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Mammalian target of rapamycin is essential for cardiomyocyte survival and heart development in mice

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Abstract

Mammalian target of rapamycin (mTOR) is a critical regulator of protein synthesis, cell proliferation and energy metabolism. As constitutive knockout of *Mtor* leads to embryonic lethality, the *in vivo* function of mTOR in perinatal development and postnatal growth of heart is not well defined. In this study, we established a muscle-specific mTOR conditional knockout mouse model (mTOR-mKO) by crossing MCK-Cre and *Mtor*$^{flox/flox}$ mice. Although the mTOR-mKO mice survived embryonic and perinatal development, they exhibited severe postnatal growth retardation, cardiac muscle pathology and premature death. At the cellular level, the cardiac muscle of mTOR-mKO mice had fewer cardiomyocytes due to apoptosis and necrosis, leading to dilated cardiomyopathy. At the molecular level, the cardiac muscle of mTOR-mKO mice expressed lower levels of fatty acid oxidation and glycolysis related genes compared to the WT littermates. In addition, the mTOR-mKO cardiac muscle had reduced Myh6 but elevated Myh7 expression, indicating cardiac muscle degeneration. Furthermore, deletion of *Mtor* dramatically decreased the phosphorylation of S6 and AKT, two key targets downstream of mTORC1 and mTORC2 mediating the normal function of mTOR. These results demonstrate that mTOR is essential for cardiomyocyte survival and cardiac muscle function.

Keywords

mTOR; heart failure; cardiomyocyte

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Introduction

Dilated cardiomyopathy (DCM), characterized by an enlarged heart volume, thinner myocardium and weakened blood pumping is a worldwide disease that can lead to congestive heart failure and sudden death [1]. DCM can be caused by both genetic and non-genetic factors. Understanding the molecular mechanisms underlying development of DCM is the key to successful treatment of the disease.

Mammalian (or mechanistic) target of rapamycin (mTOR) is a conserved serine-threonine kinase that plays a critical role in cell growth and metabolism [2,3]. In mammals, mTOR functions as the catalytic subunit of both mTOR complex 1 (mTORC1) and mTORC2 [4]. The mTORC1, consisted of mTOR, mLST8 and raptor, is sensitive to rapamycin. The major substrates of mTORC1 are S6 kinase and 4E-BP through which mTORC1 regulates protein translation, cell proliferation, apoptosis and autophagy [5,6,7,8]. The mTORC2, containing mTOR, mLST8, rictor, mSIN1, and PRR5, is insensitive to rapamycin. mTORC2 regulates cell growth and survival, actin cytoskeleton reorganization, protein synthesis and maturation, and metabolism via AKT and PKC-α pathway [9,10].

Previous studies involving genetic mutation of *Mtor*, *raptor* and *rictor* in mice have provided insights into the role of mTOR in heart development and function. *Mtor* null embryos die shortly after implantation due to defects in cell proliferation of the inner cell mass [11]. Myh6-Cre induced deletion of *Mtor* leads to late gestation lethality due to cardiomyocyte apoptosis [12]. Myh6-Cre^{ER} mediated postnatal deletion of *raptor*, a key component of mTORC1, in adult mice leads to cardiomyocyte apoptosis and heart failure due to inhibition of 4E-BP1 and protein translation [13,14]. In contrast, cardiac muscle-specific knockout of *rictor*, a key component of mTORC2, has no effect on heart development, but the *rictor* conditional knockout mice develop cardiac hypertrophy under pressure overload [15]. All these conditional knockouts have used the Myh6 promoter to drive cardiac muscle specific-deletion of mTOR-related genes. However, the mosaic activation of Myh6-Cre in only a subset of cardiomyocytes during development confounded data interpretation. In addition, late gestational lethality of the Myh6-Cre/$Mtor^{flox/flox}$ mice precluded further analysis of the role of mTOR in neonatal and postnatal heart.

In the present study, we used MCK-Cre to drive deletion of *Mtor* in cardiac muscles. The resulting mTOR conditional knockout mice exhibited growth retardation, cardiac muscle remodeling and premature death. The myopathy phenotype is accompanied by cell apoptosis, revertant expression of neonatal Myh7 expression and reduced phosphorylation of mTOR downstream substrates S6 and AKT. Our results demonstrate that mTOR plays a critical role in cadiomyocyte survival and neonatal heart development.

Materials and methods

Animals

All procedures involving mice were reviewed and approved by Purdue University Animal Care and Use Committee. Mice were from Jackson Laboratory under Stock #011009

($Mtor^{flox/flox}$) and #006475 (MCK-Cre). PCR genotyping was performed following protocols described by the supplier.

Total RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted by using Trizol (Invitrogen) according to the manufacture's instruction. The quality and concentration of RNA was measured by spectrophotometer (Nanodrop 2000c, Thermo Fisher). M-MLV (Invitrogen) and random primer were used to convert RNA to cDNA. Then Real-time PCR was carried out by using SYBR Green PCR Master Mix (Roche) on a light cycler 480 (Roche) instrument. *18s* was used as control of mRNA expression analysis. The fold change for all the samples was calculated by $2^{-\Delta\Delta ct}$ methods.

Western blot analysis and antobodies

Western blot analysis were carried out to detect heart protein levels following standard procedures as described before [16]. Primary antibodies Phospho-S6 (Ser240/244), S6, Phospho-AKT (Ser473), and AKT were from Cell Signaling Technology. The other antibodies were from Santa Cruz Biotechnology Inc.

Hematoxylin-eosin (H&E) and immunofluorescence staining

Heart from WT and mTOR-KO mice were dissected and frozen in OCT compound (Sakura Finetek). Heart cryosection (10μm) were performed on a Leica CM1850 cryostat. For H&E staining, the sections were stained in haematoxylin for 30 minutes and eosin for 5 minutes. Then the sections were mounted and the images were captured with a Nikon D90 digital camera. For immunofluorescence staining, the sections were fixed with 4% Paraformaldehyde and blocked in blocking buffer. Then the section was incubated with primary antibodies diluted by blocking buffer at 4 ° overnight. After that, the sections were washed and incubated with second antibody and DAPI for 50min. Fluorescent images were taken using Leica DM 6000B fluorescent microscope.

Evans blue (EB) dye uptake analysis

EB (20 mg/ml in PBS) was administered to WT and mTOR-mKO mice intraperitoneally (0.04 ml per 10 g body mass). After 24 hours, heart samples were harvested and sectioned using a cryostat (Leica CM1850). EB was detected as red auto fluorescence under the fluorescent microscope.

Statistical analysis

All experimental data are presented as means ± SEM. Comparisons were made by two-tailed Student's *t*-tests or one-way ANOVA, as appropriate. Effects were considered significant at $P < 0.05$.

Results

Cardiac deletion of mTOR leads to postnatal growth retardation and premature death

We established the MCK-Cre/Mtor$^{flox/flox}$ mice, abbreviated as mTOR-mKO, by crossing Mtor$^{flox/flox}$ and MCK-Cre mice (Fig. 1A). Previous studies have established that MCK is expressed in heart and skeletal muscles, and MCK-Cre has been used to delete genes in cardiac muscles [17,18]. Thus, in the mTOR-mKO mice, heart and skeletal muscles should be deleted of exons 1–5 in the *Mtor* gene (Fig. 1A). A 533-bp DNA fragment corresponding to the deleted *Mtor* allele was specifically amplified by PCR genotyping (Fig 1B). Real-time PCR analyses further confirmed that the mRNA level of *Mtor* is reduced by ~60% in the heart of mTOR-mKO mice compared to control littermates (Fig. 1C).

Mice of all genotypes were born at the expected Mendelian ratio (Data not shown), indicating that mTOR-mKO did not result in embryonic lethality. During the first 2 postnatal weeks, the mTOR-mKO mice appeared normal and indistinguishable from the control littermates. However, after weaning (at 3-week old), the mTOR-mKO mice started to grow more slowly than did their control littermates (Fig. 1D). The mTOR-mKO mice were also weak, trembling and had decreased voluntary activity (Fig. 1E). On postnatal day 27, the mTOR-mKO mice were obviously smaller and weighted only two thirds of the weight of the control littermates (KO = 10.3±0.7 g; control = 15.0±0.6 g) (Fig. 1D–E). Notably, the mTOR-mKO mice began to die on postnatal day 22, with a median life-span of 26.4±0.6 day, and all of the mTOR-mKO mice died within 33 day (Fig. 1F). Taken together, these results indicate that muscle-specific knockout of *Mtor* results in postnatal growth retardation and premature death.

mTOR deficiency results in cardiac muscle pathology resembling dilated cardiomyopathy

To determine the cause of sudden death in the mTOR-mKO mice, we examined their heart morphology and pathology. At 2 weeks of age, the hearts from mTOR-mKO mice were visually indistinguishable from their littermate control (Fig. 2A). However, at 4 weeks, the mTOR-mKO mice had an obvious enlarged heart with darker appearance (Fig. 2A). Accumulation of blood clots and/or thrombi were always visible in the hearts of mTOR-mKO mice (Fig. 2A), suggestive of a possible cardiac contractility defection. Of note, H&E staining revealed that the mTOR-mKO mice had enlarged ventricular chambers and thinned myocardium walls (Fig. 2B). In addition, immunostaining of cell surface with a laminin antibody showed that cardiomyocytes of the mTOR-mKO mice were disorganized, malformed and variable in sizes, compared to tightly-aligned and uniformly sized cardiomyocytes in the control littermates (Supplementary Fig. 1A).

To determine whether the mTOR-mKO mice undergoes heart remodeling, we first checked the expression levels of fetal genes including atrial natriuretic peptide (Anp) and brain natriuretic peptide (Bnp). We found that *Anp* and *Bnp* expression was 10- and 25-times higher in the heart of mTOR-mKO mice than in control littermates (Fig. 2C), indicating that pathological heart remodeling occurs in the mTOR-mKO mice. To further confirm this notion, we examined the expression of α-myosin heavy chain (Myh6) and β-myosin heavy chain (Myh7). Heart remodeling is usually accompanied by a shift from adult-specific Myh6 to fetal-specific Myh7 [19]. Indeed, the mRNA levels of *Myh6* was decreased by 4-fold, while *Myh7* was increased by over 1,000 times in the mTOR-mKO compared to WT heart muscles (Fig. 2D). The changes at mRNA levels were confirmed by immunostaining using an antibody against Myh7, which showed a robust shift from $Myh7^-$ to $Myh7^+$ cardiomyocytes in the mTOR-mKO mice (Fig. 2E). Specifically, over 90% of the cardiomyocytes in the mTOR-mKO mice were $Myh7^+$ at 4-week old, compared to only 5% $Myh7^+$ cells in control mice at the same age (Fig. 2E). Together, these data demonstrated that deletion of *Mtor* leads to heart remodeling and cardiomyocyte degeneration.

Deletion of mTOR induces cardiomyocytes death and cardiac fibrosis

One character of heart failure is the progressive loss of cardiomyocytes, the fundamental contractile unit of cardiac muscle [20]. To determine whether deletion of *Mtor* affects cardiomyocyte survival, we first examined the apoptosis using cleaved caspase 3 as a marker. Notably, cleaved caspase 3 positive cells were readily detectable in mTOR-mKO heart sections (Fig. 3A), indicating occurrence of cell apoptosis. Dystrophin is a membrane structural and signaling protein important for proper function of skeletal and cardiac muscles. In skeletal muscle, dystrophin expression was decreased in response to deletion of mTOR [21]. Consistently we found decreased expression and uneven distribution of dystrophin in mTOR-mKO heart muscle (Fig. 3B). In addition, we also evaluated whether the disruption of dystrophin expression affected the membrane integrity of cardiomyocytes. Mice were I. P. injected with Evans blue (EB) dye, which penetrates cells that have membrane leakage. Of note, numerous EBD positive cardiomyocytes were found in heart muscles of the mTOR-mKO mice, but not WT control heart muscles (Fig. 3C). These results indicate that deletion of *Mtor* increased cell membrane permeability, loss of cardiomyocyte integrity and necrosis.

Furthermore, we tested whether the death of cardiomyocytes results in fibrosis by Masson's trichrome staining. We found that heart muscles of mTOR-mKO mice had more prominent fibrotic staining than those of control mice (Supplementary Fig. 1B). It has been reported that the cardiac fibrosis in mice resulted from proliferation of non-cardiomyocytes [22]. with the proliferation maker Ki67 antibody showed that there is no difference in the abundance of $Ki67^+$ cells between mTOR-mKO and WT mice at 2-week old (data not shown). However, there were nearly 2 times more $Ki67^+$ cells in the heart muscle of mTOR-KO mice (40.5±3.5 cell/field) than those in control (24.0±1.3 cell/field) at 4-week old (Supplementary Fig. 1C). These results provide compelling evidence that knockout of *Mtor* leads to fibrotic tissue infiltration.

mTOR deletion decreases energy metabolism and phosphorylation of S6 and AKT

Cardiac muscle has a constant high demand for energy to sustain its contractile activity. Thus alterations of energy metabolism contribute to heart failure [21]. Fatty acid oxidation provides about 70% ATP in the heart [23]. To understand the mechanism through which mTOR deletion causes heart myopathy, we evaluated the expression of several key genes related to fatty acid oxidation. We found that the mRNA levels of *Ppar*α and *Pgc1*α were reduced by ~50% in mTOR-mKO mice heart tissue compared to that of the control mice (Fig. 4A). The expression of *carnitine palmitoyltransferase type 1* (CPT1), the rate-limiting enzyme that transfers fatty acid into mitochondria for further oxidation, was also decreased significantly (Fig. 4A). Glycolysis is another key biochemical process that provides energy source for heart function. We found that the expression of *glucose transporter 4* (Glut4) was reduced by ~70% in the heart of mTOR-mKO compared with that of control mice (Fig. 4B). Expression of the rate-limiting enzymes in glycolysis, *hexokinase 2* (HK2) and *pyruvate kinase muscle isozyme* (PKM2), was also reduced significantly after deletion of mTOR (Fig. 4B). Furthermore, Western blot results showed that deletion of mTOR decreased the expression of mTORC1 target S6 and abolished its phosphorylation at Ser240/244 (Fig. 4C). In addition, the phosphorylation level of AKT (S473), a direct target of mTORC2, was also dramatically decreased (Fig. 4C). Taken together, these data suggest that conditional knockout of *Mtor* decreased energy metabolism and inhibited the phosphorylation of mTOR downstream targets.

Discussion

Previous studies reported that cardiac-specific deletion of *Mtor* using Myh6-Cre at early embryonic stage results in embryonic death [11,12], whereas tamoxifen-induced deletion of *Mtor* in adult heart using Myh6-Cre^{ER} resulted in cardiomyocyte apoptosis [13]. However, the role of mTOR in fetal heart development and perinatal heart growth are unclear. Here we used MCK-Cre which is expressed from E12.5 in mice, to drive deletion of *Mtor* in skeletal and cardiac muscle [24]. The resulting mTOR-mKO mice were born at the expected Mendelian distribution, indicating that the mice undergo normal embryonic development. Although MCK-Cre also drives deletion of *Mtor* in the skeletal muscle, we did not observe any obvious abnormalities in skeletal muscles of the mTOR-mKO mice prior to their death (Data not shown). Consistently, skeletal muscle restricted knockout of *Mtor* using HSA-Cre did not lead to death until 24 weeks after birth, and the knockout mice can survive up to 35 weeks [21]. By contrast, our skeletal and cardiac muscle specific knockout mice began to die after 3 weeks and no animals survived beyond 5 weeks. Therefore, the main cause of the premature lethality of the mTOR-mKO mice is most likely due to cardiac myopathy.

We found that loss of mTOR in heart results in a progressive cardiac myopathy similar to that observed in the cardiac specific *raptor* knockout mice [14]. In addition, there is a dramatic switch of cardiac myosin from Myh6 to Myh7. Relative expression of cardiac myosin heavy chain (Myh) isoforms is developmentally regulated. Cardiac muscles predominantly (>90%) express Myh7 at birth and then switch to Myh6 (>85%) after 3 weeks. Myh isoform expression can also be altered by a variety of pathophysiological conditions [25]. For example, ablation of *raptor* robustly increased Myh7 expression prior to heart failure [14]. Consistently, we found that more than 90% of cardiomyocytes are Myh7^{+} in the heart muscle of the mTOR-mKO mice, compared to <5% Myh7^{+} cells in WT animals at 4 weeks. Such alteration in myosin isoform composition may affect the physiological and energetic properties of the heart. As Myh7 has a lower ATPase activity and slower shortening speed than Myh6 [25], a shift to Myh7 expression can reduce energy consumption at the expense of insufficient contraction force and blood pumping, leading to heart failure.

Our results also provide support for a role of mTOR in cardiomyocyte survival. Progressive loss of cardiomyocytes due to apoptosis contributes to heart failure [8,26]. We also found cardiomyocyte apoptosis in mTOR-mKO mice using cleaved caspase3 as a marker. In addition, we found that cardiomyocytes of mTOR-mKO mice also undergo necrosis, supported by reduced expression of dystrophin and increased EB dye uptake. The necrosis may enhance the loss of cadiomyocytes and accelerate the heart failure progress [27].

mTOR has been shown as a nutrient and energy sensor, consequently the mTOR-mKO heart muscles expressed reduced levels of fatty acid β-oxidation related genes including *Pgc1*α, *Ppar*α and *Cpt1*, indicating that mTOR regulates fatty acid β-oxidation in cardiomyocytes. Considering the constant energy demand during heart contraction, we checked whether other energy production pathway was affected by mTOR. Glycolysis has been shown to increase to meet the demand of energy in previous heart failure models [28]. To our surprise, *Glut4, Pk2* and *Hkm2* expression were all down regulated. This suggests that the glycolysis pathway is inhibited in the mTOR-mKO mice. These abnormalities in metabolism may have resulted in decreased production and contributed to dysfunctions in cardiomyocyte contraction and restricted blood pumping.

Consistent with previous reports [12], our results showed that phosphorylation levels of S6(S240/244) and AKT(S473) were reduced in the mTOR-mKO mice, indicating both mTORC1 and mTORC2 activity were inhibited. In contrast, Zhang et.al reported that only mTORC1 activity was inhibited, whereas mTORC2 activity was elevated in adult mTOR knockout mice [13]. Given that mTORC2 is the well-established kinase for AKT, they interpreted that there might be another unknown kinase that phosphorylates AKT [21,29]. Since all of the mTOR-related KO mice with elevated pAKT(S473) were reported at adult stage, whereas reduced pAKT(S473) were detected in premature (in our results) or embryonic period [12], this unknown kinase may specifically function in the adult.

Blocking mTORC1 pathway inhibits protein translation and triggers apoptosis. As activation of mTORC2 signaling promotes cell survival and suppresses apoptosis [30,31], blocking the mTOR2 pathway should accelerate the apoptosis process and result in more rapid loss of cardiomyocytes. Considering both mTORC1 and mTORC2 pathways were inhibited in our mTOR-mKO mice, this may partially explain the more severe cardiomyopathy than the reported adult *Mtor* KO mice. In conclusion, our data demonstrate the critical role of mTOR in cardiomyocyte survival, energy metabolism and heart function maintaining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- mTOR is a critical regulator of many biological processes yet its function in heart is not well understood.
- MCK-Cre/Mtor$^{flox/flox}$ mice were established to delete Mtor in cardiomyocytes.
- The mTOR-mKO mice developed normally but die prematurely within 5 weeks due to heart disease.
- The mTOR-mKO mice had dilated myocardium and increased cell death.
- mTOR-mKO hearts had reduced expression of metabolic genes and activation of mTOR target proteins.

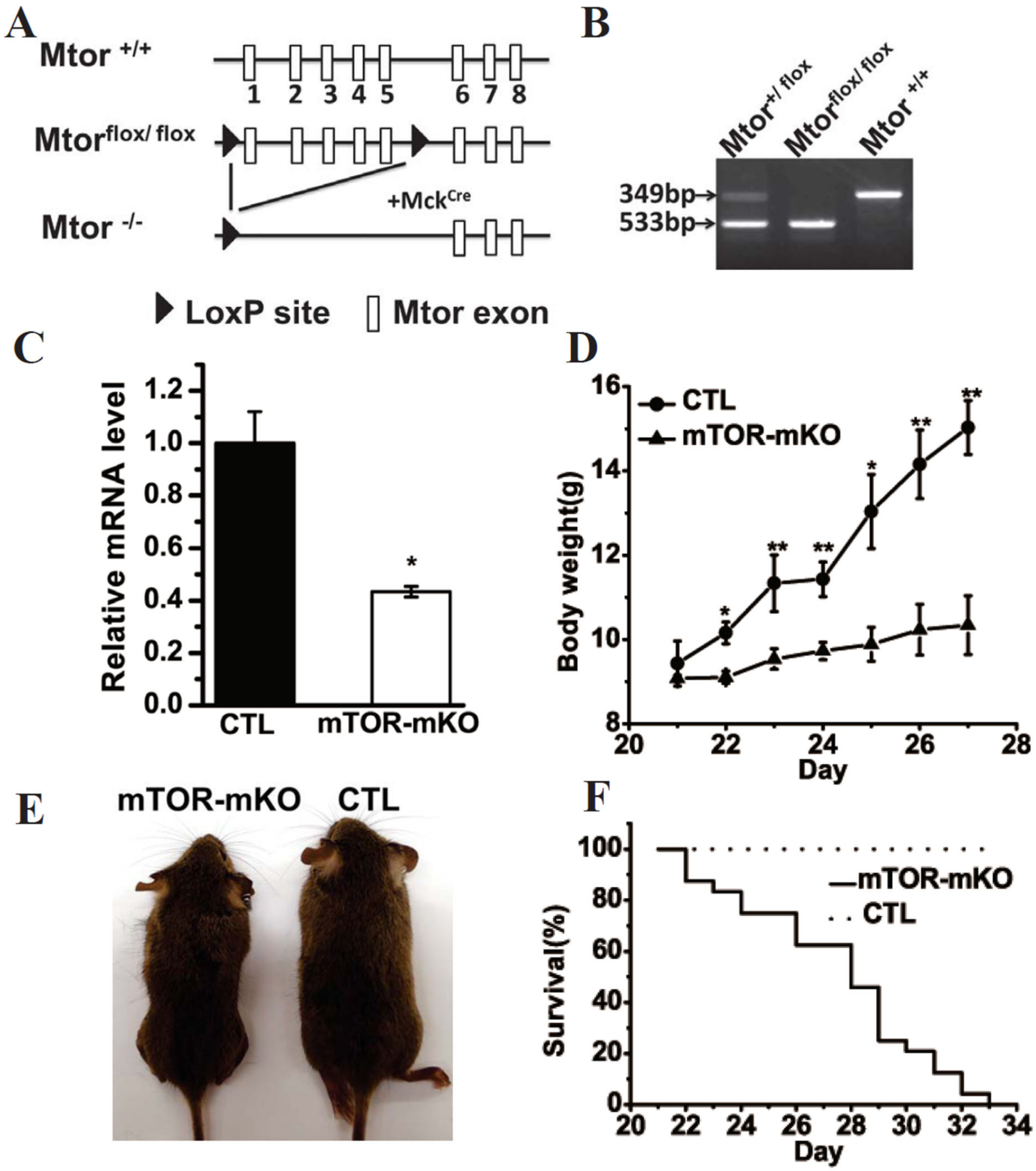

Fig. 1.

Cardiac specific knockout of *Mtor* leads to growth retardation and premature death. (A) Strategy of MCK-Cre mediated knockout of *Mtor* in cardiomyocytes. (B) PCR genotyping of Cre-meadiated deletion *Mtor* exons. (C) RNA was extracted from heart tissue of control (CTL) and mTOR-mKO mice, relative mRNA expression of *Mtor* was analyzed by real-time PCR. n=4 for each group. (D) Growth curves of CTL and mTOR-mKO mice. n=7 for each group. (E) Representative images of mTOR-mKO and CTL mice. (F) Kaplan-Meier survival curves of in CTL and mTOR-mKO mice. n=24 for each group.

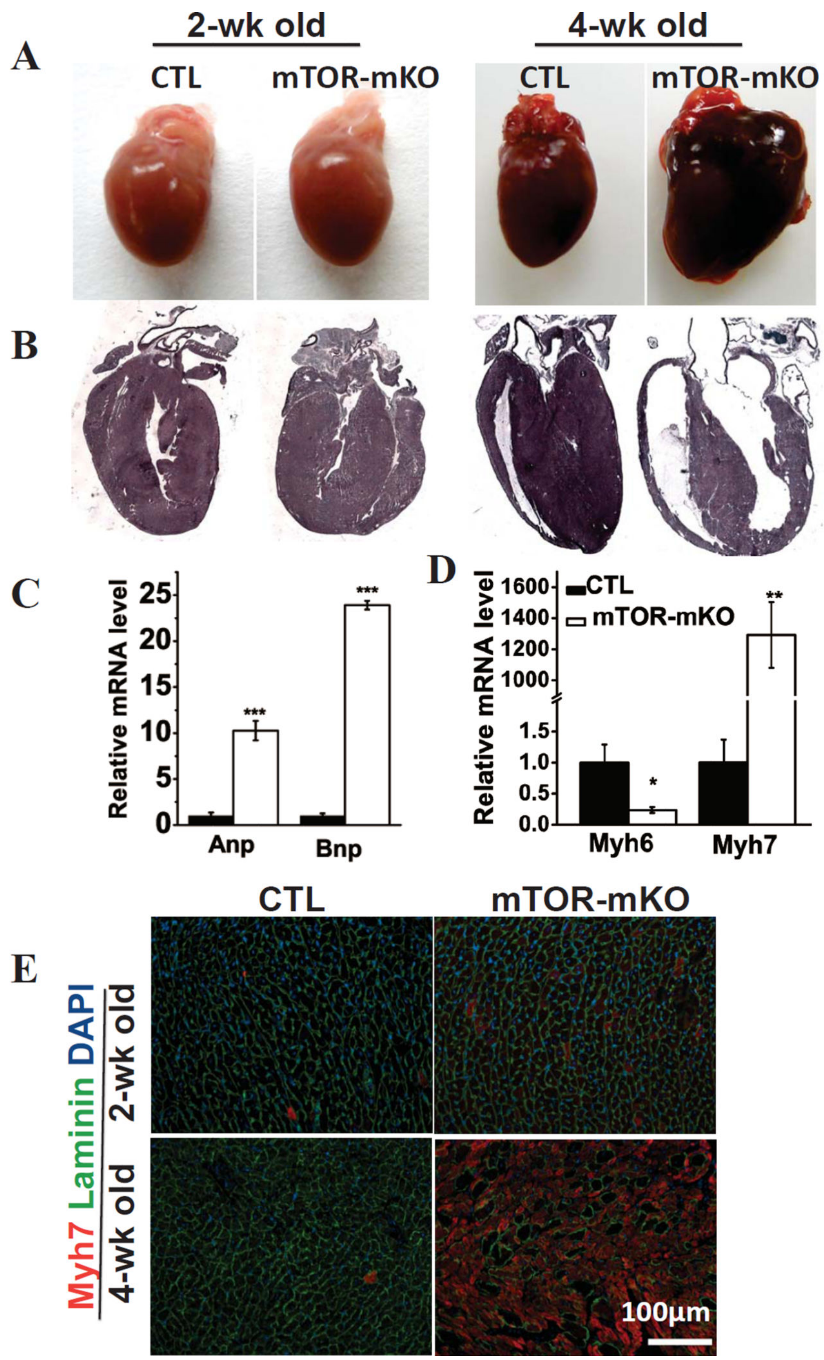

Fig. 2.

mTOR-mKO mice develop heart dilation. (A) Gross morphology of whole hearts from CTL and mTOR-mKO mice at the indicated ages. (B) Histological assessment of cardiac pathology by H&E. (C and D) Relative mRNA levles of Anp, Bnp, and Myh6, Myh7 in CTL and mTOR-mKO mice hearts. (E) Representative images of heart sections labeled with Myh7 (red), DAPI (blue) and laminin (green) in CTL and mTOR-mKO mice. Values are expressed as mean±S.E. *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$. n=6 in each group.

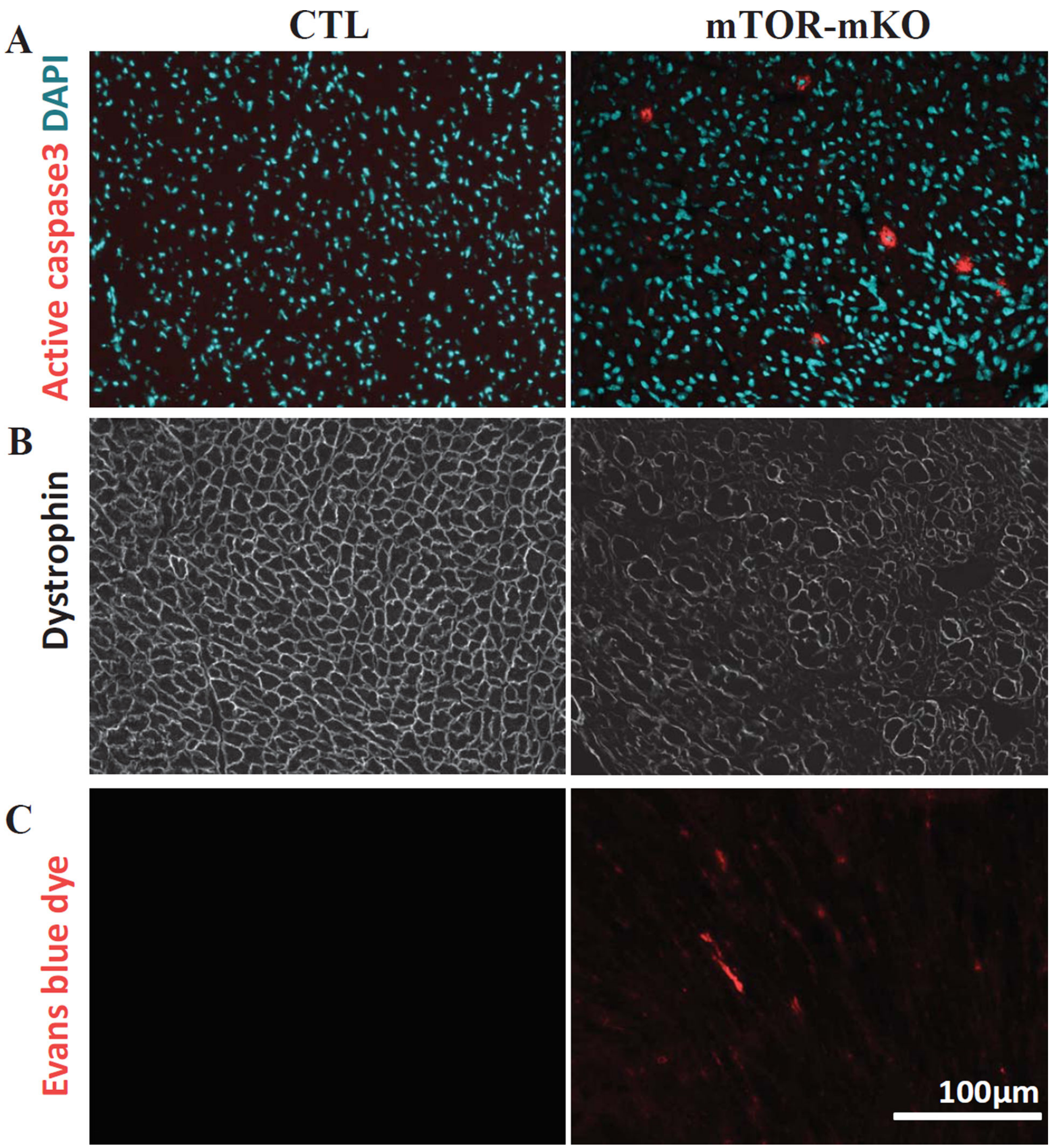

Fig. 3.

Deletion of mTOR induces crdiomyocyte death. (A) Representative images of heart sections labeled with cleaved caspase 3(red) and DAPI (blue) in 4-week old mTOR-mKO and CTL mice. (B) Representative images of heart section labeled with a dystrophin antibody. (C) Evans blue dye was injected into 4-week old CTL and mTOR-mKO mice, and positive signal (red) was examined by fluorescence microscopy.

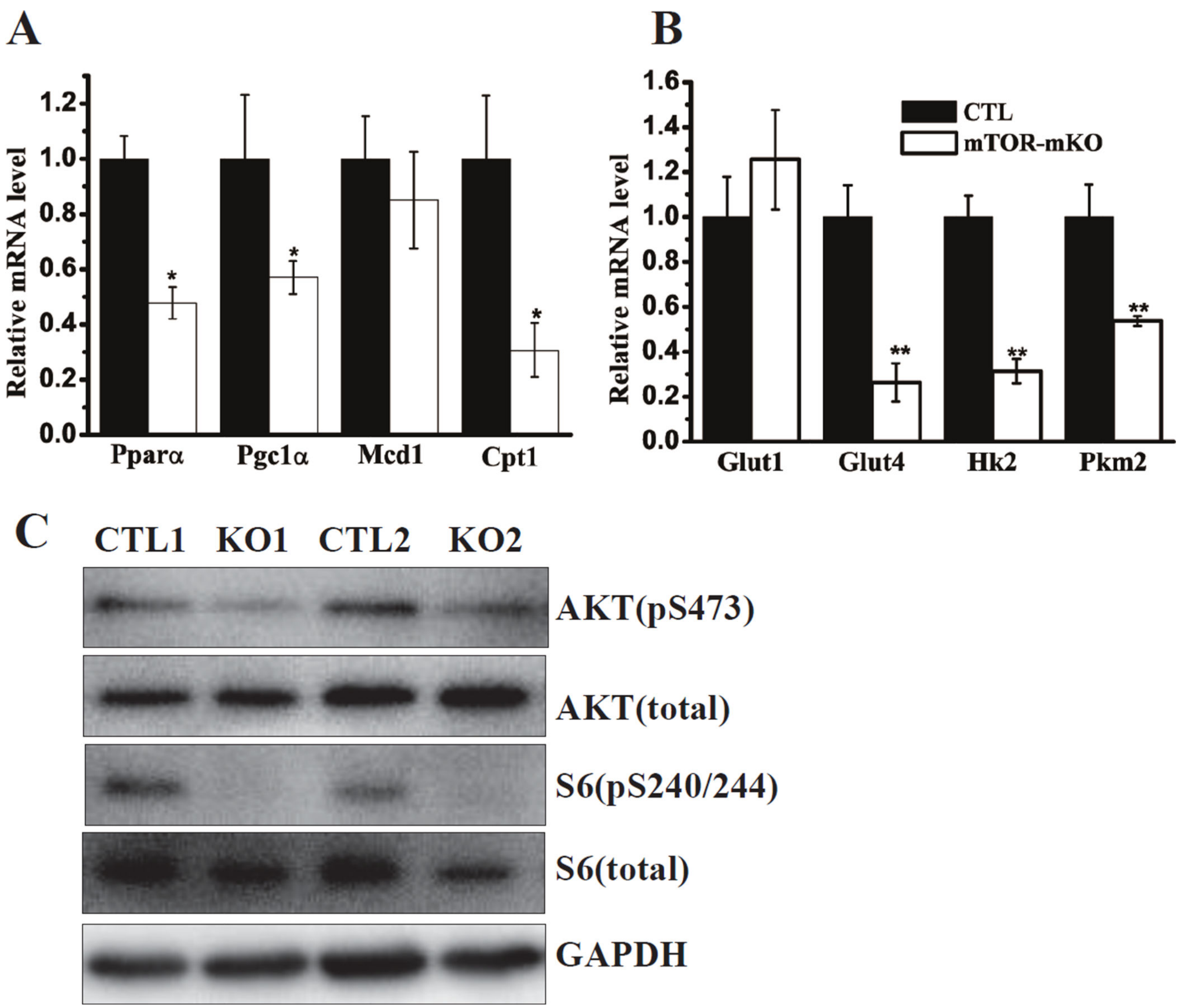

Fig. 4.

Alterations in the expression of genes related to energy metabolism and protein translation in mTOR-mKO mice. (A and B) Relative mRNA levels of fatty acid oxidation related genes (A) and glycolysis related genes (B) determined by realtime PCR. Values are expressed as mean±S.E. *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$. n=6 in each group. (C) Protein was extracted from hearts of CTL and mTOR-mKO mice and Phospho-S6 (S240/244) and phospho-AKT (S473) levels were determined by Western blot analysis.