

AKT-mTOR Signaling-Mediated Rescue of PRKAG2 R302Q Mutant-Induced Familial Hypertrophic Cardiomyopathy by Treatment with β -AR Blocker Metoprolol

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Abstract

PRKAG2 cardiac syndrome, as a common form of metabolic hypertrophic cardiomyopathy (HCM) caused by mutations in PRKAG2 gene, often shows myocardial hypertrophy and abnormal glycogen deposition in cardiomyocytes. However, it remains incurable due to lacking of a management guideline for treatment. Herein, a β 1-AR blocker Metoprolol was applied to 5 patients with PRKAG2 cardiac syndrome identified from a PRKAG2 R302Q mutant family, resulting in significantly postponed progression of cardiac hypertrophy. Overexpression of PRKAG2 R302Q in primary cardiomyocytes increased the activity of AMPK, induced cellular hypertrophy and glycogen storage, and promoted the phosphorylation levels of AKT-mTOR signaling. Application of either β 1-AR blocker metoprolol or protein kinase A (PKA) inhibitor H89 to the cardiomyocytes rescued the HCM-like phenotypes induced by PRKAG2 R302Q, including suppression of both AKT-mTOR phosphorylation and AMPK activity. In conclusion, the current study not only determined the mechanism regulating HCM induced by PRKAG2 R302Q mutant, but also demonstrated a therapeutic strategy using β 1-AR blocker to treat the patients with PRKAG2 cardiac syndrome.

Introduction

Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disorder with characteristics of increased ventricular wall thickness, cardiomyocyte hypertrophy and myocardial fibrosis [1]. PRKAG2 cardiac syndrome, as a common form of metabolic HCM caused by mutations in the PRKAG2 gene encoding the AMP-activated protein kinase (AMPK) γ 2 subunit, often shows abnormal glycogen deposition in cardiomyocytes, ventricular preexcitation and progressive cardiac conduction disturbances in addition to myocardial hypertrophy [2]. AMPK is a heterotrimeric protein composed of α , β , and γ subunits, acting as the key enzyme responsible for regulating cellular energy homeostasis. AMPK activation turns on the catabolic pathway to produce ATP and turns off the anabolic pathway that requires ATP consumption, maintaining the dynamic energy balance [3]. Genetic mutants in PRKAG2 have been demonstrated to cause inappropriate activation of AMPK, leading to arrhythmias and cardiac insufficiency. For example, elevated activity of AMPK and decreased sensitivity to AMP were reported in the cardiomyotic cells with PRKAG2 K475E mutant and in the myocardium of PRKAG2 N488I mutant mice, associated with activation of mTOR/p70S6K/4EBP1 and/or Akt-mTOR-FOXO3A pathways [4,5]. In consistence, PRKAG2 mutants-induced myocardial hypertrophy can be rescued by application of the mTOR inhibitor rapamycin.

The β -adrenergic receptor (β -AR) signaling pathway is one of the pathways mediating induction of cardiac hypertrophy, which has been suggested to be associated with signaling pathways such as cAMP-PKA, MAPK, and PI3K. A phosphoproteomic analysis in vivo revealed that the kinases AMPK, AKT, and mTOR are also involved in β -AR signaling regulation of HCM [6]. In addition, β -AR showed regulation of glucose uptake and glycogen synthesis [7]. β 1-AR agonists reduced insulin-induced glucose uptake and glycogen synthesis [8]. Glycogen storage in cardiomyocytes has been shown to be related with myocardial hypertrophy. Reduction of glycogen was associated with improvement of cardiac function,

suggesting the therapeutic values for HCM of preventing glucose uptake by cardiomyocytes. However, Maengjo et al found that inhibition of glycogen deposition in N488I mutation model reversed ventricular preexcitation but showed limited effect on rescuing cardiac hypertrophy, suggesting the correlation between myocardial hypertrophy and glycogen storage might be in a genetic disorder-dependent manner [4].

To date, effects of all kinds of clinic treatment to PRKAG2 cardiac syndrome patients remain very limited yet mainly due to lacking of a well-accepted management guideline for the disease. Metoprolol, as a selective β 1-AR blocker, interacts with β 1-AR to inhibit β 1-AR signaling, leading to improvement of myocardial remodeling, and rescue of cardiac hypertrophy caused by infarction or hypertensive [9,10]. Our previous study treated 5 patients in a PRKAG2 R302Q-induced HCM family with a long-term oral administration of metoprolol, resulting in significant prevention of myocardial hypertrophy progression [11]. However, the regulatory mechanism remains unclear.

In order to validate the function and mechanism of β -blocker in regulating myocardial hypertrophy, a fluorescent-labeled adenoviral virus carrying either wild type or R302Q mutant of PRKAG2 gene was infected into neonatal rat cardiomyocytes (NRCMs) and H9C2 cell line, followed by analyses of AMPK activity, cellular hypertrophy, glycogen storage and cell proliferation with or without treatment of metoprolol or protein kinase A (PKA) inhibitor H-89. The activity of AKT-mTOR signaling was assessed as well. As a result, increased AMPK activity was indicated in the PRKAG2 R302Q-overexpressing cells, accompanied with increase of glycogen storage, cell size and expression levels of myocardial hypertrophy markers ANP, BNP and β -MHC. Phosphorylation of AKT, mTOR, p70S6K and 4EBP1 was suggested to mediate the PRKAG2 R302Q-induced HCM phenotype. The current study not only determined the mechanism regulating HCM induction by PRKAG2 R302Q mutant, but also demonstrated a therapeutic strategy using β -AR blocker to treat the patients.

Materials And Methods

Reagents The following primary antibodies were used in our experiments: Anti- β -actin(#3700), anti-AMPK α (#2535), anti-phospho-AMPK α (#5831), anti-mTOR (#2983), anti-phospho-mTOR (#2971), anti-4E-BP1 (#9644), anti-phospho-4E-BP1 (#2855), anti-P70S6K (#2708), and anti-phospho-P70S6K (#9208) were purchased from Cell Signaling Technology (USA). Primary antibodies were diluted 1:2000 for hybridization. 1:8000 dilution was applied to the second antibody. BCA protein assay kit was purchased from Beyotime (Beyotime, Shanghai).

Cardiomyocyte cell culture and Infection H9C2 cells were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (FBS, Sigma), and 1% penicillin–streptomycin (Gibco) at 5% CO₂ and 37°C. The Neonatal rat cardiomyocytes (NRCMs) were isolated from the heart of newborn (1 day old) rat with PBS containing 0.01% collagenase type II. The cardiomyocytes were then seeded at a density of 1×10^6 cells/well in six-well culture plates coated with fibronectin in plating medium, which consisted of F12 medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were grown to 70%

confluence before infection with PRKAG2 gene γ_2 WT- (Ad γ_2 WT), or γ_2 R302Q- (Ad γ_2 R302Q) overexpressing adenoviruses at a multiplicity of infection of 36. Cells were harvested after forty-eight hours for further analysis.

AMPK activity analysis To assess the effect of PRKAG2 R302Q mutant on the activity of AMPK, a Cell AMPK Kinase Activity Colorimetric Quantitative Detection Kit (Haring Creature, China) was used following the manufacturer's instructions. Mean values of the absorbance from triplicates were calculated for each sample.

Cellular glycogen analysis Glycogen staining was performed using a Periodic Acid-Schiff kit (PAS, beyotime, China). The amount of glycogen storage in cells was quantified using a glycogen content assay kit (Solarbio, BC0340, China) according to the manufacturer's instructions.

Immunofluorescence analysis Cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 40 min, blocked with a 10% BSA solution for 1 hour at room temperature and then incubated with an primary antibody (1:50 dilution). Nuclei were stained with DAPI (Invitrogen, USA) for 10 minutes in the dark. Images were taken with a fluorescence confocal microscope. Image-Pro Plus 6.0 software was used for quantitative analysis.

Cell proliferation assay Cell proliferation was analyzed using cell counting kit-8 (CCK-8, Beyotime, China) according to the manufacturer's instructions. For each group, mean values of the absorbance from three wells were calculated. EdU staining was performed using an EdU kit (BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488, Beyotime, China). Briefly, harvested cells were seeded in 24-well plates. Subsequently, cells were incubated with EdU for 3 hours, fixed with 4% paraformaldehyde for 15 min, and permeated with 0.3% Triton X-100 for another 15 min. Then cells were incubated with the Click Reaction Mixture for 30 min at room temperature in a dark place and then incubated with Hoechst 33342 for 10 min.

Quantitative real-time polymerase chain reaction (qRT-PCR) Total RNA was isolated using Trizol Reagent (Invitrogen, USA). First Strand cDNA Synthesis Kit (Roche, USA) was used to synthesize cDNA. The mRNA levels of the indicated genes were quantified with real-time PCR using SYBR Green (Roche, USA). Sequence of primers (5' to 3') are: ANP, Forward CGGAAGCTGTTGCAGCCTA, Reverse GCCCTGAGCGAGCAGACCGA; BNP, Forward TTTGGGCAGAAGATAGACCG, Reverse TGGCAAGTTTGTGCTGGAA; β -MHC, Forward GCCTACCTCATGGGACTGAA Reverse ACATTCTGCCCTTTGGTGAC; GAPDH, Forward AGTGCCAGCCTCGTCTCAT, Reverse AGGGGCCATCCACAGTCTTC.

Western Blotting Whole cell lysates were obtained by homogenizing cells in RIPA buffer. 30 μ g of protein lysate was separated via running SDS-PAGE. PVDF membrane (Millipore) was used for transfer. 10% non-fat milk was used for blocking. Primary antibodies were applied for incubation overnight at 4°C, followed by an incubation with secondary antibody for 1.5 h at room temperature. ECL reagents (170-

5061, Bio-Rad) were applied for bands staining. Fluor Chem E imager (Cell Biosciences) was used to capture images.

Statistical Analysis The data are represented as the mean \pm standard deviation (SD). Student's two-tailed t test was used for statistical analysis using SPSS software (version 17.0, SPSS Inc.). $P < 0.05$ was considered a statistically significant difference.

Results

Treatment of PRKAG2 cardiac syndrome with β 1-AR blocker metoprolol. As described in our recent publication, 5 members in a family with PRKAG2 R302Q mutant were diagnosed as PRKAG2 cardiac syndrome, showing complete atrioventricular block and asymmetric ventricular septal hypertrophy [11] (Figure 1A). Metoprolol, as a selective β 1-AR blocker, was applied to these patients for a long-term treatment. As a result, progression of the cardiac hypertrophy was significantly postponed in all the 5 patients upon metoprolol treatment (Figure 1B,1C), including decreased ventricular hypertrophy rate measured by the thickness of the intraventricular septum (IVSth) (Figure 1B) and increased size of left ventricular end-diastolic dimension (LVEDD) (Figure 1C). The patients' information and other clinical parameters have been described recently [11].

Activation of AMPK in cardiomyocytes by overexpression of PRKAG2 R302Q. In order to determine the regulation of AMPK activity by PRKAG2 R302Q mutant in cardiomyocytes, wild type (WT) or R302Q mutant (MUT) PRKAG2 gene were cloned into an adenovirus vector, and then transfected into primary NRCMs for further analysis (Figure 2A). As shown in Figure 2B and 2C, overexpression of PRKAG2 was confirmed at mRNA and protein levels in both WT and MUT vector-transfected cells. Moreover, AMPK activity showed upregulation by both WT and MUT PRKAG2 (Figure 2D). In consistence, phosphorylation of AMPK was promoted by both WT and MUT PRKAG2 (Figure 2E). Notably, MUT PRKAG2 promoted phosphorylation of AMPK and activity of AMPK with a higher level than WT PRKAG2 (Figure 2D, 2E).

PRKAG2 R302Q induced myocardial hypertrophy and glycogen storage in cardiomyocytes. In order to determine the mechanism regulating PRKAG2 R302Q mutant-induced myocardial hypertrophy, NRCMs and H9C2 cells overexpressing WT or MUT PRKAG2 were analyzed with myocardial hypertrophy, cell proliferation and glycogen storage. Cell hypertrophy showed induction by MUT PRKAG2, which was measured through α -SMA staining (Figure 3A, 3B). Glycogen storage in cardiomyocytes was increased by WT or MUT PRKAG2 (Figure 3C, 3D). In consistence, increased expression levels of myocardial hypertrophy markers, such as ANP, BNP and β -MHC, were observed in those cells (Figure 3E). To determine the regulation of cell proliferation by PRKAG2, both EDU staining and CCK8 assay were applied to the cardiomyocytes. As shown in Figure 3F and 3G, PRKAG2 overexpression significantly promoted the proliferation of cardiomyocytes.

Rescue of PRKAG2 R302Q-induced myocardial hypertrophy by β 1-AR blocker and PKA inhibitor. In order to validate the therapeutic effects and explore the mechanisms of β 1-AR blockers to cure the patients with PRKAG2 cardiac syndrome, either β 1-AR blocker metoprolol or PKA inhibitor H89 was applied to the

PRKAG2 R302Q MUT-expressing cardiomyocytes, followed by analysis of cellular hypertrophy, glycogen storage and cell proliferation. As shown in Figure 4, both metoprolol or H89 treatment can partly or completely rescue the PRKAG2 R302Q MUT-induced phenotypes including AMPK activity (Figure 4A), cellular size (Figure 4B, 4C), glycogen storage (Figure 4D, 4E), expression levels of ANP, BNP and β -MHC (Figure 4F), and cell proliferation (Figure 4G-4I).

PRKAG2 R302Q activated AKT-mTOR signaling in cardiomyocytes. In order to explore the molecular mechanisms through which PRKAG2 R302Q induces myocardial hypertrophy, we firstly analyzed AKT-mTOR signaling in cardiomyocytes overexpressing WT or MUT PRKAG2. As shown in Figures 5A-5D, MUT PRKAG2 significantly activated AKT-mTOR signaling by promoting the phosphorylation levels of AKT, mTOR and downstream target genes p70S6K and 4EBP1. In consistence with the cellular phenotypes in Figure 4, both β 1-AR blocker metoprolol and PKA inhibitor H89 treatment reversed the MUT PRKAG2-induced phosphorylation of AKT, mTOR, p70S6K and 4EBP1 (Figure 5E-5H).

Discussion

As a highly genetically heterogeneous disease, 50%~60% of HCM are caused by mutations in myosin genes [12]. Recent evidence indicated that metabolism-related mutants can also result in myocardial hypertrophy, called "metabolic HCM". PRKAG2 cardiac syndrome is a typical form of metabolic HCM caused by mutations in the PRKAG2 gene, which encodes the γ 2 subunit of AMPK [13]. PRKAG2 cardiac syndrome has a high incidence of sudden cardiac death [14-15]. However, there is still no official guidelines available for management and treatment of PRKAG2 cardiac syndrome [16]. In the present study, we are the first to apply β 1-AR blocker metoprolol to five patients with PRKAG2 cardiac syndrome, resulting in great therapeutic effects. In vitro studies using NRCMs and H9C2 cell line demonstrated rescue of the PRKAG2 R302Q-induced cardiomyocyte hypertrophy and glycogen storage by treatment with β -AR inhibitors. Furthermore, AKT-mTOR signaling pathway was demonstrated to involve in the regulation of PRKAG2 cardiac syndrome. AKT is a serine/threonine kinase involved in the regulation of multiple cellular functions, including metabolism, glucose uptake, proliferation and protein synthesis [17,18]. In the heart, mTOR is a large-molecule serine-threonine protein kinase that is activated by phosphorylation of TSC2 by AKT [19]. Activation of mTOR leads to increased proliferation and elevated size of cardiomyocytes, which are associated with cardiac hypertrophy [20]. p70S6K and 4EBP1, as two of the main target genes downstream of mTOR, are important regulators of protein synthesis in the heart [21,22]. In the current study, overexpression of PRKAG2 R302Q in cardiomyocytes activated AKT-mTOR-p70S6K/4EBP1 signaling, leading to increased cell size and promoted cell proliferation (Figure 6).

The cardio-protective effects of β -blockers have been well defined in treatment of patients with coronary heart disease, heart failure, and hypertension, showing clinical outcomes including anti-myocardial ischemia, anti-hypertension, anti-arrhythmias, and increased left ventricular ejection fraction [23,24]. In the model of pressure overload-induced myocardial hypertrophy, β 1-AR was highly activated and enriched in the heart, accounting for about 70% of the total cardiac β -ARs [25]. Continuous activation of β 1-AR led to myocardial remodeling, myocardial hypertrophy and even heart failure [26]. In consistence, metoprolol

showed therapeutic effects here in our study to PRKAG2 R302Q-induced HCM, adding a node to the regulatory network between β -blockers and heart diseases.

Abnormal activation of AMPK has been considered as a main reason causing myocardial hypertrophy, cardiac conduction disturbances, arrhythmias and even sudden death [27,28]. Contradictory results about the effect of PRKAG2 mutants on the AMPK activity were reported. Introduction of PRKAG2 mutants into CCL13 cell line decreased the AMPK activity [29]. However, PRKAG2 cardiac syndrome was shown in the PRKAG2 mutants-transgenic mice, associated with increased AMPK activity in the heart of young mice and decreased sensitivity to AMP [30]. In consistence, here we demonstrated activation of AMPK by PRKAG2 R302Q in cardiomyocytes,

In conclusion, the current study demonstrated that application of β 1-AR blocker metoprolol is able to ameliorate or even reverse myocardial hypertrophy and glycogen storage induced by PAKAG2 R302Q mutant via activation of the AKT-mTOR signaling pathway, suggesting the potential of metoprolol to be developed as a clinical first-line drug in treatment of PRKAG2 cardiac syndrome. Nevertheless, there is no doubt that further study through clinical trials will be still required to confirm the therapeutic effects and administrative strategy.

Declarations

Acknowledgements

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Authors' contributions

HS and JY designed the research and wrote the paper. JZ, HG, XW, MF and LY performed experiments and did data analysis.

Data availability Not applicable.

Code availability Not applicable.

Conflict of interest The authors declare that they have no conflict of Interest.

Ethical approval Not applicable.

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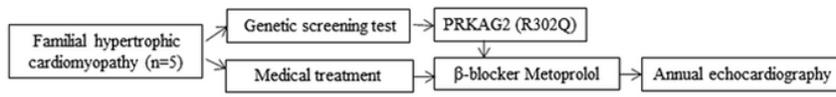
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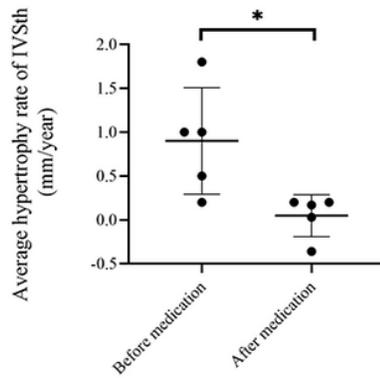
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Figures

A



B



C

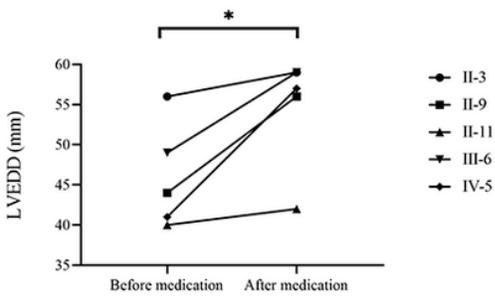


Figure 1

β 1-AR blocker metoprolol suppressed the cardiac hypertrophy in patients with PRKAG2 cardiac syndrome. A: Schematic representation of the work flow to treat the patients with metoprolol. B: Annual thickness change of the intraventricular septum (IVSth) in the 5 patients before and after treatment with metoprolol. C: Change of left ventricular end-diastolic dimension (LVEDD) in the 5 patients before and after treatment with metoprolol. Data are presented as the mean \pm SD. * $p < 0.05$ (n=5).

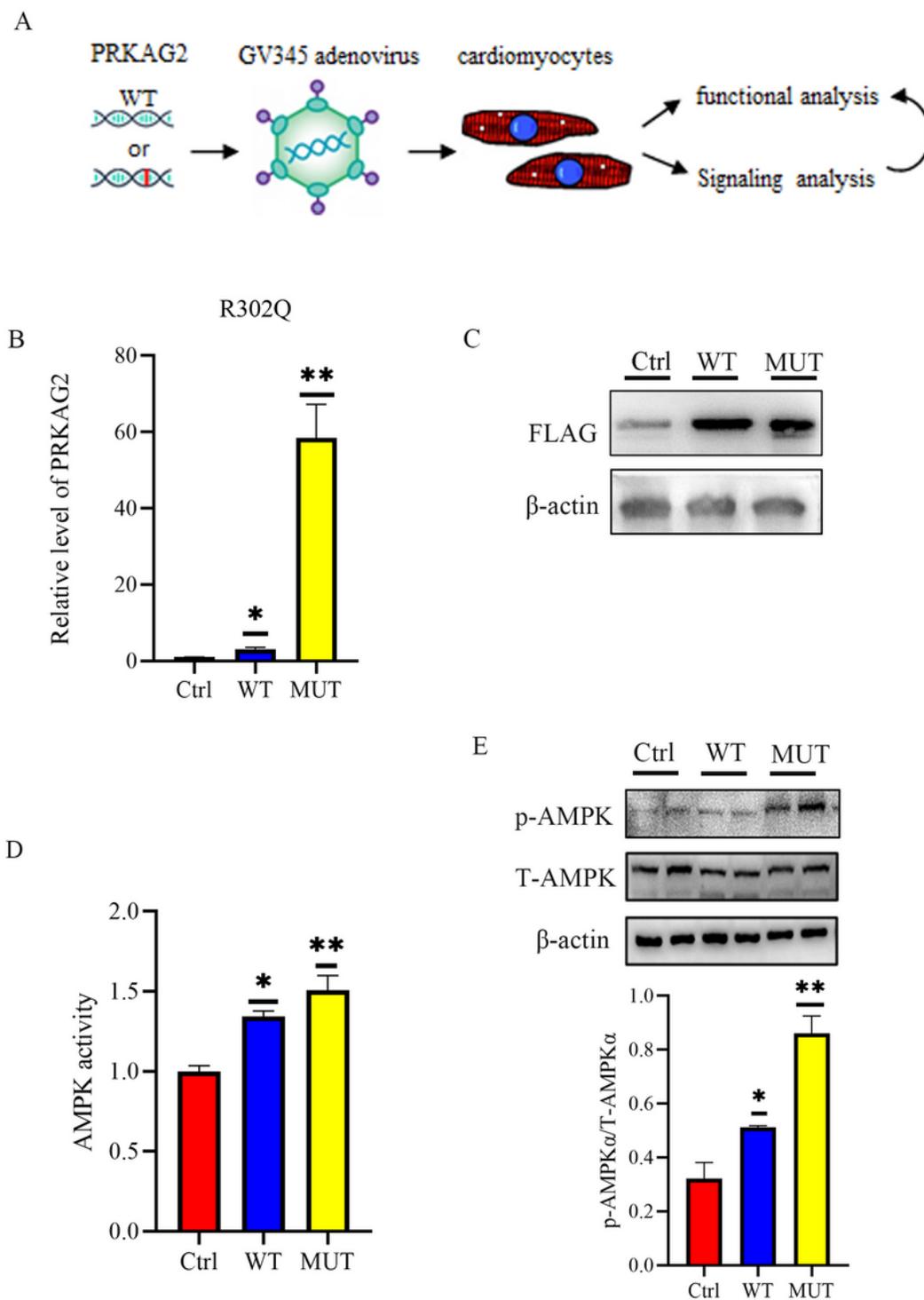


Figure 2

Overexpression of PRKAG2 R302Q in cardiomyocytes increased the AMPK activity. A: Schematic representation of the work flow to determine the function of wild type (WT) and R302Q mutant (MUT) PRKAG2 in cardiomyocytes. B, C: Overexpression of WT and PRKAG2 R302Q MUT in neonatal rat cardiomyocytes (NRCMs) was confirmed by QRT-PCR (B) and western blot (C). C: Increased AMPK activity in cardiomyocytes overexpressing either WT or PRKAG2 R302Q MUT. E: Western blot showing

increased phosphorylation of AMPK by both WT and PRKAG2 R302Q MUT in cardiomyocytes. Data are presented as the mean \pm SD (n=3). *p<0.05, **p<0.01.

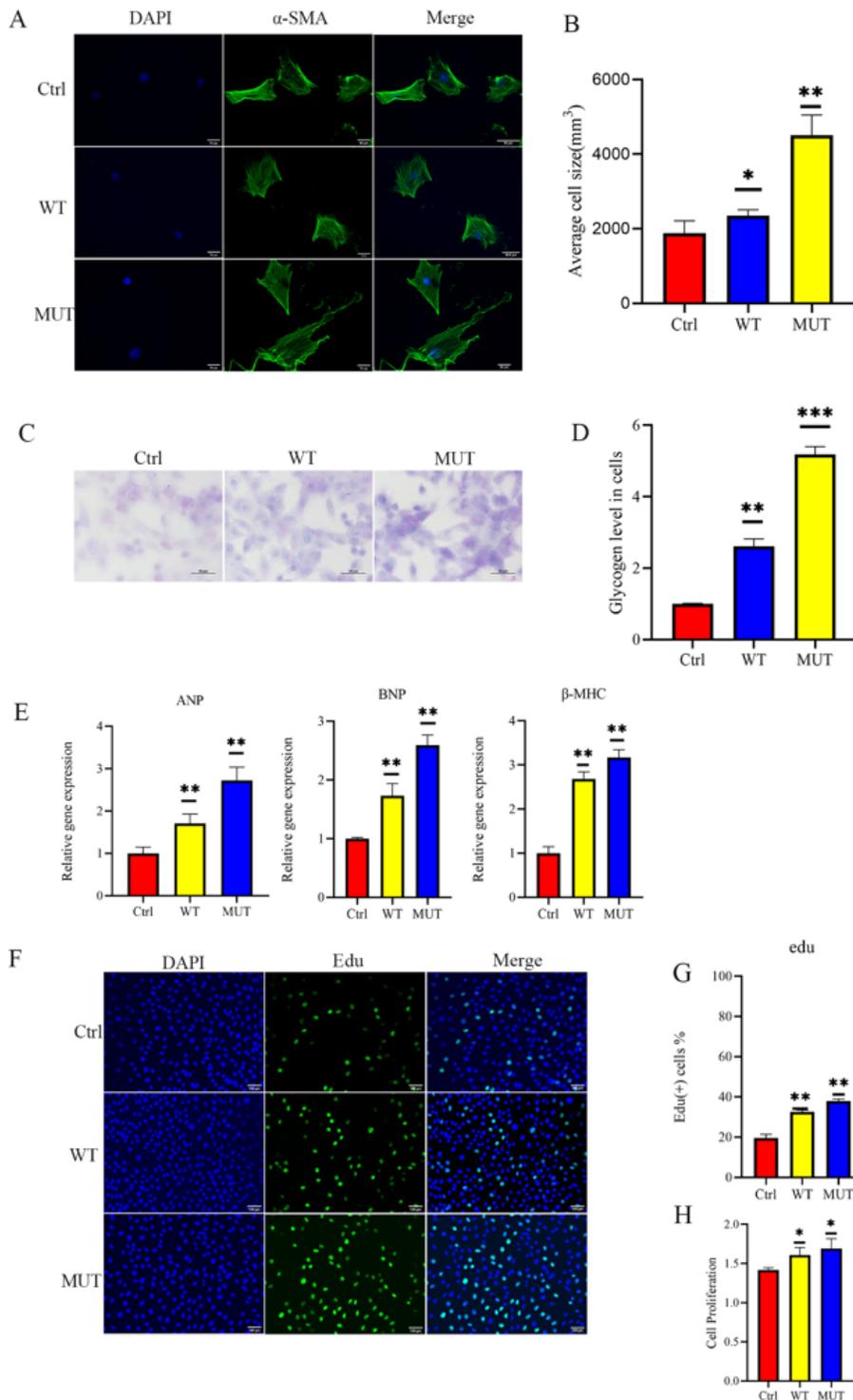


Figure 3

PRKAG2 R302Q induced myocardial hypertrophy, glycogen storage and proliferation in cardiomyocytes. A, B: α -SMA staining of neonatal rat cardiomyocytes showing induced cell hypertrophy by WT and MUT PRKAG2. C, D: Increased glycogen storage in cardiomyocytes by WT and MUT PRKAG2. E: Increased

expression of myocardial hypertrophy markers ANP, BNP and β -MHC in cardiomyocytes by WT and MUT PRKAG2. F, G: EDU staining showing increased cell proliferation in cardiomyocytes by WT and MUT PRKAG2. H: CCK8 assay further validated the increased cell proliferation in cardiomyocytes by WT and MUT. All assays were performed in neonatal rat cardiomyocytes. Data are presented as the mean \pm SD (n=3). *p<0.05, **p<0.01, ***p<0.001.

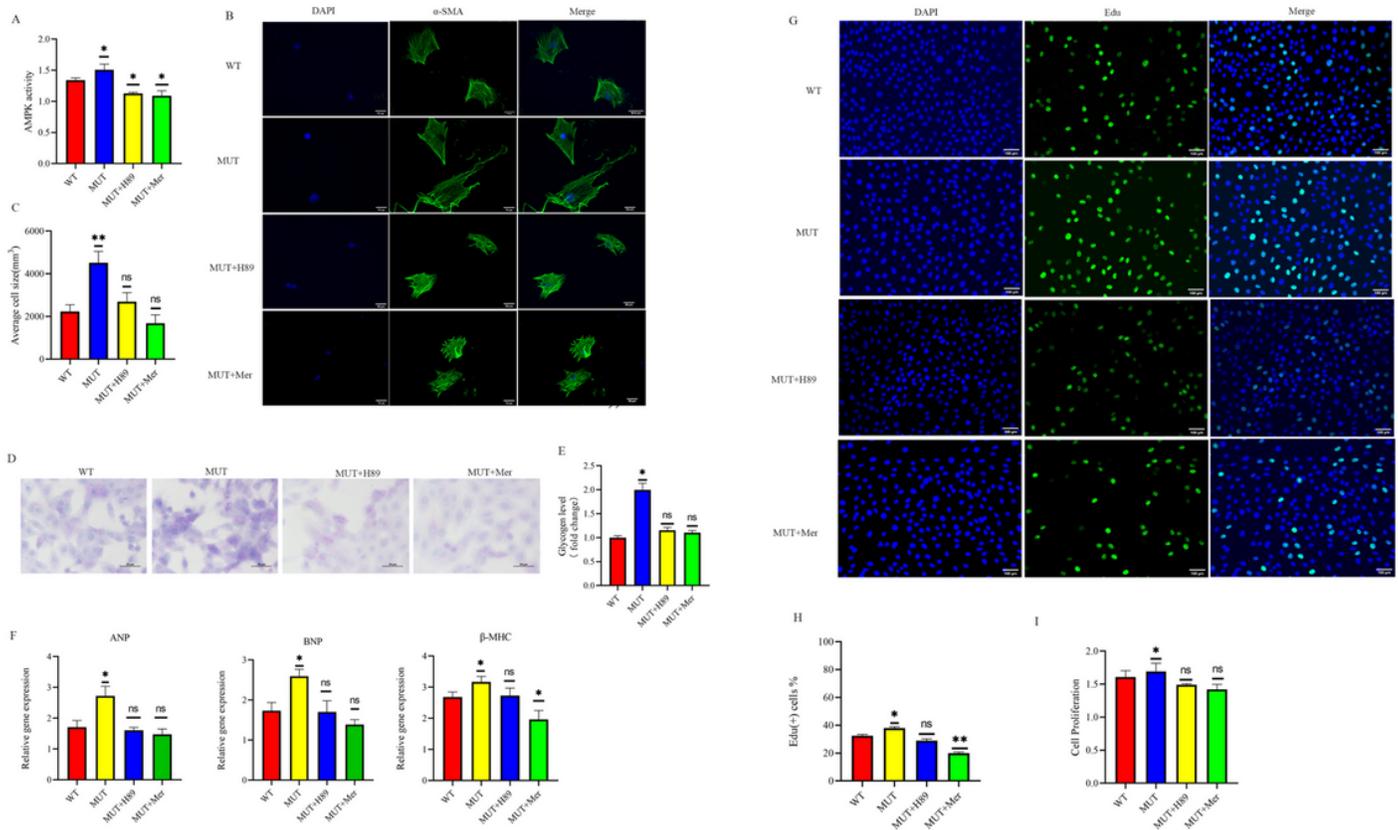


Figure 4

Rescue of PRKAG2 R302Q-induced myocardial hypertrophy by β 1-AR blocker or protein kinase A (PKA) inhibitor. A: Application of β 1-AR blocker metoprolol or PKA inhibitor H89 rescued the PRKAG2 R302Q-induced AMPK activity in cardiomyocytes. B, C: Application of metoprolol or H89 rescued the PRKAG2 R302Q-induced cell hypertrophy in cardiomyocytes. D, E: Application of metoprolol or H89 rescued the PRKAG2 R302Q-induced glycogen storage in cardiomyocytes. F: Application of metoprolol or H89 rescued the PRKAG2 R302Q-induced expression of ANP, BNP and β -MHC in cardiomyocytes. G-I: Application of metoprolol or H89 rescued the PRKAG2 R302Q-induced cell proliferation in cardiomyocytes. Both EDU staining and CCK8 assays were performed. Data are presented as the mean \pm SD (n=3). *p<0.05, **p<0.01. ns means non-significant. All comparisons were made versus WT group.

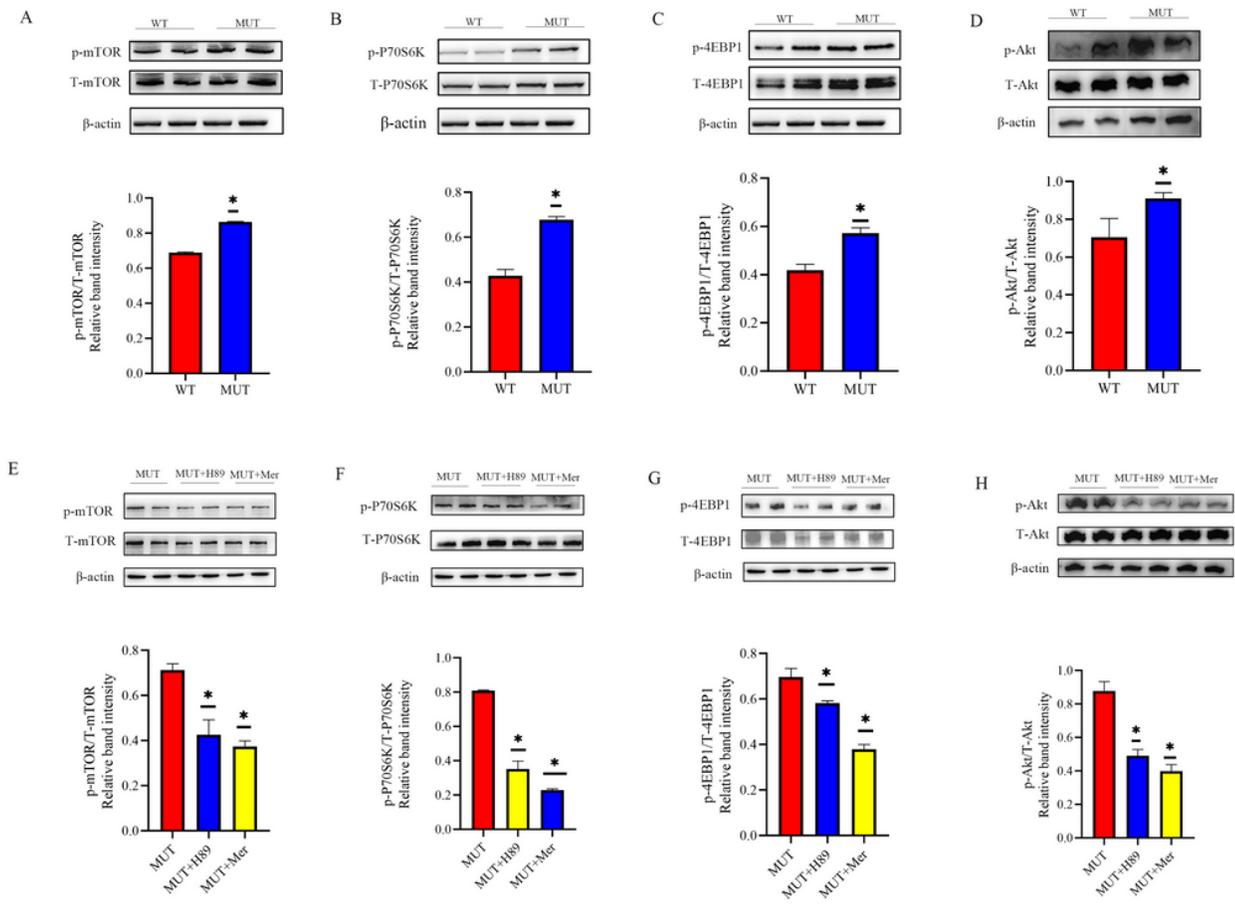


Figure 5

PRKAG2 R302Q activated AKT-mTOR signaling in cardiomyocytes. A-D: PRKAG2 R302Q MUT significantly activated AKT-mTOR signaling in cardiomyocytes by promoting the phosphorylation levels of mTOR (A), p70S6K (B), 4EBP1 (C) and AKT (D). E-H: Application of β 1-AR blocker metoprolol or PKA inhibitor H89 treatment rescued the PRKAG2 R302Q-induced phosphorylation of mTOR (E), p70S6K (F), 4EBP1 (G) and AKT (H). Western blot analyses were performed. Data are presented as the mean \pm SD (n=3). *p<0.05,

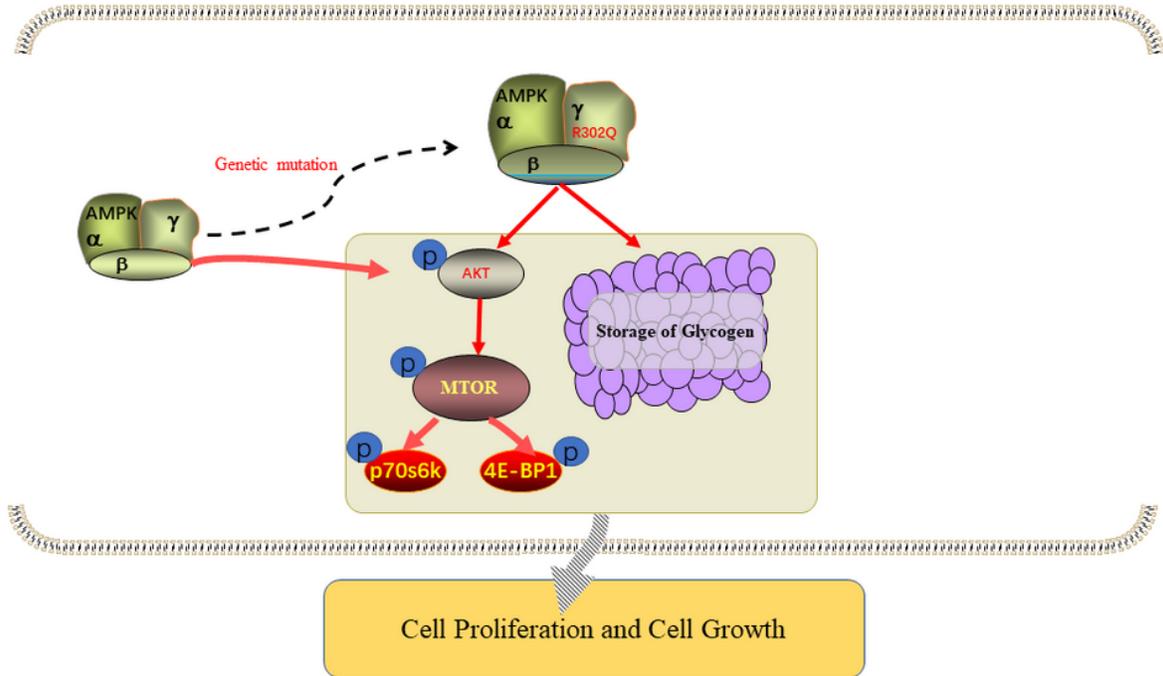


Figure 6

In the current study, overexpression of PRKAG2 R302Q in cardiomyocytes activated AKT-mTOR-p70S6K/4EBP1 signaling, leading to increased cell size and promoted cell proliferation (Figure 6).