THE ROLE OF TEL2 IN MTOR-MEDIATED CARDIAC FUNCTION

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOSCIENCES & BIOENGINEERING

DECEMBER 2013

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Keywords: mTOR, Tel2, Ischemia reperfusion injury, cardiac function

Acknowledgements

This master thesis would not have been possible without the help of so many people in many ways. Luckily, I met many great people, who inspired me and led me into the field of molecular biology and cardiovascular research.

First of all, I would like to thank the department of Molecular Biosciences & Bioengineering (MBBE) and University of HawaI‘i at Manoa for giving me the opportunity to study in this program. I would like to thank Dr. Dulal Borthakur, a former chair of the MBBE department, for giving me the opportunity to study in this program. As a graduate chair, he always made time for me and helped me get through many difficult times. His stories inspired me to continue pursuing my master degree. I would also like to thank the current MBBE department chair, Dr. Gernot Presting for helping me solve any issues I had throughout the year.

Next, I would like to give special thanks to my PI, Dr. Takashi Matsui. Even though I had no research experience and knowledge, he kindly offered to become my supervisor and accepted me into his lab as a graduate assistant. Without his kindness, I would not have gained the opportunity to learn about cardiovascular research. Throughout the years, Dr. Matsui has been an excellent supervisor and role model. During difficult times, he would always make time and helped me overcome the issues. He always gave me lectures on backgrounds and answered any questions I had. If I didn’t have a supervisor like him, I would not have come this far. I truly appreciate all the help and support he has given me. I am so lucky to have worked in his lab.

I would also like to give special thanks to my committee members, Dr. William Boisvert and Dr. Olivier Le Saux. I truly appreciate their time spent reading this thesis and attending my defense. They provided useful suggestions on my experiments and this thesis. I am so grateful to have such talented professors in my committee.

Last but not least, I owe more than thanks to my lab members, friends and family. I am so grateful to have such great lab members who were always there to support me. My friends have also been a great support. They have given me joy and the courage to pursue my master degree. Most importantly, I would like to appreciate my parents for their consistent support. They have taught me and showed me that whatever you do, you must give the best effort. Without them, I could not have come this far. I am so lucky to be their daughter.

Abstract

Previously we showed that the expression of the mechanistic target of rapamycin (mTOR) increases in heart failure, and the cardiac mTOR is sufficient to protect the heart against ischemic stresses.

Recent studies demonstrated that telomere maintenance 2 (Tel2) stabilizes mTOR in other tissues. In this study, we examined whether Tel2 is sufficient to stabilize cardiac mTOR and prevent cell death against hypoxia. Overexpression of Tel2 in cardiomyocytes (CMs) increases mTOR and activates both mTORC1 and mTORC2. Overexpression of Tel2 protects CM against hypoxia. Gene silencing by siRNA targeting Tel2 showed that Tel2 is necessary for stabilizing mTOR in cardiomyocytes.

We assessed cell shortening and Ca^{2+} transients as indicators of cardiac function. Preliminary studies of Tel2-overexpressed mTOR-Tg suggest that Tel2 positively regulates contraction and Ca^{2+} transients via stabilization of mTOR.

A study of Tel2-mediated mTOR activation would provide a novel therapeutic target for a therapy of heart failure.

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Specific Aims

Left ventricular (LV) remodeling, characterized by LV dilatation and fibrosis, is an important determinant of the development of heart failure following acute myocardial infarction (MI) [1]. The magnitude of LV remodeling is directly proportional to the initial infarct size (cell death) [2]. The mechanistic target of rapamycin (mTOR) is known as a key downstream molecule of insulin-like growth factor 1 (IGF-1), a cardioprotective factor. The mTOR consists of two complexes: mTORC1 and mTORC2. The rapamycin sensitive mTORC1 phosphorylates S6K and 4E-BP-1, whereas mTORC2 phosphorylates Akt [3]. We previously demonstrated that overexpression of mTOR inhibits cell death and attenuates LV remodeling in mouse models of pathological hypertrophy [4] and ischemia-reperfusion injury [5]. We also found that the expression level of cardiac mTOR is changed in pathological settings such as heart failure [4]. These findings suggest that the amount of the mTOR protein level is a critical mediator for cardioprotection. However, the regulatory mechanism of mTOR expression remains undefined**.**

Recent studies have demonstrated that telomere maintenance 2 (Tel2) stabilizes mTOR by mediating the maturation of mTOR complex [6] [7]. Tel2 deletion elicited depletion of mTOR expression and reduced mTOR activity [8, 9]. Since Tel2 is sufficient for the stability of mTOR activity, Tel2 may be sufficient for the further cardioprotective effect of mTOR in the heart. Based on these reports and our findings, we hypothesized that Tel2 protects cardiomyocytes (CM) against ischemic injury by stabilization of both mTORC1 and mTORC2. To test this hypothesis, we performed experiments in the following two specific aims.

Specific Aim1: To examine the role of Tel2 on the mTOR signaling pathway and cell survival in *in vitro* CMs.

We examined the effect of Tel2 in mTOR signaling using a CM cell line, HL-1 cells. To examine the requirement of Tel2 interaction, CMs were treated with Tel2-specific siRNA. To study cell death induced by hypoxia, we assessed apoptosis in CMs exposed to hypoxia.

Specific Aim 2: To examine the role of Tel2 on CM function at baseline.

We examined the effect of Tel2 in cardiac functions using isolated adult mouse CMs. Prior to the cardiac function measurement, Tel2 was overexpressed in CMs isolated from mTOR transgenic (Tg) mice by using recombinant adenovirus. Sarcomere length (SL) shortenings and intracellular calcium (Ca^{2+}) transients were assessed as indicators of cardiac function using IonOptix (Milton, MA).

Background and Significance

1) Cardiomyocyte cell death:

Left ventricular (LV) remodeling, which is characterized by enlarged LV chamber and dysfunctional wall motion, plays a critical role in the subsequent development of heart failure [10]. The magnitude of LV remodeling is directly proportional to the initial infarct size (cell death) after an ischemic coronary injury [2]. Apoptosis or programmed cell death in cardiomyocytes (CMs) has been identified in a variety of cardiac conditions in patients, including myocardial infarction [11-14]. Animal models showed that CM apoptosis is a critical pathological feature in ischemia-reperfusion injury [15].

A number of studies have shown that cardiac stem cells and progenitor cells contribute to cardiac cell repair in some settings [16,17]. One study showed that roughly 3.2% of the CMs adjacent to the infarct had undergone division 8 weeks after myocardial infarction [18]. However, endogenous CMs regeneration still appears inadequate to compensate for the lost function of injured myocardium post myocardial infarction. For these reasons, identifying possible methods to prevent cell death could provide a novel strategy for the therapy of heart failure after myocardial infarction.

2) Akt signaling in cardioprotection and cell survival

The serine-threonine kinase Akt is a powerful survival signal in many systems and is activated by several cardioprotective ligand-receptor systems, including insulin, and insulin-like growth factor (IGF)-1 [19]. Binding of insulin or IGF-1 to insulin receptor tyrosine kinase leads to phosphorylation of insulin receptor substrate (IRS)-1.

Phosphoinositide-3-kinase (PI3K) binds to the phosphorylated IRS1 by SH2 domain in the regulatory domain p85 of PI3K. The p110-catalytic subunit of PI3K is then activated, catalyzing the phosphorylation of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP_2) to generate phosphatidylinositol (3,4,5)-triphosphate (PIP_3), some of which is converted to phosphatidylinositol 3,4-biphosphate (PIP_2) by an inositol phosphatase. Accumulated PIP_2 and PIP_3 in the cell membrane recruit Akt to the cell membrane by binding to its pleckstrin homology (PH) domains, leading to phosphorylation and activation of Akt [20].

We previously reported that Akt regulates CM function and survival [19, 21]. A study using recombinant adenovirus containing constitutively active form of Akt (Ad. myr-Akt-HA) showed that Akt activation significantly inhibits hypoxia-induced apoptosis in *in vitro* neonatal CMs [21]. In *in vivo* rat models of ischemia reperfusion (I/R), constitutively active Akt was overexpressed in the myocardium by direct injection with Ad.myr-Akt-HA [19]. The study demonstrated that Akt activation significantly prevents apoptosis after I/R injury [19]. In addition, the assessment of cardiac function indicated that Akt activation preserves contractile function and calcium handling under hypoxia compared to Ad.GFP injected CMs (controls) [19]. Therefore, these results indicate that Akt activation mediates favorable effects on cardiomyocyte survival and cardioprotection [19, 22].

3) mTOR signaling pathway

Previously, the mechanistic target of rapamycin (mTOR) was known only as a key downstream molecule of Akt. But cumulative studies indicated that mTOR is not only a downstream molecule but also an upstream "kinase" [3]. The mTOR is a large protein (2548 amino isoforms) consisting of two complexes: mTOR complex 1

(mTORC1) and mTOR complex 2 (mTORC2) (Figure1). The rapamycin sensitive mTORC1 consists of raptor [23], mLST8, and mTOR. The rapamycin-insensitive mTORC2 consists of rictor [24], mLST8, and mTOR [3]. The mTOR is regulated by growth factors, such as insulin and insulin-like growth factor 1 (IGF1), as well as amino acids. Binding of growth factors to the tyrosine receptor leads to the phosphorylation of PIP_2 to generate PIP_3. PIP_3 recruits Akt from cytosol to membrane, generating an active form via phosphorylation at Thr^{308} site by phosphoinositide-dependent kinase (PDK1) [25]. Akt activates tuberous sclerosis complex (TSC) 1 and 2, disinhibiting Rheb and further activates mTORC1. The mTORC1 stimulates protein synthesis and translation via phosphorylation of p70S6 and activation of elF4E. P70S6 kinase also phosphorylates IRS-1, leading to inhibition of IRS-1. The mTORC2 activates Akt by phosphorylation at Ser^{473}. The fully activated Akt phosphorylates GSK-3 and FOXO, regulating cell survival.

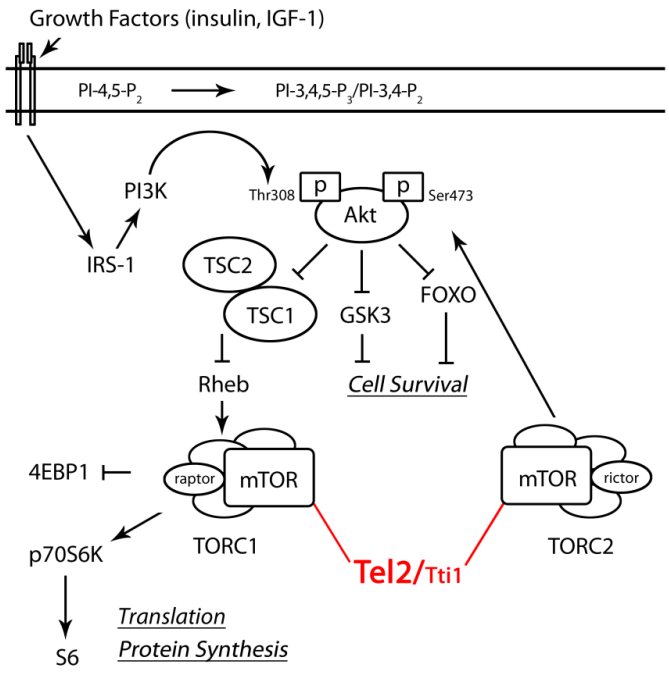

Figure 1 A scheme of mTOR signaling pathway.

Our previous study using heart tissue samples demonstrated that mTOR protein level was increased by 60% in heart failure patients compared to donors (control) [4]. In addition, the expression level of mTOR and phosphorylation of S6 and Akt were both increased in pressure-overload induced hypertrophy mice[4]. These results indicate that cardiac mTOR expression is increased in heart failure. However, a regulatory mechanism of mTOR expression in the heart remains undefined.

4) The role of mTOR in cardiac protection

Previously, several groups reported that treatment with an mTOR inhibitor rapamycin inhibits cardiac hypertrophy and preserves cardiac function in mouse models of pathological cardiac hypertrophy [26] [27]. A study using cardiac hypertrophy mice model showed increase in ratio of weight/tibial length by 35% compared to sham-operated mice [26]. However, the ratio of heart weight/tibial length was increased by only 12% compared to sham when the hypertrophy mice were treated with rapamycin [26]. The phosphorylation level of S6 was significantly increased in hypertrophy mice compared to sham-operated mice, but it was inhibited with rapamycin treatment[26].

Another group showed that pretreatment with rapamycin protects the heart in *ex vivo* mouse model of IR injury [28]. Pre-treatment with rapamycin prior to I/R using Langendorff system showed a decrease in infarct size to 10%, whereas that of DMSO pre-treated mice was 28% (64% decrease as compared to DMSO controls) [28]. In addition, the isolated adult mice CMs subjected to I/R indicated an increase in cell viability with rapamycin [28].

In other tissues, many studies demonstrated that mTORC1 signaling through S6K negatively regulates Akt by inhibiting IRS-1 [29]. A study using high fat diet mice showed

that diet-induced obesity results in an impairment of insulin-induced Akt activation, but it was not seen in S6K1-deficient mice [29]. S6K1-deficient mice also showed hypersensitivity to insulin in an activation of PI3K [29]. This is due to the inhibitory regulatory relationships of S6K and PI3K [3]. Activation of mTORC1 by growth factors or amino acids results in activation of S6K, which leads to negative regulation of IRS-1 [29]. Since IRS-1 and PI3K positively regulates activation of Akt, cardiac protection by rapamycin may be due to the inhibition of negative feedback, enhancing the activation of Akt. In fact, a study using hypertrophy rat model demonstrated an increase in Akt phosphorylation at Ser^{473} with pretreatment of rapamycin [28]. Reduced activity of mTORC1-mediated S6K1 was also seen in the isolated rat CMs pretreated with rapamycin [28]. Stimulation with insulin alone enhanced phosphorylation of both S6K1 and Akt at Ser^{473}, but treatment with rapamycin further enhanced phosphorylation of Akt at Ser^{473} [28].

To study the role of mTOR in cardiac protection, cardiac-specific transgenic mice overexpressing wild-type mTOR driven by αMHC promoter (mTOR-Tg) were generated [4]. A study using mTOR-Tg hypertrophy mice model showed little indication of cardiac dysfunction and less pathological hypertrophy in mTOR-Tg compared to WT [4]. In addition, mTOR-Tg mice subjected to I/R injury (ligation of left anterior descending coronary artery for 30 minutes followed by reperfusion) demonstrated higher survival rate and preserved cardiac function compared to WT mice [5]. These data indicated that overexpression of mTOR protects the heart in pressure-overload induced cardiac hypertrophy and against I/R injury [4, 5]. The preserved cardiac function shown in our studies using mTOR-Tg following I/R is accounted by overexpression of both mTORC1

and mTORC2 [4, 5]. Furthermore, immunoblotting showed further increases in expression of mTOR and phosphorylation of both S6 and Akt in mTOR-Tg mice following I/R [5]. These indicated that although rapamycin exhibits CM survival via Akt activation, cardiac protection by mTOR overexpression is done by both mTORC1 and mTORC2 signaling pathways.

5) Tel2

Tel2 is a protein first discovered by Lustig and Petes in 1986 from the mutant strains of the budding yeast *Saccharomyces cerevisiae* that had abnormally short telomeres [30]. Although Tel2 is known to be essential for maintaining telomere length in *S. cerevisiae*, many non-overlapping functions of Tel2 have been reported in several organisms, thus making the function of Tel2 in the cell a complex mystery. In fission yeast *Schizosaccharomyces pombe,* Tel2 is involved in DNA replication checkpoint, but in humans it plays a role in coupling the onset of cell division with the successful replication of DNA [31-33]. In the nematode *Caenorphabditis elegans*, hypomorphic alleles, which encode protein with reduced function, cause stress during DNA replication, trigger hypersensitivity to DNA double-stranded breaks, and increase life span [31, 34, 35].

To investigate the role of mammalian Tel2, Takai *et.al* studied the possibility of human and mouse Tel2 orthologs having telomere-related functions [8]. The immunofluorescence using Flag-tagged human Tel2 showed that Tel2 expression was localized in the cytoplasm and nucleus but not in telomere [8]. A study using shRNA knockdown of Tel2 or overexpression of Tel2 showed no significant effect on telomere-length homeostasis in human cell lines [8]**.** These findings suggest that Tel2 might have other functions in the mammalian cells.

To further address the function of Tel2, Takai *et.al* looked at the effect of Tel2 deletion in mice. Mouse embryonic fibroblasts (MEFs) prepared from heterozygous $Tel2^{DEL/+}$ showed normal embryonic growth and showed expected 2-fold reduction in Tel2 protein levels [8]. However, the Tel2 null mutation (DEL/DEL) led to embryonic lethality [8]. A study using retrovirus Cre-induced Tel2 knockout MEFs showed that cells lacking Tel2 gene arrested after 3 to 4 days, but cell cycle was rescued by ectopic expression of either mouse or human Tel2 cDNA [8]. Their findings indicated that Tel2 is involved in embryonic development and cellular growth.

Further studies on the role of mammalian Tel2 demonstrated that Tel2-diminished cells failed to show increases in phosphorylation of p53 and Checkpoint kinase 2 (Chk2) following genotoxic stress, which indicates that Tel2 is involved in DNA-damage signaling pathways [8]. The evaluation on the effect of Tel2 on taxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR), which regulate the response to genome damage, revealed that Tel2-deficient cells showed reduction in ATM and ATR [8]. Since ATM and ATR are the members of phosphatidylinositol 3-kinase-related protein kinase (PIKKs), the effect of Tel2 on other members of PIKKs (mTOR, DNA-PKcs, SMG1, and TRRAP) were examined. Their studies showed significantly diminished expression of all four PIKK members in Tel2-diminished cells, indicating that Tel2 is involved in the stability of PIKKs [8].

Several groups have done studies on Tel2-mediated mTOR stabilization [6,9,8]. Kaizuka *et. al* reported that Tel2-interacting protein (Tti1) binds to Tel2 and maintains the mTOR complex assembly [6]. A study using Tti1 or Tel2-depleted HeLa cells showed decreases in the expression level of both mTORC1 and mTORC2 [6]. The double

knockdown of Tti1 and Tel2 did not further reduce the mTOR proteins expression, which may indicate that Tti1 and Tel2 work cooperatively within the same pathway [6]. Additionally, Takai *et. al* reported that Tel2 preferentially interacts with newly synthesized mTOR [8]. Their study using the labeling of newly synthesized mTOR demonstrated that the association of newly synthesized mTOR with raptor and rictor is decreased in the absence of Tel2 [8]. This indicates that Tel2 is important for the assembly of the mTOR complex.

Several groups have reported some possible mechanisms on the interaction of Tel2 and mTOR [36],[8],[7]. The studies done by Takai *et. al* reported that Tti1-Tel2 complex acts with Hsp90 chaperone ,which is involved in protein folding, and mediates maturation of the mTOR complex [8]. The formation of mTOR complex 1 and 2 was impaired with inhibition of Tel2 or Hsp90[8]. They also reported that Tel2 is constitutively phosphorylated on Ser [487] and Ser[491] by serine/threonine kinase casein kinase 2 (CK2)[36]. CK2 phosphorylation leads to binding of the R2TP/prefoldin-like complex, in which R2TP contains chaperones and prefoldins involved in protein complex assembly[36]. The interaction of Tel2 with R2TP/prefoldin-like complex through CK2 phosphosite is essential for Tel2-mediated mTOR stability[36]. However, the mechanism of how Tel2 interacts with mTOR still remains unclear. Especially regarding Tel2 in the heart, no studies have been reported.

6) Regulation of Cardiac Muscle Contraction

Cardiac muscle contraction is tightly regulated by Calcium (Ca^{2+}) ion (Figure 2). When the myocyte membrane depolarizes, extracellular Ca^{2+} enters the cell through the L type voltage dependent Ca^{2+} channel. The initial influx of Ca^{2+} triggers the opening of

Ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR), resulting in a release of Ca^{2+} from the SR. RyR2 are localized on the SR membrane adjacent to L type voltage dependent calcium channel [37]. At the time of Ca^{2+} influx, RyR2 channels are activated, causing a synchronized release of Ca^{2+} release, which is known as Ca^{2+} sparks [38]. Multiple Ca^{2+} sparks lead to a sufficient intracellular Ca^{2+} transient to trigger contraction.

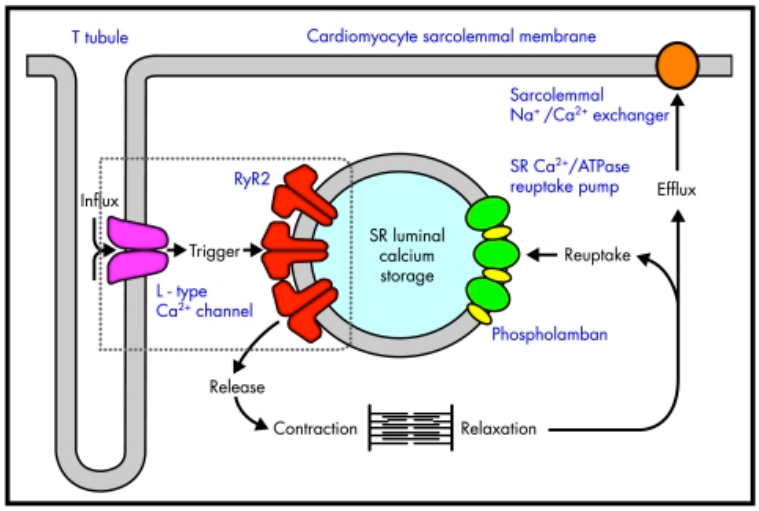

Figure 2 A scheme of Excitation-Contraction coupling.

The movement of Ca^{2+} is indicated by arrows. *Scoote et. al*

Contraction is triggered by the release of Ca^{2+} into the myofibrillar space followed by the cross-bridge formation of tropomyosin-troponin complex [39-42]. Myofilaments consist of a regular array of thick and thin filaments. Sarcomere is composed of one unit of interacting thin and thick filaments. The thick filaments consist of myosin heavy chains and two pairs of light chains [39, 40,42]. The tail of the molecule is coiled with two myosin heavy chains wound around each other. Each myosin head consists of ATP-binding area near the myosin ATPase, which breaks down ATP. The thin filaments are composed of actin and troponin complex. The troponin complex is carried on a long molecule called tropomyosin. The troponin complex is composed of

one molecule each of troponin C, T, and I. The strength of the bond linking troponin I and actin varies depending on the intracellular Ca^{2+} level [43].

When there is a low level of cytosolic Ca^{2+}, the actin-binding site is covered by tropomyosin, which makes the myosin impossible to bind to the actin [44]. This is due to the bond linking troponin I to actin. When the level of cytosolic Ca^{2+} increases due to the Ca^{2+} release from the SR, Ca^{2+} binds to troponin C and weakens the bond linking of troponin I to actin. This leads to the conformational change of the tropomyosin-troponin C complex, allowing the myosin head to bind to actin [44]. The ATP hydrolysis triggers a strong binding of the myosin head to actin followed by a power stroke, which moves the actin molecule [44]. The myosin head releases ADP to accept new ATP for the new cycle. The binding of new ATP to the myosin head causes detachment of myosin from actin, and the relaxation occurs.

Relaxation occurs by the closure of RyRs and the rapid removal of cytosolic Ca^{2+} [45]. The Ca^{2+} ions are either recollected back into the SR by the cardiac isoform of the Ca^{2+}-ATPase pump (SERCA2) or effluxed out of the cell by sarcolemmal Na^{+}/Ca^{2+} exchanger [45]. In humans, about 75% of the Ca^{2+} are recollected back into the SR and about 25% of Ca^{2+} are removed by the Na^{+}/Ca^{2+} exchanger [43].

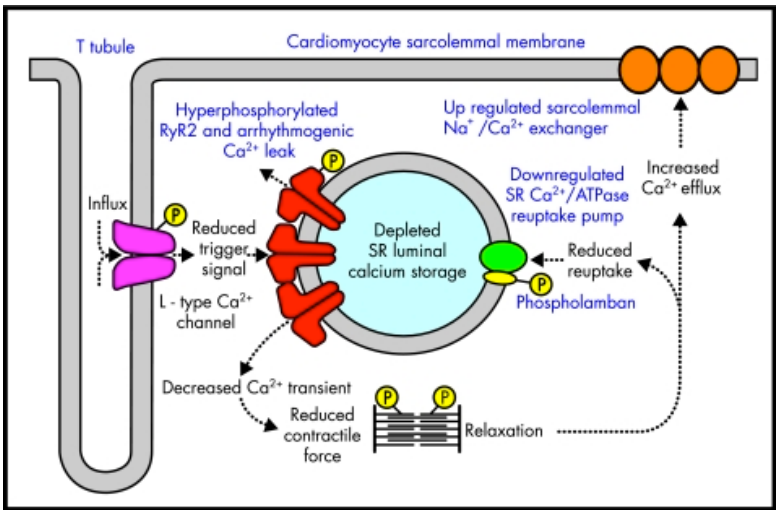

Figure 3 A scheme of Excitation-Contraction coupling in heart failure.

The altered regulation of Ca^{2+} is indicated by dotted arrows. *Scoote et. al*

In heart failure, defect in regulation of Ca^{2+} appears to be a major cause of contractile dysfunction (Figure 3). It has been shown that the amount of SR Ca^{2+} is significantly reduced [43]. Since the contraction is dependent on the release of Ca^{2+} from SR, insufficient amount of Ca^{2+} from the SR results in reduced contractile force [46]. The likely cause of this Ca^{2+} reduction is the downregulation of SERCA activity. [36]. Another study has shown that the cause of SR Ca^{2+} depletion may be due to upregulation of NCX, which may promote the removal of Ca^{2+} from the cell [47]. Furthermore, the depletion of stored SR Ca^{2+} leads to hyperphosphorylation of RyR2 as a compensatory effect in heart failure. Such hyperactivation of RyR2 causes increase in open probability of the channels and Ca^{2+} leak [46].

As mentioned above, the expression level of mTOR in the heart is altered in certain situation such as pathological cardiac hypertrophy. Because Tel2 is involved in mTOR complex assembly, we investigated the role of mTOR-Tel2 signaling pathway on

cardiomyocyte contraction to test whether the change in mTOR activity is a compensatory or decompensatory reaction.

Research Design and Method

Specific Aim1: To examine the role of Tel2 on the mTOR signaling pathway and cell survival in vitro CMs.

Hypotheses:

Overexpression of Tel2 increases mTOR stabilization *in vitro* CMs. Tel2 protects CMs against ischemic injury via stabilization of both mTORC1 and mTORC2.

Approach:

To investigate whether Tel2 activates cardiac mTOR, Tel2 was transiently overexpressed in HL-1 cells. Tel2 overexpressed HL-1 cells were harvested 48 hours after the transfection and were subjected to western blot to examine the change in cardiac mTOR expression. The downstream molecules of mTOR were also examined to study the stabilization of mTORC1 and mTORC2. To study whether Tel2 is sufficient to mediate mTOR expression and activation, Tel2 was silenced by Tel2-specific siRNA (Santa Cruz). To examine the cell survival effect of Tel2 in cardiac mTOR, cell death ELISA (Roche) was used to detect the fragmented DNA as an indicator of apoptosis.

Rationale:

Rationale for using CM cell line HL-1 cells:

In *in vitro* experiments with primary CMs from mouse hearts, the low transfection efficiency in transient transfection limits the available experimental strategy. In our experiments, HL-1 cells were used for transient transfection. HL-1 cells are currently the only CM cell line available that continuously divides and spontaneously tracts while maintaining a differentiated cardiac phenotype [29]. Although derived from the derivative

of AT-1 mouse atrial cardiomyocyte tumor lineage, HL-1 cells have been used for studies of cardiac biology at the cellular and molecular levels [18]. For genetic manipulation using HL-1 cells, transient transfection using cationic lipid reagents yields transfection efficiency of about 75-80% [18]. Infection of HL-1 cells with recombinant adenovirus provides close to 100% efficiency [18]. In our previous report, mTOR overexpression in HL-1 cells showed the same signaling pattern seen in mTOR-Tg mice, suggesting that these cells provide an *in vitro* system that parallels the mTOR-Tg mice[4].

Rationale behind assays for cell death:

Cell death ELISA (Roche) was used to quantify the level of cell death. Apoptosis is characterized by condensation of cytoplasm and cleavage of double stranded DNA by endonuclease, generating histone-associated DNA fragments (mono- and oligonucleosomes). Cell death ELISA is commonly used for detection of such DNA fragments by using mouse monoclonal antibodies against DNA and histones.

Experiments:

1.1). Effects of Tel2 on mTOR stability at baseline

To look at the role of Tel2 in cardiac mTOR *in vitro*, HL-1 cell line was used to overexpress Tel2. Before passaging the cells, the appropriate culture dish was coated with 5ug/ml of fibronectin in 0.02% of gelatin in water. Gelatin was purchased from Difco and fibronectin was purchased from Sigma Aldrich. A 10cm culture dish was used for passaging the cells, and a 6-well plate was used for transient transfections of Tel2. The cells were maintained in Claycomb medium (JRH Biosciences, Cat#51800C-500ml) supplemented with 10% fetal bovine serum (Sigma Aldrich, Lot#128K8423), 100ug/ml

penicillin/streptomycin (Life Technologies Cat#15140-122), 0.1mM norepinephrine (Sigma Aldrich, A-0937), and 2mg/ml L-glutamine (Life Technologies Cat#25030-081). The medium was changed approximately every 24hr. The cells were grown at 37ºC in an atmosphere of 5% CO_2 and 95% air at a relative humidity of approximately 95%. Once the cells reached confluence, the cells were split 1 to 3. Prior to transient transfections, the cells were plated on a pre-coated 6-well plate. A day before the transfection, the cells were incubated in pre-transfection medium (Claycomb media, 10% Fetal bovine serum, 2mg/ml L-glutamine).

To determine whether Tel2 stabilizes cardiac mTOR, Tel2 plasmid (a generous gift from Dr. Noboru Mizushima, Tokyo Medical and Dental University, Japan) was transiently transfected in HL-1 cells using lipid transfection agent (Lipofectamine 2000, Invitrogen). HL-1 cells were transfected with either 10ug of p3x-FLAG CMV10 plasmid (control) or 10ug of human Tel2 plasmid (a generous gift from Dr. Mizushima). Before transfections, 8ul of Lipofectamine 2000 (per well) was incubated in 250ul of transfection media (Claycomb medium, 10mg/ml L-glutamine). Following 5 minutes of incubation, each transfecting plasmid in 250ul of Claycomb medium was added to Lipofectamine 2000. The plasmids were incubated for 20 minutes prior to the application to HL-1 cells. The cells were incubated at 5% CO_2 and 95% air for 6 hours, and the media was changed to the supplemented Claycomb media without norepinephrine. 24 hours after transfection, the HL-1 cells were serum-deprived and were incubated for another 24 hours. The cells were harvested 48 hours after the transfection, and the expression levels of mTOR, S6, and Akt were assessed by Western blot. Anti-TEL2 Polyclonal antibody (NBP1-81761) used to detect human Tel2 was

purchased from Novus Biologicals. Anti-TELO2 polyclonal antibody (15975-1-AP) was purchased from Proteintech to detect endogenous Tel2 protein expression in mouse HL-1 cells. Antibodies against mTOR (#2983), phospho-S6 (#4856), total S6 (#2217), phospho-Akt (#9271), total Akt (#9272) were purchased from Cell Signaling Technology. Anti-GAPDH polyclonal antibody (ab9485) was purchased from Abcam.

1.2) Effect of hypoxic stressors on Tel2 in cardiac mTOR

To determine whether Tel2 has cell survival effect against hypoxic stress via cardiac mTOR stabilization, we assessed cell death ELISA on Tel2-overexpressed HL-1 cells. Transient transfection of Tel2 in HL-1 cells was performed as described previously. 24 hours later, the cells were serum-starved and were subjected to either normoxia (95% O_2/5% CO_2) or hypoxia (95% N_2/5% CO_2). The cells were harvested 24 hours after the incubation and apoptosis was measured using cell death ELISA.

1.3) Knockdown of Tel2. (Scrambled-siRNA) vs. (siRNA-Tel2)

To further assess the effect of Tel2 on cardiac mTOR stability, Tel2 was silenced by introducing Tel2-specific siRNA (siRNA-Tel2) into HL-1 cells. The HL-1 cells were transiently transfected with 16ug of 10uM siRNA-Tel2 (Santa Cruz) using 4ul of Lipofectamine 2000 (Invitrogen) in 6-well plate. The scrambled siRNA (Santa Cruz) was also transfected into HL-1 cells as a negative control. Before the transfection, 4ul of Lipofectamine (per well) was incubated in 100ul of transfection medium. After 5 minutes of incubation, the siRNA Tel2 or scrambled siRNA in 100ul of transfection was added to Lipofectamine 2000. The plasmids were incubated for 20 minutes before applying to the cells. Additional 600ul of transfection medium was added to each well along with the siRNA plasmids and the cells were incubated for 4 hours at 5% CO_2 and 95% air. After 4

hours, the medium was changed to supplemented Claycomb medium without norepinephrine. The cells were harvested 72 hours after the transfection, and the expression levels of mTOR, S6, and Akt were assessed by Western blot.

Research Results

Overexpression of Tel2 activates mTOR via stabilization of mTORC1 and mTORC2:

To examine the role of Tel2 in cardiac mTOR at baseline, Tel2-overexpressed CMs were subjected to Western blot to check the mTOR signaling (Figure 4). We detected the expression of human Tel2 only in the Tel2-overexpressed CMs, indicating that Tel2 was successfully transfected in HL-1 cells. The expression of Tel2 detected by anti-TELO2, which detects both human and mouse Tel2, showed endogenous Tel2 protein expression in mock and increased expression of Tel2 in Tel2-overexpressed CMs (4-fold increase). Tel2 overexpression showed an increase in mTOR protein expression by 1.5-fold increase. The phosphorylation level of S6, which is the downstream molecule of mTORC1, was increased by almost 2-fold increase in Tel2 overexpression. The phosphorylation level of Akt at Ser^{473}, which is the downstream molecule of mTORC2, was also increased by 1.5-fold increase. These indicate that Tel2 overexpression activates cardiac mTOR through stabilization of both mTORC1 and mTORC2 at baseline.

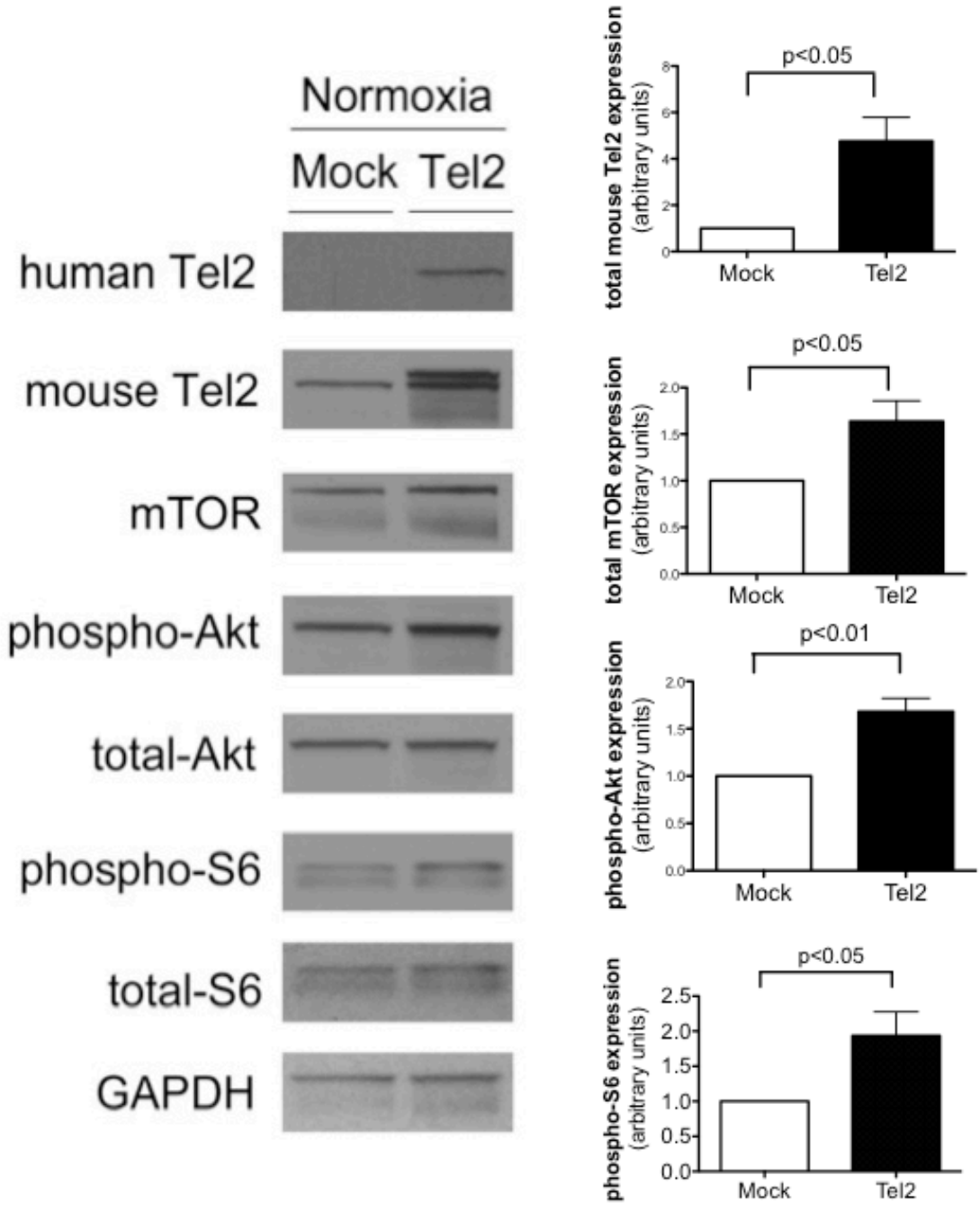

Figure 4 A representative immunoblots of mTOR signaling molecules in Tel2-overexpressed HL-1 cells.

Left: Representative immunoblots of mTOR signaling molecules in Tel2-overexpressed HL-1 cells. Immunoblotting was performed with antibodies of downstream molecules of mTOR: S6, Akt. GAPDH was used as a loading control. (This is a representative data of 5 individual experiments). Right: Densitometric quantification of immunoblots. (n=5)

Tel2 protects CMs against hypoxia via mTOR stabilization:

To determine whether Tel2 protects CMs from hypoxic stress, Tel2-overexpressed CM cells were incubated in either normoxic or hypoxic conditions. The morphology of the CM cells 48 hours after the transfection showed no difference in control and Tel2-overexpressed cells under normoxic condition (Figure 5). However, there was significantly reduced number of dead cells in Tel2-overexpressed cells compared to control under hypoxic condition. To further investigate the effect of Tel2 in cardiac cell death, Cell death ELISA was assessed to quantify the degree of apoptosis. We found that the fragmented DNAs were reduced by almost half in Tel2-overexpressed CMs under hypoxic condition compared to control (Figure 6). There was no significant change in the control and Tel2 overexpression under normoxic condition as we expected. These data indicated that Tel2 is sufficient to protect CMs against hypoxic stress.

Depletion of Tel2 decreases mTOR activity via destabilization of both mTORC1 and mTORC2:

To investigate whether Tel2 is necessary for mTOR stability, the endogenous Tel2 in HL-1 cells were knocked down by transient transfection of siRNA targeting Tel2. Immunoblotting showed that the expression level of Tel2 was diminished in siRNA-Tel2 transfected CMs compared to control (Figure 7). The expression level of mTOR was slightly decreased, indicating the diminished mTOR activity Tel2 depletion. The phosphorylation levels of S6 and Akt at Ser^{473} were decreased in Tel2-diminished CMs, which indicates decreased stability of both mTORC1 and mTORC2. These data demonstrated that Tel2 knockdown diminishes the activity of cardiac mTOR via destabilization of both mTORC1 and mTORC2.

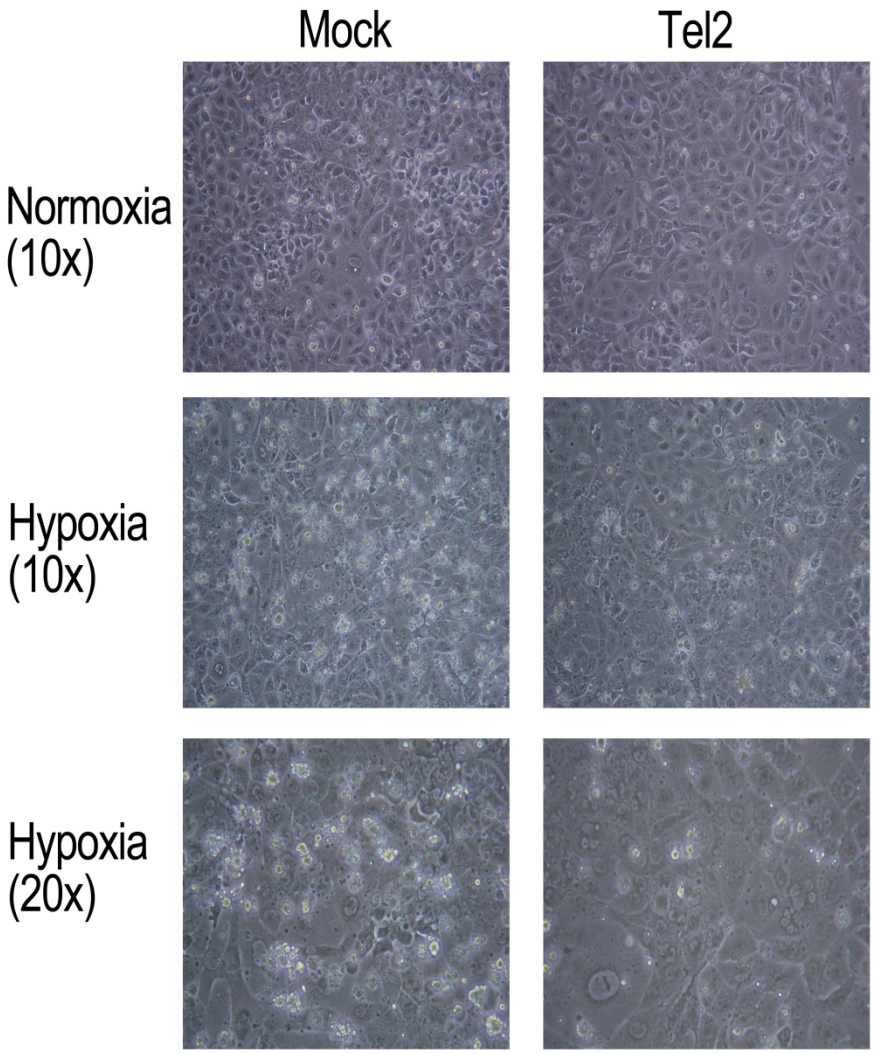

Figure 5 A representative morphology of Tel2-overexpressed HL-1 cells.

The HL-1 cells were subjected to hypoxia (95% N_2, 5% CO_2) 24 hours after overexpression of Tel2. The cells were harvested 48 hours after transfection for the morphological assessment of cell death.

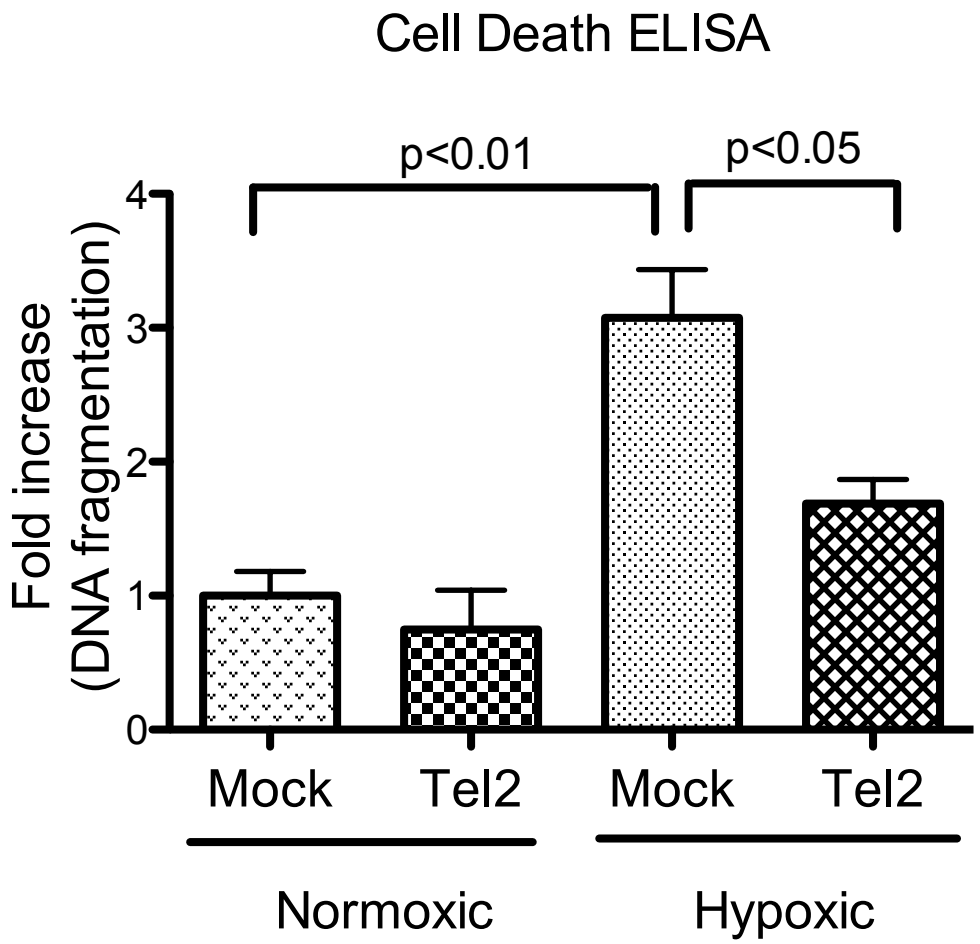

Figure 6 A quantification of apoptosis in Tel2-overexpressed CM cells under hypoxic stress.

Cell Death ELISA (Roche) was used to observe the fragmented DNA as an indicator of apoptosis in CM cells 48 hours after transfection. (n=4)

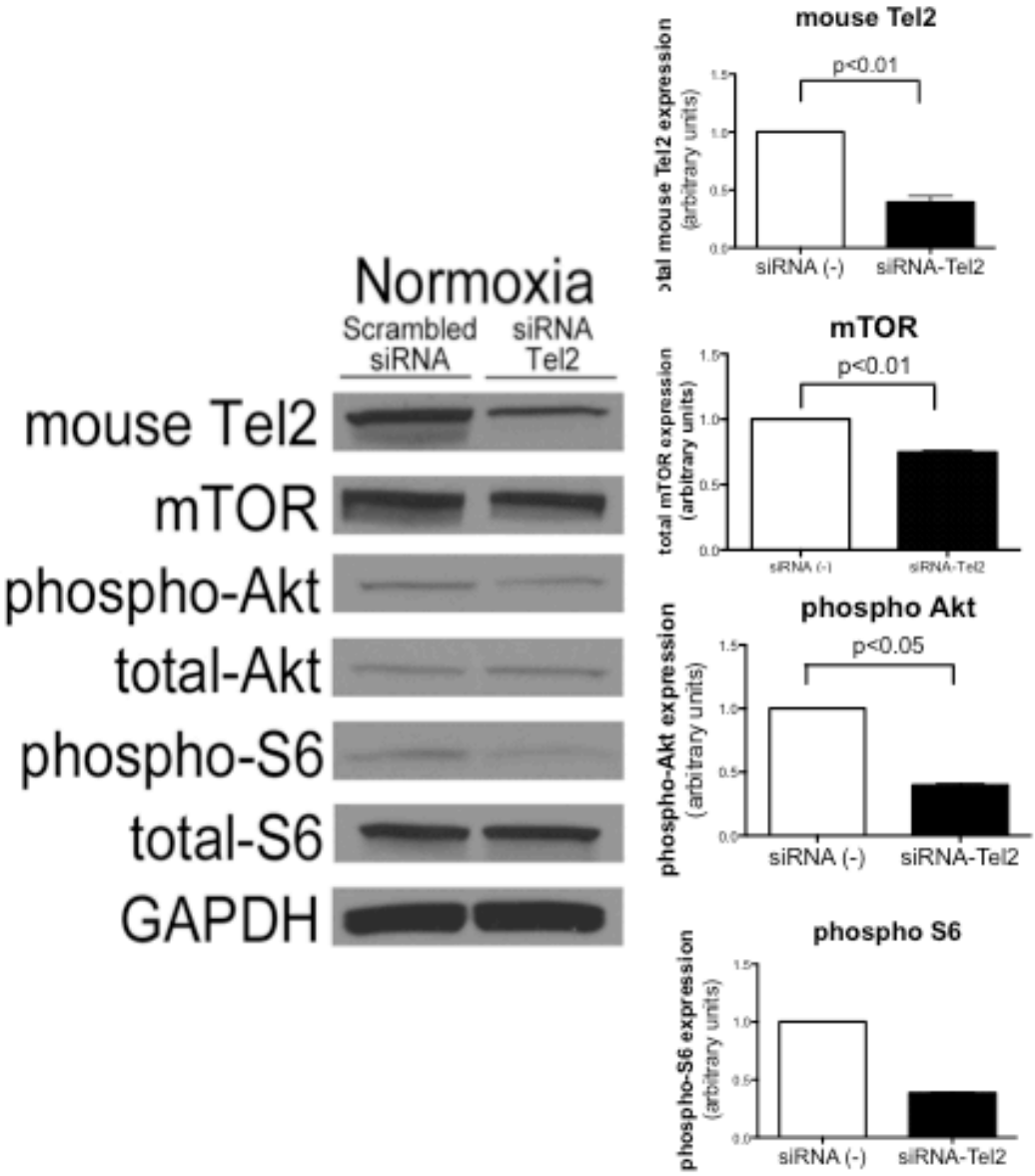

Figure 7 Representative immunoblots of mTOR signaling molecules in Tel2-diminished HL-1 cells.

Immunoblotting was performed with antibodies of downstream molecules of mTOR: S6, Akt. GAPDH was used as a loading control. Left: Representative immunoblots of mTOR signaliing molecules in Tel2-diminished HL-1 cells. (This is a representative data of 3 individual experiements). Right: Preliminary densitometric quantification of immunoblots. (n=3 for mTOR, mouse Tel2, and phosphor-Akt, n=2 for phosphor-S6)

Experimental Design and Method

Specific Aim 2: To examine the role of Tel2 on CM function at baseline.

Hypotheses:

Overexpression of Tel2 in mTOR-Tg CMs further improves cardiac function via stabilization of mTOR.

Approach:

We examined the effect of Tel2 in cardiac functions using isolated adult mouse CMs. Prior to the cardiac function measurement, Tel2 was overexpressed in isolated adult mTOR-Tg mice. To obtain higher transfection efficiency, adenovirus containing human Tel2 was generated for overexpression of Tel2 in mTOR-Tg CMs. Sarcomere length shortenings and intracellular Ca^{2+} transients were examined as indicators of cardiac function using IonOptix (Milton, MA).

Rationale:

Rationale for using adult mouse CMs:

While HL-1 CM cell line is a useful tool for manipulating genes because of its high efficiency in transient transfections, it is not an ideal model for analyzing CM function. We have established isolation of adult mouse CMs and physiological assays, including cell contraction and intracellular Ca^{2+} transients. Therefore, Tel2 overexpression using adult mouse CMs will allow us to explore the role of Tel2 in cardiac function.

Rationale for using recombinant Adenovirus:

We have a great deal of experience using recombinant adenovirus (Ad) in CMs and generating adenoviruses [21, 33, 34]. Recombinant adenoviral vectors offer several significant advantages. The viruses can be prepared at extremely high titer, infect non-replicating cells, and provide high transfection efficiency of CMs both *in vitro* and *in vivo*. We have successfully generated Ad. Tel2 using Ad. Easy system.

Rationale for culturing adult mouse CMs:

In our study, Ad.Tel2 was infected in isolated adult mouse CMs to examine the role of Tel2 in CM function. Based on preliminary studies we planned to infect CMs with Ad.Tel2 48 hours before examining CM function to have sufficient expression of the protein. In addition, maintaining the clear sarcomere band structures and the undamaged CM membrane is necessary for the measurement of contraction following 48 hours of incubation. However, although many laboratories have been successful in using adult mouse CMs in acute studies within the same day of isolation, maintaining the adult mouse CMs in primary culture for gene transfer has proved to be challenging due to a rapid decline in cell viability[48]. To overcome this difficulty, we established adult mouse CMs cultures using Blebbistatin, which is a specific myosin II inhibitor[48]. Our study showed that the viability of adult mouse CMs was increased when the media was supplemented with blebbistatin (data not shown). In addition, overdigested adult mouse CMs during perfusion of digestion buffer damages the CM membrane, reducing the cell viability during cultures. We also established the isolation of adult mouse CMs with minimal CM damage by adjusting the digestion time.

Rationale for using Langendorff system

In our study, Langendorff method was used to isolate cardiomyocytes from small animals using retrograde perfusion through the aorta with enzyme-containing solutions (Figure 8) [49]. In the basic Langendorff apparatus, tubing from the solution reservoirs leads to a cannula, on which the heart is mounted via the aorta (Figure9) [49]. Isolation of cardiomyocytes can be done by either constant pressure perfusion or constant flow perfusion. In a constant pressure perfusion, the solutions are suspended above the apparatus and gravity fed to the heart. On the other hand, a constant flow perfusion is employed by pumping the perfusate. In both methods, the perfusate are kept at 37ºC. The Isolation of adult cardiomyocytes using the Langendorff method provides much higher quality cardiomyocytes and is much more suitable especially for studying cardiac function compared to simple enzymatic digestion in a Petri dish[49].

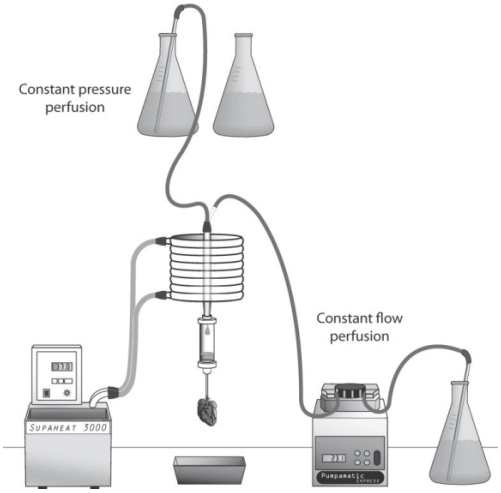

Figure 8 A scheme of the Langendorff system

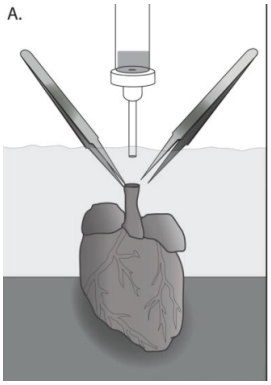

Figure 9 Cannulation of a heart via aorta

Rationale for measuring cardiac function

In present study, cardiac function of isolated adult mouse CMs was measured using IonOptix (Milton, MA) (Figure 10). For the measurement of myocyte contraction, the cells were field-stimulated by MyoPacer Field Stimulator (IonOptix, Milton, MA) at 2 Hz, 10 Volts. Sarcomere length was recorded using a video edge detector and MyoCam-S (IonOptix, Milton, MA). IonWizard Core Analysis software (IonOptix) was used to analyze the obtained data.

Intracellular Ca^{2+} transients were measured using the fluorescent calcium indicator fura-2 pentacetoxymethyl AM ester (Fura-2AM) (Molecular Probes). When fura-2AM enters the cell, the esterase enzyme cleaves the AM groups to leave fura-2 inside the cell for binding to Ca^{2+} ions. The Ca^{2+}-bound fura-2 can be detected by the dual-excitation fluorescence photomultiplier system (IonOptix). A photomultiplier tube detects the fluorescence at 510nm emission. In the presence of Ca^{2+} in cytosol, fura-2 fluorescence is excited by light at 340nm. In the absence of Ca^{2+}, it is excited by light at 380nm. The concentration of free intracellular Ca^{2+} corresponds to the ratio of fluorescence at 340/380. IonWizard Core Analysis software (IonOptix) was used to analyze the obtained data.

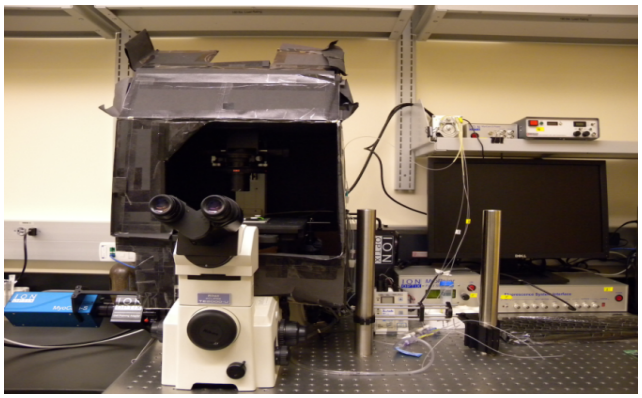

Figure 10 IonOptix system

Experiments:

2.1) Generation of Adenovirus containing Tel2

In order to achieve high overexpression efficiency using primary CMs, recombinant adenovirus containing Tel2 will be generated. The methods on generating the recombinant adenovirus will be discussed in the result section.

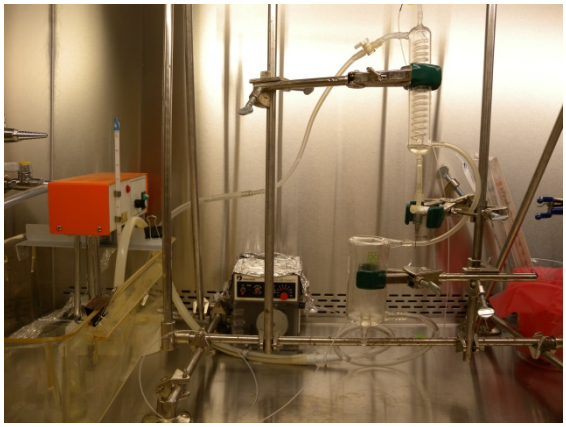

Figure 11 Langendorff System

2.2) Isolation of adult mouse CMs

In order to measure the cardiac function of CMs overexpressed with Tel2, adult mouse CMs were isolated using Langendorff system (Figure 11). Prior to isolation, mice were injected with heparin (250IU/ml). 5 minutes later, the mice were anesthetized with tribromoethanol (250mg/kg). The heart was quickly excised in ice-cold Ca^{2+} free perfusion buffer (10mM glucose, 135mM NaCl, 4mM KCl, $MgCl_2$, 10mM HEPES pH7.4, 0.33mM NaH_2PO_4, 10mM BDM, and 5mM Taurine), and the isolated heart was cannulated through aorta (Figure12). The cannulated heart was perfused with Ca^{2+} free perfusion buffer for 2 minutes at a constant flow rate of 3ml/min to wash out the remaining Ca^{2+} in

the heart. The heart was then digested by perfusion of digestion buffer (0.4mg/ml collagenase B (Roche), 0.3mg/ml collagenase D (Roche), 0.03mg/ml protease (Sigma

Aldrich) in 50ml of Ca^{2+} free perfusion buffer) for approximately 15 minutes depending on the heart condition. The digested heart was perfused once again with Ca^{2+} free perfusion buffer to wash out the digestion buffer and was mechanically teased apart and gently triturated with a plastic transfer pipette (Figure 13). The isolated CMs were filtered through a 250um filter top and were settled by gravity for 10 minutes. The Ca^{2+} concentration of the CMs were gradually brought back up to 1.2mM by incubating in Ca^{2+} transfer buffers with Ca^{2+} concentration of 0.06mM, 0.24mM, 0.6mM and 1.2mM for 10 minutes each.

Figure 12 Cannulated mouse heart via aorta.

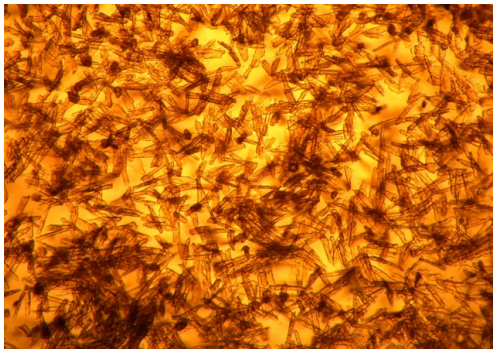

Figure 13 Isolated cardiomyocytes

2.3) Primary Cardiomyocyte plating and culture

Prior to plating, a laminin-coated culture dish was prepared. Laminin was diluted with phosphate-buffered saline to achieve a final concentration of 10ug/ml. The isolated CMs with Ca^{2+} concentration of 1.2mM were resuspended with plating media (M199, 5% FBS, 2mM L-glutamine, 100IU/ml Penicillin/Streptomycin, and 25uM Blebbistatin) and were plated on the laminin-coated dish. The plated cells were incubated in 5%CO_2 and 95% N_2 at 37% for 2 hours to allow the cells to attach to the bottom. After 2 hours, the

medium was changed to culture media (M199, 0.1mg/ml BSA, 1/100 of Insulin/Transferrin/sodium, 2mM L-glutamine, 100IU/ml Penicillin/Streptomycin, and 25uM blebbistatin). The CMs were incubated for 48 hours.

2.4) Adenovirus infection using primary CMs

At the time of media change, the CMs were infected with 20ul of Ad.GFP or 40ul of Ad.Tel2 in a 3.5cm dish. The CMs were incubated for 48 hours and the adenovirus infection was confirmed by the expression of GFP using fluorescence microscope (Figure 14).

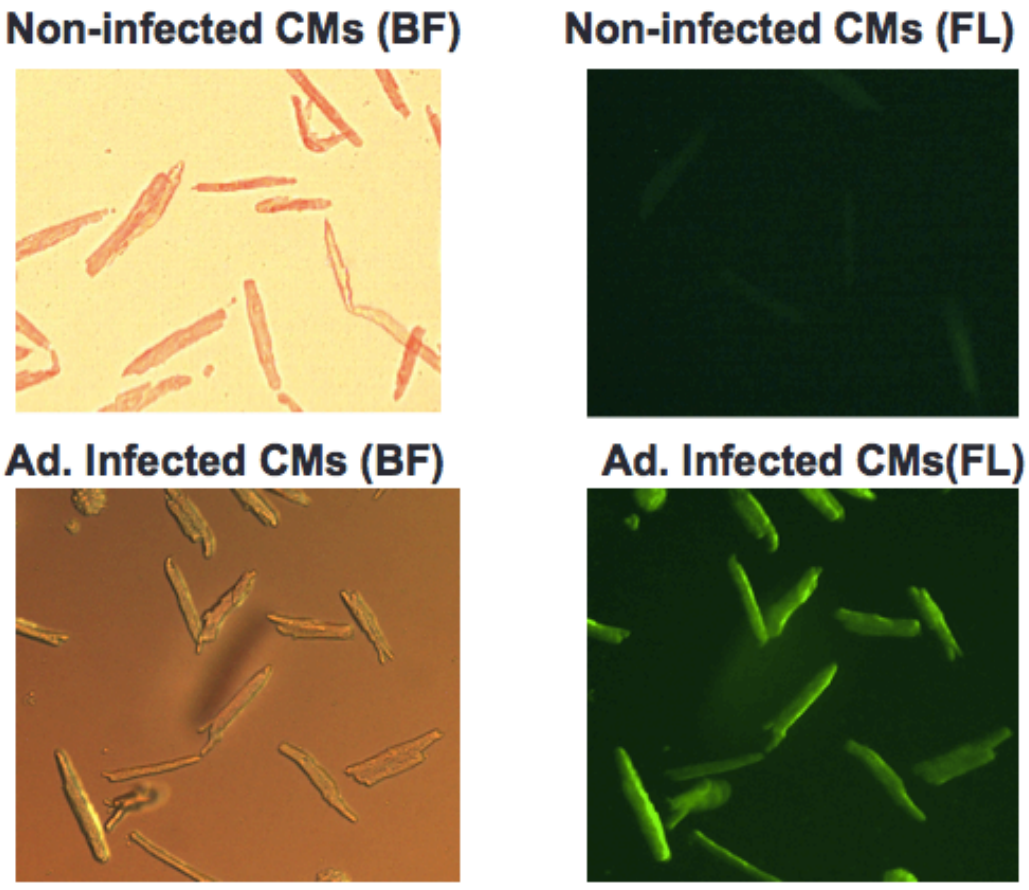

Figure 14 Cultured adult mouse CMs 48 hours after Adenovirus infection.

2.5) Measurement of Cardiac function

Cardiac function of a single cardiomyocyte was measured using IonOptix (Figure 15). In order to measure the Ca^{2+} transients, the CMs were incubated in Fura 2-AM loading buffer (1mM Fura 2-AM) for 15 minutes. The CMs were washed with 1.2mM Ca^{2+} tyrode buffer to remove the excess free Fura 2-AM three times. The 1.2mM Ca^{2+} tyrode buffer contains 10mM glucose, 137mM NaCl, 5.4mM KCl, 0.5mM MgCl2, 10mM HEPES pH7.4, and 1.2mM CaCl2. The Fura 2-loaded CMs were placed on the IonOptix stage and were electrically stimulated at 2Hz. The 1.2mM Ca^{2+} tyrode buffer was perfused through the IonOptix stage throughout the cardiac function measurement. The amplitude of Ca^{2+} transients, sarcomere length shortening, and the time to maximum departure and return velocity of Ca^{2+} transients and sarcomere length shortening were determined as indicators of cardiac function (Figure 16).

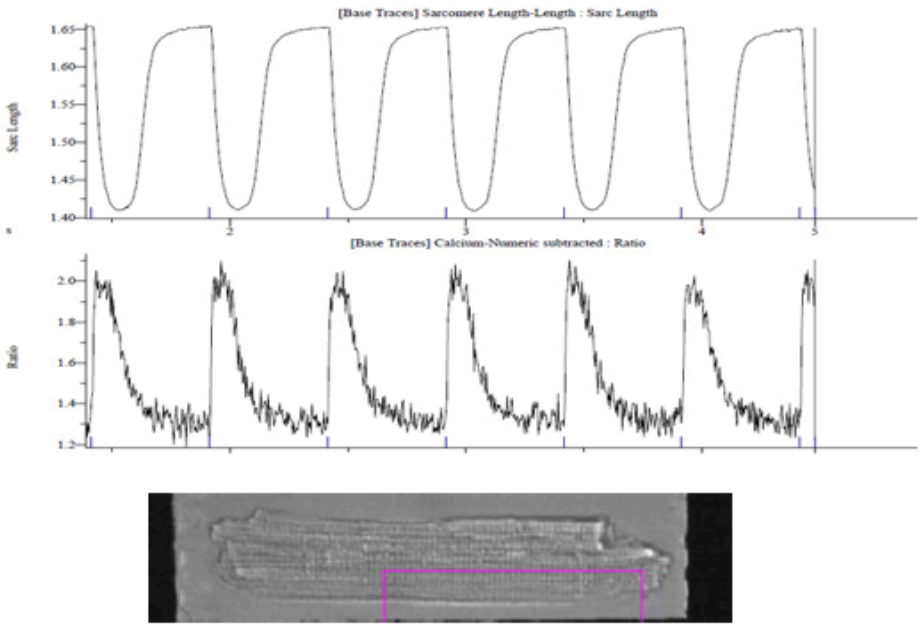

Figure 15 Measurement of cardiac function using IonOptix.

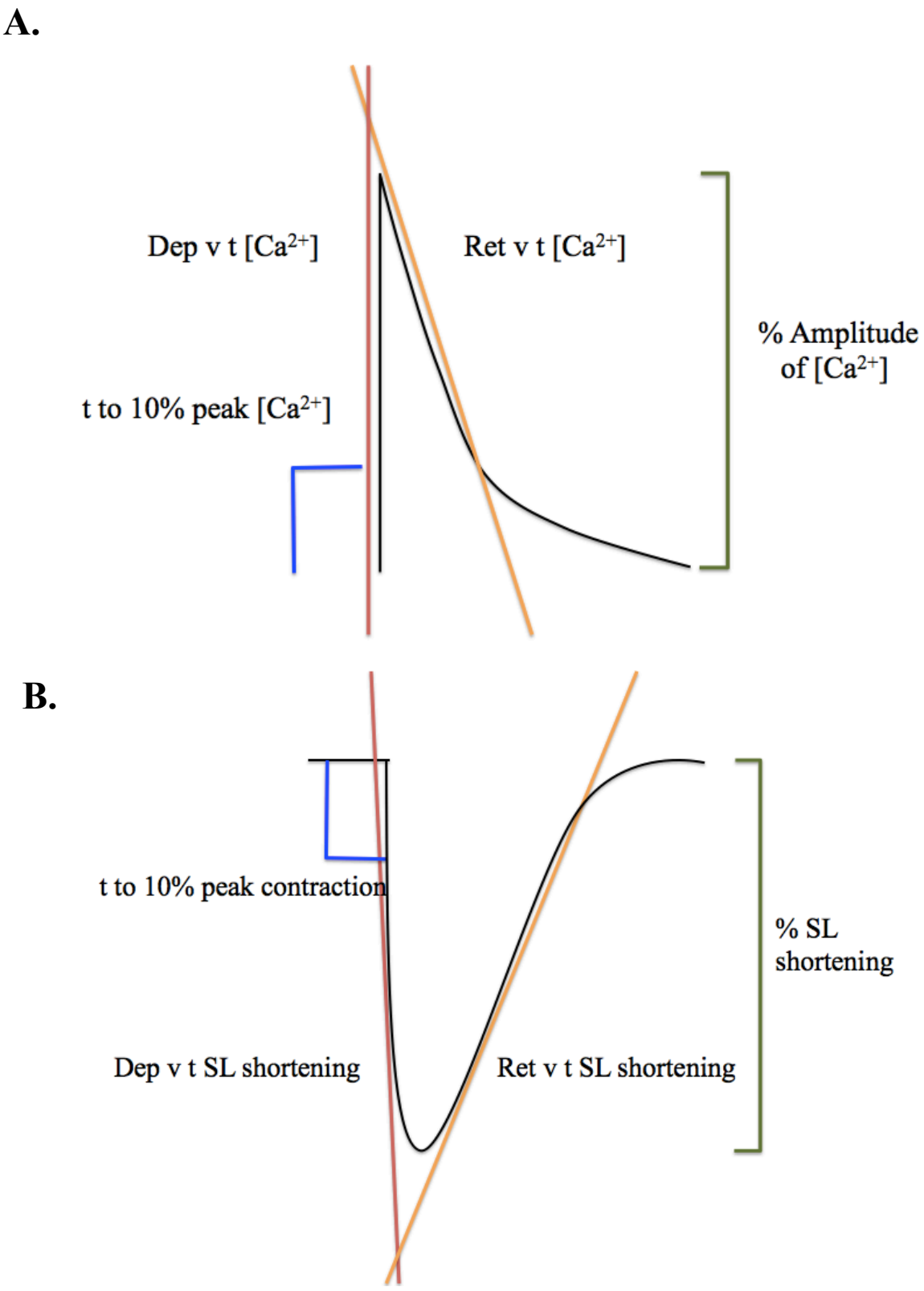

Figure 16 Overview of cardiac function measurements by IonOptix

Research Results

Generation of Recombinant Adenovirus Containing Tel2:

To obtain higher transfection efficiency, adenovirus containing human Tel2 was generated for the overexpression of Tel2 in primary CMs. A recombinant type 5 adenovirus containing human Tel2 was generated using Ad.Easy system. Adenovirus type 5 used for recombinant adenovirus does not contain E1 and E3, which are part of the viral transcription units. A gene of interest, in this case human Tel2, is inserted into E1 region (Figure 17). Generation of recombinant adenovirus using the AdEasy consists of: subcloning human Tel2 into a shuttle vector (pAdTrack-CMV), introducing the linearized shuttle vector into BJ5183 bacterial cells, and transfecting the linearized recombinant adenoviral DNA into HEK293 cells for generation of adenovirus. Introducing the shuttle vector into BJ5183 bacterial cells leads to the homologous recombination of the shuttle vector containing Tel2 and the adenoviral backbone plasmid contained in BJ5183. Transfection of the homologous recombinant into HEK293 cells is required for generation of adenovirus since the deleted E1 in the vector is necessary for replication of viruses. The detailed steps on generating recombinant adenovirus containing Tel2 are discussed below.

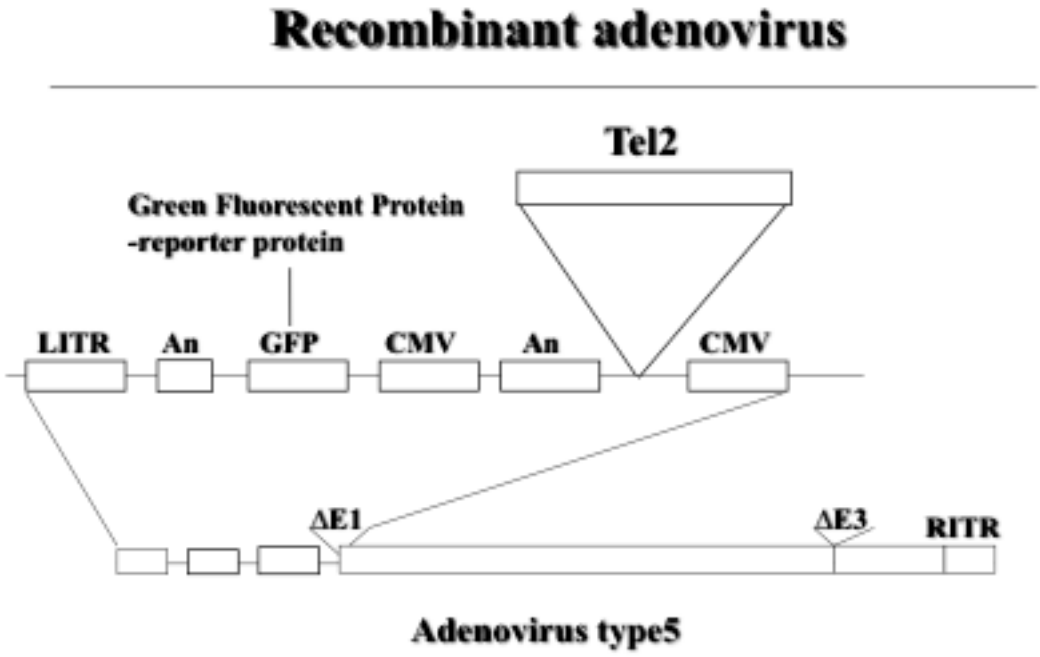

Figure 17 A scheme of recombinant adenovirus containing Tel2.

The human Tel2 plasmid was subcloned into a shuttle vector (pAdTrack CMV). For ligation, the shuttle vector and the insert ratio of 1:2 and 1:6 were used. After the ligation, the plasmid was inoculated into the competent cells DH5α and was purified by a plasmid purification kit, Nucleobond Miniprep (Macherey-Nagel). To confirm the successful ligation, the resultant plasmid was linearized by digesting with restriction endonuclease Not I. The selected plasmids were amplified and purified using Nucleobond Maxiprep kit (Macherey-Nagel). To further confirm the expression of Tel2 in the shuttle vector, the purified plasmid was transfected into HEK293 cells using Lipofectamine 2000. After 2 days of incubation, the cells were harvested and the expression of Tel2 was confirmed by Western Blot.

The plasmid was linearized by digestion with restriction endonuclease Pme I, and the linearization was confirmed by gel. The linearized plasmid was then purified using phenol chloroform purification and was resuspended in water. The purified plasmid was then transformed into 40ul of competent AdEasier cells using electroporation. AdEasier cells are BJ5183 derivatives containing the adenoviral backbone plasmid AdEasy-1 [50]. For transformation, the pulse electroporator was set at 2,500V, 200Ω, and 25uF. After transformation, the mix was resuspended in 500ul LB medium and plated in kanamycin-plate. The next day, smallest colonies were picked and were amplified in LB medium containing kanamycin. Following the amplification and purification by Miniprep, the candidate clones were digested with restriction endonuclease Pac I. The successful homologous recombination was confirmed by gel. The correct recombinants usually yield a large fragment (approximately 30kb) and a smaller fragment (3.0 or 4.5kb)[50]. To further confirm the homologous recombination and the presence of Tel2, the candidate was transfected into HEK293 cells following PacI restriction digestion. The expression of Tel2 was detected by Western blot on all the final candidate clones. The confirmed candidates were then retransformed into DH10B competent cells for amplification and were purified using maxiprep kit.

After confirming the candidate clones, these were linearized by PacI restriction digestion and were purified by phenol chloroform purification method. The linearized candidates were then transfected into HEK293 cells using Lipofectamine 2000. HEK293 cells contain recombinant adenovirus E1, allowing them to produce adenoviruses from backbone vectors without the E1 gene [50]. The transfected cells were incubated for 7 days, and 5ml of fresh complete DMEM medium was added every 3 days. Once the GFP

expression was confirmed in majority of the cells, the cells were harvested and freeze-thaw-vortex cycles were performed to release adenoviruses from the cells. Unlike uninfected HEK293 cells, infected cells showed round morphology and were detached from the bottom. During the freeze-thaw-vortex cycles, the aliquoted cells were put in dry-ice/ethanol and 37ºC water bath repeatedly for 4 times. At each cycle, the cells were vortexed thoroughly for the release of adenovirus from the cells.

For the amplification of adenovirus, the viruses were infected into HEK293 cells in 6 x 15cm^2 culture dishes to enhance the efficiency of amplification. The infected cells were harvested and were purified using an adenovirus purification kit, Vivapure AdenoPACK 100 (Sartorius). After the purification, the generated Ad.Tel2 was infected in HL-1 cells, and the expression of Tel2 was confirmed by immunoblotting (Figure 18).

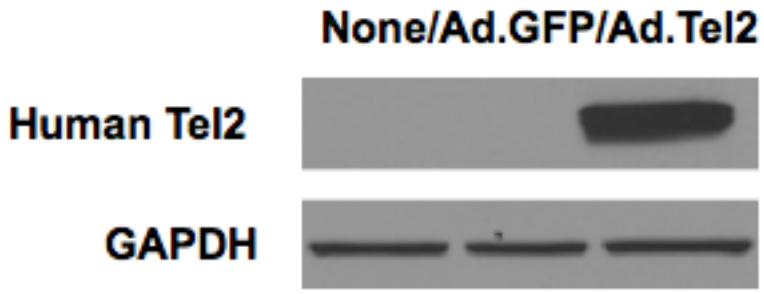

Figure 18 Tel2 expression of Ad.Tel2 was confirmed by immunoblotting.

Cardiomyocyte Function In Vitro:

Based on the results shown in specific aim I, we suggest that the cardiac protection against hypoxia by Tel2 is through the stabilization of mTOR complexes. Therefore, we examined the cardiac functions of isolated Wild Type (WT) and mTOR-Tg CMs at baseline before the cardiac function assessment of Tel2-overexpressed mTOR-Tg CMs. The measurement of sarcomere length shortening indicated that WT mice had higher percentage of SL shortening compared to mTOR-Tg (Figure 19). However, the time to 10% peak of contraction was significantly faster in mTOR-Tg compared to control. The time to maximal departure velocity (dep v t) and the time to maximal return velocity (ret v t) were both significantly decreased in mTOR-Tg compared to control. These indicated that although the amplitude of contraction is lower, the speed of contraction is positively regulated in mTOR-Tg.

The measurement of Ca^{2+} transients using Fura-2AM indicated that the time to 10% peak of Ca^{2+} release via RyR of SR was significantly decreased in mTOR-Tg compared to control (Figure 20). In addition, the time to maximal Ca^{2+} release (dep v t. $[Ca^{2+}]$) and the time to maximal Ca^{2+} reuptake (ret v t. $[Ca^{2+}]$) were both significantly decreased in mTOR-Tg. As we have shown in the measurement of SL shortening, the amplitude of $[Ca^{2+}]$ was also decreased in mTOR-Tg compared to control.

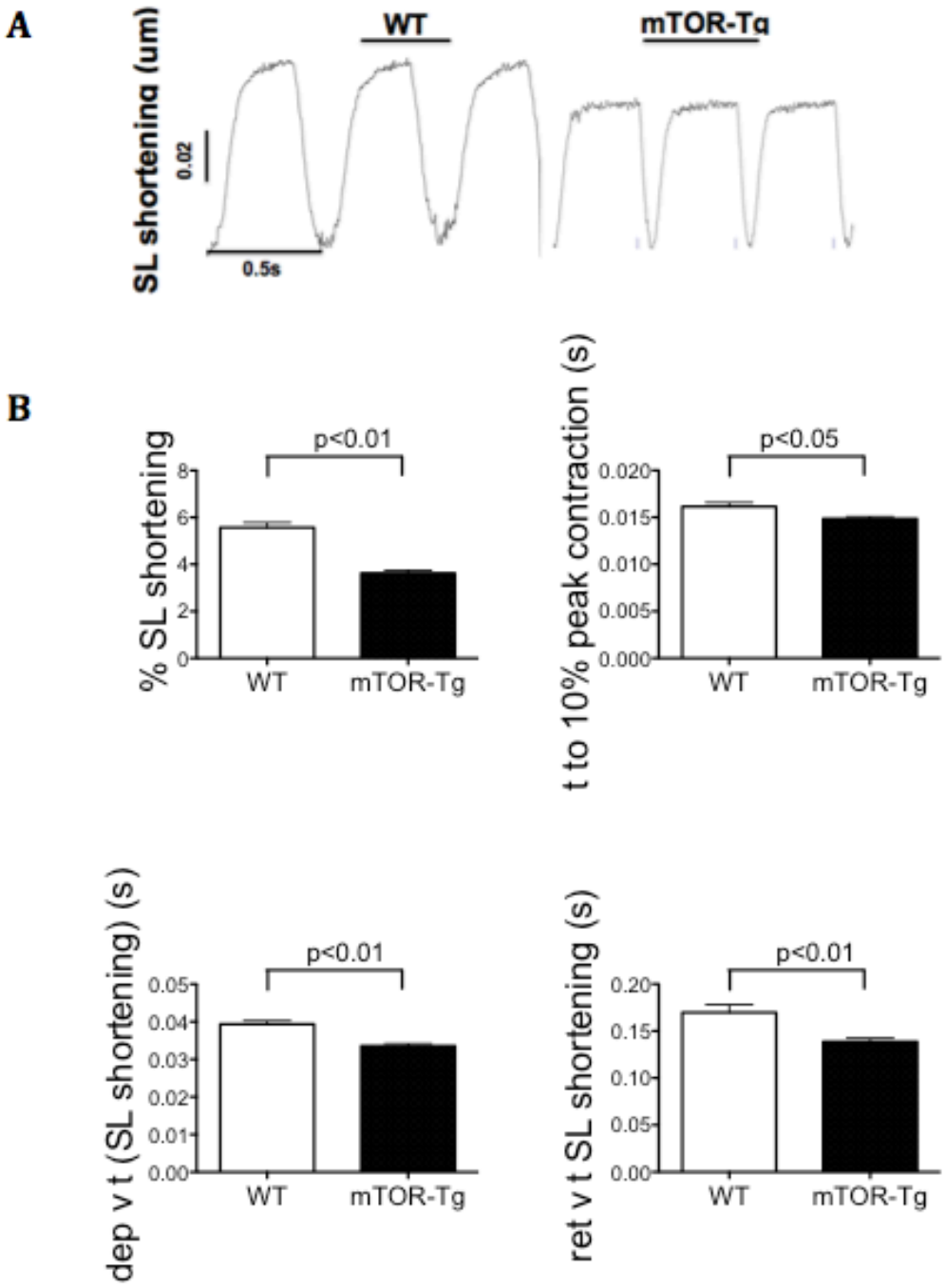

Figure 19 SL shortening of mTOR-Tg at baseline.

Isolated WT-CMs were used as control. Isolated myocytes were subjected to the measurement of SL shortening using IonOptix. A: Representative SL shortening traces of isolated mTOR-Tg CMs and WT-CMs(control). B: Graphs illustrating the % amplitude of SL shortening, time to 10% peak of SL shortening, time to maximum velocity of contraction (Dep v t. SL), and time to maximum velocity of relaxation (Ret v t. SL). (n=15-22)

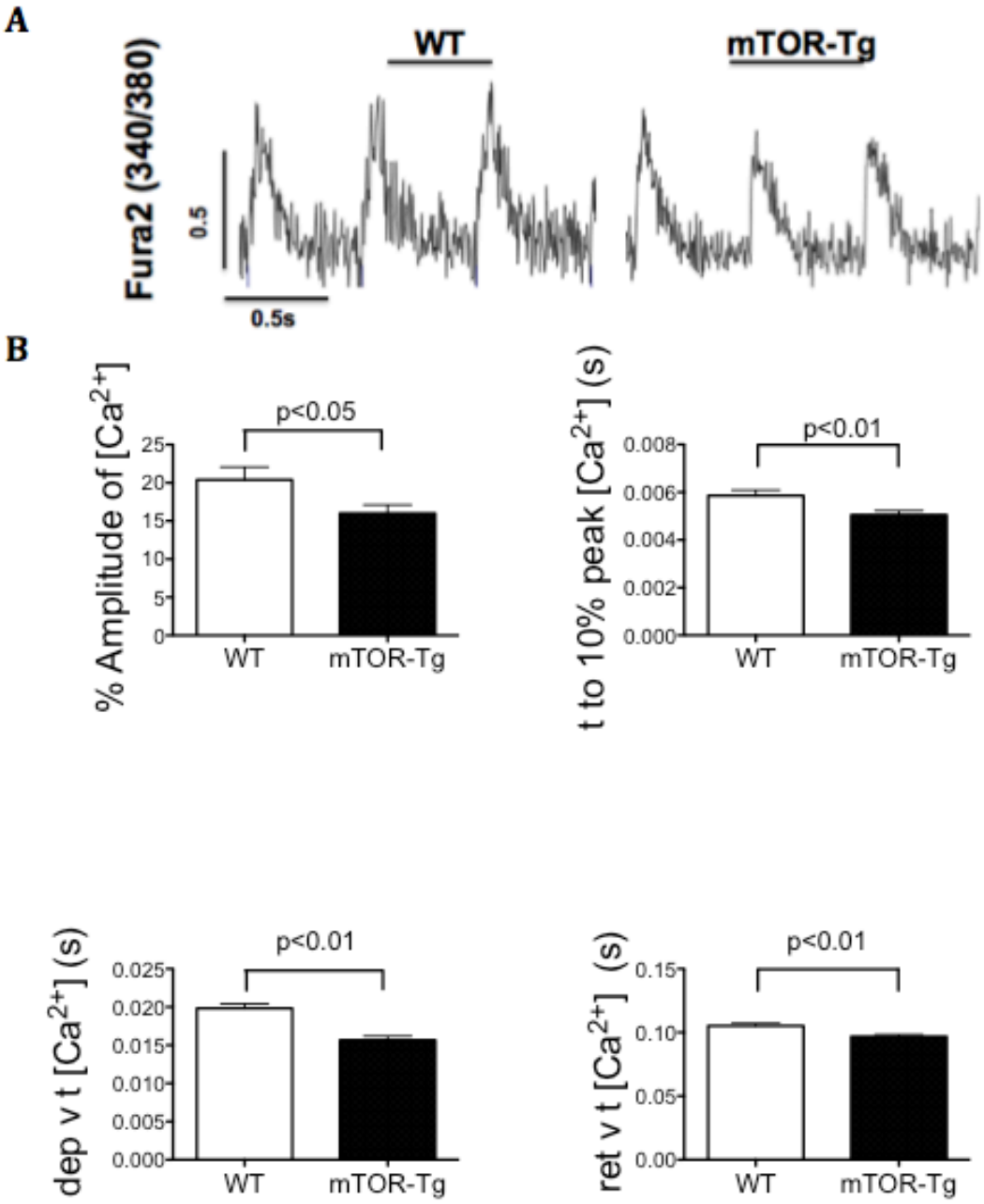

Figure 20 Ca^{2+} transients of mTOR-Tg at baseline.

Isolated WT-CMs were used as control Isolated myocytes were subjected to the measurement of Ca^{2+} transients using IonOptix. A: Representative Ca^{2+} Transient traces of isolated mTOR-Tg CMs and WT-CMs. B: Graphs illustrating the % amplitude of Ca^{2+} Transients, time to 10% peak of Ca^{2+} transients, time to maximum velocity of Ca^{2+} release (Dep v t. Ca^{2+}), and time to maximum velocity of Ca^{2+} reuptake (Ret v t. Ca^{2+}). (n=15-22)

To determine the cardioprotection effect of Tel2 via mTOR stabilization, Ad.Tel2 was used to infect cultured CMs isolated from the mTOR-Tg mice. Ad. GFP was used to infect the mTOR-Tg CMs as a control. The preliminary SL shortening measurement of Tel2-overexpressed mTOR-Tg CMs indicated that the time to maximal rate of contraction (dep v t) and the time to maximal return velocity (ret v t) were both decreased in Tel2-overexpressed mTOR-Tg compared to control (Figure 21). The time to 10% peak of contraction was similar in control and Tel2-infected CMs. However, the amplitude of SL shortening was lower in Tel2 infected CMs compared to control as we have shown in baseline study.

The measurement of Ca^{2+} transients showed that the time to maximal 10% peak of Ca^{2+} release via RyR of SR and the percentage amplitude of Ca^{2+} transients were similar in Tel2-infected mTOR-Tg CMs and control. However, the time to maximal Ca^{2+} release (dep v t. [Ca^{2+}]) and the time to maximal Ca^{2+} reuptake (ret v t. [Ca^{2+}]) were both decreased in Tel2-infected mTOR-Tg CMs compared to control. These data suggest that Tel2 positively regulates Ca^{2+} transients via mTOR stabilization during contraction.

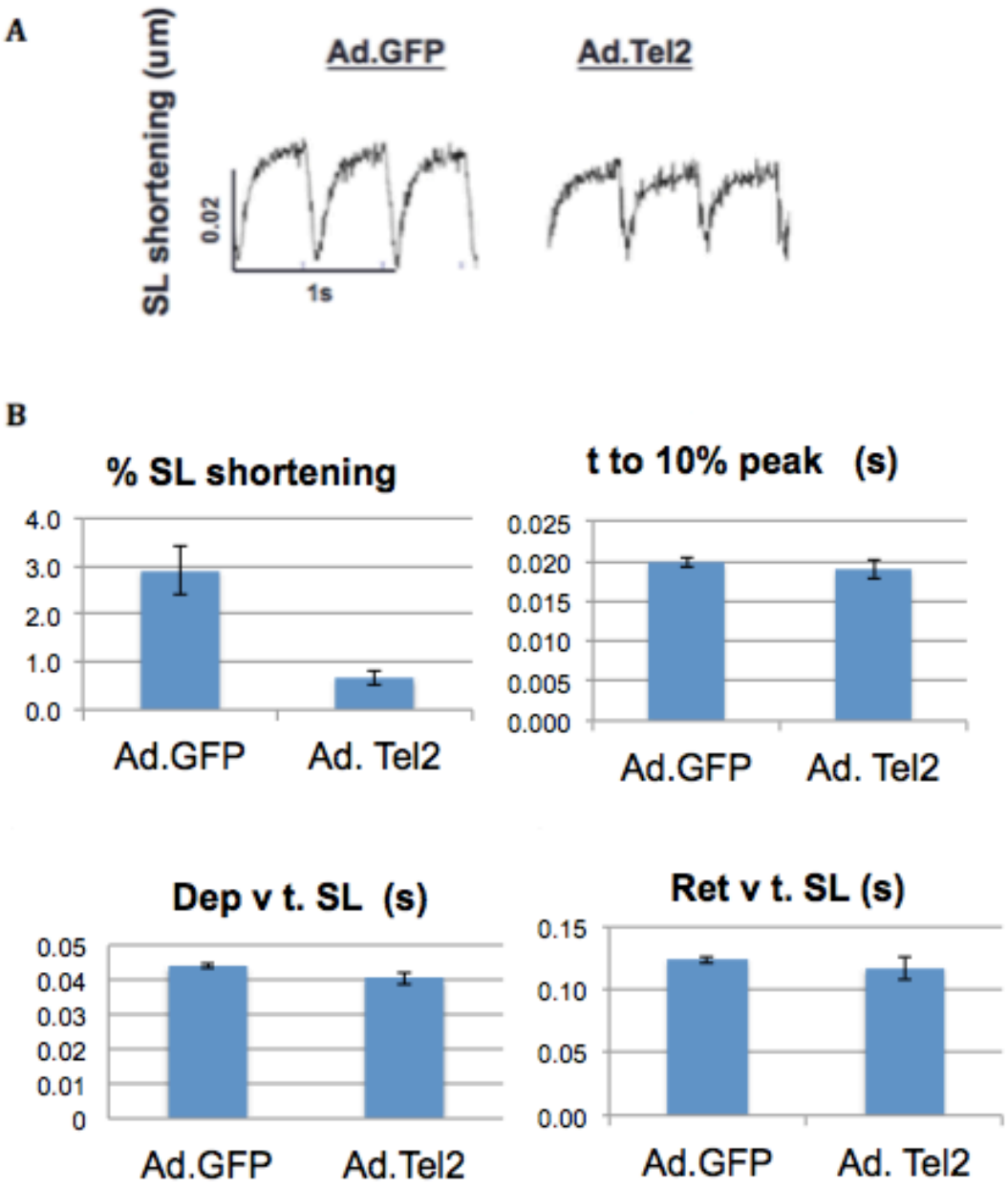

Figure 21 SL shortening of Tel2-overexpressed mTOR-Tg CMs.

Ad. GFP-infected mTOR-Tg CMs were used as control. Infected myocytes were subjected to the measurement of SL shortening using IonOptix. A: Representative SL shortening traces of mTOR-Tg CMs infected with Ad.Tel2 or Ad.GFP (control). B: Graphs illustrating the % amplitude of SL shortening, time to 10% peak of SL shortening, time to maximum velocity of contraction (Dep v t. SL), and time to maximum velocity of relaxation (Ret v t. SL). (n=7-11)

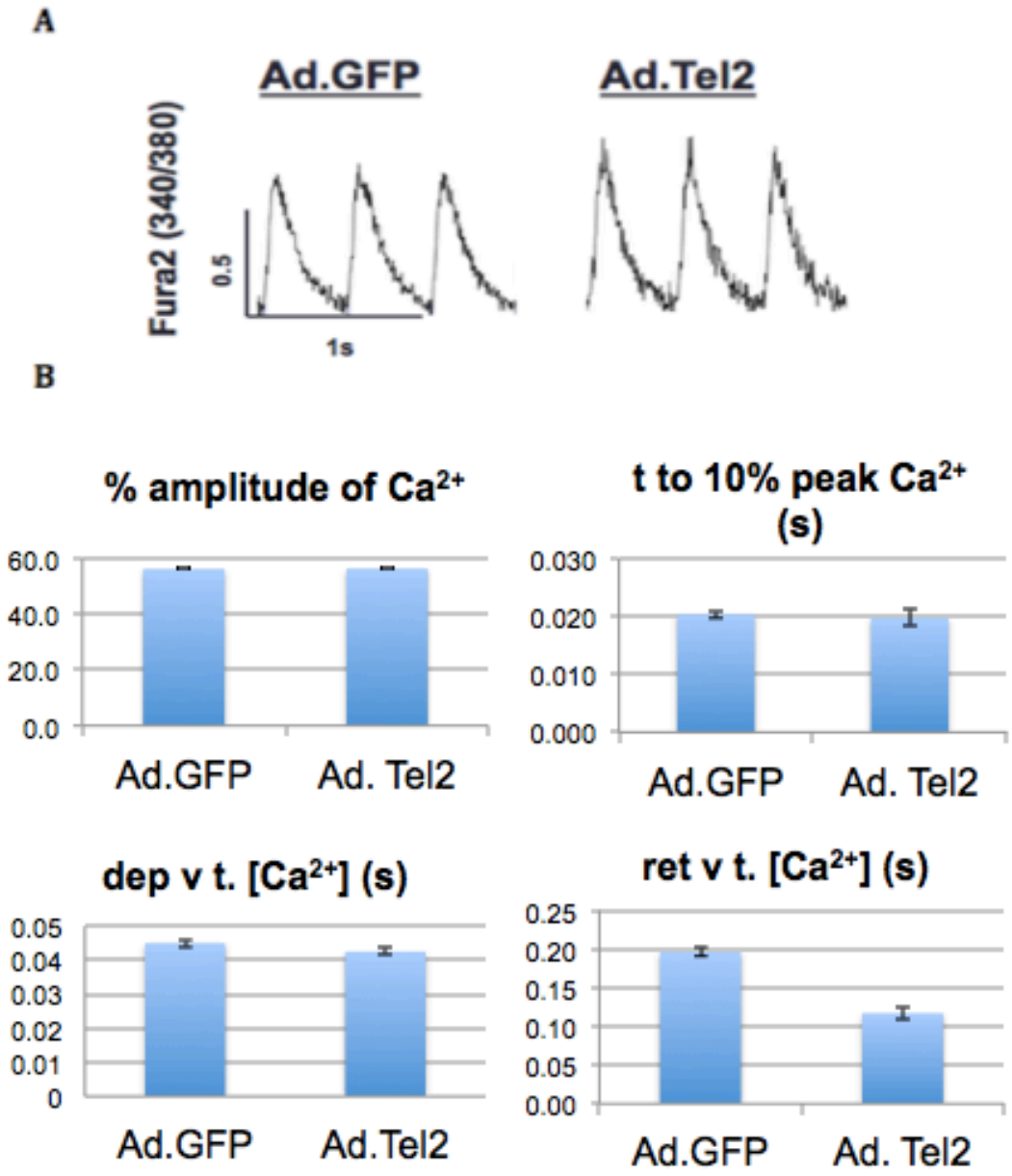

Figure 22 Ca^{2+} Transients of Tel2-overexpressed mTOR-Tg CMs

Ad. GFP-infected mTOR-Tg CMs were used as control. Infected myocytes were subjected to the measurement of Ca^{2+} transients using IonOptix . A: Representative Ca^{2+} Transient traces of mTOR-Tg CMs infected with Ad.Tel2 or Ad.GFP (control). B: Graphs illustrating the % amplitude of Ca^{2+} Transients, time to 10% peak of Ca^{2+} transients, time to maximum velocity of Ca^{2+} release (Dep v t. Ca^{2+}), and time to maximum velocity of Ca^{2+} reuptake (Ret v t. Ca^{2+}). (n=7-11)

Discussion

The present study demonstrates that Tel2 is sufficient to protect CMs against ischemic stress and positively regulates contraction via stabilization of both mTORC1 and mTORC2. *In vitro* experiments using HL-1 cells showed that overexpression of Tel2 increases phosphorylation of Akt and S6, indicating that Tel2-mediated activation of mTOR is via stabilization of both mTORC1 and mTORC2. The assessment of cell death using morphological assay and ELISA indicated that Tel2 is sufficient to inhibit cardiac cell death following ischemic stress *in vitro* experiments. These data suggest that Tel2-mediated activation of mTOR via stabilization of both mTOR complexes at baseline is sufficient to protect CMs against ischemia *in vitro*.

To examine the requirement of Tel2 in mTOR stabilization, we investigated the change in mTOR activity when Tel2 is depleted. Our *in vitro* experiments using siRNA targeting Tel2 demonstrated that a decrease in Tel2 expression reduced cardiac mTOR activity. The phosphorylation of S6 and Akt were both diminished in Tel2 depleted CMs, indicating that a decrease in cardiac mTOR activity is via destabilization of both mTORC1 and mTORC2. Our finding is consistent with the report by Takai *et al.* on destabilization of both mTORC1 and mTORC2 when Tel2 is depleted in HeLa cells. Our findings suggest that Tel2 is necessary for activation of cardiac mTOR. A study using mTOR-KO mice indicates that mTOR ablation leads to fetal dilated cardiomyopathy and an increase in apoptosis in CMs [35]**.** In the paper, the authors showed that 4EBP, a downstream of mTORC1, plays a key role in mTOR-mediated cardioprotection. Recently a report using transgenic mice overexpressing PRAS40, a negative regulatory protein of mTORC1, demonstrated that mTORC2 is a critical component on mTOR-mediated

cardioprotection in ischemia-reperfusion injury [51]. As shown in the present study, Tel2 activates both mTORC1 and mTORC2 with an increase in mTOR stabilization. These findings strongly suggest thatTel2 is a potential molecule for cardioprotection via stabilization of mTOR complexes.

Our previous study demonstrated that mTOR is sufficient to preserve cardiac function against I/R injury [5]. To examine this effect further, we investigated the role in cardiac mTOR in regulation of contraction at baseline. Our *in vitro* experiment using mTOR-Tg demonstrated reduced contraction percentage but significantly decreased speed of contraction and relaxation compared to WT. Although the percentage of Ca^{2+} transients was decreased, the speed of Ca^{2+} transients indicated faster Ca^{2+} release from the SR and faster Ca^{2+} reuptake into the SR in mTOR-Tg. Previously we reported that Akt activation blocks hypoxia-induced myocyte dysfunction and preserves contractile function and calcium handling [19]. Since Akt activity is increased in mTOR overexpression [4], these findings suggest that mTOR positively regulates Ca^{2+} transients and enhances cardiac contraction.

In present study, we investigated whether Tel2-mediated mTOR stabilization further enhances cardiac contraction using Tel2-overexpressed primary CMs. For this in vitro experiment, we generated recombinant Ad.Tel2. Overexpression of Tel2 by Ad.Tel2 provides much higher transfection efficiency in isolated adult mouse CMs compared to the transient transfection of Tel2 plasmid. The recombinant Ad.Tel2 also contains GFP under different a promoter, which allows us to confirm the overexpression of Tel2 in primary CMs. Our previous studies using the same adenovirus construct demonstrated that GFP itself does not affect cardiac function or cell viability[21, 52]. Therefore, generation

of Ad.Tel2 is favorable in our studies. The preliminary data of cardiac function measurement on Tel2-overexpressed mTOR-Tg CMs did not show any change in the amplitude percentage of SL shortening but demonstrated rapid contraction and relaxation. The speed of Ca^{2+} release and reuptake was also faster in Tel2-overexpressed CMs. Previous report indicated that Tel2 is important for the assembly of newly synthesized mTOR into the mTORC1 and mTORC2 complexes [7]. Therefore, these data suggest that Tel2-mediated mTOR stability may further regulate Ca^{2+} transients in cardiac contraction. However, these sample numbers are still not enough to determine the effect of Tel2 in cardiac function. Further examination is necessary to prove our hypothesis.

In summary, we found that overexpression of Tel2 enhances the activity of cardiac mTOR via stabilization of both mTORC1 and mTORC2 at baseline. Tel2 is sufficient to protect CMs and inhibit cell death against ischemic stress via the stabilization of mTOR complexes. Tel2 is necessary for the stabilization of mTOR complexes, and the depletion of Tel2 reduces the stability of both mTORC1 and mTORC2. The results of our experiments using isolated mTOR-Tg CMs suggest that mTOR enhances cardiac function via positive regulation of contraction and Ca^{2+} transients. Overexpression of Tel2 further enhances regulation of myocyte contraction and the Ca^{2+} transients via mTOR stabilization.

Our previous report using *in vivo* I/R mice model showed higher percentage of fractioning shortening and ejection fraction in mTOR-Tg following I/R, indicating that mTOR overexpression protected hearts after ischemia [5]. In this study, the measurement of cardiac function on WT and mTOR-Tg at baseline and Tel2-overexpressed mTOR-Tg CMs was done under normoxic condition. Therefore, we expect to see much more

significant improvement on regulation of contraction and Ca^{2+} transients under hypoxic condition.

In this study, we did not look at the mechanism of Ca^{2+} transient regulation by mTOR and Tel2. We demonstrated increased rate of contraction and Ca^{2+} transients in mTOR-Tg at baseline. We suggest that such effects may be due to an increase in SERCA2 activity. In fact, several groups have demonstrated that overexpression of SERCA2 results in increased contraction and relaxation rate of the Ca^{2+} transient [53 54]. To explain this possibility, we will investigate the activity level of SERCA2. Since the preliminary data on Tel2-overexpressed mTOR-Tg CMs showed a similar trend, Tel2 may be further enhancing such cardioprotective effect of mTOR. Therefore, we will further examine the myocyte function and the activity of SERCA2 in Tel2-overexpressed CMs.

Generation of recombinant Ad.Tel2 allowed us to apply our finding on Tel2-mediated cardiac mTOR stability into the investigation on the effect of Tel2 in cardiac function. However, the assessment of cardiac function using adult mouse CMs requires culturing of adenovirus infected CMs for 48 hours to overexpress Tel2. The measurement of cardiac function also requires CMs to be in a healthy condition for the infected CMs to contract under stimulation. The challenge to meet such requirements gives us the limitation on obtaining sufficient data to prove our hypothesis. A possible solution to this difficulty is to infect the adult mouse CMs with the minimum amount of adenovirus for maintaining the physiological condition. Generally, excess protein overexpression by adenovirus gene-transfer causes toxic effects into the cells including CMs. Therefore, the optimal amount of protein should be determined. In the constructor of Ad.Tel2, GFP is

co-expressed with independent promoter. Thus, it is feasible to adjust the optimal protein level by comparing the level of GFP expression in live cells.

In this study, we investigated the role of Tel2 in cardiac mTOR signaling. The cardioprotection of mTOR, a crucial mediator in cell survival and cell growth, brings us the potential target for preserving myocardial function in many cardiac diseases. However, its large molecular size makes it difficult to manipulate into gene therapy. Therefore, understanding the Tel2-dependent mTOR regulatory pathway in the heart may help identify a novel therapeutic target for a therapy of heart failure following myocardial infarction.

Future Directions

We demonstrated that Tel2 inhibits cardiac cell death against hypoxia via mTOR stabilization in HL-1 cells. To further confirm its cardioprotection against ischemia, the cell death assessment using adult mouse CMs is necessary. We will examine cell death in Tel2-overexpressed adult mouse CMs subjected to hypoxia. To determine whether Tel2 is necessary to protect CMs from ischemia, we will also examine cell death in Tel2-depleted CMs.

To further examine whether Tel2 preserves cardiac function via mTOR stabilization, the assessment of CM contraction in hypoxia is necessary. We will treat Tel2-overexpressed mTOR-Tg CMs with hydrogen peroxide to induce hypoxia and will examine the myocyte contraction.

To determine the necessity of Tel2-mediated mTOR stability, we will overexpress Tel2 in mTOR-KO CMs. To investigate the mechanism of Ca^{2+} transient regulation by mTOR and Tel2, the activity levels of SR proteins such as SERCA2 will be assessed by immunoblotting. A greater understanding of these mechanisms is likely to identify new targets for therapies that can maximize the benefits of cardioprotection by mTOR in heart failure.

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