Effect of adenosine monophosphate-activated protein kinase–p53–Krüppel-like factor 2a pathway in hyperglycemia-induced cardiac remodeling in adult zebrafish

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ABSTRACT
Aims/Introduction: Diabetic cardiomyopathy is a type of myocardial disease. It causes left ventricular hypertrophy, followed by diastolic and systolic dysfunction, eventually leading to congestive heart failure. However, the underlying mechanism still requires further elucidation.

Materials and Methods: A high-glucose zebrafish model was constructed by administering streptozocin intraperitoneally to enhance the development of cardiomyopathy and then treated with adenosine monophosphate-activated protein kinase (AMPK) activator. Cardiac structure and function, and protein and gene expression were then analyzed. Cardiomyocytes (CMs) culture in vitro using lentivirus were used for detection of AMPK, p53 and Krüppel-like factor 2a (klf2a) gene expression.

Results: In the hyperglycemia group, electrocardiogram findings showed arrhythmia, echocardiography results showed heart enlargement and dysfunction, and many differences, such as increased apoptosis and myocardial fiber loss, were observed. The phospho-AMPK and klf2a expression were downregulated, and p53 expression was upregulated. Activation of phospho-AMPK reduced p53 and increased klf2a expression, alleviated apoptosis in CMs and improved cardiac function in the hyperglycemic zebrafish.

In vitro knockdown system of AMPK, p53 and klf2a using lentivirus illustrated an increased p53 expression and decreased klf2a expression, but no changes in the expression of AMPK and its phosphorylated type.

Conclusions: In the model of streptozocin-induced hyperglycemia zebrafish, the reduction of phosphorylated AMPK increased p53, which led to KLF2a decrease to facilitate apoptosis of CMs, inducing the cardiac remodeling and cardiac dysfunction. These results can be reversed by AMPK activator, which means the AMPK–p53–klf2a pathway might be a potential target for diabetic cardiomyopathy intervention.

INTRODUCTION
Heart failure in diabetes mellitus patients without any comorbidities, such as coronary artery disease and hypertension, was first defined by Rubler et al. in 1972, and this was referred to as diabetic cardiomyopathy (DCM), which is a general complication of diabetes mellitus. DCM has become a serious public health issue in worldwide, and is associated with a high incidence of heart failure and mortality in diabetes patients. Mechanisms, such as inflammation, cardiomyocyte apoptosis, oxidative stress, myocardial fibrosis and mitochondrial dysfunction, have been reported to occur in the pathogenesis of DCM. However, the underlying mechanism responsible for DCM still remains unclear and requires further elucidation.

Zebrafish are tropical fish that have recently emerged as a new and attractive model for studying physiological and
pathological processes, and this is because of its high similarity to the human genome, transparent embryos and suitable for genetic manipulation. The zebrafish animal model has been established as a disease model for cardiovascular diseases, and this is because of the fact that adult humans suffer from a high rate of cardiovascular diseases. To date, there are several challenges posed in establishing the heart disease models in zebrafish, limiting their use in this field. Streptozocin (STZ), a diabetogenic drug, induces diabetes mellitus and exerts a special function on cardiomyopathy in several animal models, and so zebrafish were used to establish a hyperglycemic model. Sarras et al. reported that a type 1 diabetes mellitus model of adult zebrafish induced by STZ was applied in research that focused on tissue regeneration. The underlying mechanisms of how STZ induces DCM in adult zebrafish still remain unclear.

Previous studies have documented that apoptosis of cardiomyocytes (CMs) has great significance in DCM. Increased apoptosis of CMs in diabetic animal models or patients has been the leading cause of loss of contractile tissues, resulting in systolic and diastolic dysfunction. Thus, inhibiting cardiomyocyte apoptosis can be an efficient way to inhibit myocardial remodeling in DCM.

Adenosine monophosphate-activated protein kinase (AMPK) plays a vital role in adenosine triphosphate-deprived conditions by sensing cell energy changes. However, the role of AMPK in cell proliferation and apoptosis is more complex than originally thought, and often remains controversial. Guo et al. showed that resveratrol relieves cardiac cell injury and apoptosis by activating AMPK. Recent studies have shown that AMPK regulates cell proliferation and apoptosis by phosphorylating and stabilizing the tumor suppressor, p53. Krüppel-like factor 2a (klf2a), a member of the family of Cys2/His2 zinc-finger transcriptional factors, exerts an important function in cell proliferation and differentiation. Wang et al. showed that the levels of klf2a could affect deoxyribonucleic acid damage-induced apoptosis. AMPK, p53 and klf2a play vital roles in cardiovascular diseases, but the mechanism of progression in cardiomyopathy still requires extensive research.

Hence, in the present study, the hyperglycemia model was successfully established by STZ injection in zebrafish. The myocardial structural collapse and dysfunction of the heart in the hyperglycemic model showed that apoptosis plays an important role in the cardiac remodeling mechanism, participating in the pathogenesis of DCM. Furthermore, the activated AMPK pathway attenuates apoptosis in CMs, and restores structural collapse and dysfunction of the heart in the model. The in vitro knockdown system of AMPK, p53 and klf2a using lentivirus has shown an upstream and downstream relationship among them. Above all, these results suggested that inhibition of myocardial cell apoptosis by activating the AMPK–p53–klf2a pathway might be a potential method for treating DCM.

METHODS

Zebrafish care and husbandry

The adult AB strain zebrafish (Danio rerio) were maintained in the laboratory of Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine. The fish were maintained in water for 10 h dark and 14 h light cycle at a temperature of 28.5°C. The fish used in the present study were approximately 15–18 months-of-age and were randomly chosen from different clusters. The experiments were carried out according to the approved ethical principles provided by the Rui Jin Hospital Animal Care and Use Committee animal protocols.

STZ injection

STZ (S0130; Sigma, St. Louis, MO, USA) was administered through intraperitoneal injection by using a 29-G insulin syringe needle. After that, 0.3% STZ was dissolved in 0.09% NaCl and 350 mg/kg (70–150 µL dependent on weight) on days 1, 3 and 5. The control fish were injected with the same volume of 0.09% NaCl solution on days 1, 3 and 5. The corresponding indexes were detected on days 7, 14 and 21.

A-769662 injection

Zebrafish (aged 15–18 months) were randomly selected, followed by administration of 0.16 mg/mL tricaine liquid anesthesia to cease the whole-body activity, and the anesthesia time was approximately 3 min and 40 s. A-769662 (Gene Operation, ISY1061). Next, phosphate-buffered saline 1:1 dilution with 50% dimethyl sulfoxide was injected by using a 28.5-G insulin syringe needle intraperitoneally at a dose of 100 µg/g on days 2, 4 and 6. The zebrafish in the normal control group were injected with a similar volume of 0.09% NaCl on days 2, 4 and 6. Zebrafish were also tested on days 7, 14 and 21.

Glucose measurements

The zebrafish were anesthetized with 0.16 g/L of tricaine (MS-222; SIGMA-ALDRICH, St. Louis, MO, USA), the blood (1–2 µL) from each fish was harvested, and then, the blood glucose was measured using the glucose (HK) assay kit (DIGL-200; BioAssay Systems, Taichung City, Taiwan, China) according to the manufacturer’s instructions.

Western blot analysis

Primary antibodies, such as anti-cleaved P53 (ab1101; Abcam, Shanghai, China), anti-AMPK alpha 1 (ab32047; Abcam), anti-KLF2 (ab203591; Abcam) and anti-glucose transporter (GLUT) 12 (ab75441; Abcam) were obtained. The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; DH0261#) was purchased from Donghuan Biotech Co., Ltd (Shanghai, China). All antibodies were diluted according to the manufacturer’s instructions, and immunoreactivity was visualized using a chemiluminescence detection kit (DH0101). Dissection of zebrafish cardiac muscle was manually homogenized and then lysed in tissue lysis buffer with protease inhibitor (Roche, Basel, Switzerland).
Ribonucleic acid extraction and quantitative real-time polymerase chain reaction

Total ribonucleic acid (RNA) from cultured cells or tissues was extracted using Trizol reagent according to the manufacturer’s instructions. The total RNA was measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and retrotranscribed immediately by the FastQuant RT Super Mix (KR108; TIANGEN Biotech, Beijing, China). Quantitative polymerase chain reaction (qPCR) was used with ABI PRISM 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the SuperReal PreMix (FP204; TIANGEN Biotech). GAPDH messenger RNA was considered the control. The primers for AMPK in quantitative real-time PCR experiments were as follows: the forward primer F; 5'-GGATCC TTCTTACGCGTCAG-3', and reverse primer R; 5'-TCCTC CCGAATCTTTTGT ATG-3'. Primers for p53 were the forward primer F; 5'-TCTCTCCCTCAGAATTG CAGGGCCTACGCGA-3', and reverse primer R; 5'-AGTATGATGGTTGAGGTGTTGCCC-3'. Primers for Ki2 were the forward primer F; 5'-CAGAATACAC GAGACGACAGA-3', and reverse primer R; 5'-CGATCCCGA GATTGTCAGA-3'. Primers for GLUT12 were the forward primer F; 5'-TGAGACGACGACTTACCA-3', and reverse primer R; 5'-GCCACTGACATCCCAACCA-3'.

Immunostaining and fluorescence microscopy

The hearts were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 5-µm thick slices and then underwent immunofluorescence. Antibodies used in this experiment included anti-MEF2 (sc-17785; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and anti-proliferating cell nuclear antigen (PCNA; sc-790; Santa Cruz Biotechnology; Santa Cruz, CA, USA). The CMs underwent different treatments were placed on a glass. Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) assays were operated using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. The images obtained were visualized under an OLYMPUS DP70 microscope.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling assay

The hearts were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 5-µm thick slices. The CMs that underwent different treatments were placed on a glass. Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) assays were operated using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s protocol.

Transmission electron microscopy

The heart tissues were fixed in 2.5% glutaric dialdehyde for approximately 4 h and washed three times with 0.1 M phosphate buffer. Next, the samples were embedded in Epon 812 and cut into 100-nm thick slices using UC7 Ultramicrotome (Leica, Heerbrugg, Switzerland). The sections were then stained with uranyl acetate and lead citrate, and the images were captured by using an electron microscopy core facility with Philips (Amsterdam, the Netherlands) CM-120 TEM (Amsterdam, the Netherlands).

Electrocardiogram and echocardiography

Electrocardiogram (ECG) and echocardiography were operated as described previously, and the fish were anesthetized as described previously. The fish were fixed and placed by facing the ventral side upwards and gently squeezing the caudal fin. ECG was recorded after stabilizing for 2 min using the BIO-PAC ECG100C System (Goleta, CA, USA). ECG was measured and statistically analyzed, which was as follows: the amplitude of QRS-wave alternating between high and low was referred to as “voltage alteration”; the ST-T depression or T-wave inversion was defined as “ST-T change”; and the “prolonged P-P interval” was referred to as the “sinus arrest”. For echocardiography, imaging was carried out in the short axis (i.e., perpendicular to the fish) and long axis (i.e., parallel to the fish) using an MS-5508 transducer (VisualSonics, Toronto, Canada) coupled to a 50-MHz ultrasound probe. The ejection fraction (EF) was calculated by the formula, (end-diastolic volume minus end-systolic volume) / end-diastolic volume. The time parameters, including isovolumic relaxation time (IVRT), ventricular ejection time (VET), and isovolumetric contraction time (IVCT), were calculated, and the parameters after adjusting the heart rate (HR; IVRTa, VETa and IVCTa, respectively) represent the percentage of heartbeat time.

Primary CM culture

The CMs from the ventricles were obtained for the study. CMs were cultured in L-15 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The CMs were maintained in a humidified atmosphere at 28.5°C containing 5% CO2.

Plasmid construction production of lentivirus and cell infection

The specific short hairpin RNAs (shRNA) against P53, klf2a, prkaa1 and control shRNA were designed and synthesized by GenePharma (Shanghai, China). The shRNAs were ligated into the Ad5/EcoRI sites in the lentiviral vector pLKO.1. The shuttle vectors and HEK 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the protocol. Medium containing the virus was harvested and filtered after 72 h of transfection, and was used for further research.

Statistical analysis

All data are expressed as the mean ± standard deviation, and each experiment was repeated at least three times. P < 0.05 was considered to be significant, whereas P < 0.01
represented a highly significant difference in a two-tailed Student’s t-test.

RESULTS

STZ induces hyperglycemia model in zebrafish
Previous studies have shown that STZ treatment induced DCM in several animal models. To determine whether STZ induces hyperglycemia in zebrafish, STZ 350 mg/kg was administered intraperitoneally32. The blood glucose levels of zebrafish in the STZ-injected group were significantly increased the next day, and high levels were maintained until 30 days without intervention (Figure 1a). The zebrafish had a fasting plasma glucose level of ≥200 mg/dL, which was considered hyperglycemic33. Previous studies reported that the GLUT family, which are the main carriers of glucose, was involved in cell apoptosis through regulating p5334,35. Our qPCR and western blot analysis results showed a significant decrease in the expression of GLUTs, such as GLUT1, GLUT2 and GLUT12, in the hyperglycemic fish group (Figure 1b,c). In addition, the number of zebrafish was counted daily, and zebrafish in both groups before treatment showed a 100% survival rate. The fish count was recorded after every 3 days in each group, and the survival of zebrafish in the hyperglycemia group was decreased when compared with the control group (P < 0.01; Figure 1d). Furthermore, the weight

![Graphs and images showing blood glucose levels, fold expression of GLUTs, survival analysis, and body mass analysis.](image)

Figure 1 | Streptozocin (STZ) induces hyperglycemia in zebrafish. (a) Blood glucose assay analysis of blood glucose in vivo continues for 30 days. The fish were injected with STZ in a dose of 350 mg/kg and maintained in different days. Values >200 mg/dL were considered hyperglycemic. (b) Quantitative polymerase chain reaction analysis of glucose transporter (GLUT)1, GLUT2 and GLUT12 in hyperglycemic zebrafish hearts. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured as the control. (c) Western blot analysis of GLUT1, GLUT2 and GLUT12 in hyperglycemic zebrafish injected with STZ. GAPDH was used as the control. (d) Survival analysis of the hyperglycemic fish and control fish in 30 days. (e) Body mass analysis of the hyperglycemia zebrafish model. Each experiment was repeated a minimum of three times. **Highly significant difference (P < 0.01) in a two-tailed Student’s t-test.
of zebrafish in both groups was measured on day 21, and the fish injected with STZ lost weight compared with the control group (Figure 1e).

Expression of AMPK, p53 and klf2a in the heart of hyperglycemic zebrafish

A previous study showed that AMPK regulation could affect apoptosis in CMs and a hyperglycemic zebrafish model. Thus, to elucidate the possible mechanism of DCM, the expression levels of components of the AMPK pathway were examined in our models. Western blotting results showed that the protein levels of phospho-AMPK and klf2a were reduced, but the expression of p53 was elevated in the STZ-induced DCM (Figure 2b). Recently, many compounds have been found to activate AMPK, such as A769662, 5-aminoimidazole-4-carboxamide-1-b-4-ribofuranoside, non-steroidal anti-inflammatory drugs and so on. In the present study, A769662 was used and increased the phosphorylation level of AMPK and the expression of klf2a, whereas p53 was suppressed (Figure 2a,b).

Hyperglycemia induces muscular disarray, myofibril loss, vacuolization, mitochondrial condensation and apoptosis activation

In the present study, transmission electron microscopy analysis confirmed muscular disarray and myofibril loss in the hearts of hyperglycemic zebrafish. Meanwhile, vacuolation and mitochondrial condensation were found in the hearts (Figure 3a). Previous work documented apoptosis as one of the main causes of DCM, and so hematoxylin–eosin, TUNEL and western blotting were carried out to assess the apoptosis in the zebrafish model of DCM. Hematoxylin–eosin staining showed marked muscular disarray, myofibril loss and reduced myocardial density in the heart of hyperglycemic fish (Figure 3b). TUNEL assay showed that the apoptosis was increased in the hearts of hyperglycemic fish hearts when compared with the control group (Figure 3c).

Hyperglycemia induces cardiac dysfunction in adult zebrafish

ECG was carried out to detect the hyperglycemia-induced electrophysiological changes in the myocardium of zebrafish, and
Figure 3 | Hyperglycemia induced muscular disarray, myofibril loss, vacuolization, mitochondrial condensation and apoptosis activation. (a) Transmission electron microscopy image verified muscular disarray, myofibril loss, vacuolization and mitochondrial condensation in hyperglycemic zebrafish compared with the control group (n = 21 field, repeated five times); scale bars, 2 µm. (b) Hematoxylin–eosin staining analysis of the heart in hyperglycemic zebrafish and A769662 intervention zebrafish compared with the control group after 21 days; scale bars, 100 µm. Quantification analysis of ventricular myocardial density in hematoxylin–eosin-stained hearts between the three groups. (c) Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) assay analysis of cell apoptosis in the heart of hyperglycemia zebrafish and A769662 intervention zebrafish compared with the control group zebrafish; scale bars, 50 µm. Each experiment was repeated a minimum of three times. ** Highly significant difference (P < 0.01) in a two-tailed Student’s t-test. STZ, streptozocin.
the results showed a marked decrease in the HR of hyperglycemia fish. The incidence of “ST-T change” and “voltage alternation” in hyperglycemia fish was markedly increased. However, the PR and QT intervals after adjusting the HR showed no significant differences (Figure 4c). A normal ECG recording from the untreated fish is shown in Figure 4b(i). The representative diagram of escape rhythm is shown in Figure 4b(ii), representative T-wave inversion is shown in Figure 4b(iii). Other abnormal ECG changes, such as “voltage alternation”, is shown in Figure 4b(iv).

Echocardiographic parameters showed significant differences between the hyperglycemia group and control group (Table 1). The B-mode echocardiography images are presented in 4-D. The ventricular function and morphology were automatically generated using the Vevo 2100 Workstation Software package (Toronto, Canada).

Cardiac function was dynamically monitored by echocardiography, and the results showed that the stroke volume (SV), cardiac output (CO) and EF in the hyperglycemia group were lower than those in the control group. After 14 days of STZ intervention, the IVRT adjusted by HR (IVRTa) of the zebrafish in the hyperglycemia group was shorter than the control group, which indicated that the diastolic function was impaired, while the systolic function showed no significant change. After 21 days of STZ intervention, a significant reduction of SV and EF showed impaired myocardial contractility and systolic dysfunction (Tables 1 and 2). Systolic indices showed shortened VET adjusted by HR (VETa) and IVCT adjusted by HR (IVCTa) in hyperglycemia group. Furthermore, a significant reduction of HR and SV contributed to decreased CO. The remarkable decrease in EF was used to estimate the cardiac function, showing an impaired systolic function in the hyperglycemic zebrafish (Table 1).

In addition, echocardiography was carried out to examine the effect of A769662 on the hearts of hyperglycemic zebrafish. After treatment with A769662, the echocardiography results

| Table 1 | Doppler echocardiography measurement in the hyperglycemic zebrafish |
|---------|----------------------------------|----------------|----------------|----------------|
|         | Wild-type                         | STZ-treated 7 days | STZ-treated 14 days | STZ-treated 21 days |
|         | (n = 21)                          | (n = 21)        | (n = 21)        | (n = 21)        |
| IVCTa (ms) | 40.57 ± 6.831                  | 38.276 ± 6.787  | 37.373 ± 6.845  | 31.010 ± 5.769**|
| VETa (%)   | 55.5 ± 14                      | 51.0 ± 09       | 51.0 ± 13       | 42.0 ± 0.5**    |
| VET (ms)   | 232.548 ± 36.197                | 229.752 ± 30.664 | 224.710 ± 36.845 | 220.643 ± 37.459 |
| VETa (%)   | 320.0 ± 8.1                    | 309.5 ± 5.7     | 304.8 ± 6.8     | 300.0 ± 7.4     |
| E (mm/s)   | 25.737 ± 11.547                 | 34.477 ± 9.112** | 35.619 ± 8.235** | 38.431 ± 8.687**|
| A (mm/s)   | 123.994 ± 39.484                | 121.369 ± 20.476 | 117.764 ± 26.910 | 116.459 ± 26.403 |
| E/A        | 0.205 ± 0.039                   | 0.285 ± 0.067**  | 0.304 ± 0.033**  | 0.332 ± 0.049** |
| IVRT (ms)  | 91.133 ± 16.042                 | 90.805 ± 16.117  | 85.443 ± 9.190*  | 82.238 ± 8.401* |
| IVRTa (%)  | 124.2 ± 29                     | 121.1 ± 18       | 116.0 ± 21       | 111.1 ± 1.6     |
| SV (μL)    | 0.270 ± 0.095                   | 0.263 ± 0.061    | 0.259 ± 0.046    | 0.255 ± 0.054   |
| EF (%)     | 50.379 ± 5.106                  | 47.268 ± 8.081   | 45.648 ± 7.093*  | 40.063 ± 8.154**|
| CO (μL/min)| 21.709 ± 7.434                  | 21.586 ± 7.053   | 21.143 ± 5.290   | 20.858 ± 5.958  |
| Volume s (μL)| 0.264 ± 0.054               | 0.326 ± 0.049**  | 0.343 ± 0.058**  | 0.369 ± 0.06**  |
| Volume d (μL)| 0.533 ± 0.114                | 0.627 ± 0.124*   | 0.666 ± 0.068**  | 0.674 ± 0.094** |

Values are the mean ± standard deviation, n = 21 in each group. *P < 0.05 and **P < 0.01 as compared with wild-type zebrafish. Image data were automatically generated using the Vevo 2100 Workstation Software package. A, A-wave peak velocity; E, E-wave peak velocity; E/A, E to A ratio; EF, ejection fraction defined as (end-diastolic volume minus end-systolic volume) / end-diastolic volume; IVCT, isovolumic contraction time; IVCTa, adjusted isovolumic contraction time, calculated by the formula, isovolumic contraction time ms / (60 s × 1000 / heart rate) × 100%; IVRT, isovolumic relaxation time; IVRTa, adjusted isovolumic relaxation time, calculated by the formula, isovolumic relaxation time ms / (60 s × 1000 / heart rate) × 100%; STZ, streptozocin; VET, ventricular ejection time; VETa, adjusted ventricular ejection time, calculated by the formula, ventricular ejection time ms / (60 s × 1000/HR) × 100%; Volume d, end-diastolic volume; Volume s, end-systolic volume.
showed that the SV, CO, and EF were improved in the hyperglycemic fish group, IVRTa also showed significant prolongation than the untreated hyperglycemia group, and the diastolic function was improved as well (Table 2). The volume and the end-diastolic volume were restored in hyperglycemia that A769662 alleviated the STZ-induced apoptosis in the hyperglycemic fish heart (Figure 3c), which was consistent with the echocardiography results above. These findings suggested that STZ could induce DCM in zebrafish, and activation of AMPK by A769662 could inhibit cardiac remodeling.

**AMPK–p53–klf2a pathway is involved in hyperglycemia zebrafish cardiomyocytes**

To further confirm the inhibition of AMPK–p53–klf2a pathway by hyperglycemia, the expression of the AMPK pathway was detected in hyperglycemic zebrafish CMs cultured in vitro. As expected, activation of AMPK in vitro inhibited the expression of p53 and increased klf2a (Figure 5a,b). Furthermore, qPCR analysis showed that A769662 restored STZ-induced repression of GLUT1, GLUT2 and GLUT12 (Figure 5c). In addition, AMPK activation alleviated STZ-induced apoptosis in CMs (Figure 5d), which was consistent with the results obtained in vivo.

The above data showed that the AMPK–P53–klf2a pathway was involved in the hyperglycemic zebrafish CMs model. Thus, it was necessary to identify the relationships among them. The primary cultured CMs of zebrafish were isolated and cultured, followed by suppressing the expressions of AMPK, p53 and klf2a by shRNA that are specific to them, respectively. qPCR and western blotting results showed that the AMPK and klf2a expression were inhibited, and p53 expression was elevated in zebrafish cardiomyocytes transfected with shAMPK and shklf2a (Figure 6a,b). Furthermore, the echocardiography results above. These findings suggested that STZ could induce DCM in zebrafish, and activation of AMPK by A769662 could inhibit cardiac remodeling.

**Table 2** | Doppler echocardiography results after treatment with A769662 in the hyperglycemic zebrafish

| n        | STZ     | STZ + A769662 |
|----------|---------|---------------|
| (n = 21) | (n = 21) | (n = 21)      |
| IVCT (ms)     | 41.762 ± 6.518 | 31.024 ± 6.904** | 38.300 ± 12.353* |
| IVCTa (%)     | 58 ± 1.1 | 42 ± 1.3** | 52 ± 1.4* |
| VET (ms)      | 230.692 ± 54.900 | 220.875 ± 41.426 | 237.678 ± 41.536 |
| VETa (%)      | 32.2 ± 9.8 | 29.6 ± 6.8 | 32.4 ± 4.2 |
| E (mm/s)      | 25.643 ± 7.607 | 38.648 ± 13.277** | 31.531 ± 12.130 |
| A (mm/s)      | 125.714 ± 21.648 | 116.786 ± 40.499 | 133.297 ± 25.144 |
| E/A           | 0.203 ± 0.047 | 0.337 ± 0.073** | 0.249 ± 0.126** |
| IVRT (ms)     | 91.905 ± 23.076 | 82.286 ± 11.032* | 90.238 ± 16.791 |
| IVRTa (%)     | 12.7 ± 3.2 | 11.1 ± 2.5 | 12.3 ± 1.8 |
| SV (µL)       | 0.271 ± 0.100 | 0.256 ± 0.079 | 0.266 ± 0.085 |
| EF (%)        | 51.0 ± 6.2 | 406 ± 9.1 ** | 48.1 ± 10.9* |
| CO (µL/min)   | 22.672 ± 8.777 | 20.111 ± 5.225 | 21.404 ± 5.183 |
| Volume s (µL) | 0.262 ± 0.049 | 0.376 ± 0.055 ** | 0.305 ± 0.091 ** |
| Volume d (µL) | 0.533 ± 0.132 | 0.681 ± 0.115** | 0.614 ± 0.208 |

Values are the mean ± standard deviation, n = 21 in each group. *P < 0.05 and **P < 0.01 as compared with wild-type zebrafish. Image data were automatically generated using the Vevo 2100 Workstation Software package. A: A-wave peak velocity; E: E-wave peak velocity; E/A: E to A ratio; EF: Ejection fraction; IVCT, adjusted isovolumic contraction time, calculated by the formula, isovolumic contraction time ms/(60 s / (60 s + 1000/HR)) × 100%; IVRT, isovolumic relaxation time; IVRTa, adjusted isovolumic relaxation time, calculated by the formula, isovolumic relaxation time ms / (60 s + 1000/HR) × 100%; STZ, streptozocin; VET, ventricular ejection time; VETa, adjusted ventricular ejection time, calculated by the formula, ventricular ejection time ms / (60 s × 1000/HR) × 100%; Volume d, end-diastolic volume; Volume s, end-systolic volume.
AMPK-p53-klf2a pathway in DCM zebrafish

(a) Western blot analysis showing the expression of p-AMPK, AMPK, and GAPDH. The gray level of protein/GAPDH was quantified.

(b) Relative expression of p53 and klf2a following treatment with NaCl, STZ, and STZ+A769662.

(c) Relative expression of Glut1, Glut2, and Glut12 under the same treatment conditions.

(d) Immunofluorescence staining for Tunel, Mef2a, and Merge. The ratio of TUNEL/Mef2a was calculated.

** Note: Statistical significance indicated by * and **.
qPCR and western blotting analysis showed that p53 expression was inhibited and klf2a expression was elevated without altering AMPK expression in the cells transfected with shp53 (Figure 6c,d). In addition, qPCR and western blotting analysis showed that klf2a expression was inhibited, but showed no alteration in the expression of AMPK and p53 in cells transfected with shklf2a (Figure 6e,f).

DISCUSSION

Our previous work included zebrafish model mimics of type 2 diabetes by inducing high glucose33. The blood glucose levels in vivo were elevated, the body mass of zebrafish treated with STZ was decreased and cardiomyopathy structural remodeling was observed in hyperglycemic zebrafish, which was consistent with the metabolic disturbances in type 1 diabetes
(Figures 1,3,4). The levels of GLUTs expression in zebrafish hearts indicated the decreased ability of zebrafish myocardium to utilize glucose. (Figure 1b,c). Studies have suggested that the changes in ECG in high-glucose models usually occurred early in the disease. ECG results in the present study showed that the HR in high-glucose models was slowed down, which was consistent with the rodent diabetes model. ECG changes occurred after 21 days of STZ injection, including ST-T changes, increased electrical alterations and QT interval prolongation, suggesting myocardial ischemia and impaired cardiac function. Furthermore, non-invasive ECG was used to detect cardiac function of zebrafish, and reduced results of SV, CO and EF after 21 days of STZ-intervention suggested damage of myocardial contractility in the hyperglycemic zebrafish group (Table 1). Meanwhile, after 14 days of STZ intervention, the IVRTa of the zebrafish hearts in the hyperglycemia group was shorter than the control group, whereas IVCTa showed no marked reduction, indicating diastolic dysfunction before systolic dysfunction. This was consistent with the majority of evidence on DCM, and patients with diabetes usually have impaired diastolic function followed by contraction function. Furthermore, as shown in Table 1, the variations in the early diastolic flow velocity (peak E), and the late diastolic/atrial flow velocity (peak A) and E/A ratio, are opposite to what is seen in humans and other higher vertebrates. This is probably because of the size and construction of zebrafish hearts, that are used for assessing cardiac diastolic function through E/A ratio alteration in zebrafish, are limited.

Previous studies have shown that AMPK, a sensor of cellular energy status, is involved in cell apoptosis, and its function in cell apoptosis is often controversial. AMPK is considered as a key regulator of cellular and systemic energy homeostasis, and is involved in various metabolic functions in diseases. Thus, it is necessary to directly activate AMPK with the help of drugs, and understand the mechanism of its role in many diseases, such as cardiovascular disease, type 2 diabetes mellitus, obesity and cancers, as well as DCM. In the present study, AMPK expression was decreased in STZ-induced apoptosis along with apoptosis, activated p53 and suppressed klf2a (Figures 2b,3c). The present study found that A769662 could activate AMPK (Figure 2a,b), which in turn attenuates apoptosis, and restores pathological conditions and dysfunction of hyperglycemic fish model hearts (Figures 4d,3c; Table 2). Also, regulation of AMPK could affect apoptosis in CMs and the zebrafish model of hyperglycemia. Thus, ECG analysis showed activation of AMPK in hyperglycemic model zebrafish, resulting in more increased SV, CO and EF, and prolonged IVRTa and IVCTa in the AMPK-activated group than hyperglycemic zebrafish. This is an improvement in diastolic and contraction functions. Above all, activation of AMPK could inhibit remodeling of the heart. Apoptosis is defined as a programmed cell death, and exerts an important function in eliminating excessive, damaged or harmful cells. Previous studies have documented that apoptosis caused loss of contractile tissue, cardiac remodeling and dysfunction in DCM. In our studies, TUNEL analysis showed that STZ induced cell apoptosis in hyperglycemic zebrafish hearts (Figure 3c), indicating that anti-apoptosis might be a potential treatment strategy for DCM. The most noteworthy point is that cardiac remodeling of the heart in hyperglycemic zebrafish was attenuated when the apoptosis was inhibited (Figure 3c). Taken together, these results suggested that the AMPK pathway might be a potentially new therapeutic target for treating DCM. It is important to understand whether upregulation of AMPK acts as a compensatory change to protect against cardiomyopathy. In summary, the results showed significant advances in the development of AMPK-targeting therapies and a reliable method to treat DCM.

Furthermore, an in vitro study of hyperglycemic zebrafish CMs was carried out to determine the direct effects of high glucose, and the roles of AMPK, p53 and klf2a. Messenger RNA expression of AMPK, p53 and klf2a was determined when the CMs of hyperglycemic zebrafish were incubated. To support this, the lentivirus knockdown system showed an upstream and downstream relationship among AMPK, p53 and klf2a in vitro CMs. qPCR and western blotting analyses showed that AMPK reversed the regulation of p53, and that p53 was considered as an upstream activator of klf2a, which is vital for the progression of hyperglycemia-induced cardiomyopathy of adult zebrafish. In summary, these results showed that apoptosis and the AMPK pathway was included in DCM, and the AMPK–p53–klf2a pathway might be considered as a suitable method for progression, development and treatment of DCM.

We might be able to propose that intervention with AMPK activators can increase AMPK phosphorylation, which down-regulates p53, then upregulates klf2a, thereby reducing apoptosis, and improving ventricular remodeling and cardiac dysfunction. The limitations of our research are as follows: the therapeutic benefit of AMPK was built on a single compound A-769662, and only one experiment was carried out on apoptosis, which should be further studied in the future.

In conclusion, a DCM model was successfully established. A new molecular pathway of the AMPK–p53–klf2a pathway plays an important role in hyperglycemia-induced cardiac remodeling in adult zebrafish, which might be regarded as a potential method to treat DCM.

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DISCLOSURE
The authors declare no conflict of interest.

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