Phosphoinositide-Dependent Kinase 1 and mTORC2 Synergistically Maintain Postnatal Heart Growth and Heart Function in Mice

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The protein kinase Akt plays a critical role in heart function and is activated by phosphorylation of threonine 308 (T308) and serine 473 (S473). While phosphoinositide-dependent kinase 1 (PDK1) is responsible for Akt T308 phosphorylation, the identities of the kinases for Akt S473 phosphorylation in the heart remain controversial. Here, we disrupted mTOR complex 2 (mTORC2) through deletion of *Rictor* in the heart and found normal heart growth and function. *Rictor* deletion caused significant reduction of Akt S473 phosphorylation but enhanced Akt T308 phosphorylation, suggesting that a high level of Akt T308 phosphorylation maintains Akt activity and heart function. Deletion of *Pdk1* in the heart caused significantly enhanced Akt S473 phosphorylation that was suppressed by removal of *Rictor*, leading to worsened dilated cardiomyopathy (DCM) and accelerated heart failure in *Pdk1*-deficient mice. In addition, we found that increasing Akt S473 phosphorylation through deletion of *Pten* or chemical inhibition of PTEN reversed DCM and heart failure in *Pdk1*-deficient mice. Investigation of heart samples from human DCM patients revealed changes similar to those in the mouse models. These results demonstrated that PDK1 and mTORC2 synergistically promote postnatal heart growth and maintain heart function in postnatal mice.

Dilated cardiomyopathy (DCM), usually diagnosed as idiopathic dilated cardiomyopathy (IDC), is characterized as ventricular dilation and impaired heart contractility (systolic and diastolic functions) (1). The causes of DCM are not well defined, and from human studies, sarcomere and cytoskeletal gene mutations, abnormalities of the dystrophin-sarcoglycan complex, myocarditis, toxics, and muscle dystrophy are regarded as some of the inducing factors of DCM (1–3). DCM is often the long-term outcome of cardiac hypertrophy due to increased workload, such as hypertension and valve disease (4).

In mice and humans, heart weight increases by approximately 20-fold from birth to adulthood (the period of postnatal heart growth). Therefore, cardiomyocyte growth contributes substantially to heart weight increase during postnatal heart development (5). One of the features of DCM is a thinned ventricular myocardium, which may result from defective cardiomyocyte growth in the postnatal period. However, the relationship between postnatal heart growth and DCM has not been well investigated.

The protein kinase Akt is phosphorylated at threonine 308 (T308) and serine 473 (S473) for activation (6–10). Akt T308 is phosphorylated by the only upstream kinase of phosphoinositide-dependent kinase 1 (PDK1), and mTOR complex 2 (mTORC2) is the major regulator of Akt S473 phosphorylation (7, 11–14). mTORC2 consists of mTOR, Rictor, Sin1, and mLST8, and deletion of each of them abolishes Akt S473 phosphorylation in murine embryonic fibroblasts (MEFs) (15–17). However, a recent study indicated that mTOR was dispensable for Akt S473 phosphorylation in the heart (18). Zhang and colleagues deleted *mTor* in cardiomyocytes and found markedly enhanced Akt S473 phosphorylation levels in the heart tissue (18). Later, it was reported that the atypical I κ B kinase ϵ and TANK-binding kinase 1 (IKK ϵ /TBK1) regulated Akt S473 phosphorylation (19). Therefore, there is strong debate on the subject of Akt S473 phosphorylation reg-

ulation. Further careful investigation of how Akt S473 phosphorylation is regulated in the heart is needed.

Previously, we found that long-term treatment of mice with rapamycin abolished Akt S473 in the heart while enhancing Akt T308 phosphorylation, which is cardiac protective (20). This study suggests that mTOR is responsible for Akt S473 phosphorylation. To test whether mTORC2 phosphorylates Akt S473 in the heart, we disrupted mTORC2 through deletion of *Rictor* in the heart and found normal heart growth and function. *Rictor* deletion caused significant reduction of Akt S473 phosphorylation but enhanced Akt T308 phosphorylation, suggesting that a high level of Akt T308 phosphorylation maintains Akt activity and heart function. Deletion of *Pdk1* in the heart caused significantly enhanced Akt S473 phosphorylation that was suppressed by removal of *Rictor*. Deletion of *Rictor* caused deteriorated myocardial growth and accelerated heart failure in *Pdk1*-deficient mice. These results demonstrated a protective role of enhanced Akt S473 phosphorylation in *Pdk1* deletion mice. Furthermore, we augmented Akt S473 phosphorylation in *Pdk1* deletion mice through removal of *Pten* and found normal heart function and survival of *Pdk1*-*Pten* double-deletion mice. Our study may help to develop a new therapeutic approach to treat heart growth defects and defective myocardial growth in humans.

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MATERIALS AND METHODS

Mice. The mice in this study were on a C57BL/6 genetic background and were housed in groups with 12-h dark/light cycles and with free access to food in accordance with the regulations on mouse welfare and ethics of Nanjing University. All procedures were conducted with the approval of the IACUC of the Model Animal Research Center of Nanjing University. *Akt1* floxed mice were generated through homologous recombination as previously described (21, 22). Briefly, the *Akt1* allele was modified on a 129 bacterial artificial chromosome (BAC) DNA with exons 3 to 11 floxed and with two LoxP sites. Correctly targeted embryonic stem (ES) cell clones were characterized using PCR and Southern blotting. The Neo cassette was removed after crossing with mice carrying flippase (data not shown). Genotyping of the mice was performed with the following primers: *Akt1*-5F, 5'-GGGATCAGCAGTTGAAGGACAGA3'; *Akt1*-5R, 5'-GC CAGGAATACAGCATGAGCCAC3'. The PCR product band for the wild type is 196 bp and for the deletion is 302 bp. The *Akt1* floxed mice were maintained on a C57BL/6 genetic background. *Pdk1* (encoding phosphoinositide-dependent kinase 1) floxed mice were as described previously and were maintained on a C57BL/6 genetic background (23, 24). *Rictor* floxed mice were as previously reported and were on a C57BL/6 genetic background (25). Deletion of *Pdk1*, *Pten*, and *Rictor* in cardiomyocytes in mice was accomplished through crossing the conditional floxed mice with α MHC-Cre mice.

Echocardiography. Echocardiography was performed using a Vevo 770 UBM system (VisualSonics) that possesses a single-element mechanical transducer with a center frequency of 30 MHz and a frame rate of 30 Hz. The spatial resolution of B-mode imaging was ~ 115 μ m (lateral) by ~ 55 μ m (axial). The body temperature of mice was monitored using a rectal thermometer and was maintained between 36 and 38°C. The heart rate was maintained between 350 and 450 beats/min. After measurement, the cardiac output values, such as the ejection fraction (EF), fractional shortening (FS), and left ventricular internal diameter at end diastole (LVIDD), were calculated according to the guidelines accompanying the Vevo 770 UBM system.

Administration of rapamycin and bpV. Rapamycin treatment of mice was as previously described (20). Potassium bisperoxo(1,10-phenanthroline)oxovanadate [bpV(phen)] was purchased from Santa Cruz (sc-221378A) and was administered to mice intraperitoneally at 2 weeks after birth for 7 days at a dose of 6.6 mg/kg body weight.

Western blotting. Heart tissues were dissected and snap-frozen in liquid nitrogen until use. Tissue lysates were prepared in lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 20 mM 2-glycerophosphate, 1% Nonidet P40, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM benzamidine, 1 mM dithiothreitol [DTT], 50 mM sodium fluoride, and 4 μ M leupeptin). Proteins were resolved by SDS-PAGE (10% gels) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% milk in TBST (Tris-buffered saline containing Tween; 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.5 mM Tween 20) and then incubated overnight with primary antibodies. The following antibodies were purchased from Cell Signaling Technology: S6 (catalog no. 2317), phospho-S6 (Ser235/Ser236; catalog no. 2211), total Akt (catalog no. 9272), phospho-Akt (Ser473; catalog no. 9271), PRAS40 (proline-rich Akt substrate of 40 kDa) (catalog no. 2610), phospho-PRAS40 (Thr246; catalog no. 2640), Rictor (catalog no. 240), and α / β -tubulin (catalog no. 2148). Antibodies against mouse Akt1, Akt2, and Akt3 were described previously (26). The following rabbit monoclonal antibodies were purchased from Epitomics: phospho-Akt (Thr308; catalog no. 2214-1) and PDK1 (catalog no. 1624-1). The panactin antibody (catalog no. MS-1295-P0) and horseradish peroxidase (HRP)-linked secondary antibodies (catalog no. 31460 and 31430) were purchased from Thermo Scientific.

Histology. The protocols for hematoxylin and eosin (H&E) staining, Masson's trichrome staining, and immunofluorescence (IF) were as described previously (23, 27). Briefly, heart samples were first washed with

ice-cold phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde (PFA) at 4°C. The samples were processed successively by (i) a 30-min wash in PBS at 4°C; (ii) 15 min each in 30%, 50%, 75%, and 85% ethanol and then twice for 10 min each time in 95% and 100% ethanol at room temperature (25°C); (iii) 3 times for 10 min each time in xylene at room temperature; (iv) 20 min in paraffin-xylene (1:1) at 65°C; and (v) 3 times for 30 min each time in fresh paraffin at 65°C. The processed samples were then embedded in paraffin and sectioned (6- μ m-thick sections), and the sections were stained.

TUNEL assay. The TUNEL (TdT [terminal deoxynucleotidyltransferase]-mediated dUTP nick end labeling) assay was performed as described previously (23, 27). Briefly, sections were treated with proteinase K (20 μ g/ml) and incubated with TdT and biotinylated dUTP.

Statistical analysis. Results are shown as means $\pm$ standard errors of the mean (SEM). For comparisons between two groups, statistical significance was determined using an unpaired two-tailed Student's *t* test. A *P* value of <0.05 was considered statistically significant, and *P* values of <0.01 and <0.001 were considered statistically very significant.

RESULTS

Deletion of *Rictor* abolished Akt S473 phosphorylation but enhanced Akt T308 phosphorylation in the heart. We first tested the deletion efficiency of α MHC-Cre mice using Rosa-mTmG reporter mice. Rosa-mTmG mice express tomato (T [red]) ubiquitously, and the *tomato* cassette can be removed by the Cre enzyme. Upon expression of Cre, the *tomato* cassette is deleted and green fluorescent protein (GFP) (G [green]) starts to be expressed. Using this strategy, we found that Cre was expressed in some ventricular cardiomyocytes by postnatal day 3 (P3), in a large number of ventricular cardiomyocytes by postnatal day 5, and in nearly all cardiomyocytes by postnatal day 7 (Fig. 1A to C and A' to C').

mTORC2 consists of mTOR, Rictor, mLST8, and Sin1 (11, 15–17). Previously, it has been reported that removal of *Rictor*, *mLST8*, or *Sin1*, encoding the key components of mTORC2, disrupted Akt S473 phosphorylation in cells and tissues, such as MEFs, B cells, skeletal muscle, adipose tissue, and prostate (13, 15, 28–30). However, a recent study suggested that mTOR was dispensable for Akt S473 phosphorylation in the heart because cardiomyocyte-specific deletion of *mTor* caused substantial enhancement of Akt S473 phosphorylation (18).

To test whether mTORC2 regulates Akt S473 phosphorylation in the heart, we deleted *Rictor* in the heart. We found that the phosphorylation levels of Akt S473 were drastically reduced in *Rictor*-deficient heart tissue (Fig. 1D and E). Removal of *Rictor* in the heart had little effect on heart growth and function (Fig. 1F and G and Table 1). Akt T308 phosphorylation levels were markedly increased in the *Rictor* deletion heart (Fig. 1D and E). Previously, we found that long-term (1-week) treatment of mice with rapamycin impaired Akt S473 phosphorylation in the heart while it enhanced Akt T308 phosphorylation, which is cardiac protective (Fig. 1H). Taken together, these results demonstrated that mTORC2 might be responsible for Akt S473 phosphorylation in the heart and that enhanced Akt T308 phosphorylation maintains Akt activity and heart function.

Although Rictor is dispensable for postnatal heart function, we found that it is essential for embryonic heart development. Deletion of *Rictor* in embryonic heart tissue using *Mesp1*-Cre caused heart defects and embryonic lethality by approximately embryonic day 12 (E12) (Fig. 1I).

Deletion of *Pdk1* resulted in significant enhancement of Akt S473 phosphorylation. We deleted *Pdk1* in the heart and

FIG 1 Deletion of *Rictor* in the heart and analysis of Akt signaling and heart function. (A to C) Deletion efficiency of α MHC-Cre using Rosa-mTmG reporter mice. (A' to C') Histological analysis of heart tissue in panels A to C. (D) Western blotting. Mice were fasted overnight and injected with insulin for 15 min as reported previously (46). (E) Quantitation of the data in panel D. The error bars indicate SEM. (F) Comparison of control and *Rictor* deletion hearts at 7 months. (G) Heart weight/body weight ratios. (H) Western blotting after rapamycin treatment for 1 week. The mice were fasted overnight and injected with insulin for 15 min. (I) Histological analysis of embryonic-heart-specific *Rictor* deletion mice at E10.5.

found PDK1 levels were dramatically decreased in the mice (Fig. 2A). Accordingly, the phosphorylation levels of Akt T308 were markedly reduced following *Pdk1* deletion (Fig. 2B and C).

Surprisingly, we found that Akt S473 phosphorylation levels were increased significantly upon *Pdk1* deletion (Fig. 2B and C). One month after *Pdk1* deletion, the enhancement of Akt S473 phosphorylation was more apparent and the protein levels of Akt were also increased slightly (Fig. 2C). These observations prompted us to investigate the physiological function of enhanced Akt S473 phosphorylation in the heart.

Deletion of *Rictor* increased heart growth defects and accelerated DCM and heart failure in *Pdk1*-deficient mice. To understand the physiological function of enhanced Akt S473 phosphorylation in *Pdk1*-deficient mice, we deleted *Rictor* to abolish Akt S473 phosphorylation in the mice and generated *Pdk1*-*Rictor* double-knockout (DKO) mice. Western blotting revealed nearly undetectable Akt S473 phosphorylation in the *Pdk1*-*Rictor* DKO heart (Fig. 3A). The levels of phospho-PRAS40, representing Akt activity, were also drastically reduced compared to the control (*Pdk1*^{F/F} *Rictor*^{F/F}) (Fig. 3A). These results demonstrated that the increased Akt S473 phosphorylation was mediated by mTORC2

TABLE 1 Echocardiography (7 months)

Parameter ^a	Value	
	<i>Rictor</i> ^{F/F} (n = 6)	<i>Rictor</i> ^{F/F} α MHC-Cre (n = 6)
IVS;d (mm)	0.75 $\pm$ 0.08	0.78 $\pm$ 0.13
LVID;d (mm)	4.05 $\pm$ 0.29	4.04 $\pm$ 0.27
LVPW;d (mm)	0.61 $\pm$ 0.06	0.63 $\pm$ 0.07
IVS;s (mm)	1.20 $\pm$ 0.12	1.10 $\pm$ 0.12
LVID;s (mm)	2.59 $\pm$ 0.29	2.70 $\pm$ 0.20
LVPW;s (mm)	0.71 $\pm$ 0.07	0.67 $\pm$ 0.11
LV Vol;d (ml)	72.53 $\pm$ 12.16	72.36 $\pm$ 11.58
LV Vol;s (ml)	25.02 $\pm$ 6.47	27.27 $\pm$ 5.08
EF (%)	65.79 $\pm$ 5.74	62.27 $\pm$ 4.35
FS (%)	35.91 $\pm$ 4.32	33.22 $\pm$ 3.06

^a IVS, interventricular septum; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall; d, diastolic; s, systolic; LV, left ventricle; EF, ejection fraction; FS, fractional shortening.

and further confirmed the function of mTORC2 in phosphorylating Akt S473.

Deletion of *Pdk1* in postnatal cardiomyocytes impaired heart growth. At 2 weeks (P14), the heart weight/body weight ratio of *Pdk1*-deficient mice was significantly smaller than that of control mice, and the difference was further increased by 3 weeks (P21) (Fig. 3B and C). Two to 3 months after *Pdk1* deletion, a majority of the mice displayed DCM and died from heart failure (Fig. 3D and E).

We found that deletion of *Rictor* increased DCM and accelerated heart failure in *Pdk1*-deficient mice. The *Pdk1-Rictor* DKO mice were all lost within 1 month after birth (Fig. 3E). Echocardiographic measurements displayed substantial reduction of heart function in the *Pdk1-Rictor* DKO mice at approximately 20 days (Table 2). Histological analysis of *Pdk1-Rictor* DKO hearts showed thin myocardium and ventricular dilation, which are typical changes in DCM (Fig. 3F).

Cell biological study revealed reduced cardiomyocyte size in *Pdk1*-deficient mice at 25 days (Fig. 3G and H). However, the size of DKO cardiomyocytes was strikingly smaller than that of *Pdk1*-deficient mice (Fig. 3G and H).

We also examined cardiomyocyte apoptosis in the DKO mice and found very few apoptotic cells (Fig. 3G).

Collectively, these results demonstrated that PDK1 and mTORC2 synergistically promote postnatal heart growth. Enhanced Akt S473 phosphorylation in the *Pdk1*-deficient heart was cardiac protective. Meanwhile, these results indicated that disruption of PDK1-mTORC2 signaling gave rise to defective postnatal heart growth, which could be the cause of DCM and heart failure.

Loss of *Pten* reversed DCM and heart failure in *Pdk1*-deficient mice. So far, our work had revealed the critical function of Akt S473 phosphorylation in sustaining Akt activity and heart function. Next, we investigated whether further enhancement of Akt S473 phosphorylation could augment Akt activity and improve heart function in *Pdk1*-deficient mice. Previously, it was shown that deletion of *Pten* in cardiomyocytes caused increased Akt S473 phosphorylation (31–33). Therefore, we removed *Pten* in *Pdk1*-deficient mice to enhance Akt S473 phosphorylation. Western blotting detected significantly augmented Akt S473 phosphorylation in *Pten-Pdk1* DKO mice compared to *Pdk1*-deficient mice (Fig. 4A and B). Using MEFs,

we found that deletion of *Pten* increased Akt activity in *Pdk1*-deficient cells (Fig. 4C and D).

Half-deletion of *Pten* prolonged the survival of the *Pdk1*-deficient mice for up to 5 months (Fig. 4E, blue line). Nearly all *Pten-Pdk1* DKO mice could survive normally (Fig. 4E, red line). Histological and echocardiographic analyses displayed normal heart morphology and function (Fig. 4F and Table 3). Thus, loss of *Pten* reversed DCM and heart failure in *Pdk1*-deficient mice.

Cardiac protection of *Pdk1*-deficient mice conferred by *Pten* loss was mediated by Akt. To test whether cardiac protection of *Pdk1*-deficient mice conferred by *Pten* loss was due to enhanced Akt S473 phosphorylation, we deleted *Akt1* from *Pten-Pdk1* DKO mice. Half-deletion of *Akt1* resulted in mortality of these mice within 6 months, and *Pdk1-Pten-Akt1* triple-knockout (TKO) mice died at an age similar to that of *Pdk1*-deficient mice (Fig. 5A). Histological analysis showed defective heart growth of TKO mice at 4 weeks (Fig. 5B). Heart function was impaired in TKO mice compared to *Pten-Pdk1* DKO mice (Fig. 5C and D). Western blotting revealed substantial reduction of total Akt and Akt S473 phosphorylation levels in the TKO mice (Fig. 5E and F). The levels of phospho-PRAS40, representing Akt activity, were also greatly decreased (Fig. 5E and F). Taken together, these results indicated that cardiac protection of *Pdk1*-deficient mice conferred by *Pten* loss was attributable to enhanced Akt S473 phosphorylation.

Cardiac protection of *Pdk1*-deficient mice conferred by *Pten* loss was mediated by mTORC2. To test whether cardiac protec-

FIG 2 Deletion of *Pdk1* in the heart and analysis of Akt phosphorylation. (A) Western blot showing that *Pdk1* was effectively deleted in the heart. (B) Detection of Akt T308 phosphorylation levels in the heart by Western blotting. Mice were fasted overnight and injected with insulin for 15 min. Akt S473 phosphorylation levels were markedly increased in the *Pdk1*-deficient heart at 18 postnatal days compared to the control. (C) At 42 postnatal days, enhancement of Akt S473 phosphorylation in the *Pdk1*-deficient heart was more apparent, and Akt levels in the *Pdk1*-deficient heart were slightly higher than in the control.

FIG 3 Study of *Pdk1*-deficient and *Pdk1-Rictor* double-deficient mice. (A) Western blotting of heart tissues at ~20 days. Mice were fasted overnight and injected with insulin for 15 min. The hearts were then dissected and frozen in liquid nitrogen. (B) Histological study of hearts at different ages. (C) Heart weight/body weight ratios at different ages. The error bars indicate SEM. (D) Histological and echocardiographic analyses at ~2 months. *Pdk1*-deficient hearts showed dilation. (E) Survival curves. (F) Histological analysis of hearts at 25 postnatal days. The *Pdk1-Rictor* double-deficient heart showed DCM. (G) Immunofluorescence and TUNEL staining. Topro3 stained the nuclei. TUNEL-positive cells are in red, and WGA staining displayed the sizes of cardiomyocytes. (H) Quantitation of cardiomyocyte size. Three mice were used for each genotype for analysis. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

tion of *Pdk1*-deficient mice conferred by *Pten* loss was mediated by mTORC2, we removed *Rictor* from *Pten-Pdk1* compound-deletion mice. We found that removal of *Rictor* caused mortality of these mice within 2 months due to DCM and heart failure (Fig. 6A and B). Western blotting displayed markedly decreased Akt S473

phosphorylation (Fig. 6C and D). The phosphorylation levels of PRAS40 and S6, representing Akt activity, were all reduced significantly (Fig. 6C and D). Collectively, these results demonstrated that mTORC2 activation of Akt conferred cardiac protection on *Pten-Pdk1* DKO mice.

TABLE 2 Echocardiography (20 to 21 days)

Parameter ^a	Value	
	<i>Pdk1</i> ^{F/F} <i>Rictor</i> ^{F/F} α MHC-Cre (n = 5)	<i>Pdk1</i> ^{F/F} <i>Rictor</i> ^{F/F} (n = 5)
IVS;d (mm)	0.45 ± 0.13	0.53 ± 0.05
LVID;d (mm)	2.60 ± 0.35	2.49 ± 0.27
LVPW;d (mm)	0.49 ± 0.093	0.53 ± 0.1
IVS;s (mm)	0.64 ± 0.17	0.78 ± 0.16
LVID;s (mm)	1.85 ± 0.35 ^b	1.39 ± 0.24
LVPW;s (mm)	0.67 ± 0.09	0.85 ± 0.17
LV vol;d (μl)	25.33 ± 7.49	22.52 ± 5.38
LV vol;s (μl)	11.18 ± 4.68 ^b	5.32 ± 2.45
EF (%)	57.07 ± 10.51 ^c	76.05 ± 9.22
FS (%)	28.98 ± 6.69 ^c	43.82 ± 8.56

^a IVS, interventricular septum; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall; d, diastolic; s, systolic; LV, left ventricle; EF, ejection fraction; FS, fractional shortening.

^b $P < 0.01$.

^c $P < 0.05$.

Chemical inhibition of PTEN prolonged the survival of *Pdk1*-deficient mice. Next, we tested whether chemical inhibition of PTEN could improve heart function in *Pdk1*-deficient mice. bpV has been reported to inhibit PTEN in mice, and we therefore,

TABLE 3 Echocardiography (18 months)

Parameter ^a	Value	
	<i>Pdk1</i> ^{F/F} <i>Pten</i> ^{F/F} (n = 5)	<i>Pdk1</i> ^{F/F} <i>Pten</i> ^{F/F} α MHC-Cre (n = 5)
LV vol;d (μl)	48.29 ± 3.03	46.47 ± 8.08
LV vol;s (μl)	14.60 ± 3.29	13.72 ± 2.32
EF (%)	70.08 ± 5.27	69.46 ± 8.58
FS (%)	38.88 ± 4.47	38.88 ± 6.44
LV mass (mg)	79.10 ± 6.90	97.49 ± 26.75
LV mass corrected (mg)	63.28 ± 5.52	77.99 ± 21.40

^a LV, left ventricle; d, diastolic; s, systolic; EF, ejection fraction; FS, fractional shortening.

administered bpV to *Pdk1*-deficient mice at the age of 2 weeks in a treatment lasting 1 week (34). This treatment significantly enhanced Akt S473 phosphorylation and prolonged the survival of *Pdk1*-deficient mice (Fig. 7A to C). The subsequent study failed to find tumors in these mice (unpublished observations). These results suggest that a novel approach could be developed to enhance Akt S473 phosphorylation in order to effectively treat DCM in humans.

We studied the heart samples from human DCM patients. Out

FIG 4 Study of *Pdk1*- and *Pdk1*-*Pten* double-deficient mice. (A) Western blotting. Mice were fasted overnight and injected with insulin for 15 min. (B) Quantitation of panel A data. Error bars, SEM. (C and D) Examination of Akt activity in MEFs. Akt was pulled down and incubated with glutathione S-transferase (GST)—glycogen synthase kinase 3 (GSK3) protein. The samples were then analyzed by Western blotting. (E) Survival curve. ***, $P < 0.001$. (F) Histological analysis. Bars, 0.1 mm.

FIG 5 Study of *Pdk1*-deficient, *Pdk1*-*Pten* double-deficient, and *Pdk1*-*Pten*-*Akt1* triple-deficient mice. (A) Survival curve. (B) Histological analysis. Bars, 0.1 mm. (C and D) Echocardiographic analysis. (E) Western blotting. Mice were fasted overnight and injected with insulin for 15 min. (F) Quantitation of the data in panel E. The error bars indicate SEM. *, $P < 0.05$; ***, $P < 0.001$.

of 5 samples, we found changes in PDK1 and Akt S473 phosphorylation levels similar to those in the mouse models (Fig. 7D). Thus, it is possible that impairment of PDK1-Akt signaling is one of the causes of DCM in humans.

DISCUSSION

In this study, we first demonstrated that deletion of *Rictor* abolished Akt S473 phosphorylation. Deletion of *Pdk1* resulted in enhanced mTORC2 activation that in turn gave rise to increased Akt S473 phosphorylation. Upon removal of *Rictor*, Akt S473 phosphorylation was abolished, which caused deterioration of postnatal cardiomyocyte growth and accelerated DCM in *Pdk1*-deficient mice. In contrast, enhancement of Akt S473 phosphorylation through PTEN loss reversed DCM and heart failure in *Pdk1*-deficient mice.

Phosphorylation and activation of Akt by mTORC2. In 2005, Sarbassov et al. reported the identification of a Rictor-mTOR complex (mTORC2) as the direct regulator of Akt S473 phos-

phorylation in *Drosophila* and human cell lines (35). Subsequent studies revealed that mTORC2 consists of mTOR, Rictor, mLST8, and Sin1 (16, 17, 35, 36). Knockdown or deletion of these components of mTORC2 resulted in substantial reduction of Akt S473 phosphorylation (15, 16, 25, 28). Although it was shown that disruption of mTORC2 through removal of Sin1 or Rictor abolished Akt S473 phosphorylation in MEFs, adipose tissue, skeletal muscle, and immune cells, a recent report by Zhang et al. revealed that mTOR was dispensable for Akt S473 phosphorylation in the heart (18). In this study, we have provided clear evidence to show that mTORC2 is the upstream regulator for Akt S473 phosphorylation in cardiomyocytes. The explanation for the discrepancy between our study and that of Condorelli et al. is as follows: (i) mTORC2 was somehow hyperactivated in non-*mTor*-deficient cells, such as cardiac fibroblasts, which make up nearly half of the cells in the heart; (ii) mTORC2 is the major upstream regulator for Akt S473 phosphorylation, and in the absence of mTOR, other kinases, such as the atypical IKK ϵ /TBK1, ILK, or DNA-PK, may substitute for

FIG 6 Study of *Pdk1*^{F/F} *Pten*^{F/+} *Rictor*^{F/+} αMHC-Cre mice. (A) Histological analysis; the hearts show DCM. The arrows indicate blood clotting in the atria due to impaired heart contraction. (B) Survival curves. ***, $P < 0.005$. (C) Western blotting study. Mice were fasted overnight and injected with insulin for 15 min. (D) Quantitation of the data in panel C. *, $P < 0.05$.

mTOR and phosphorylate Akt at S473 (19, 37–39). Interestingly, another heart-specific *mTor* knockout mouse line was generated, and *mTor* was deleted in embryonic cardiomyocytes, resulting in late-embryonic lethality (40). In that work, it was shown that the

levels of Akt S473 phosphorylation were reduced in the heart, which is consistent with our results.

One of the important findings in this study is increased Akt S473 phosphorylation resulting from PDK1 loss in the myocardium. The underlying mechanism for this could be that loss of PDK1 caused reduced S6K activation that in turn relieved the negative feedback between S6K and mTORC2-Akt signaling (41). PDK1 phosphorylation of S6K at T229 is critical for S6K activation, and the results of this study indicated that S6K was low in *Pdk1* deletion mice (14). The S6K-Akt negative feedback was originally reported by Um and colleagues in S6K1 mutant-mouse models (41). This needs to be investigated in the future.

Phosphorylation of Akt T308 and S473 and Akt activation. In this study, as well as our previous work, we found that reducing or abolishing the phosphorylation of either Akt T308 or S473 resulted in enhanced phosphorylation of the other site. This suggests that in the absence of phosphorylation of one site, phosphorylation levels of the other site would be increased in order to maintain Akt activity, although the underlying mechanisms are not clearly defined.

Function of Akt in the postnatal heart. In this study, we have demonstrated that the function of Akt in postnatal cardiomyocytes is to maintain cell growth but not cell survival. In both *Pdk1*-deficient and *Pdk1*-*Rictor* double-deficient cardiomyocytes, the most significant phenomenon was impaired cell growth, and we failed to observe significantly increased cell apoptosis. We also deleted *Akt1* and *Akt2* in cardiomyocytes from adult mice, and we made similar observations (unpublished data). Only at a late stage close to heart failure did we find a large number of apoptotic cells in the hearts of these mice.

We deleted all three *Akt* genes (*Akt1*-*Akt2*-*Akt3* TKO) in the cardiomyocytes using αMHC-Cre mice and found that the phenotype is similar to that of *Pdk1*-*Rictor* DKO mice, suggesting the critical roles of Akt proteins downstream of PDK1-Rictor in the heart.

We performed phosphoproteomics analysis of the heart samples. PDK1 is a kinase with Akt proteins as critical downstream effectors. PTEN loss rescues heart failure and mortality of *Pdk1* KO mice, so we examined the differences in phosphorylation of Akt substrates between the *Pdk1* KO heart and the *Pdk1*-*Pten* DKO heart in order to find the proteins whose enhanced phosphorylation in *Pdk1*-*Pten* DKO mice may contribute to the rescue of *Pdk1* DKO mice. One of the identified proteins is PFK-2, an important regulator of glucose metabolism. In the *Pdk1* KO heart, the phosphorylation of PFK-2 was undetectable, but phospho-PFK-2 was identified in the *Pdk1*-*Pten* DKO heart by mass spectrometry. Mora et al. reported loss of PFK-2 phosphorylation and activity in the *Pdk1* KO heart (24). Therefore, we believe that PFK-2-regulated metabolism might play an important role in postnatal heart growth and that the recovery of its function after *Pten* loss might rescue the *Pdk1* KO phenotype in the heart. Other Akt substrates we identified include IRS1, myofibril protein, and histone modifiers. We will study these proteins in the future.

Postnatal cardiomyocyte growth defects and DCM. Currently, our understanding of the causes of DCM remains largely incomplete. Our work suggests that defective postnatal cardiomyocyte growth or disrupted cardiomyocyte growth homeostasis is one of the causes of DCM. In this study, we showed that PDK1-mTORC2-Akt signaling controlled cardiomyocyte growth and that impairment of this signaling pathway, such as insulin resis-

FIG 7 Treatment of *Pdk1*-deficient mice with bpV and study of human DCM hearts. (A) Survival curves. (B to D) Western blotting and quantitation. The error bars indicate SEM. *, $P < 0.05$.

tance in diabetes mellitus, might cause DCM in humans. In the future, it will be necessary to investigate this signaling pathway in human DCM.

Treatment of DCM in humans. In this study, we found that deletion of *Pten* could reverse DCM in *Pdk1* deletion mice in a dose-dependent manner. Pharmacological inhibition of PTEN showed similar protective effects. One major concern about PTEN inhibition is initiation of tumorigenesis in mice or humans. However, we did not find any tumors in mice after PTEN inhibitor treatment. The reason could be that the PTEN inhibitor blocked PTEN phosphatase activity and the PTEN protein was intact. In human PTEN-related tumors, *Pten* alleles were frequently deleted or mutated (42).

One of the intriguing findings in this work is markedly increased Akt S473 phosphorylation levels in the *Pdk1*-deficient heart, which is consistent with previous studies on human DCM (43, 44). In some DCM patients, Akt S473 phosphorylation levels were higher than those of controls (43, 44). Using the heart samples from human DCM patients, we observed similar changes. In two DCM samples, PDK1 levels were low but both total Akt and Akt S473 phosphorylation levels became high. These observations suggest that a mechanism similar to that in *Pdk1*-deficient mice may exist in some DCM patients (45). Therefore, a novel approach to enhancing mTORC2-Akt activity may be developed to treat human DCM, as seen in *Pdk1*-deficient mice.

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