKNOWLEDGEMENTS

I cannot tell how much I appreciate my family to support and give me positive energy to pursue my goals in life especially when we lost Dad. Thank you, Mom, for everything you have done for me my entire life. There is no way for me to express my utmost gratitude and thanks to you too. Through, the good times and the bad, you have always been there for me guiding me on the right path.

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## LIST of ABBREVATION

| | |
|---|---|
| **Mef2** | Myocyte Enhancer Factor-2 |
| **GATA4** | GATA Binding Protein 4 |
| **Tbx5** | T-box Transcription factor TBX5 |
| **NFAT** | Nuclear Factor of Activated T-cell |
| **HATs** | Histone Acetylates |
| **HDACs** | Histone Deacetylases |
| **bHLH** | basic helix-loop-helix |
| **MAPK** | Mitogen-Activated Protein Kinases 5 |
| **GPCRs** | G-Protein Coupled Receptors |
| **βAR** | β-Adrenergic Receptors |
| **CaMK** | calcium / calmodulin-dependent kinase |
| **SERCA** | Sarcoplasmic Reticulum $Ca^{2+}$ ATAPase |
| **MyHC** | Myosin Heavy Chain |
| **ANF** | Atrial Natriuretic Factor |
| **Klf2** | Kruppel like factor 2 |
| **Rarres2** | Retinoic Acid Receptor Responder Protein 2 |
| **Alas2** | delta-aminolevolinic acid synthase 2 |
| **PKA** | Protein Kinase A |

| | |
|---|---|
| **PKC** | Protein Kinase C |
| **TAD** | Transcriptional Activation Domain |
| **MADS** | Mcm1 Agamous Deficiens Srf |
| **cAMP** | Cyclic Adenosine Monophophate |
| **ISO** | Isoproterenol |
| **Ate** | Atenolol |
| **TAC** | Transverse Aortic Constriction |
| **HF** | Heart Failure |
| **MLC1/3** | myosin light chain 1/3 |
| **MLC2v** | ventricular myosin light chain 2 |
| **GLUT4** | insulin-sensitive glucose transporter 4 |
| **ET-1** | endothelin I |
| **AngII** | angiotensin II |
| **PLC** | phospholipase C |
| **CO** | cardiac output |
| **IP** | immunoprecipitation |
| **WGA** | wheat germ agglutinin |
| **MMPs** | matrix metalloproteinase |
| **Junb** | Jun-B proto-oncogene |

| | |
|---|---|
| **WB** | western blot |
| **SRF** | serum response factor |
| **GATA4** | GATA binding protein 4 |
| **Nkx2-5** | NK2 transcription factor related, locus 5 |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **Q-PCR** | Quantitative polymerase chain reaction |
| **ChIP** | Chromatin immunoprecipitation |

## Literature Review,

### 1. General Overview

Regulation of gene expression is fundamental in cell growth, differentiation, and disease. Signal-dependent on gene regulation is modulated by transcription factors. Transcription factors are a very different family of proteins and generally function in multi-subunit protein complexes. They may bind to the specific DNA sequences such as promoter and enhancer to regulate turn off or turn on genes (1). Cardiac transcription factors govern the complex process of heart development during embryogenesis and cardiogenesis and are involved in stress regulation of the adult heart in response to hypertrophic stimulation in the generation of cardiac hypertrophy or cardiac protection from cytotoxic stress. Pathological heart diseases activate pathological signaling pathways that target transcription factors and reprogram cardiac gene expression, which then translated into the production of proteins involved in cardiac hypertrophy, leading to heart failure (2). Thus, the regulation of a network of transcriptional regulatory proteins in the heart has indeed been a central theme in understanding the molecular control of physiology and pathology of the heart. A number of transcriptional regulators such as the GATA family, Myocyte Enhancer Factor 2 (MEF2), the home box transcription factor CSX/NKX 2-5, and nuclear factor of activated T-cell (NFAT). They have been identified as playing prominent roles in the heart via control of cardiac gene expression particularly MEF2 as a core transcription factor involved in cardiac physiological and pathological pathways (3).

## 2. MEF2 role and regulation

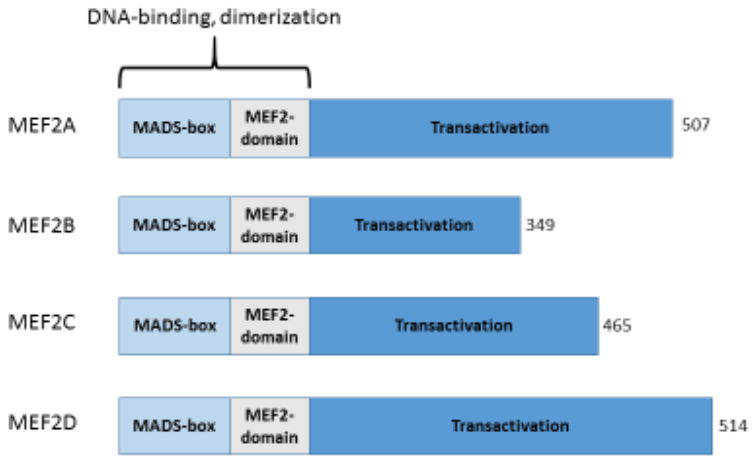
### 2.1 MEF2 Overview

MEF2 transcription factors have prominent roles in cell differentiation, proliferation, apoptosis, migration, shape, and metabolism in cardiac muscle, skeletal muscle, vascular, neural, blood and immune system (4). MEF2 family factors belong to the MADS (Mcm1, Agamous, Deficiens, Serum response factor) superfamily of sequence-specific DNA-binding transcription factors (5). They were initially identified as a prominent factor in skeletal muscle development that can bind A/T-rich sequence within gene promoter of muscle creatine kinase (*mck*). These MEF2 dependent genes can encode a wide array of proteins, including structural proteins like α-cardiac myosin heavy chain (α-MHC), myosin light chain 1/3 (MLC1/3), ventricular myosin light chain 2 (MLC2v), sarcoplasmic reticulum $Ca^{2+}$ ATPase (SERCA), cardiac troponin (-T,-C, and –I), desmin, dystrophin and insulin-sensitive glucose transporter 4 (GLUT4) which regulate cardiac metabolism (6).

Vertebrate MEF2 is encoded by four genes, *mef2a*, *-b*, *-c*, and *–d*, that are expressed in cardiac muscle. These proteins play predominant roles in the differentiation, morphogenesis, and maintenance of vertebrate tissue types. MEF2 proteins contain three domains: an N-terminal DNA-binding MADS domain, a central MEF2 domain and a C-terminal transactivation domain. The MADS and MEF2 domains are well conserved among MEF2 family proteins, unlike the transactivation domain which is divergent across MEF2 proteins (5).

## 2.2 The structure of MEF2 family proteins

MEF2 family proteins from different species share similar N-terminal that contains a highly conserved MADS-box domain and an immediate adjacent MEF2 domain, it consists of 58-amino acids MADS domain and an adjacent 28 amino-acids MEF2 domain. This region plays a key role in the recognition of their target sequences. The main roles of these invariable residues are combined with abundant A/T DNA sequences and they regulate dimerization of MADS-box proteins and provide places for the interaction of MEF2 with other co-factors. Due to the conserved sequence within MEF2 domain, MEF2A, B, C, and D can form homodimers or heterodimers structures with themselves or hetero-dimerization with other molecules (5). Collectively, these two domains mediate dimerization, co-factor interactions and DNA binding to the consensus DNA sequence (T/C) TA $(A/T)_4$ TA(G/A). MEF2 from C-terminal has conserved regions containing potential phosphorylation sites recognized by some kinases, however, the remainder is less conserved (7) (8).




Adapted from (Chen et al. 2017)

**Figure 1. Schematic diagrams of MEF2 isoforms.** MEF2 family proteins, including MEF2A, B, C, and D share a similar N-terminal containing a highly conserved MADS-box domain and immediate adjacent MEF2 domain. Both domains contribute to DNA binding and dimerization. However, there is a diversity in the structure of transactivation domain of MEF2 family members in C-terminal along with the number of amino acids in the longer form of each protein.

**2.3 MEF2 isoforms and their function in the heart**

In the heart, MEF2 family proteins have been recognized as prominent regulators to control cardiac muscle differentiation, vascular integrity and heart development (9). To determine the in vivo role of the mammalian MEF2 transcription factor, global deletion of MEF2A showed that the majority of MEF2A mutant mice die suddenly in the initial perinatal period with cardiac enlargement (10). Deleted MEF2A in cardiomyocytes also revealed severe myofibrillar defects causing the misregulation of a MEF2A-dependent costamere gene program (11). Neonatal cardiomyocytes depleted MEF2A also indicate widespread apoptosis probably resulting from deficiencies in focal adhesion contacts (3). Furthermore, dysregulated expression of MEF2A has been reported in the embryonic heart of a cardiac-specific knockout of focal adhesion kinase, FAK and cause thin ventricular walls, disorganized myofibrils, and decreased cardiomyocyte proliferation (12). Further, previous studies indicated that MEF2A knockdown in zebrafish causes cardiac morphology defects, impairments of sarcomere assembly and cardiac contractility. In addition, expression of cardiac contractile genes, including troponin C, troponin T, atrial myosin heavy chain (aMHC) and cardiac myosin light chain 2 (MLC2,) which are expressed in the cardiac contractile organization were down-regulated in absence of MEF2A transcription factor (9). Taken together, MEF2A plays important roles in a focal adhesion/costamere regulatory circuit in cardiomyocyte differentiation and mediates survival and control of cell growth in cardiomyocytes (3). MEF2C plays an important role in embryogenesis and myogenesis of cardiovascular cells. Of the mammalian *mef2* genes, depleted MEF2C causes embryonic lethality and displays defective cardiac looping morphogenesis and vascular malformation, however, MEF2B expression was enhanced, possibly to compensate for the loss of MEF2C (13). Lack of Mef2d resulted in viable

mice with normal cardiac function, however, when they were subjected to stress, these hearts displayed attenuated hypertrophy and fibrosis (9). Acute depletion of MEF2D in cardiomyocytes cells result in cell cycle re-entry programmed cell death (3). Studies also showed that MEF2D has two alternative spliced isoforms, α1 and α2, are expressed in skeletal muscle, but the MEF2D α2 isoform appears to be required for differentiation and includes a coding exon that is resistant to inhibitory phosphorylation mediated by protein kinase A (PKA). MEF2B is highly expressed in cardiac precursors and primitive heart tube, however, MEF2B knock out mice are viable and its absence in heart could be compensated by either MEF2A or MEF2C in cardiac development (13).

**2.4 Regulation of MEF2 activity by post-translational modification**

**2.4.1 MEF2 functions as a transcriptional co-factor**

The MEF2 transcription factor can interact with a diverse array of co-factors that regulate MEF2 activity as an activator or as a repressor, depending on co-factor interactions to facilitate the ability of MEF2 to respond to intracellular signaling. MEF2 function as a transcriptional co-factor was first recognized in skeletal muscle, where MEF2 proteins were shown to function as essential co-factors for the myogenic proteins, including Myod and myogenin (14).

Myogenic bHLH proteins have the ability to convert non-muscle cells to muscle cells in culture and it was identified that this activity relied on interaction with MEF2. MEF2 and MyoD physically associate and their binding sites are coordinately positioned in the enhancers and promoters of muscle-specific genes (15). In the heart, MEF2C cooperatively activates transcription of the *Nppa* gene with the bHLH proteins HAND1 and HAND2, which like MEF2, are necessary modulators of cardiac development (16). GATA4 is another cardiac transcription factor that interacts with MEF2 to activate the *nppa* promoter. Given the broad overlap in the

expression of GATA4 and MEF2 transcription factors and the prevalence of GATA4 and MEF2 sites in cardiac promoters, these members of these two families of transcription factors might participate in the co-activation of numerous other genes in the heart (17). Thyroid hormone receptor (TR) is another MEF2 co-factor that interacts with MEF2 in the heart and this interaction is facilitated by another factor called p300/CBP, which is considered to bridge the two factors and promote transcriptional activation (18). TR and MEF2 interaction have been recognized to be crucial for the activation of the *α-mhc* gene via closely-positioned binding sites for the two factors in the proximal promoter region. Interestingly, the interaction of MEF2 and MyoD in skeletal muscle is also facilitated by P300. In details, P300 acetylated MEF2 and subsequently cause chromatin relaxation and promote MEF2 transactivation (19).

**2.4.2 MEF2 and chromatin remodeling**

In the heart, MEF2 activity is regulated by associating with histone acetylase (HATs) and deacetylase (HDACs) as chromatin remodeling enzymes which bind to MEF2 and cause chromatin relaxation with transcriptional activation and chromatin condensation with transcriptional repression (20). In this context, MEF2 forms a complex with class II histone deacetylases (HDACs), which include HDACs 4, 5, 6, 7, and 9. Interaction with class II HDACs occurs through the MADS domain at the N-terminus of MEF2 and similarly, a conserved N-terminal domain in HDAC dictates interaction with MEF2. This complex can repress transcription by deacetylating histones, resulting in chromatin condensation and reduced accessibility of core-transcriptional machinery to promoter and enhancer regions of MEF2 target genes (21).

Class II HDACs are prominent mediators of transcription in the developing and postnatal heart that help regulate the hypertrophic response. Normal growth of the myocardium needs

large amounts of structural proteins to be synthesized as cells enlarge, however excessive enlargement of the heart can lead to pathologic hypertrophy, which ultimately causes heart failure. Thus, HDACs serve as a kind of regulated braking mechanism, keeping the MEF2-dependent transcriptional response in check until signals stimulating myocardial growth are received. MEF2 also interacts with several histone acetyltransferases, including P300, which serve to balance the repressive effects of HDAC on MEF2 and allow MEF2 to function as a transcriptional switch (3).

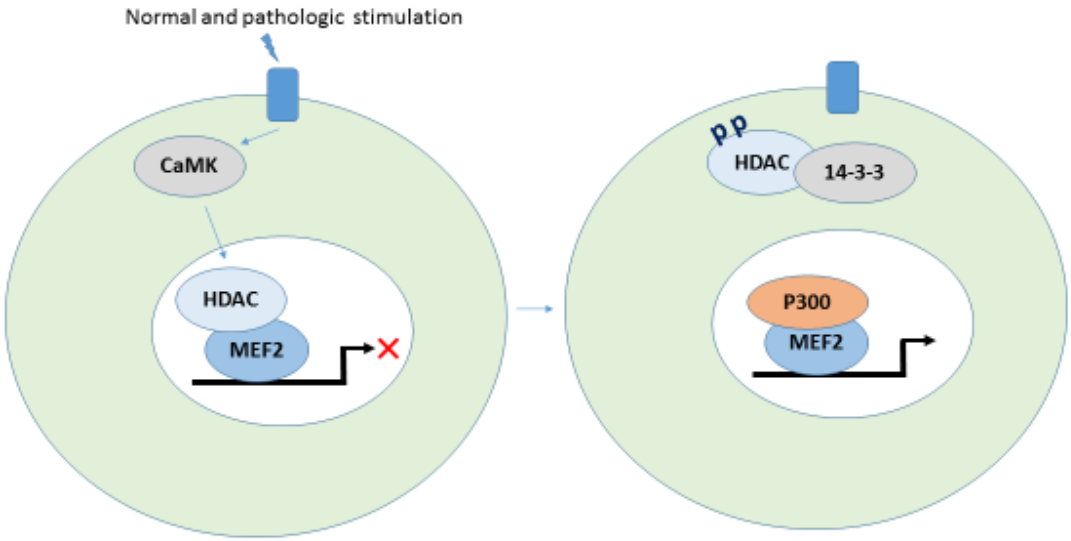



**Figure 2. MEF2 functions as a signal dependent transcriptional switch.** MEF2 functions as a repressor by recruiting class II HDACs to promoter and enhancer regions of target genes. In response to a variety of developmental and pathological signals, CaMK signaling is activated.

Phosphorylation of HDAC results in association with 14-3-3 protein and nuclear export. MEF2 associates with HATs (p300) and potent co-activators.

### 2.4.3 MEF2 regulation upon signaling pathways in cardiac cells

MEF2 transcriptional regulatory machinery plays a crucial function in cardiac gene expression during physiological and pathologic adaption of the heart (22). MEF2 is involved in activating many genetic programs such as those that control and regulate cell morphogenesis, proliferation, differentiation, survival, and apoptosis. These different roles for MEF2 transcriptional activity are regulated by chromatin remodeling enzymes and several kinase signaling cascades. They control MEF2 functions as both a repressor and an activator, depending on the gene, cell type and cellular differentiation state to control of physiology and pathology of the heart.

The interaction of MEF2 with HDACs as one type of chromatin remodeling enzyme, emphasizes the function of MEF2 proteins as both positive and negative regulators of transcription. Furthermore, Class II HDACs are phosphorylated in response to a variety of extracellular signals, including pressure and adrenergic signaling. These signals can result in an increase in the concentration of $Ca^{2+}$ in the cytoplasm, which activates the phosphatase calcineurin and stimulates the activity of calcium/-calmodulin-dependent kinases (CaMK). Phosphorylation of class II HDACs by CaMK happens on the N-terminus of the HDAC protein and subsequently, CaMK causes the disruption of MEF2-HDAC complexes upon mediating nuclear export of the transcriptional repressor, HDAC II, which binds to 14-3-3 proteins. This interaction allows MEF2 to associate with histone acetylases (HATs) and to activate downstream genes (23).

MEF2 factors themselves are considerably phosphorylated in response to a host of intracellular cues. The P38 and BMK1/ERK5 mitogen-activated protein kinases (MAPK) can phosphorylate MEF2 and play a role in MEF2 regulation (24). ERK5 signaling was found to associate with MEF2 through N-terminal region, which contains the MADS-MEF2 domain and not the C-terminal domain that P38 binds (25). ERK5 phosphorylates MEF2C at serine-387 which is a conserved residue in MEF2A and MEF2C, but not in MEF2B or MEF2D in response to a variety of extracellular signals, including adrenergic signaling and pressure overload in the myocardium (26). The p38 MAPK signaling pathway also plays a crucial role in the post-translational modification of MEF2 in myocytes. In fact, p38 can directly phosphorylate MEF2A and MEF2C and enhance its transcriptional activity, but not MEF2B and MEF2D. The p38 phosphorylation promotes the role of MEF2 as a transcriptional activator in response to normal developmental and postnatal hypertrophic growth of the heart, as well as to pathologic hypertrophic cue (25,27).

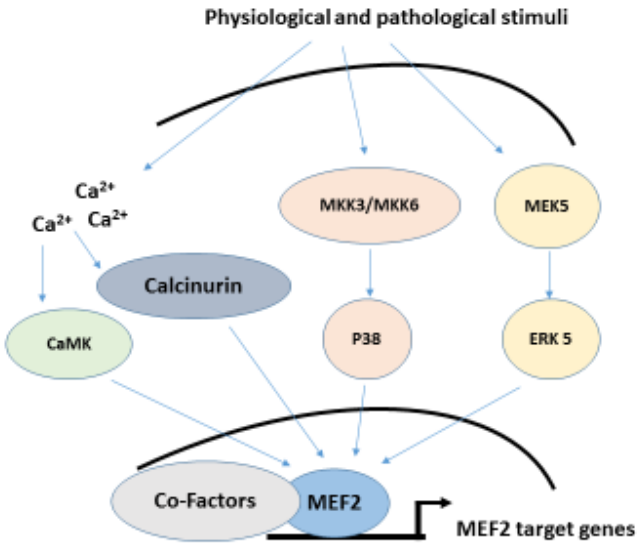


**Figure 3**. **MEF2 regulation by signaling pathways.** Calcium-dependent signals activate MEF2 by stimulating calcium-calmodulin dependent kinases (CaMK) and calcineurin. MAP kinases signaling and the subfamilies include the extracellular regulated kinase 5 (ERK5) and the p38 MAPK can activate MEF2.

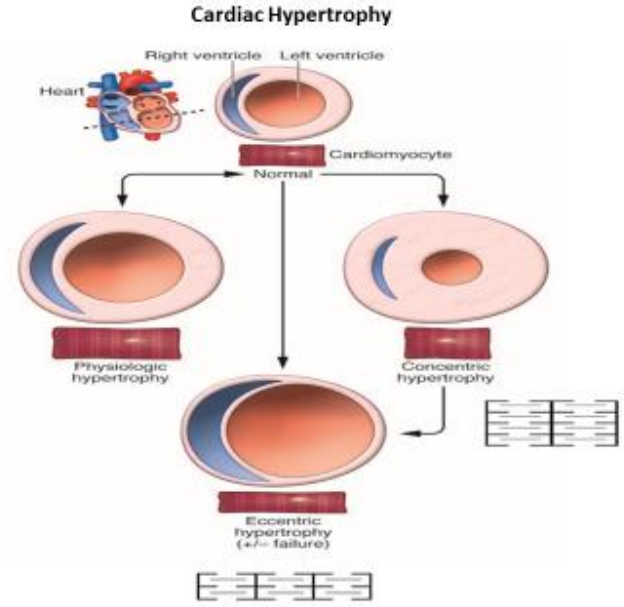
## 3. Myocardial remodeling

Myocardial remodeling is a process by which ventricular size, shape, and function are regulated by mechanical, neuro-hormonal, and genetic factors. In this context, cardiac remodeling might be physiological during normal growth or maladaptive due to pressure overload, volume overload, hypertrophy, cardiomyopathy, hypertension, myocardial infarction or valvular heart disease (28). Left ventricular remodeling is also a step on the progression to heart failure, refers to the acute loss of myocardium which results in an abrupt increase in loading conditions that induce the infarcted border zone and remote non-infarcted myocardium. The underlying molecular and cellular processes leading to cardiac remodeling consist of myocyte growth (cardiac enlargement/cardiac hypertrophy), myocyte death, and remodeling of the extra-cellular matrix (ECM) (29).

### 3.1 Cardiac Hypertrophy

Cardiac hypertrophy refers to the abnormal enlargement, or thickening of the heart muscle resulting from an increase in cardiomyocyte size and changes in heart muscle components, such as enhancing protein synthesis, and re-organization of sarcomeres (30). Cardiac hypertrophy has two forms; physiological and pathological hypertrophy. Physiological hypertrophy happens during normal growth. The size of chamber and thickness of the wall grow in response to natural reactions, such as pregnancy and exercises. In physiological hypertrophy,

cardiomyocytes are increased in the length and width and eventually lead to ventricular hypertrophy with enhanced cardiac performance, however, there is no cardiac dysfunction, fibrosis, and heart failure (31) (32). In pathological cardiac hypertrophy, cardiomyocytes initially undergo compensatory hypertrophy which is an adaptive response to pressure or volume overload, however, they eventually increase in size with a significant thickening of the ventricular walls and lose their elasticity in long standing of cardiomyocyte hypertrophy during development and deterioration of heart failure (28). Pathological hypertrophy commonly happens as a result of myocardial infarction, hypertension, and myocardial valve diseases. According to cellular and molecular level, it is characterized by sarcomere re-organization, activation of transcriptional machinery, re-expression of immediate early genes, such as (*c-jun*, *c-fos*), enhanced protein synthesis and increased expression of cardiac fetal genes such as β myosin heavy chain (*β-MHC*) and atrial natriuretic factor (*ANF*) (2). There are two different pathological hypertrophy phenotype, concentric hypertrophy, and eccentric hypertrophy. Concentric hypertrophy caused by pressure overload in conditions such as hypertension. Sarcomeres are added in parallel and growing in lateral. The size of the chamber goes down, however, the thickness of the wall goes up. In contrast, eccentric hypertrophy caused by volume overload in conditions such as myocardial infarction, cardiac dilation, and cardiomyopathy. Sarcomeres are added in series and growing in longitudinal. The thickness of the wall goes down, however, the size of the chamber goes up (Hunter et al. 1999) (33).




Adopted from Van Berlo, J. H. et al. 2013

**Figure 4**. **Different types of cardiac hypertrophy.** The normal heart can develop various types of cardiac remodeling depending on the stress. Physiological hypertrophy caused from natural actions, such as exercises and pregnancy, in which individual cardiomyocytes increase in length and width and the heart stands a balanced type of eccentric hypertrophy (Chamber walls, and septum enlarge in size). Pathological cardiac hypertrophy caused by pathologic stress and neuroendocrine factors which lead to concentric hypertrophy, in which cardiomyocytes increase in width compared with length, resulting in the wall and septal thickening and a loss of chamber area. However, long-standing of this state can deteriorate into eccentric hypertrophy, in which cardiomyocytes reduce in width and lengthening, leading to extreme chamber enlargement with loss of wall and septal thickness.

### 3.2 Signaling pathway in cardiac hypertrophy

Pathological cardiac hypertrophy characterized by abnormal growth of cardiac myocyte upon re-organization of the sarcomeres and activation of fetal cardiac genes (32). At the molecular level, these effects lead to activation of intracellular signaling pathways that ultimately regulate pathological gene expression programs. Briefly, signaling pathways involved in cardiac

hypertrophy include G-protein coupled receptor, mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and C (PKC) signaling, $Ca^{2+}$ dependent pathways; calmodulin-dependent kinase (CaMK), and calcineurin. (34) (31).

### 3.2.1 Myocardial G-protein-coupled receptors (GPCRs)

Myocardial G-protein-coupled receptors (GPCRs)**,** including adrenergic, angiotensin and endothelin (ET-1) receptors mediate a prominent role in the regulation of cardiac function and hypertrophic growth (35). GPCRs are coupled to three classes of heterotrimeric GTP-binding proteins; Gs, Gq/G11, and Gi which transduce agonist-induced signals to intracellular effectors such as enzymes and ion channels. $G_q/_{11}$- coupled receptors are stimulated through interaction with pathological cardiac hypertrophy agonists, such as angiotensin II (AngII), endothelin I (ET-1), and α-adrenergic which activates phospholipase C (PLC), and downstream kinase PKC, and develop concentric cardiac hypertrophy (31). Stimulating of the β-adrenergic receptors (βAR) activates Gαs and adenylate cyclase (AC) activity. Accumulation of cAMP and the subsequent activation of PKA lead to phosphorylation of downstream effectors that govern the cardiac contraction pathway and thus regulating chronotropy (heart rate) and inotropy (cardiac contractility) (36). Acute stimulation of βAR in response to epinephrine and norepinephrine is correlated with physiological hypertrophy and increased contractile function but chronic stimulation of βAR eventually results in cardiomyocyte hypertrophy, fibrosis, and progressive deterioration of cardiac performance (31). We have three different β-adrenergic subtypes, including $\beta_1$, $\beta_2$, and $\beta_3$ in mammalian. $B_1$-Adrenergic receptor is highly expressed in cardiac tissue and plays an important role to control of heart rate and contraction. The $\beta_2$-Adrenergic receptor is predominantly expressed in the smooth muscle tissue and acts as a crucial factor to control

vasodilatory response. The last subtype, $\beta_3$-Adrenergic receptor is mainly expressed in adipose tissue (37) (Brum et al. 2006). In the heart, $\beta_1$ adrenergic receptors are the most abundant adrenergic receptors and its downstream effector initially increases contractile function but eventually results in cardiomyocyte hypertrophy, fibrosis, and progressive deterioration of cardiac performance (38). In normal condition, $\beta_1$-ARs are activated in response to sympathetic activation, such as norepinephrine and play an important role to increase chronotropy and inotropy. Acute activation of $\beta_1$-ARs causes to increase cardiac output (CO) during the sympathetic response, however, chronic stimulation of the $\beta_1$-ARs is cytotoxic and leads to myocyte death and eventually eccentric cardiac remodeling (37).

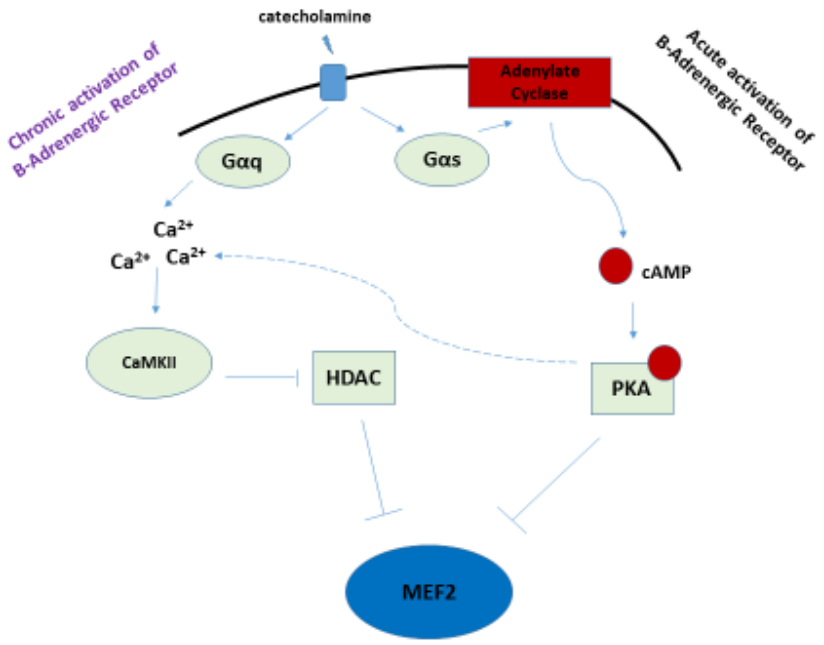



**Figure 5. MEF2 and its regulation by GPCRs (β-Adrenergic Receptor).** In acute activation of β-adrenergic signaling, MEF2 is repressed by activation of PKA. Chronic activation of β-adrenergic signaling leads to the activation of CaMKII and nuclear exclusion of HDAC which results in enhancing the activity of MEF2 inside the nucleus.

### 3.2.2 $Ca2^{+}$ / Calcineurin/ Nuclear Factor of Activated T cells (NFAT) and Ca2+/calmodulin-dependent protein kinase II (CaMKII)

In cardiac hypertrophy, an increase in intracellular $Ca^{2+}$ concentration can activate many $Ca^{2+}$-dependent signaling pathways which are involved in the progression of the heart failure. Calcineurin, a $Ca^{2+}$/-calmodulin (CaM)-activated phosphatase is one of them. Calcineurin is a protein phosphatase that dephosphorylates the nuclear activated T cells (NFAT) transcription factor, which leads to its translocation to the nucleus to regulate genes which are involved in pathological cardiac hypertrophy and heart failure (39). $Ca^{2+}$ /-calmodulin-dependent protein

kinase II (CaMKII) is another protein which phosphorylates HDAC. Class II HDAC is dissociated from MEF2. MEF2 transcriptional activity is enhanced inside the nucleus and mediate progression of cardiac hypertrophy genes (30).

### 3.2.3 Myocyte Enhancer Factor-2 (MEF2)/ Histone Deacetylases (HDACs)

MEF2 activity can be regulated by direct association with histone acetylates (HATs) and deacetylates (HDACs) which are chromatin remodeling enzymes. These enzymes are recruited to the target genes through binding to particular transcription factors such as MEF2. In details, HATs acetylate nucleosomal histones and leads to chromatin relaxation and transcriptional activation, however, HDAC does the reverse work and causes chromatin condensation and repression of the transcriptional activation. In response to cardiac hypertrophy, phosphorylation of class II HDACs by CaMKII disrupts the complex of HDAC and MEF2 and it leads to exposure of a nuclear export of HDAC through recruiting with 14.3.3 protein and activation of MEF2 transcriptional activity contributed to the genes observed in cardiac failure (3).

## Statement of purpose

Our group previously showed that Myocyte enhancer factor 2 (MEF2) has a prominent pro-survival role in cardiomyocytes and this property can be regulated through β-Adrenergic / PKA signaling (40).

Although MEF2 proteins are important transcription factors to regulate the expression of genes in the physiological and pathological adaption of the heart, there is a lack of information about protein-protein interaction and post-translational modifications of MEF2. These interactions may regulate hypertrophic gene transcription in response to activating β-adrenergic signaling. We speculate that studying the regulation of MEF2 interactome in cardiomyocytes may develop the approaches to recognize MEF2 as an activator or a repressor to regulate survival and hypertrophy gene transcriptions. Our work developed the strategies to study and uncover MEF2 interactome in HL-1 cardiomyocytes for the next step, using quantitative proteomics approaches. In our work we also mimicked an experimental hypertrophy condition using isoproterenol treatment through acute activating of β-adrenergic receptors in HL-1 cardiomyocytes.

In previous research, transcriptome analysis (RNA-seq) from left ventricular RNA samples and MEF2A depleted cardiomyocytes identified some genes, including *klf2, junb, alas2* and *rarres2* which may have implications in cardiac hypertrophy (41). Our ChIP-qPCR result indicated that MEF2A is recruited to the *rarres2* promoter in primary cardiomyocytes. Thus, *rarres2* is a novel MEF2A target gene and it will be interesting to study *rarres2* gene regulation by MEF2A interactome characterization.

## Rationale of study

Cardiomyocytes are terminally differentiated cells of the myocardium that lose their ability to proliferate after birth. Thus, they grow in cell size without cell division to adapt to a demand for increased workload of the heart. In this context, cardiac transcription factors are defined as transcriptional activators which are expressed predominantly in the myocardium. They regulate the expression of the cardiac genes encoding structural proteins which are central to cell growth, regulate of survival, and differentiation. MEF2 is one of the most prominent cardiac transcription factors which controls cardiac muscle differentiation, heart development, and vascular integrity. In previous research, our group identified that MEF2 activity is repressed upon acute activation of β-adrenergic/ PKA signaling pathway. β-blockade treatment upregulates MEF2 activity and promoting a pro-survival function of MEF2 in cardiac myocytes (40). Previous studies also identified that MEF2 transcription factors are associated with the regulation of genes expressed during cardiac hypertrophy. We suspect that changes in MEF2 activity may be caused by alterations in protein-protein interaction and post-translational modifications. These interactions may regulate hypertrophic gene transcription in response to activating β-adrenergic signaling.

To study the regulation of MEF2A interactome in cardiomyocytes, we chose HL-1 cardiac muscle cells for the model of study. HL-1 cardiac muscle cell line is an immortalized mouse cardiomyocyte cell line derived from an AT-1 mouse atrial cardiomyocyte tumor. They can be serially passaged unlike neonatal primary cardiomyocytes, they maintain the ability to contract and retain differentiated cardiac morphological, biochemical, and electrophysiological properties (42).

The rationale for choosing MEF2A was that it is predominantly expressed in the adult heart and forms heterodimers with MEF2D. It also targets genes which may be involved in survival and control of cell growth. Previous research also revealed that depleted MEF2A is associated with severe myofibrillar defects and cardiac enlargement which may regulate many hypertrophy genes. To study regulation of MEF2A interactome in cardiomyocytes, first we optimized two different methods for immunoprecipitation (IP) of MEF2A interacting proteins (interactome), IP of endogenous MEF2A interactome proteins and immunoprecipitation of Flag-MEF2A interactome proteins. Induction of cardiac hypertrophy in cardiac HL-1 muscle cells have been done with an agonist (isoproterenol). Isoproterenol binds to GPCRs and causes activation of β-adrenergic / PKA signaling pathway in cardiomyocytes. Hypertrophy in cardiomyocytes observed by visualizing and quantitating the cell surface area of cells as an index of cellular hypertrophy upon wheat germ agglutinin (WGA) staining. Taken together, we developed two systems to precipitate MEF2A interactome in normal and hypertrophy conditions. Besides, we established our in-vitro model under experimental hypertrophy condition by isoproterenol treatment. These experimental achievements will allow state of the art quantitative proteomics approaches to study MEF2A interactome in both normal and hypertrophy conditions.

The previous study, transcriptome analysis (RNA-seq) from left ventricular RNA samples and MEF2A depleted cardiomyocytes identified 65 differentially expressed genes (DEGs) upon transverse aortic constriction (TAC) treatment. Of the 65 TAC regulated DEGs, atenolol (AT) reversed the expression of the 28 mRNAs. Some of them, including *klf2, junb, alas2,* and *rarres2* are selected for further analysis. The rationale for choosing these particular genes was that they were dysregulated under TAC conditions and, prominently, their expression pattern was

substantially reversed with Atenolol treatment. Thus, they may be involved in pathological hypertrophy that can be potentially changed by drug therapy (41). Since two fundamental features of heart failure are cardiomyocyte hypertrophy and apoptosis, we attempted to further test the capacity of some of the identified genes in vitro using gain and loss of function analysis. For the gain of function (GOF) analysis, we tested exogenously *klf2, junb,* and *alas2* alone and in combination to assess their effects on induction of hypertrophy by visualizing and quantitating the cell surface area as an index of cellular hypertrophy in HL-1 cardiac muscle cells (41).

We also identified that *rarres2* is a novel MEF2 target gene which MEF2A is recruited to the *rarres2* promoter in primary cardiomyocytes. This result provides us with an opportunity to further study gene regulation of *rarres2* by some characterized MEF2A interactome in cardiac diseases. Previous studies revealed that *rarres2* gene may affect CASPASE 9 and promote cardiac apoptosis in cardiomyocytes. We speculated that *rarres2* gene may also play a role to develop cardiomyocytes hypertrophy. Thus, we tested the potential role of *rarres2* gene to promote cardiac hypertrophy in primary neonatal cardiomyocytes and HL-1 cardiac cells. Our result showed that cross-sectional area of only transfected cells with exogenous expression of *Rarres2* is significantly enhanced compared to that of in control condition. Thus, *rarres2* highly likely plays an important role to promote cardiomyocyte hypertrophy. Overall, we studied the potential role of some genes including *rarres2* gene to develop cardiac hypertrophy in the HL-1 cardiac cell, however, our observations show that further analysis needs to be done to study MEF2A regulation of *rarres2* gene in heart disease.

## Materials and Methods

## Cell Culture

### A) Primary neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes prepared from 1 to 3 days old Sprague Dawley rats using the Neonatal Cardiomyocyte Isolation system (Worthington Biochemical Corp, Lakewood, NJ, USA). Briefly, whole hearts (8-12) were dissociated with trypsin (Promega, Madison, WI, USA) and collagenase (Worthington Biochemical Corp). The cells were re-suspended in Dulbecco's Modified Eagle's medium F12 (Gibco, Burlington, ON, Canada) supplemented with 10 % fetal bovine serum, 1% penicillin/streptomycin and 50 mg/l gentamycin sulfate (Invitrogen, Burlington, ON, Canada). The isolated cells were plated for 60 min in 37 °C humidified incubator with a 5% CO2 in air, allowing differential attachment of non-myocardial cells. The cardiomyocytes were counted and transferred to gelatin-coated plates. The following day, the culture media was removed and replaced with fresh media. For drug treatment and transfection, cells were serum starved for the indicated time and replenished with fresh medium every 24 hours.

### B) HL-1 Cardiac muscle cell line

The HL-1 cell line was originally established from an AT-1 subcutaneous tumor from an adult female Jackson Laboratory inbred C57BLY6J mouse. HL-1 Cardiac cells were cultured by Clay-comb medium (Sigma) including; 10 % fetal bovine serum, 100 µM nor-epinephrine (Sigma), 100 µg/ml Hyclone penicillin-streptomycin in 70-% confluency plates. In detail, to prepare 100 ml supplemented Claycomb Medium, we added 87 ml Claycomb Medium, 10 ml 10% fetal bovine serum (FBS), 1 ml 100 µg/ml Hyclone penicillin-

streptomycin, 1 ml 0.1 mM norepinephrine (10 mM stock), and 1 ml 2 mM L-glutamine (200 mM stock).

**Freezing Medium**

Freezing medium is made up of 95% FBS/5% DMSO. This can be stored up to a week at 4 °C.

**Pre-Coating Flasks (Gelatin/Fibronectin)**

Gelatin (0.1 g) was dissolved in 500 ml distilled water and autoclaved. The concentration of the gelatin was 0.02%. Fibronectin (1mg/ml) is received in a tube as a liquid and 1 ml fibronectin was diluted in 199 ml of 0.02% gelatin. Before culturing cells, tissue culture plates were coated with gelatin/fibronectin and incubated at 37 °C for at least one hour. The gelatin/fibronectin was removed by aspiration just before adding the cells to the plates.

**Culturing Cells**

Cultures were fed with supplemented Claycomb Medium every weekday. To avoid feeding the cells on weekends, 10 ml of supplemented Claycomb Medium was added to each plate Friday afternoon; the Medium is not changed until the following Monday morning.

**Passaging- Procedure for a 1:3 Split**

When the cells in the remaining plates reached confluency three days later, they were split 1:3.

**Treatment with isoproterenol**

HL-1 cells were cultured upon Clay-comb media under serum starvation condition and were treated with 10 µM isoproterenol for 72 hours.

## Transformation

Stock expression plasmids were diluted to 10 ng/µl and stored at -20°C freezer for later transformation or 1 µl (10 ng) of expression plasmid was added to 50 µl of *E. coli* XL1Blue and incubated on ice for 30 minutes. Heat shock was performed for 1 min at 42°C water bath follows up with 2 min on ice. At the end, the complex was added to the agar plate and incubated at 37 °C degrees overnight.

## Western Blot

Plastic cell scrapers were used to collect cells on ice in 1X PBS. NP-40 lysis buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCL pH 8.0, 1 mM sodium vanadate, 1 mM PMSF, 0.5 % deoxycholate, supplemented with a protease inhibitor cocktail (Sigma, P-8340) was used for biochemical lysis. Protein concentration was determined by standard Bradford assay. Equal amounts of proteins were loaded on 10 % SDS-PAGE gels. Subsequently, the electrophoretic transfer was done on Immobilon-P membranes (Millipore). Blocking was done for 1-2 hours in 5% milk in PBS or TBS dependings on the antibody procedures. Next, primary antibody in 1% milk in PBS or TBS-T was incubated at 4°C overnight. HRP-conjugated secondary antibodies in 1% milk in PBS or TBS were incubated at room temperature for 1 hour (Santa Cruz Biotechnology, Cell Signaling Technology). Enhanced chemiluminescence reagent (Amersham) was used to detect

immuno-reactive secondary antibodies which still bound to the membrane. The membrane were then exposed to Biomax film (Kodak).

## Harvesting Cells

The old media was aspirated and replaced with 1x PBS cold for washing. After aspirating the last PBS wash, 1.0 mL of cold 1x PBS was added to cells for scrapping with a rubber policeman and transferred to a new tube.

## Protein extraction

Protein samples were kept on ice during the entire procedure. Cells were washed three times with cold 1x PBS. After aspirating the last PBS wash, 1.0 mL of cold 1xPBS was added to the cells. Cells were then gently scraped with a rubber policeman and transferred to a new tube and then centrifuged at 1500xg for 5 min at 4°C. After removing the PBS, the pellet was diluted with three times its volume in NP-40 lysis buffer supplemented with 1 mM sodium orthovanadate, 1 mM PMSF and protease inhibitor cocktail (Sigma, P-8340).

Cells were vortexed briefly every 10 minutes for a total of 30-40 min and centrifuged at high speed (>10 000xg) and the supernatant was transferred to a fresh tube. Protein concentration was determined by Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as a standard. An equal amount of protein was diluted with 3X SDS sample buffer and samples were boiled for 5 min chilled on ice and centrifuge to use for SDS-PAGE.

## Transfection

For transfection in HL-1 cardiomyocytes and primary neonatal rat cardiomyocytes, Lipofectamine 2000 (Invitrogen) was employed. Cells were seeded around 80-90 % confluence in 10 ml plates or 6-well plates 24 hours prior to transfection. For 6-well plates, for each well, dilute a total of 2.5 µg of DNA in 150 µl in Opti-MEM medium (Invitrogen), and in a separate tube dilute 2.5X of Lipofectamine 2000 reagent in 150 µl Opti-MEM medium. The DNA and Lipofectamine mixtures (300 µl) were combined, mix and incubated for a minimum of 5 min at RT. The DNA/Lipofectamine mixture was added to the cells in a serum starved culture medium, and incubated at 37 °C overnight. Following the incubation, the cells were refed with new medium and allowed to recover for a minimum 24 hours prior to harvesting or pharmacological treatment.

For siRNA knockdown of HL-1 cardiomyocytes, Lipofectamine RNAiMAX (Invitrogen) was employed and cells were seeded at 80-90% confluence in 6-well plates for 24 hours prior to transfection according to manufacturer's instruction. For each well, Lipofectamine RNAiMAX reagent was diluted into 150 µl in Opti-MEM medium, and in a separate tube, siRNA (100 nM) was added to 150 µl Opti-MEM medium. The reagent were mixed and incubated for a minimum 5 min at RT. The siRNAiMAX / Lipofectamine mixture was added to the cells and incubated at 37 °C overnight.

Following the incubation, the cell were re-fed with new media and harvested 24 to 48 hours later for western immunoblotting analysis to determine the efficacy of protein knockdown.

## Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For primary neonatal cardiomyocytes, total RNA was extracted from cells by using the RNeasy plus Kit (Qiagen) and Qiashredder (Qiagen). RNA was converted to cDNA using Superscript III (Invitrogen) according to manufacturer's instruction.

## Quantitative Polymerase Chain Reaction (Q-PCR)

2.5 µl cDNA was mixed with SyberGreen (BioRad) and 500 nM primers in a final volume of 20 µl. cDNA was diluted 1:10 before to use. Each sample was prepared in triplicate analyzed using Rotor-Gene Q (Qiagen). Parameters for ChIP-qPCR: 5 min 95 °C, [5s 95°C, 15s 60°C] x 40cycles. Fold change (qRT-PCR) was quantified using the ΔΔCt method.

## Chromatin Immunoprecipitation (ChIP)

ChIP experiment was performed followed the guidelines set by EZ ChIP ™ with minor modifications. The protocol was set up for four possible IPs, 2-4 confluent 10 ml plates were used to collect approximately $1 \times 10^7 - 5 \times 10^7$ per each treatment. Confluent plates were washed by PBS at RT. Cells were fixed with 1 % formaldehyde (Sigma) for 10-15 min. Fixing was quenched by adding 1.25 M glycine (10X Glycine) dropwise to each plate for a final concentration of 0.125 M glycine or 1 ml per 10 ml plate for 5 min with slow rocking. Plates were placed on the ice and washed with ice-cold 3x PBS. Cells were scraped on ice into 1 ml of ice-cold PBS containing Roche tablet and PMSF (10.5 ml PBS + 1 Roche tablet + 105 µl PMSF. Cells were pelleted by centrifugation at 5000 rpm for 5 minutes at 4 degrees. The supernatant was removed and the subsequent pellet was washed with 1 ml of wash buffer I (10 mM HEPES pH 6.5, 0.5 M ethylene glycol tetraacetic acid (EGTA), 10 mM EDTA, 10 mM EDTA, 0.25% Triton X-100, protease inhibitor cocktail, PMSF) for 5 min incubation on ice. Nuclei were collected by centrifugation at 5000 rpm for 5 min at 4°C. The supernatant was removed and the nuclei was resuspend in 1 ml of wash buffer 2 (10 mM HEPES pH 6.5, 0.5 mM EDTA, 200 mM NaCl, protease inhibitor cocktail, PMSF) for 10 minutes on ice. The nuclei was centrifuge at 3000-5000 rpm for 5 minutes at 4°C, the supernatant was removed, and the collected nuclei was lysed with fresh lysis buffer (50 mM Tris–HCl pH 8.1, 1 mM EDTA, 1% SDS, plus Roche tablet and PMSF). Chromatin was sheared to approximately 500 bp fragments using sonicator. Crosslinked sheared chromatin was collected following a 15-minute spin at maximum speed and transferred to the new tubes. Twenty percent of total sheared chromatin was set aside as input.

Sheared crosslinked chromatin was diluted 1:10 with IP dilution buffer (0.01% SDS, 1.1%Triton-X 100, 1.2 mM EDTA, 16.7 Mm Tris-HCL pH 8.1, 167 mM NaCl) and incubated with antibody overnight at 4°C with rocking. Protein G Dynabeads (Invitrogen) also were blocked with 20 µg salmon sperm DNA in IP dilution buffer (15 µl of beads + 135 ul IP dilution buffer + 20 µg salmon sperm DNA per IP) overnight at 4°C with rocking. Next, 152 µl of pre-blocked beads were incubated with the IP reaction at 4°C for 1h. Dynabeads-bound antibody-chromatin complexes were washed using IP wash buffer I (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton-X 100, 0.1% SDS) and then with wash buffer II (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), for 10 minutes incubation at 4°C, and followed with two more washes in Tris-EDTA (TE) buffer at 4°C. DNA-protein complexes were separated from the Dynabeads through adding the elution buffer (0.1 M NaHCO3, 1% SDS) for 30 minutes incubation at RT. To separate protein from DNA, samples were treated with 12 µl of 5 M NaCl (BioShop) at 65°C for 4 h or overnight. Protein was further degraded using of Proteinase K (Sigma), EDTA, Tris Ph 6.5 FOR 1 h at 45°C, and DNA samples were purified using a PCR clean up kit (Qiagen, Mississauga, ON, Canada).

## Immunoprecipitation (IP)

HL-1 cells were collected and harvested by using NP-40 lysis buffer including, 100 mM Tris-HCL pH 8.3, 150 mM NaCL, 0.5% (v/v) NP-40, 0.5 % deoxycholate (DOC), 1x Protease inhibitor cocktail. Protein concentration was measured using Bradford assay. 500 µgr protein used for the Immunoprecipitation. Immunoprecipitation was performed using the Immuno Cruz Optima kit (Santa Cruz Biotechnology), according to the instructions of the manufacture. Beads were washed three times with PBS. Proteins eluted from the beads by SDS loading buffer at 100 °C for 5 minutes incubation and run into the 10 % SDS –PAGE for separation.

## MyHC Staining

Primary neonatal rat cardiomyocytes were washed with PBS, pH 7.4 and fixed with 4 % formaldehyde for 10 minutes at RT followed by membrane permeabilization by 90 % methanol at -20 °C for 10 minutes. The cells were blocked using 5 % milk in PBS for 1 hour at 37 °C. Cells were incubated at room temperature with MF-20 (MyHC antibody) diluted in blocking buffer (1 % milk PBS) for 2 hours. Then cells were washed three times with PBS and incubated for 60 minutes at room temperature with horseradish peroxidase (HRP)-conjugated α-mouse secondary antibody. Cells were again washed three times with PBS. Next, cells incubated with the developer which was (0.06 mg/ml DAB and 30% hydrogen peroxide in PBS) to detect α-MyHC. In the end, cell incubated for 2 min with Haematoxylin and washed. Images were taken with an Olympus CKX41 microscope.

## Wheat Germ Agglutinin (WGA) staining

Cells (primary neonatal rat cardiomyocytes and cardiac HL-1 muscle cell line) were fixed with 4 % formaldehyde for 15 minutes. Cells were washed three times with cold PBS. Attention was made not to permeabilize the cells. Sufficient amount of labeling solution (10 µg/ml) was applied to the cells and incubated for 10 minutes at RT. Cells were washed three times with cold PBS. Cells were visualized using Alexa Fluor 633 (Invitrogen) staining, fluorescence excitation (Ex 632nm) and emission (Em 647nm). Images were captured using Axio Observer, Z1 microscope, Plan-Apochromat 63x Oil objective, SDC Camera. For GFP, Ex: 488nm and Em: 509nm, DAPI, Ex: 353nm Em: 465nm, and WGA, Ex 631nm and Em 647nm. Cross-sectional area of cells was quantified using Zen Zeiss Microscopy Analysis Program.

## Results

Some of the following experiments were published in Nature Scientific Reports and in this manuscript I contributed Fig. 4b, Fig. 6b, and Fig. 6e.

### Immunoprecipitation of endogenous MEF2A

To develop the strategy to study the regulation of MEF2A interactome in cardiomyocytes, we initially chose to optimize the system using endogenous MEF2A interactome in HL-1 cardiomyocytes. In this way, the minimum stress entered into the cells and other proteins could bind to MEF2A easier. Our result in **figure 6** showed that MEF2A precipitated successfully from the protein lysate. IgG used as a negative control.

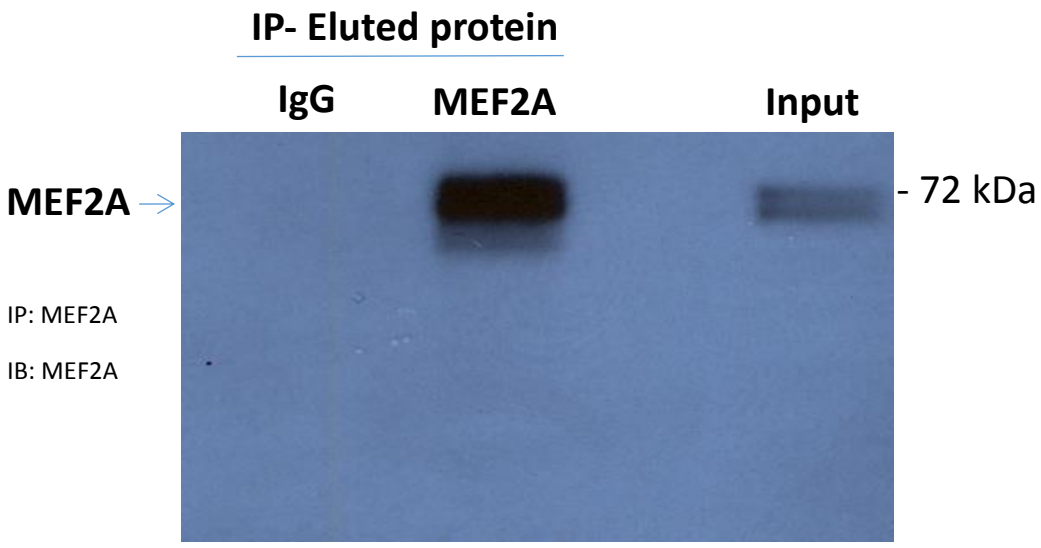



**Figure 6.** An immunoprecipitation (IP) assay was performed in HL-1 cells, using MEF2A antibody to precipitate MEF2A protein. 10 μg lysate used for the input.

**Immunoprecipitation of 3X-Flag-MEF2A in HL-1 cardiomyocytes**

Using a Flag-Tag expression system is another method to develop our strategy to study the regulation of MEF2A interactome in cardiomyocytes. The rationale for using this method is that it is very small hydrophilic tags which improve detection and purification of the recombinant fusion protein. It also facilitates the study of low-abundance protein and the optimization of difficult expression protein. However, FLAG-tag fused to our protein which may cause small hindrance for the other co-factors to bind to MEF2A from N-terminus. Last but not least, scientists believed that over-expression of MEF2A cause to activate protein kinase signaling pathways which regulate gene transcriptions involved in cardiac hypertrophy.

In this way, first we needed to show that 3X-Flag-MEF2A expression vector could be successfully transfected in our model of study. Next, tried to optimize the immunoprecipitation method for pulling down 3X-Flag-MEF2A interactome.

Our result in **figure 7 (A)** Indicated that both primary neonatal cardiomyocytes and cardiac HL-1 cells were successfully over-expressed with 3X-Flag-MEF2A. Actin was used a loading control in the western blot. **Figure 7 (B)** indicated that immunoprecipitation of 3X-Flag-MEF2A proteins has been done successfully in HL-1 cardiac muscle cells.

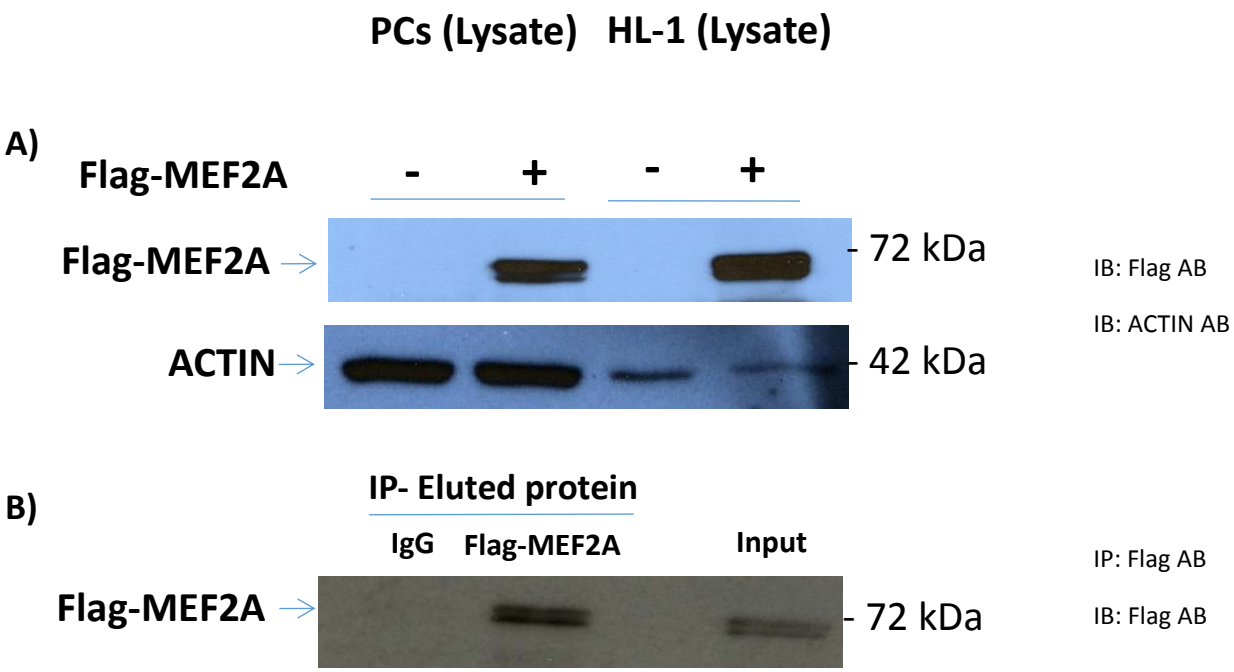
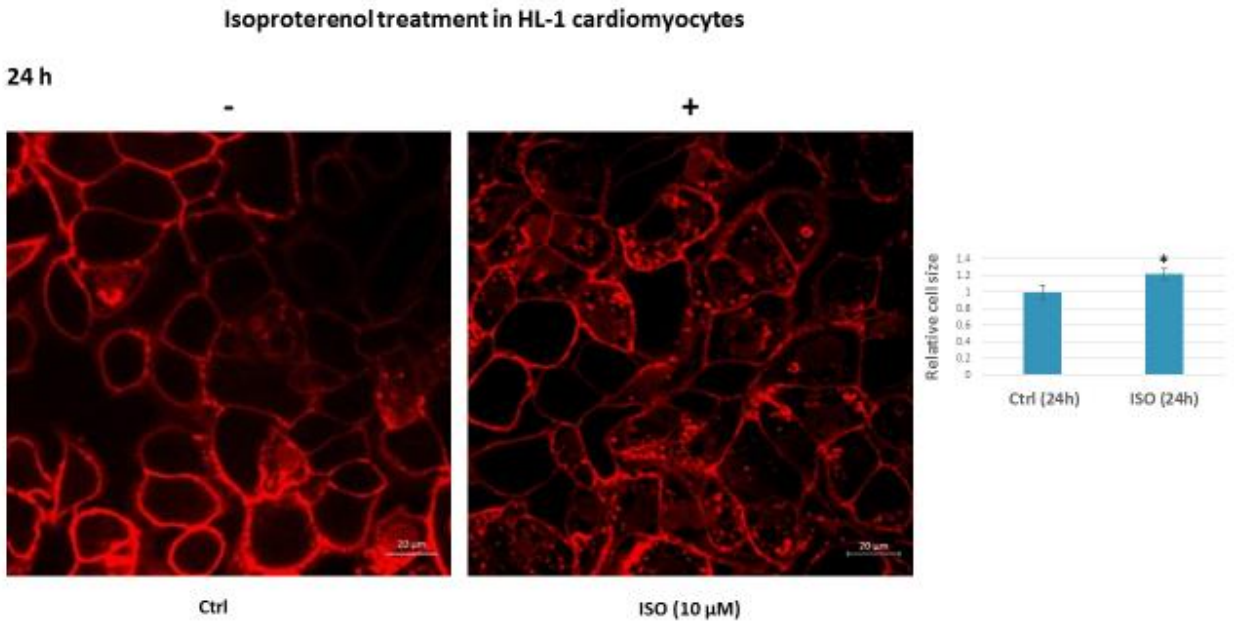



**Figure 7. (A)** Primary cardiomyocyte and HL-1 cells were over-expressed by 3x-Flag-peptide-MEF2A through transfection by Lipofectamine 2000. 20 μg protein from the lysate used for the conditions in Immuno-blotting. **(B)** IP assay was performed in HL-1 cells, using Flag antibody to precipitate Flag-MEF2A protein. 10 μg lysate used for the input.

**Inducing cardiac hypertrophy in HL-1 cardiac muscle cells by Isoproterenol treatment**

To establish an experimental cardiac hypertrophy condition in HL-1 cardiomyocytes, we employed isoproterenol (ISO). Isoproterenol binds to GPCRs and promotes the activation of the β-adrenergic/ PKA signaling pathway and causes acute cardiac hypertrophy**.** In our experiment, 10 µM Isoproterenol was added to the media and the cells were treated for 72 hours. Cells were fixed after 24, 48, and 72 hours and stained with wheat germ agglutinin (WGA) staining. Cross sectional area of cardiomyocytes was visualized and quantitated by Zen Zeiss program. We observed that there is a statistically significant enhancement in cell surface area of cardiomyocytes which means that it developed a role in promoting cardiomyocytes hypertrophy.



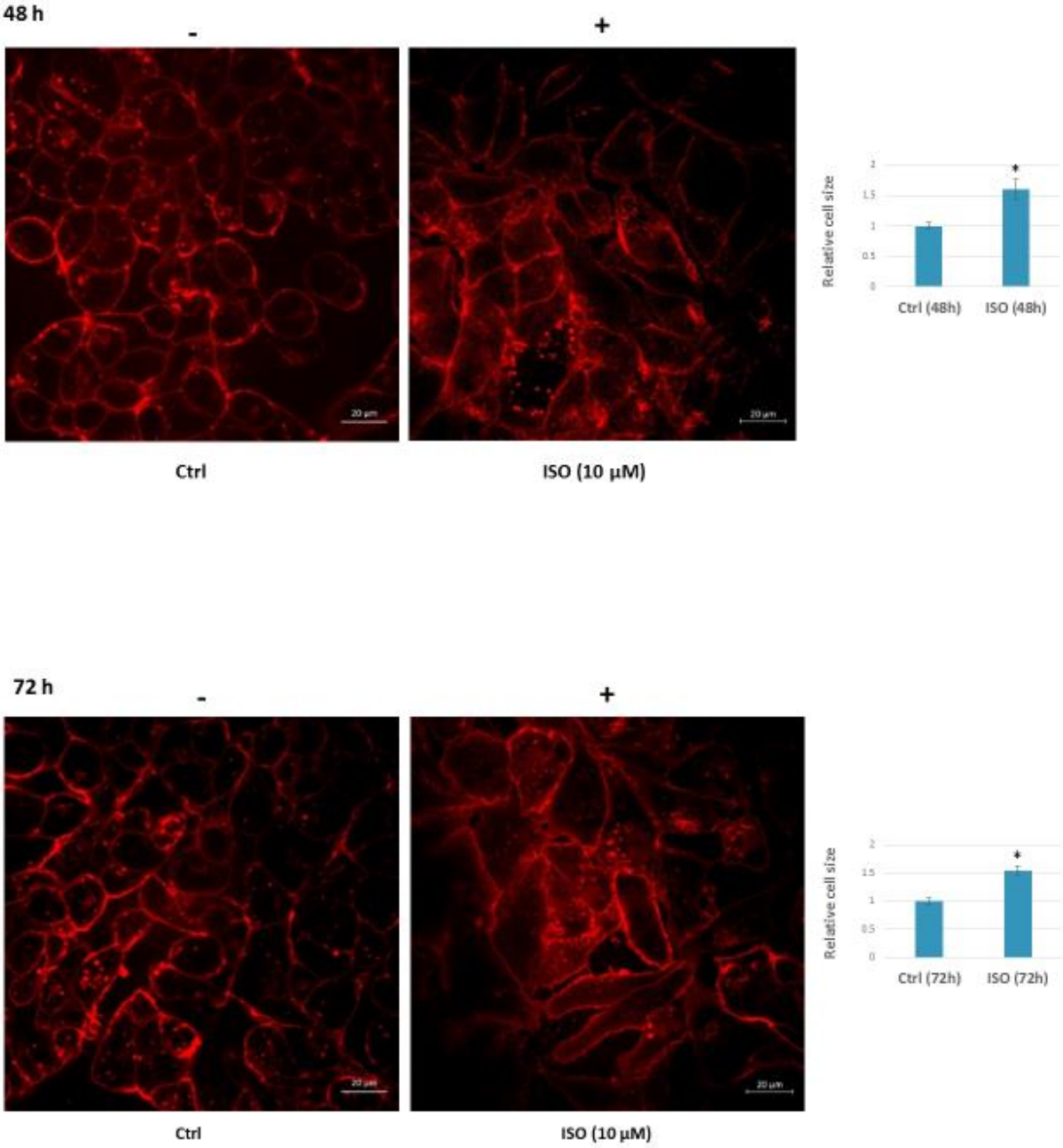



**Figure 8.** WGA staining depicting cardiac hypertrophy in HL-1 cardiac cells upon 10 µM isoproterenol treatment for 72 hours. Cross sectional area of HL-1 cells quantified based on 10 cell measurements per image. Scale bar is 20 µm. Data are presented as ± SEM.

*P < 0.01 vs control.

**Immunoprecipitation of endogenous MEF2A in cardiomyocyte hypertrophy**

To study the regulation of MEF2A interactome in cardiomyocytes hypertrophy, we mimicked an experimental hypertrophy condition in HL-1 cardiac muscle cells using 10 µM ISO treatment for 24 hours. Next, cells were harvested and proteins were extracted. Result in **Figure 9** indicated that immunoprecipitation pulled down MEF2A interactome in normal and hypertrophy conditions.

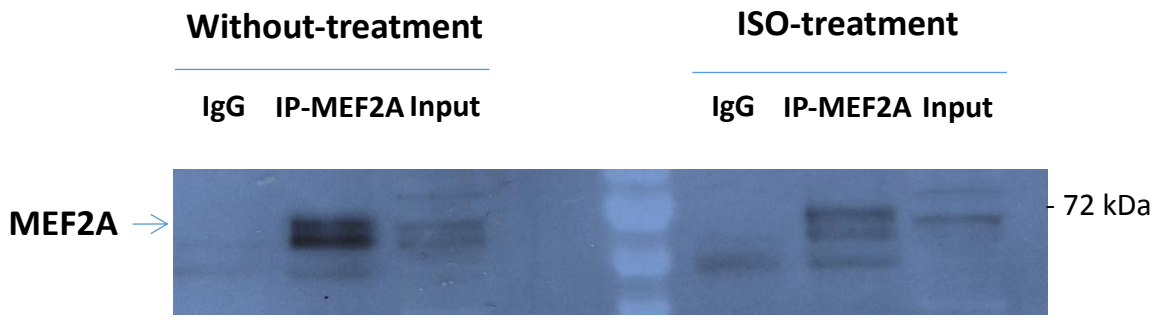



**Figure 9.** An immunoprecipitation assay was performed in serum starved cardiac HL-1 cells upon ± 10 µM isoproterenol treatment. MEF2A antibody was employed to precipitate MEF2A proteins. 10 µg lysate used for the input.

Transcriptome analysis (RNA-seq) from left ventricular RNA samples and MEF2A depleted cardiomyocytes in our previous study found some genes, including *klf2, junb,* and *alas2* which may have implications in cardiac hypertrophy (41). *junb* and *klf2* were transcription factors while *alas2* regulated heme synthesis.

Other studies shown that these genes may have a potential involvement in cardiac remodeling in heart disease and our study attempted to further test the capacity of *klf2*, *junb*, and *alas2* genes to develop cardiac hypertrophy in vitro.

**The role of *klf2*, *junb*, and *alas2* in cardiomyocyte hypertrophy**

To study cardiomyocyte hypertrophy, we chose to exogenously express *klf2*, *junb*, and *alas2*, alone or in combination to measure their effects on induction of hypertrophy in the cardiac HL-1 cell line. Cells were transfected with GFP and expression plasmids for our genes of interest individually or in combination. Next cells are stained with WGA to visualize and quantitate the cell surface area as an index of cellular hypertrophy **(figure 10)**. Only GFP+ cells were quantified for this analysis. Our result indicated that *klf2*, *junb*, and *alas2* may have a role to develop cardiomyocytes hypertrophy since their exogenous expression in cultured HL-1 cells led to a significant enhancement in cell surface area with *klf2*, *junb*, and *alas2* alone or in combination of together (41).

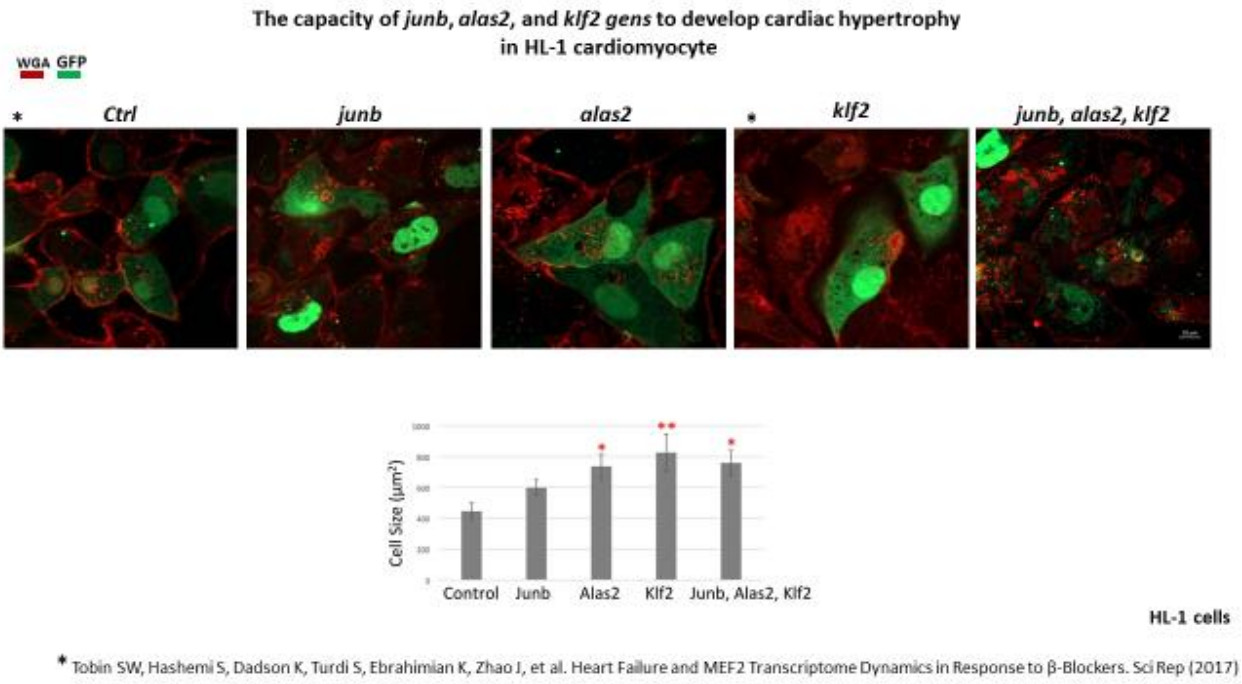
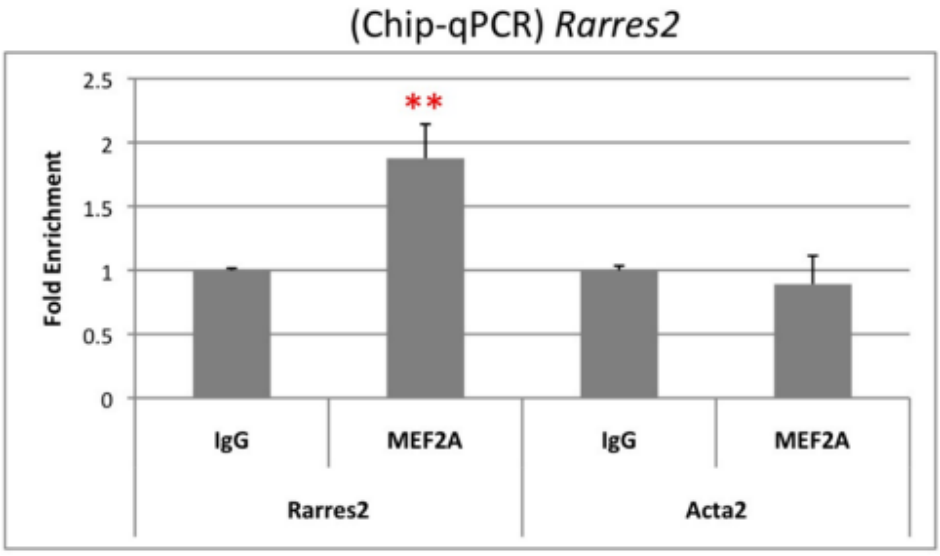



**Figure 10.** HL-1 cells were transfected with 3 µg DNA in total. WGA staining depicts cardiac hypertrophy. Cardiac HL-1 cells (upper panel) were transfected with GFP and *Alas2*, *Junb*, or *Klf2* expression plasmids individually or in a mixture and stained with Wheat Germ Agglutinin (WGA) in red. The bar graph indicates that cell size of GFP positive HL-1 cells quantified based on 5 cell measurements per image. Scale bar is 10 µm. Data are presented as ± SEM. *P < 0.05 **P < 0.01 vs control.

*rarres2* is another gene identified by transcriptome analysis (RNA-seq) from left ventricular RNA samples and Mef2a depleted cardiomyocytes. *rarres2* encoded Chemerin which was an adipokine shown to be associated with cardiac apoptosis and coronary artery disease. In our study, *rarres2* was identified as a novel MEF2 target gene which may have implications in cardiomyocyte hypertrophy (41).

**_rarres2_ is a novel MEF2A target gene**

Our ChIP-qPCR indicated that MEF2A is recruited to the promoter of *rarres2* gene in primary neonatal cardiomyocytes. Our finding opened the new window to study MEF2A functions on a *rarres2* gene regulation.




Tobin SW, Hashemi S, Dadson K, Turdi S, Ebrahimian K, Zhao J, et al. Heart Failure and MEF2 Transcriptome Dynamics in Response to β-Blockers. Sci Rep (2017)

**Figure 11.** ChIP-qPCR analysis demonstrated that Mef2a is recruited to the Rarres2 promoter in primary neonatal cardiomyocytes. Acta2 was used as a negative control. Data were presented as fold enrichment (n = 3, **P < 0.01)

**The role of *rarres2* in cardiomyocytes**

In subsequent experiment we also assessed the role of *rarres2* gene to induce cardiac hypertrophy in primary neonatal cardiomyocytes and HL-1 cardiac muscle cells. *Rarres2* gene were exogenously expressed in two models. Next, cells are stained for WGA staining to visualize and quantitate the cell surface area as an index of cellular hypertrophy **(Figure 11).** Only GFP+ cells were quantified for this analysis. Results precisely showed that there is a significant enhancement in cell surface area of transfected cardiomyocytes with our gene of interest. Thus, *rarres2* may play an important role to promote cardiomyocytes hypertrophy (41).

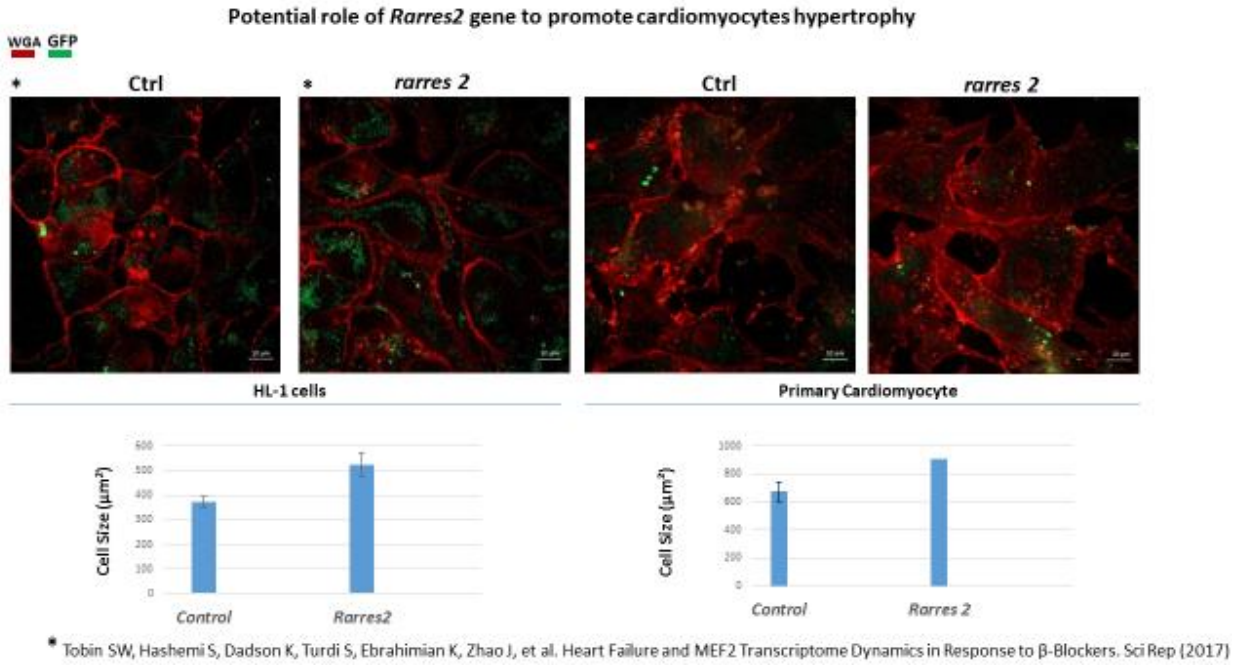



**Figure 12.** Cardiac HL-1 cells and primary neonatal cardiomyocytes were transfected with GFP and *rarres2* expression plasmids and stained with WGA in Red. The bar graph indicates cell size of GFP positive cells, quantified by 10 cell measurements per image for HL-1 cardiac cells and 5 cell measurements per image for primary neonatal cardiomyocytes. Scale bar is 10 µm. Data are presented as ± SEM. *P < 0.05, **P < 0.01.

## Discussion

In consideration of the fact that the human heart has particularly no innate capacity for regeneration, the mechanisms of cardiomyocytes cell growth and survival will become more important. In this context, MEF2 was recognized as prominent regulator of myocardial gene expression, vascular integrity and heart development (9,13). Previous work from our group and others showed that the MEF2 family of transcription factors have been implicated in the control of gene expression and differentiation in cardiac (14,43), skeletal (44,45), smooth muscle differentiation (13,46), neuronal survival and plasticity (47,48), and T cell activation (49).

MEF2 family proteins are also extremely responsive to a number of signal transduction cascades, and post-translational modulation by covalent modification by PKC (50), ERKS (51,52), p38 MAPK (15,50), and PKA which have been proved by our group (7) and others (53,54).

Previous work in our group showed that β-AR/ PKA signaling modulated MEF2 function in cardiomyocytes (40) and prolonged activation of the β-AR system regulated MEF2 cellular localization in cardiomyocytes and resulted in dilated and heart failure (53,54). Our group also observed that acute β-AR stimulation regulated inactivation of the pro-survival function of MEF2 in cardiomyocyte, thereby leading to myocyte cell death (40), however, chronic hyper-activation of β-AR stimulation may have led to a PKA mediated phosphorylation of the ryanodine receptors that resulted in calcium leakage from sarcoplasmic reticulum (SR) and stimulated the $Ca^{2+}$ signaling which eventually played prominent role in generation of cardiac hypertrophy, progression towards heart failure, and marked activation of MEF2 activity (41,55,56).

Post-translational modification of MEF2 transcription factor by targeted phosphorylation (50), acetylation and deacetylation (57,58) and also protein-protein interaction with other

transcription factors such as GATA4 (17), NFAT (59,60), SRF (45,61), and thyroid hormone receptors (62) are becoming a regulatory model in the control of gene expression in cardiac and skeletal muscle genes. However, despite considerable understanding of the transcriptional role of MEF2 proteins in the activation of cardiac muscle genes, it remains to be determined whether protein interaction network between MEF2 and other transcription factors are implicated in the generation of cardiac hypertrophy genes towards heart pathology.

Previous work showed that acute β-AR stimulation mediated inactivation of the pro-survival function of MEF2 in cardiomyocyte, however, chronic hyper-activation of β-AR stimulation may have led to enhanced activation of MEF2 activity. Besides, loss of MEF2 function resulted in up-regulation and down-regulation of many genes which are implicated in cardiomyocytes survival, apoptosis induction and cardiac hypertrophy (19,40,41,63).

Among MEF2 family members, MEF2A is the predominant subunit expressed in skeletal muscle and adult cardiac cells (64). In skeletal muscle, It has been shown that MEF2A regulate several genes associated with the actin cytoskeleton (65,66). In cardiac cells, it formed heterodimers with MEF2D and mediated diverse functions in the control of cell growth and survival (61,67). The transcriptional activation properties of MEF2A is also modulated by different post-translational mechanisms including regulation by MAPKs such as p38 (68), ERKs (61,68), and PKA (40) and also through interaction with class II HDACs which suppress MEF2-target gene activation (69). MEF2A loss of function is also associated with mitochondrial and contractile defects (10), sarcomere disorganization, and cardiac enlargement which may regulate many hypertrophy genes (3). Taken together, MEF2A functions indicated prominent roles to regulate survival and hypertrophy genes in cardiomyocytes.

The spotlight of this project was to study regulation and function of MEF2A in cardiomyocytes. Understanding MEF2A protein networks associated with cardiac hypertrophy will provide critical insight into underlying role of MEF2A protein interaction in heart disease. Thereby, we hypothesized that characterizing the regulation of MEF2A interactome in cardiomyocytes may uncover MEF2A functions as an activator or repressor to regulate survival and hypertrophy gene transcriptions. This will be achieved using state of the art quantitative proteomics approaches.

For this project, firstly we developed and optimized two different strategies for pulling down MEF2A interactome in cardiomyocytes. The first strategy was endogenous IP of MEF2A interactome in HL-1 cardiomyocytes (**figure 6).** Our endogenous IP concentrated and isolated MEF2A interactome in the natural condition successfully. Quantitative proteomics approaches will be the next step to characterize our MEF2A interactome in natural condition. We speculate that some known MEF2A protein network such as MEF2D, thyroid hormone receptors, HDACs and GATA4 were bound to Mef2a and precipitated by IP technique and will be characterized by quantitative proteomics techniques. Importantly, we will identify other transcription factors which were still unknown and bound to MEF2A and may have prominent role on MEF2A functions to regulate Mef2a cardiac survival genes.

To study regulation and function of MEF2A interactome in HL-1 cardiomyocytes hypertrophy, an experimental hypertrophy condition was mimicked in HL-1 cardiomyocytes using isoproterenol treatment to activate β-adrenergic signaling and subsequently concentric hypertrophy. Our observation and quantification in **figure 8** confirmed the hypertrophy condition in our experimental cultured cells. We expect that Mef2 transcriptional activity will be inactive

to regulate cardiac survival genes due to acute stimulating of β-adrenergic signaling at the beginning stage of the hypertrophy. Thereby, we speculate to precipitate MEF2A protein partners which were bound to MEF2A while cardiomyocytes were initially leading to hypertrophy condition.

A follow up experiment in **figure 9** showed that endogenous MEF2A protein could be isolated and concentrated in control and hypertrophy conditions using IP method. This is the first step which needed to be done to have characterized MEF2A interactome using quantitative proteomics techniques. Our precipitated interactome in hypertrophy condition will be assumed to have some known proteins bound to MEF2A such as NFAT and p300, however quantitative proteomics techniques will characterize the other global MEF2A interactome in hypertrophy and natural condition. We also think that identifying global Mef2a protein network leading to understand better MEF2A functions as an activator or repressor on MEF2 survival and hypertrophy target genes.

In the second strategy, we chose to use Flag-Tag expression system to study regulation of MEF2A interactome in cardiomyocytes. The rationale was that FLAG peptide is small hydrophilic tags which improves detection and purification of recombinant fusion protein. Pulling down our interactome with Flag antibody is more efficient with less non-specific proteins due to using a specific Flag antibody. **Figure 7 (A)** demonstrated that 3X-Flag-MEF2A transfected in primary neonatal cardiomyocytes and HL-1 cardiac cells were over expressed successfully. In next step, we optimized pulling down of 3X-Flag-MEF2A interactome using IP technique. Our result in **figure 7 (B)** showed that 3X-Flag-MEF2A protein was concentrated by IP and observed by western blot. However, over-expression of cardiac cells by Flag-MEF2A may activate protein kinase signaling

which can mediate expression of cardiac hypertrophy genes (70)(71) and subsequently may alter the interactome bound to MEF2A. Furthermore, it will be possible to lose some protein partners which could not bind to MEF2A from N-terminal due to having a barrier with Flag peptide which was fused to MEF2A from N-terminal.

In previous study, our group identified global gene transcription networks associated with HF with and without β-blocker treatment. Transcriptome analysis (RNA-seq) from left ventricular RNA samples and MEF2A depleted cardiomyocytes identified some genes, including *klf2*, *junb*, *rarres2* and *alas2* which were dysregulated under TAC condition and remarkably, their expression pattern was substantially reversed with Atenolol (β-adrenergic receptor antagonist) treatment showing that they may be involved in pathological changes that can be potentially altered by drug therapy. Two of these genes, *junb* and *klf2* were transcription factors whereas *alas2* mediated heme synthesis and *rarres2* encoded Chemerin which was an adipokine.

One of the most important features of progressive heart failure is cardiac hypertrophy (72) and interestingly, these four genes may be modulated in different aspects of heart failure. *alas2* was reported to be upregulated and associated with oxidative stress and cell deaths in failing human hearts (73). *junb* was shown as a transcriptional regulator for the upregulation of MMP's which mediated cardiac remodeling in heart disease like ischemia-reperfusion (74). *klf2* has been reported to be involved in endothelial cells by shear stress (75) and has been speculated that the upregulation of *klf2* correlated with an increased hemodynamic stress . Thus, clearly the role of *klf2* requires further characterization in cardiac disease. Lastly, *rarres2* was shown to be associated with cardiac apoptosis and coronary artery disease (76). Taken together, these data suggested that these genes may have a potential involvement in cardiac hypertrophy.

We attempted to further test the capacity of *Klf2*, *Junb*, and *Alas2* genes to develop cardiac hypertrophy in vitro. Cardiac HL-1 cells were co-transfected with GFP and expression plasmids for *Klf2*, *Junb,* and *Alas2* genes individually or in combination and cells were stained with WGA to visualize and quantitate the cross-sectional area as an index of cellular hypertrophy. Our analysis in **figure 10** confirmed that *Klf2*, *Junb*, and *Alas2* may have a role in promoting cardiomyocyte hypertrophy since their exogenous expression in cultured HL-1 cells led to a statistically significant enhancement in cell surface area (41).

ChIP-qPCR analysis in **figure 11** showed that Mef2a is recruited to the *rarres2* promoter in primary cardiomyocytes. *rarres2* is a novel Mef2a target gene (41). We also attempted to further test the capacity of *rarres2* to develop cardiac hypertrophy in vitro. Primary neonatal cardiomyocytes and HL-1 cardiac cells were co-transfected with GFP and *Rarres2* expression plasmid. Our result in **figure 12** indicated that exogenous expression of *rarres2* in cultured primary neonatal cardiomyocytes and HL-1 cardiac cells led to a statistically significant enhancement in cell surface area with *rarres2* (41). Our preliminary result concerning the possible function of *rarres2* gene to promote potential cardiac hypertrophy in cardiomyocytes.

These observation supported a cohort of genes with vast potential role on induction of cardiac hypertrophy and clearly requires further characterization in a heart context.

## Future directions

Both strategies which were used to pull down MEF2A interactome developed the methods for the next step which will be using quantitative proteomics approaches to characterize MEF2A interactome in cardiomyocytes. Our knowledge of the mechanisms modulating cardiac hypertrophy through MEF2 transcription factor is still incomplete. On the basis of our observation, we propose that characterizing MEF2A protein network in normal and hypertrophy conditions will fulfil a critical role to understand MEF2A functions as an activator or repressor on MEF2 survival and hypertrophy target genes such as *rarres2*. This achievement may have an important implications for our understanding and therapeutic targeting of cardiac pathology.

Furthermore, cardiac and skeletal muscle share many properties and are similar in their dependence on a sarcomere structure (77). MEF2 is also expressed in both skeletal and cardiac muscle cells. Thereby, it can represent a useful paradigm for studying common MEF2A protein network and characterize MEF2A functions on common MEF2A target genes in both skeletal and cardiac models.

MEF2 is a fundamental regulator of cardiac hypertrophy (78) and previous study in our group documented the transcriptomic effects of MEF2 suppression in cardiomyocytes (41). From those genes, we observed the capacity of *klf2*, *junb*, *alas2*, and *rarres2* genes to develop cardiac hypertrophy in vitro and found that *rarres2* is a MEF2 target gene. The next step will be characterizing the functions of those genes with the vast potential for the therapeutic interventions in heart failure.

We also suggest that characterizing the function of a cohort of MEF2A protein partners on regulation of the genes which were dysregulated by TAC and their expression pattern were

substantially reversed with drug therapy such as *rarres2* will provide a critical insight into the underlying etiology of heart failure and unravelling array of molecular targets for therapeutic intervention.

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