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The effects of Tel2 on cardiomyocyte survival

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ABSTRACT

Aims: Overexpression of the mechanistic target of rapamycin (mTOR), a member of the PIKK (phosphoinositide kinase-related kinase) family, protects cardiomyocytes from cell death induced by pathological stimuli such as ischemia. We previously reported that posttranslational modification of mTOR plays an important role in regulating cardiac mTOR expression. The aim of this study was to see if Tel2 (telomere maintenance 2), a protein that regulates the abundance of PIKKs, confers similar cardioprotective effects as mTOR. Tel2 is not well-characterized in cardiomyocytes, therefore we examined the effects of Tel2 on cardiomyocyte viability under ischemic stress conditions.

Materials and methods: We overexpressed Tel2 or silenced Tel2 with siRNA in the HL-1 cardiomyocyte cell line to survey the effects of Tel2 overexpression and downregulation on cell survival during hypoxia. Adult mouse cardiomyocytes transfected with Tel2 adenoviruses were used to test whether Tel2 sufficiently prevented cardiomyocyte cell death against hydrogen peroxide (H_2O_2).

Key findings: Overexpressing Tel2 increased mTOR expression with a concomitant increase in mTOR Complex 1 (mTORC1) and mTORC2 activity in HL-1 cells. Tel2 deletion decreased mTOR expression, and mTORC1 and mTORC2 activity accordingly. In both HL-1 cells and adult mouse cardiomyocytes, Tel2 overexpression protected cardiomyocytes under ischemic stress. These effects were mTOR-dependent, as mTOR inhibitors blunted the effects of Tel2. While gene silencing of Tel2 did not affect cell survival under normoxia, Tel2 silencing made cardiomyocytes more vulnerable to cell death under hypoxia.

Significance: Upregulating Tel2 expression increases mTOR-mediated cardiomyocyte survival and targeting Tel2 could be another therapeutic strategy against ischemic heart disease.

1. Introduction

mTOR is a member of the PIKK (phosphoinositide kinase-related kinase) family, and an important mediator of the insulin-PI3K-Akt axis in the heart [1,2]. mTOR serves as a critical regulator for cell growth, metabolism, and cell survival in the heart and many other organs [2–5]. mTOR signaling also plays a crucial role in regulating autophagy [6,7]. mTOR binds with multiple proteins and can form two functionally and structurally distinct signaling complexes, mTOR complex1 (mTORC1) and mTORC2 [3,4]. The two mTOR complexes each elicit their own distinct downstream pathways that modulate cellular function and metabolism. mTORC1 is known to regulate protein translation, cell growth, and autophagy, whereas mTORC2 regulates cell survival and proliferation [4]. All of these functions are highly relevant to the proper functioning, maintenance, and repair of the heart, making mTOR a powerful target in preserving cardiac function during and after pathological conditions.

mTOR is necessary for cardiomyocyte growth and viability in early development of the heart [8] and cardioprotection in pressure overload-induced cardiac hypertrophy [9]. Our previous studies used transgenic mice with cardiac-specific overexpression of wild type mTOR (mTOR-Tg) to demonstrate that cardiac mTOR protects the heart against ischemia-reperfusion (I/R) injury [10,11] and pressure overload-induced cardiac hypertrophy [12]. We also reported that mTOR expression was significantly increased in the myocardium of patients and mice with advanced heart failure compared to controls and that posttranslational modification of mTOR plays an important role in regulating cardiac mTOR expression [12]. Taken together, an increase mTOR expression and mTOR complex activity appears to be an adaptive response that promotes cardiomyocyte cell survival under

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pathological conditions, rather than a pathological biomarker and trigger for cardiac disease. Although a considerable amount of data regarding the cardiac role of mTOR have been published, the pathophysiological roles of post-translationally regulated mTOR expression in cardiomyocyte viability are not characterized well.

Tel2 (telomere maintenance 2) and Tti1 (Tel2 interacting protein 1) proteins are known to interact with all known mammalian PIKKs and essentially regulate their abundance [13,14]. mTOR is part of the PIKK family, which also comprises of ataxia telangiectasia-mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), suppressor of morphogenesis in genitalia (SMG-1), and transformation/transcription domain-associated protein (TRRAP). Tel2 and Tti1 proteins are not only crucial for the stability of mTOR proteins, but also constitute essential components of both mTORC1 and mTORC2 complexes that are vital for their assembly [15].

To study whether mTOR stabilization is a viable strategy in cardioprotection, we examined the effect of Tel2 on cardiomyocyte cell viability against hypoxic stimuli. We used two methods; transient transfection with Tel2 in cardiomyocyte cell lines, and recombinant adenoviral gene transfer carrying Tel2 into primary cardiomyocytes isolated from adult mice. Our findings demonstrate that Tel2 enhances mTOR complex activity in cardiomyocytes, protects cardiomyocytes against hypoxic stimuli, and maintains cell viability.

2. Materials and methods

2.1. Cell culture and transfection

The HL-1 cardiomyocyte cell line was a generous gift from Dr. William Claycomb (Louisiana State University Medical Center, New Orleans, LA). The cells were cultured and transfected as performed previously [12]. The Tel2 plasmid was a gift from Dr. N. Mizushima (Tokyo University, Japan) (p3xFLAG-CMV10-hTel2, Addgene, Plasmid #30214). HL-1 cells were transfected with either Tel2 plasmid or backbone p3x-FLAG CMB10 vector (mock) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One day before transfection, 1.0×10^6 HL-1 cells were transferred into each well of a six-well plate containing 2 ml of pre-transfection medium [Claycomb medium (Sigma-Aldrich, St. Louis, MO), 10% of fetal bovine serum and 2 mg/ml of L-glutamine]. The next day, HL-1 cells were transfected with 10 μg/well Tel2 or control DNA vector using Lipofectamine 2000. To examine the mTOR signaling pathway, the cells were serum-deprived 24 h after transfection. Forty-eight hours after the transfection, the cells were harvested for the assays described below.

2.2. Western blotting

Protein lysates from the cultured HL-1 cells were collected with cold cell lysis buffer (Cell Signaling, Danvers, MA) as performed previously [12]. Protein concentrations were measured by the Bradford method (Bio-Rad, Hercules, CA). SDS/PAGE was performed under reducing conditions on 4–20% gradient gels (Bio-Rad). Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Blots were incubated with primary antibodies for 24 h at 4 °C. Blots were then incubated with HRP-conjugated secondary antibodies, and signals were detected using enhanced chemiluminescence (Cell Signaling, Danvers, MA). To detect human Tel2, we used anti-TEL2 polyclonal antibody (NBP1–81761, Novus Biologicals, Centennial, CO). For endogenous Tel2 protein expression in mouse HL-1 cells, we used anti-TELO2 polyclonal antibody (15975–1-AP, Proteintech). We also used primary antibodies against S6 (#2217), phospho-S6 (Ser235/236, #4856), Akt (#9272), phospho-Akt (Ser473, #9271), mTOR (#2983) (Cell Signaling, Danvers, MA), and anti-GAPDH polyclonal antibodies (ab9485, Abcam, Cambridge, MA).

2.3. In vitro model of cardiomyocyte hypoxia

To test the effects of Tel2 on cell survival under hypoxic stress, Tel2-transfected HL-1 cells were subjected to transient hypoxia as performed previously [16]. After the transfection, the cells were incubated in Claycomb medium with 37 °C/5% CO_2 conditions for 24 h. For hypoxic conditions, cells were changed to serum-free medium and placed in a 37 °C airtight box saturated with 95%N_2/5%CO_2 for 24 h. For normoxic conditions, the transfected cells were placed in a 37 °C/5% CO_2 incubator for 24 h before the analysis.

2.4. DNA fragmentation assay

Histone-associated DNA fragments were quantified with a Cell Death Detection ELISA kit (Roche Diagnostics) [16]. All cells from each well were collected by trypsinization and pipetting, pelleted (800 rpm, Allegra 6 Centrifuge, 5 min), lysed, and subjected to a capture ELISA according to the manufacturer's protocol. Data were normalized to the amount of DNA fragmentation seen with normoxic p3x-FLAG CMB10 vector-transfected cells. Each experiment was carried out in triplicate and repeated in ≥3 independent experiments.

2.5. RNA interference

siRNA targeting the TELO2 gene was obtained from Santa Cruz Biotechnology. As a negative control, control siRNA-A consisting of a scrambled sequence (Santa Cruz Biotechnology) was used. One day before transfection, 1.0×10^6 HL-1 cells were seeded into each well of a six-well plate containing 2 ml of pre-transfection Claycomb medium with 10% fetal bovine serum and 2 mg/ml L-glutamine. After incubation in the conditioning medium, Lipofectamine2000 was used to transfect the cells with 10 μM Tel2-siRNA or control siRNA-A. Forty-eight hours after transfection, the medium was changed to the Claycomb medium with 10% fetal bovine serum, 2 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The transfected HL-1 cells were subjected to either viability assays in hypoxic conditions (described above) or cell proliferation assays (described below). Seventy-two hours after siRNA transfection, we assessed protein expression, apoptosis, and cell proliferation of the cells as described in each assay.

2.6. BrdU proliferation assay

A BrdU proliferation assay kit (Millipore, Billerica, MA) was used to quantify the level of proliferation. BrdU (5-bromo-2′-deoxyuridine) was added to the siRNA transfected HL-1 cells twenty-four hours before harvesting the cells. The level of BrdU incorporation was measured using a monoclonal BrdU antibody according to the manufacturer's instructions.

2.7. Recombinant adenovirus carrying Tel2 (Ad.Tel2) and adult mouse cardiomyocyte culture

The Ad.Tel2 adenovirus encodes separate CMV-driven expression cassettes for Tel2 and GFP, and was constructed by subcloning the Tel2 cDNA into pAdTrackCMV and obtaining homologous recombinants through co-transformation with pAdEasy1 in *E.coli* BJ 5183 cells as done before [17].

Animal experiments in this study were approved by the Institution Animal Care and Use Committees of the University of Hawaii (Honolulu, HI). Adult mouse cardiomyocytes were isolated with the perfused heart method and cultured in a special medium for adult mouse cardiomyocytes, as described previously [18]. After isolation, cardiomyocytes were cultured in culture medium [Medium 199, 100 μg/ml bovine serum albumin, insulin (5 mg/l) - transferrin (5 mg/l) - sodium selenite (5 μg/l) media supplement (ITS, Sigma-Aldrich), 25 μM blebbistatin, 100 U/ml penicillin, 100 μg/ml streptomycin] and

immediately infected with 3 μl of 1×10^9 particles (pt)/ml of control virus (Ad.GFP; green fluorescence protein) [17], or Ad.Tel2.

One day after adenoviral infection, the cardiomyocytes were exposed to 20 μM of hydrogen peroxide (H_2O_2) for 3 h. To assess the mTOR signaling pathway in Tel2-mediated cardiomyocyte cell survival, thirty minutes prior to H_2O_2 treatment, some cells were given an additional pretreatment, either with 100 nM of rapamycin (Cayman Chemical, Ann Arbor, MI), an mTORC1 inhibitor, or 100 nM of Torin-1 (Selleck Chemicals, Houston, TX), an mTORC1 & mTORC2 inhibitor. Rapamycin and Torin-1 were initially dissolved in DMSO (Dimethyl sulfoxide, Sigma-Aldrich), and diluted to a final concentration of 100 nM. Cardiomyocyte viability was assessed by morphological changes and Live/Dead Viability Assays (Thermo Fisher Scientific) as done previously [18].

2.8. Statistical analysis

Data are means ± standard error (SE). Group differences were analyzed using the two-tailed Student's *t*-test or Welch's *t*-test. For all analyses, P values < 0.05 were considered significant.

3. Results

3.1. Tel2 overexpression activates the mTORC1 and mTORC2 signaling pathways

Human Tel2 was transfected into HL-1 murine cardiomyocytes to assess the effects of Tel2 on the mTOR signaling pathway in cardiomyocytes. Western blotting using an antibody specific for human Tel2 confirmed antibody specificity and that human Tel2 was expressed only in HL-1 cells transfected with human Tel2 (Fig. 1A). Western blotting using anti-TELO2 antibodies, which detects both human and mouse Tel2, detected endogenous mouse Tel2 in mock-transfected cells, and demonstrated that overall expression of Tel2 was 4-fold greater in Tel2-overexpressed cardiomyocytes compared to the mock control ($p < 0.05$) (Fig. 1A and B). Tel2 overexpression increased mTOR protein expression by a 1.5-fold increase ($p < 0.05$) (Fig. 1A and B). Furthermore, the phosphorylation of S6 and Akt, which are downstream targets of mTORC1 and mTORC2 respectively, were significantly increased (p-S6, $p < 0.05$; p-Akt, $p < 0.01$) (Fig. 1A and B). These data suggest that overexpression of Tel2 enhances both mTORC1 and mTORC2 activity by stabilizing mTOR protein.

3.2. Tel2 overexpression protects cardiomyocytes against apoptosis induced by hypoxia

To test whether Tel2 overexpression protects cardiomyocytes against hypoxia, HL-1 cells that overexpressed Tel2 were subjected to hypoxia or normoxia for 24 h. Morphologically, there was no difference between control and Tel2-overexpressed HL-1 cells under normoxia 48 h after transfection (Fig. 1C). In mock-transfected cells, 24-h hypoxia resulted in many dead round and floating cells. In contrast, the majority of Tel2-overexpressed cells survived under hypoxia (Fig. 1C). Using a Cell Death ELISA assay, we assessed the degree of apoptosis induced by hypoxia. The hypoxic control-transfected HL-1 cells showed significantly increased DNA fragmentation compared to normoxic control-transfected HL-1 cells (3-fold increase, $p < 0.01$), indicating that transient hypoxia induced apoptosis (Fig. 1D). Under normoxic conditions, there was no significant difference in apoptotic levels between control and Tel2-overexpressed HL-1 cells (Fig. 1D). However, under hypoxic conditions, fragmented DNA were suppressed by about half in Tel2-overexpressed HL-1 cells compared to the control ($p < 0.05$) (Fig. 1D). Taken together, the findings indicate that Tel2 is sufficient for preventing hypoxia-induced apoptosis in cardiomyocytes.

3.3. Tel2 depletion suppresses the activity of mTOR

To examine the necessity of Tel2 in the activity of cardiac mTOR, expression of Tel2 protein was depleted by gene silencing via transfection of siRNA targeting endogenous Tel2 in HL-1 cells. Three days after in vitro siRNA transfection, immunoblotting showed significantly reduced Tel2 expression in Tel2 siRNA-transfected HL-1 cells compared to control siRNA-transfected cells (60% reduction, $p < 0.01$) (Fig. 2A and B). The level of mTOR protein decreased (25% reduction, $p < 0.01$) (Fig. 2A and B), which indicated diminished mTOR activity due to Tel2 depletion. Tel2 knockdown also suppressed S6 and Akt phosphorylation significantly ($p < 0.05$) (Fig. 2A and B), suggesting a decrease in both mTORC1 and mTORC2 activity. These findings showed that Tel2 depletion inhibited the activity of the mTOR signaling pathway in cardiomyocytes by suppressing both mTORC1 and mTORC2.

To determine whether Tel2 is necessary to preserve cell viability and prevent cell death under hypoxic conditions, gene silencing of Tel2 was used to see whether removal of Tel2 deteriorates cell viability and induces cell death. To accomplish this, HL-1 cells were transfected with Tel2 siRNA or control siRNA and subjected to hypoxia for 24 h. Interestingly, there was no significant difference in morphology between the two groups (Fig. 2C). Cell Death ELISAs demonstrated that the level of fragmented DNA in Tel2 siRNA-transfected cells was higher than control siRNA, but there was no statistical difference between them (Fig. 2D). In normoxic controls, we found that the number of cells in the Tel2 siRNA group was less than the control siRNA group. We assessed whether this was due to potential effects of Tel2 knockdown on proliferation, as previous studies using other cell lines demonstrated that mTOR accelerates cell proliferation. To measure Tel2-mediated proliferation, HL-1 cells were transfected with either Tel2 or control siRNA and treated with BrdU for 24 h (Fig. 3A). The level of BrdU incorporation in Tel2 siRNA-transfected cells was significantly lower than control siRNA-transfected cells (Fig. 3B). These findings suggest that the effects of Tel2 knockdown are mainly mediated by suppression of cell proliferation, rather than the unremarkable level of cell death in HL-1 cells observed in Fig. 1D. Therefore, it might be taken that Tel2 is not necessary for cell survival under normoxic conditions, but rather that Tel2 is important in preserving cardiomyocytes and promoting cell survival when hypoxic conditions do occur.

3.4. Adenoviral gene transfer with Ad.Tel2 protects adult cardiomyocyte against H_2O_2-induced cell death

To explore the role of cardiac Tel2 in adult mouse cardiomyocytes, we constructed a recombinant adenovirus carrying human Tel2 (Ad.Tel2), as done before [16]. Western blotting confirmed that human Tel2 was expressed only in cells transfected with Ad.Tel2, and that Ad.Tel2 infection increased total expression of Tel2. Hydrogen peroxide (H_2O_2), a product of reactive oxygen species (ROS), is a known key trigger of cardiomyocyte cell death during ischemia-reperfusion injury [19]. Live/Dead Cell Viability Assays demonstrated that in in vitro adult mouse cardiomyocyte cultures, Tel2 overexpression significantly suppressed H_2O_2-induced cell death (Fig. 4B and C). We used Torin-1, an mTORC1/mTORC2 inhibitor [20], to see whether this protective effect was due to Tel2-mediated stabilization of mTOR complexes. Torin-1 fully inhibited the cardioprotective effects of Tel2 against H_2O_2, while rapamycin, an mTORC1 inhibitor, did not significantly suppress the protective effect of Tel2. These findings suggest that Tel2 overexpression protected cardiomyocytes against H_2O_2-induced cell death via both the mTORC1 and mTORC2 signaling pathways.

4. Discussion

The present study demonstrates that Tel2 positively regulates the activity of mTOR complexes and is sufficient to prevent cardiomyocytes

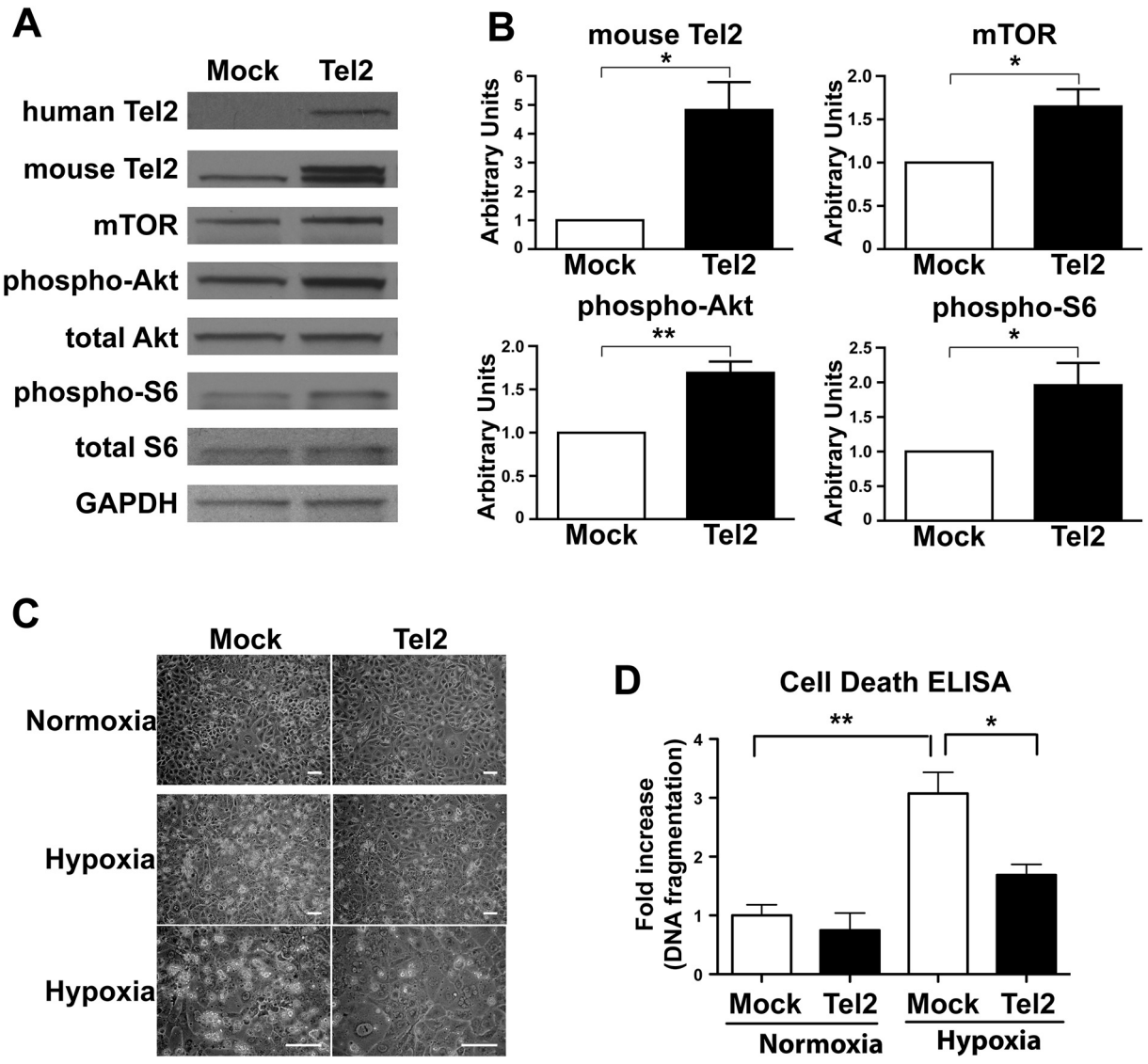

Fig. 1. Tel2 overexpression activates the mTORC1 and mTORC2 signaling pathways via stabilization of mTOR and prevents hypoxia-induced cell death.
HL-1 cells were transfected with an empty vector or human Tel2 plasmid. Two days later, cells were lysed and immunoblots were performed with primary antibodies indicated in the Materials and Methods section. A: representative immunoblots of mTOR signaling molecules. To assess the Tel2-mTOR signaling pathway, expression levels of Tel2 and mTOR, and phosphorylation levels of S6 (Ser235/236) and Akt (Ser473) were examined by immunoblotting. GAPDH was detected as a loading control. Representative blots are shown from 5 independent experiments. B: Quantification of immunoblots by densitometric assays. Data were normalized to the mean protein level in mock-transfected cells. Values are means ± SE; $n = 5$. *; $p < 0.05$, **; $p < 0.01$, Mock vs. Tel2. C: Protective effects of Tel2 overexpression against hypoxia. One day after Tel2 transfection and overexpression, the HL-1 cells were exposed to hypoxia (95% N_2, 5% CO_2). Morphological changes were assessed 48 h after transfection. The figure shows a representative view of 4 independent experiments. Scale bars, 200 μm. D: Quantitative assays of hypoxia-induced cell death in Tel2-overexpressed cells. The Tel2-overexpressing HL-1 cells were subjected to hypoxia for 24 h and harvested for the assessment of DNA fragmentation using Cell Death Detection ELISA kit (Roche Diagnostics) as an indicator of apoptosis. Data were normalized to the mean value of mock-transfected cells under normoxic condition. Values are means ± SE: $n = 4$. **; $p < 0.01$, Mock in Normoxia vs. Mock in Hypoxia, *; $p < 0.05$, Mock vs. Tel2 in Hypoxia.

against ischemic stresses. In vitro studies with the HL-1 cardiomyocyte cell line demonstrated that overexpressing Tel2 increases the phosphorylation levels of Akt and S6. The results suggest Tel2 overexpression results in activation of both mTORC1 and mTORC2 via Tel2-mediated mTOR stabilization. Cell death assessed by morphological changes and Cell Death ELISAs showed that Tel2 overexpression inhibits cardiomyocyte cell death, apoptosis, caused by ischemic stress. Knockout of Tel2 showed that the Tel2-mTOR axis is required for cell proliferation. In vitro studies with adult mouse cardiomyocytes showed that Tel2 overexpression protects cardiomyocytes against ROS-mediated cell death that mainly occurs via necrosis.

Previously, we reported that mTOR expression was significantly increased in the myocardium of patients and mice with advanced heart failure compared to controls [12]. In the study, we also demonstrated that posttranslational modification of mTOR plays an important role in regulating cardiac mTOR expression [12]. Tel2 has a profound effect on the stability of mTOR through physical binding [13]. In in vitro settings, the half-life of mTOR is approximately 18 h, while in Tel2-depleted cells, it is reduced to 2 h [13]. Tel2 binds to Tel2-interacting protein 1 (Tti1), and both Tel2 and Tti1 are necessary and sufficient to stabilize and activate both mTORC1 and mTORC2 signaling pathways [15,21]. mTOR is a member of the PIKK family, and in several cells, the Tel2-Tti1-Tti2 (TTT) complex plays a key role in PIKK stabilization [22]. On the other hand, the role of Tel2/Tti1 on mTOR stabilization in cardiomyocytes has not been reported. In this study, we found that Tel2 overexpression increased mTOR expression and activated both

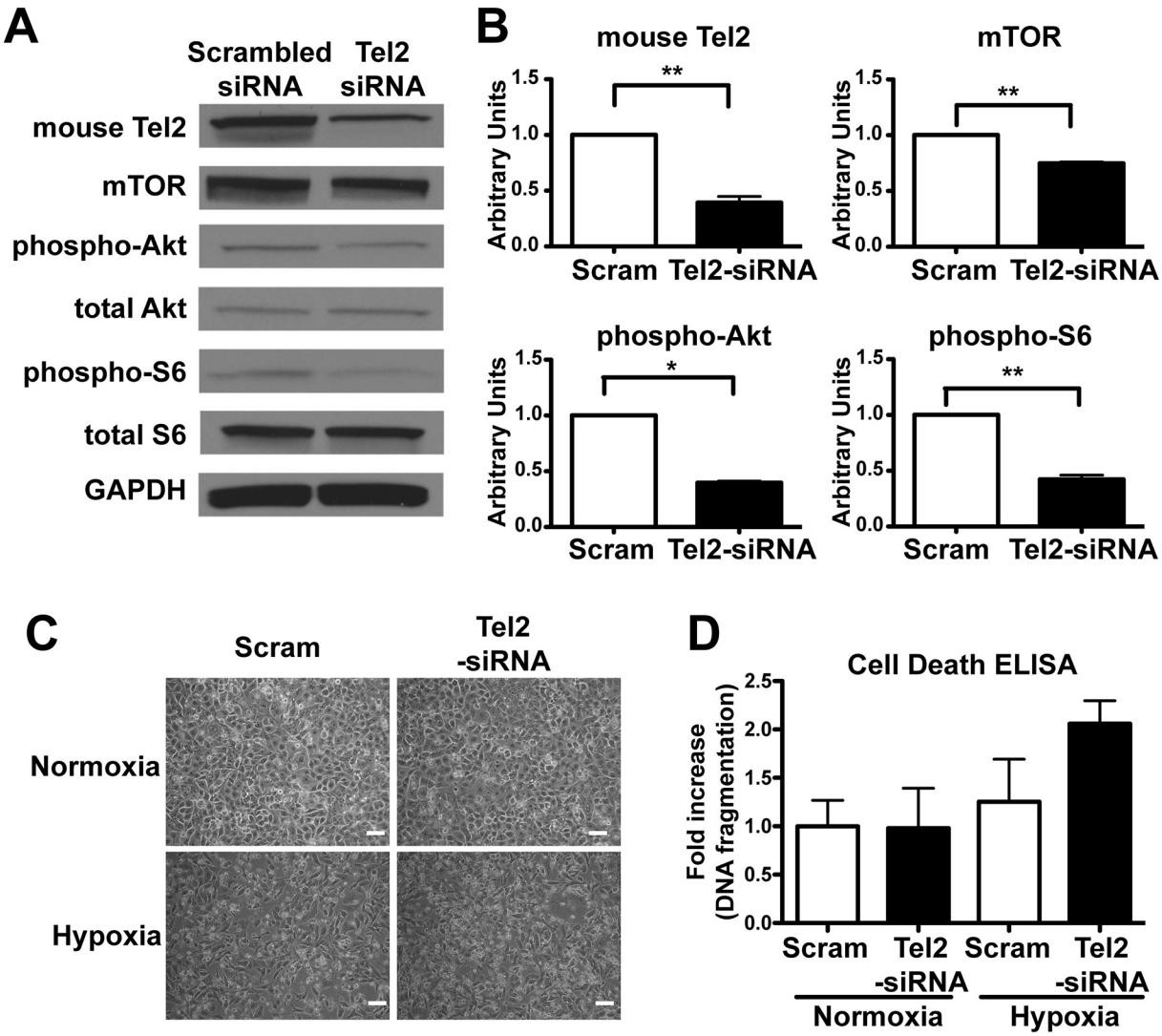

Fig. 2. Tel2 depletion suppresses activation of the mTORC1 and mTORC2 signaling pathways and exaggerates hypoxia-induced cell death.
HL-1 cells were transfected with either scrambled-siRNA or siRNA against Tel2. Three days later, cells were lysed and immunoblots were performed with primary antibodies as indicated in the Materials and Methods section. A: representative immunoblots of mTOR signaling molecules. To assess the Tel2-mTOR signaling pathway, expression levels of Tel2 and mTOR, and phosphorylation levels of S6 (Ser235/236) and Akt (Ser473) were examined by immunoblotting. GAPDH was detected as a loading control. Representative blots are shown from 5 independent experiments. B: Quantification of immunoblots by densitometric assays. Data were normalized to the mean protein level in scrambled (Scram) siRNA-transfected cells. Values are means ± SE; $n = 3$. *; $p < 0.05$, **; $p < 0.01$, Scram vs. Tel2-siRNA. C: Representative images of hypoxia-induced cell death in Tel2-depleted cells. Forty-eight hours after treatment with Tel2-siRNA, the cells were exposed to hypoxia for 24 h as done in Fig. 1. The figure is representative of 4 independent experiments. Scale bars, 200 μm. D: Quantitative assay of hypoxia-induced cell death. After observing morphological changes in Fig. 2-C, the HL-1 cells were harvested for the assessment of DNA fragmentation using a Cell Death Detection ELISA kit (Roche Diagnostics) as done in Fig. 1-D. Data were normalized to the mean value of mock-transfected cells under normoxic condition. Values are means ± SE: $n = 4$.

mTORC1 and mTORC2 signaling pathways. In addition, knockdown of Tel2 by Tel2 siRNA showed that reducing Tel2 expression suppressed mTOR expression and both mTORC1 and mTORC2 activity in cardiomyocytes, suggesting that destabilization of mTOR suppresses mTOR activity in the two mTORC1 and mTORC2 complexes. Consistent with this finding, a previous report using HeLa cells showed that Tel2 deletion destabilizes both mTORC1 and mTORC2 [13].

The current study showed that Tel2 overexpression protected cardiomyocytes under both hypoxia and H_2O_2 treatment conditions. Many groups, including us, demonstrated that activation of Akt protects cardiomyocytes against in vitro hypoxia-induced apoptosis [16,23]. The rapamycin-insensitive mTOR complex, mTORC2, is the kinase responsible for the phosphorylation and activation of Akt at Ser473 [24,25], while mTORC1 is rapamycin-sensitive and regulates protein synthesis via phosphorylation of p70S6K and 4E-BP1 (4E-binding protein1). Consistent with those findings, we observed that in HL-1 cells, Tel2 overexpression protected cardiomyocytes against hypoxia-induced apoptosis. These effects were accompanied by an increase in Akt phosphorylation, an indicator of mTORC2 activation, as well as S6 phosphorylation, which indicates mTORC1 activation, respectively. In adult cardiomyocytes, Torin-1, an mTORC1/mTORC2 inhibitor, eliminated Tel2-mediated protection against H_2O_2, while rapamycin, an mTORC1 inhibitor, partially suppressed the protective effect of Tel2. All of these combined results suggest that the effects of Tel2 in both mTORC1 and mTORC2 activation are required for cardiomyocyte survival. The results of this study are consistent with our reports using transgenic mice overexpressing cardiac-specific mTOR (mTOR-Tg mice), in which both mTORC1 and mTORC2 are activated in response to cardiac mTOR overexpression, which in turn protects the heart against I/R injury [10,11].

Necrosis is another major pathological feature in acute myocardial infarction, especially in I/R injury [26]. Previous studies suggest that

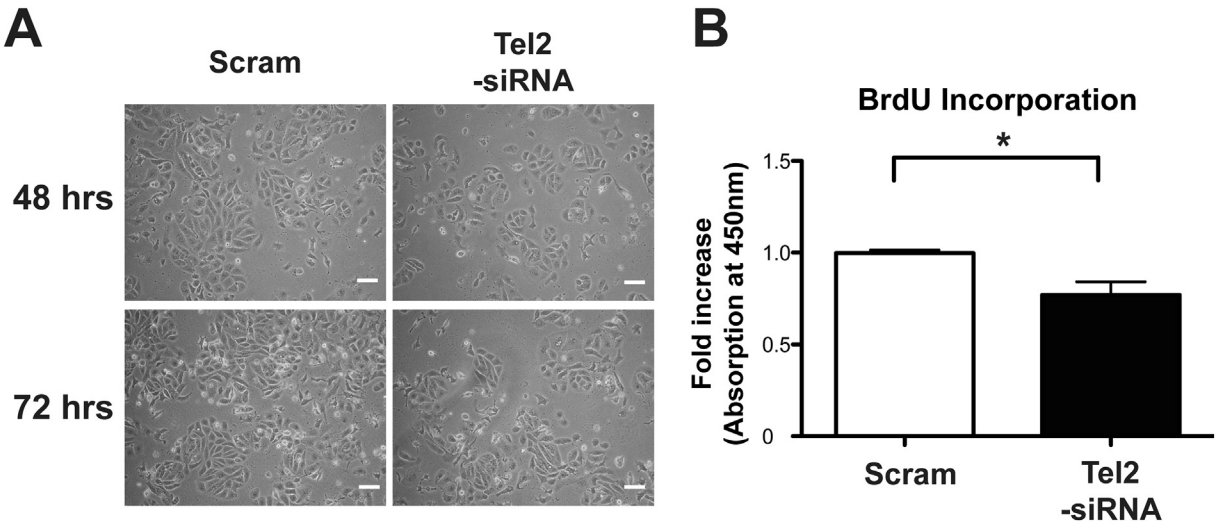

Fig. 3. Depletion of Tel2 suppresses cell proliferation.HL-1 cells were transfected with either scrambled-siRNA or siRNA against Tel2. A: Morphological changes in Tel2-depleted HL-1 cells. Morphological changes of cells were observed 48 h and 72 h after siRNA Tel2 transfection. This is a representative photo of four individual experiments. Scale bars, 200 μm. B: Tel2-depletion decreases proliferation in HL-1 cells. BrdU proliferation assay was used to observe proliferation in cardiomyocytes 72 h after transfection. Values are means ± SE: n = 4. *; $p < 0.05$, Scram vs. Tel2-siRNA.

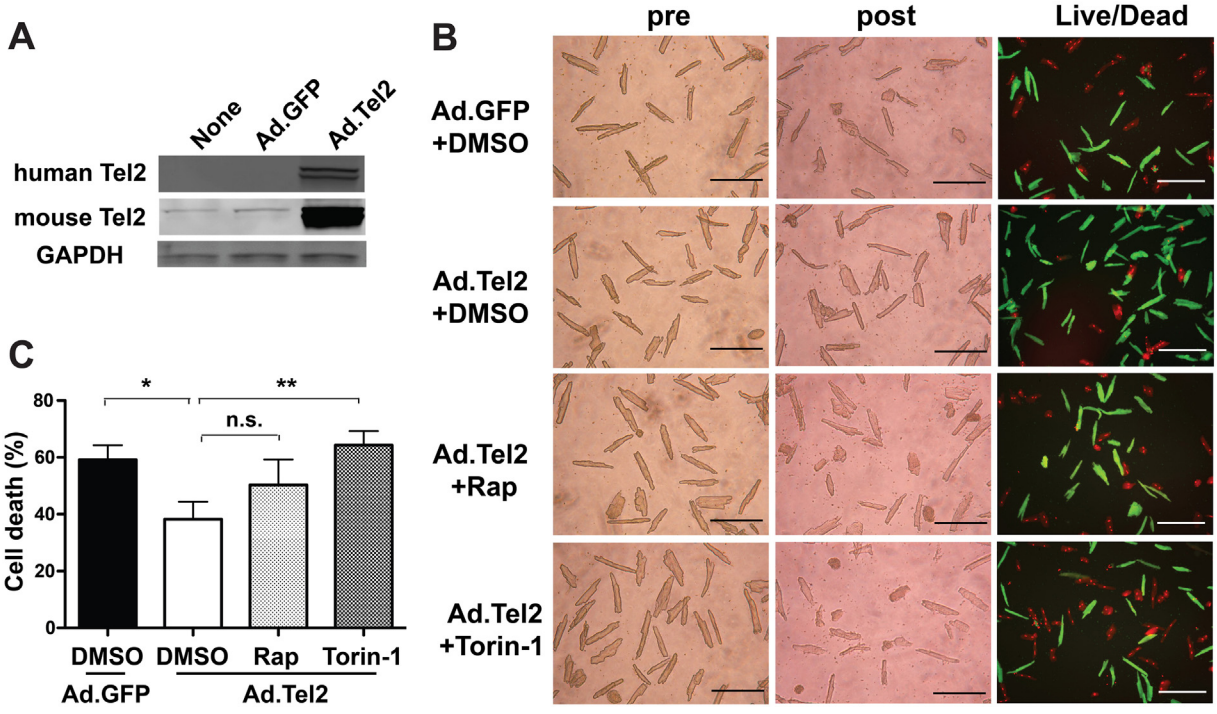

Fig. 4. Overexpression of Tel2 prevents H_2O_2-induced cell death via the mTORC1 and mTORC2 signaling pathway A: Representative immunoblots of Tel2 in adult mouse cardiomyocytes infected with Ad.Tel2. Overexpression of Tel2 was detected by western blotting with anti-human and anti-mouse Tel2 antibodies. Figure shows representative data among 3 independent experiments. B: Effects of Tel2 overexpression on H_2O_2-induced cell death in isolated cardiomyocytes. One day after infection with Ad.Tel2 or Ad.GFP, cardiomyocytes were exposed to 20 μM H_2O_2 for 3 h. To assess the mTOR signaling pathway, cardiomyocytes were pretreated with either 100 nM of rapamycin (Rap), an mTORC1 inhibitor, or 100 nM of Torin-1, an mTORC1 and mTORC2 inhibitor, 30 min prior to H_2O_2 treatment. DMSO was used as a vehicle control. Morphological changes in cultured cardiomyocytes were assessed before applying the reagents (pre) and at the end of experiments (post). Live/Dead Assays (Live/Dead), in which live cells stain with calcein AM (green) and nuclei of dead cells stain with ethidium homodimer-1 (red), were used to assess cell viability. This is a set of representative images among four independent experiments. Scale bars, 200 μm. C: Quantitative data of cardiomyocyte cell death. The total numbers of live cytosol (green) and dead nuclei (red) cells were counted in 3 low-power fields. More than 500 cells were counted in each condition. Cell Death % was calculated as dead cells/total cells. $n = 6$. *; $p < 0.05$, Ad.GFP + DMSO vs. Ad.Tel2 + DMSO, **; $p < 0.01$, Ad.Tel2 + DMSO vs. Ad.Tel2 + Torin-1, n.s.; no significance, Ad.Tel2 + DMSO vs. Ad.Tel2 + rapamycin (Rap). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ROS production in the reperfusion phase exaggerates cell death [19]. Our previous study using mTOR-Tg mice in in vivo and ex vivo I/R models showed that mTOR overexpression in cardiomyocytes prevents post-I/R myocardial injury that mostly occurs as a result of necrosis [10]. Recently, we reported that mTOR overexpression protected cardiomyocytes from ferroptosis [18], an iron-dependent form of regulated cell death [27], which is a critical pathophysiological feature in I/R injury [28]. Taken with the current study, these findings suggest that the stabilization of mTOR by Tel2 overexpression prevents cardiomyocyte cell death by protecting cardiomyocytes against ischemic stimuli.

Tel2 activates both mTORC1 and mTORC2, but its role in cardiac aging is not characterized well. mTORC1 directly and indirectly regulates autophagy, which maintains cellular function by facilitating the degradation of damaged proteins and organelles, which accumulate during aging and in the metabolic syndrome [3,4,7]. Rapamycin, an inhibitor of mTORC1, prevents aging by activating autophagy [29]. On the other hand, mTORC1 and mTORC2 inactivation by mTOR deletion is lethal [30]. Given these, Tel2 might be a key factor and potential factor in aging by balancing mTORC1 and mTORC2 activity, rather than simple upregulation and overexpression. Interestingly, a previous report showed that Tel2 contributes to life span by regulating the ATM-p53 axis via control of ATM expression [31]. Further studies on the role of the Tel2 on aging is required.

Consistent with a previous report [32], we observed the proliferation of HL-1 cells in conditioned medium with serum. In the study using BrdU, we demonstrated that deletion of Tel2 reduced the proliferation rate of HL-1 cells, rather than the unremarkable levels of cell death induced by hypoxia. Previous reports demonstrated that mTOR mediates cell proliferation rates in many cell lines [33]. An mTOR kinase inhibitor, AZD8055, which inhibits the proliferation of cancer cells, is a potential pharmacological agent for cancer therapy [34]. Compared with HL-1 cells, the proliferation rate of cardiomyocytes in the adult heart is relatively low to the point of not being significant [35]. Thus, cardiomyocyte proliferation regulated by mTOR might not be a critical factor for the physiological function of the adult heart. On the other hand, deletion of cardiac mTOR leads to dilated cardiomyopathy accompanied by increased apoptotic cell death [8]. Based on these findings, we expect that Tel2 is necessary to protect cardiomyocytes against ischemia and other pathological stimuli. Further experiments are required to verify the necessity of cardiac Tel2 in cell survival.

5. Conclusion

While previous reports suggest that mTOR in cardiomyocytes is necessary and sufficient for cardioprotection against multiple pathological settings, the role of mTOR stabilization in cardiomyocyte cell survival is not characterized well. The current study showed that overexpressing Tel2, a part of the mTOR-stabilizing TTT complex, protected cardiomyocytes from ischemic conditions. Understanding the mechanisms of TTT complex in the heart will provide a new therapy for patients with ischemic heart disease.

Acknowledgements

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