

Cardio-protective effects of VCP modulator KUS121 in murine and porcine models of myocardial infarction

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Competing interests: In relation to this manuscript, Kyoto University has applied for a patent (Tokugan 2018-078272), and Y.I., T.H., N.S., T.K., A.K., and K.O. are named as inventors on the patent. The other authors declare no competing interests.

Abstract

Background: No effective treatment has yet been made available to enhance early reperfusion therapy for acute myocardial infarction (MI) using primary percutaneous coronary intervention (PCI). Kyoto University Substance 121 (KUS121) was developed to selectively inhibit the ATPase activity of valosin-containing protein (VCP). KUS121 can prevent cardiac cell death by maintaining cellular ATP levels and reducing endoplasmic reticulum (ER) stress.

Objectives: The purpose of our study was to investigate the potential cardioprotective effects of KUS121 on ischemic heart disease.

Methods: H9C2 rat cardiomyoblast cells were cultured with tunicamycin, hydrogen peroxide, or in glucose-free medium and treated with KUS121, and the *in vitro* effects of KUS121 on the cells were evaluated. We created murine and porcine cardiac ischemia and reperfusion (I/R) injury models and then investigated the cardio-protective effects of KUS121 *in vivo*.

Results: We found that KUS121 protected H9C2 rat cardiomyoblasts from cell death by reducing ER stress, preserving ATP levels, and maintaining mitochondrial function. We revealed that KUS121 treatment was able to reduce the myocardial infarct size by administration even after reperfusion in murine I/R injury models. This improvement was also associated with reductions in ER stress and the maintenance of ATP levels, which indicated that KUS121 had cardio-protective effects *in vivo* by same mechanisms as in *in vitro*. We also confirmed in porcine I/R injury models that intracoronary administration of KUS121 attenuated the infarcted area in a dose-dependent manner.

Conclusions: Our study demonstrated the efficacy of KUS121 as a promising novel therapeutic agent for MI in conjunction with primary PCI.

Highlights

- Kyoto University Substance 121 (KUS121) was developed to selectively inhibit the ATPase activity of VCP without affecting other cellular functions of VCP.
- KUS121 preserved ATP levels, reduced ER stress, and suppressed cell death in H9C2 rat cardiomyoblast cells, treated with tunicamycin or hydrogen peroxide, or cultured in glucose-free medium.
- In murine ischemia and reperfusion (I/R) injury models, KUS121 treatment after reperfusion attenuated the infarcted size and preserves cardiac function by maintaining ATP levels and reducing ER stress.

- In porcine I/R injury models, intracoronary administration of KUS121 also attenuated the infarcted area in a dose-dependent manner.
- These results indicated that KUS121 is a promising novel therapeutic agent for myocardial infarction.

Condensed Abstract

No effective treatment is yet available to reduce infarct size and improve clinical outcomes after acute myocardial infarction (MI) by enhancing early reperfusion therapy using primary percutaneous coronary intervention (PCI). Our study showed that Kyoto University Substance 121 (KUS121) reduced ER stress, maintained ATP levels, and ameliorated the infarct size in a murine cardiac ischemia and reperfusion (I/R) injury model. We confirmed the cardio-protective effect of KUS121 in a porcine I/R injury model. These findings confirmed that KUS121 is a promising novel therapeutic agent for MI in conjunction with primary PCI.

Keywords: myocardial infarction, ATP, ER stress, KUS121

Abbreviations and acronyms

AAR, area at risk; BiP, immunoglobulin heavy chain-binding protein; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; I/R, ischemia and reperfusion; IBMPFD, inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia; KUS121, Kyoto University Substance 121; OCR, oxygen consumption rate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; VCP, valosin-containing protein.

Introduction

Ischemic heart disease (IHD) is a leading cause of death worldwide. Of the 17.5 million cardiovascular deaths in 2012, an estimated 7.4 million deaths were due to IHD (1). In the United States, more than 360,000 people died from IHD in 2015, and IHD accounted for 43.8% of deaths from cardiovascular disease (2). In Japan, IHD accounted for 35.6% of deaths from heart disease in 2016 (3).

In patients with acute myocardial infarction (MI), early reperfusion therapy using primary percutaneous coronary intervention (PCI) is performed to reduce the infarct size and improve outcomes (4). However, when a larger infarct size remains after primary PCI, the rates of all-cause mortality and hospitalization for heart failure (HF) are still high (5). High mortality from HF and rehospitalization for HF are becoming serious concerns from both healthcare and medical cost perspectives (2,6).

To further reduce infarct sizes and improve clinical outcomes, new treatments in addition to early reperfusion therapy are needed. Indeed, drugs such as an inhibitor of Na^+/H^+ exchanger (NHE) and cyclosporine A were reported to have cardioprotective effects in animal experiments (7) or in small clinical trials (8). However, these effects were not confirmed in multicenter, randomized, double-blind clinical trials (9,10). Although many other clinical trials of novel therapies for acute MI are ongoing, there are currently no therapeutic agents available to reduce infarct size and improve clinical outcomes (11).

Valosin-containing protein (VCP) is a member of the ATPase associated with diverse cellular activities family, and it is expressed ubiquitously in almost all cell types. As reported previously, in addition to ATPase activity, VCP is involved in various cellular functions, including proteasome-mediated protein degradation, endoplasmic reticulum (ER)-associated

degradation, lysosomal protein degradation, autophagy, cell cycle progression, membrane fusion, etc. (12). Gain-of-function mutations in VCP have been reported to cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) (13), and IBMPFD-causing mutations in VCP result in elevated ATPase activity (14). The major clinical phenotypes of IBMPFD are myopathy, bone lesions, and dementia, but it is notable that cardiac phenotypes, such as dilated cardiomyopathy, are also manifested in certain IBMPFD patients (15).

Kyoto University Substance 121 (KUS121) was developed to selectively inhibit the ATPase activity of VCP without affecting other cellular functions of VCP. Indeed, KUS121 has been shown to maintain cellular ATP levels, reduce ER stress, and prevent cell death *in vitro* without showing any toxic effects (16). KUS121 has also been shown to elicit neuroprotective effects in murine retinitis pigmentosa models, murine glaucoma models, rat retinal ischemic injury models, and murine Parkinson's disease models (16–19).

Considering the cardiac phenotype in IBMPFD patients and the neuroprotective effect of KUS121 *in vivo*, we anticipated that KUS121 may produce a cardioprotective effect in IHD. In this study, we found that KUS121 preserved ATP levels, reduced ER stress, and suppressed cell death in H9C2 rat cardiomyoblast cells. Furthermore, in murine and porcine ischemia and reperfusion (I/R) injury models, KUS121 ameliorated cardiac damage and preserved cardiac function. These results indicated that KUS121 is a promising novel therapeutic agent for MI.

Methods

This study was approved by the Kyoto University Ethics Review Board. Additional detailed methods are available in the Supplemental materials.

Ischemia and reperfusion injury models in mice

In 8-week-old mice, the left anterior descending (LAD) coronary artery was ligated with a PE-10 tube. After 45 min of ischemia, reperfusion was induced by untying the knot and removing the tube. At 7 days after reperfusion, Masson's trichrome staining was performed to evaluate the infarcted area.

For quantification of heart ATP levels, I/R injury protocols using GO-ATeam2 mice were performed. ATeam biosensors are a series of fluorescence resonance energy transfer (FRET)-based indicators for ATP, which are able to estimate relative ATP levels in live cells in real-time (20). Go-ATeam2 mice were developed by genetically integrating the GO-ATeam expression cassette into mice (M.Y. et al., manuscript in preparation) (19); thus, the *in vivo* orange fluorescent protein (OFP)/green fluorescent protein (GFP) FRET ratio depends on the relative cellular ATP levels (21).

Analysis in murine IR injury models was performed by an experimenter who was blinded to treatment groups.

Ischemia and reperfusion injury models in pigs

In 3-month-old pigs, the LAD coronary artery was occluded using a 3.0–20 mm balloon (Terumo, Japan). After 60 min of occlusion, reperfusion was induced by deflation of the balloon.

At 7 days after reperfusion, to evaluate the infarcted area, gadolinium enhanced cardiac magnetic resonance imaging (MRI) and double staining with triphenyltetrazolium chloride (TTC) and Evans blue were performed, and analyzed by an experimenter who was blinded to treatment groups.

Statistical analysis

Measured data are presented as mean $\pm$ standard error of the mean (SEM). For statistical comparisons between two groups, unpaired Student's t-test was used. For statistical analysis of three or more groups, one-way analysis of variance (ANOVA) was used. In one-way ANOVA, Sidak's post-hoc test was performed to compare all pairs of groups (Fig. 3b–f and 5b–e, and Supplementary Fig. 4b–e) and Dunnett's post-hoc test to compare one group as a control to the other groups (Fig. 2b–e and h, and 8c, d and f). A p-value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

Results

VCP mRNA is expressed in human and murine hearts, especially in cardiomyocytes

We first analyzed the expression levels of VCP mRNAs in various human organs (Fig. 1a) and mice (Fig. 1b). VCP expression levels in the human heart were comparable to that in central nervous tissues. VCP expression was also confirmed in the mouse heart and its expression in cardiomyocytes was 2-fold higher than in fibroblasts (Fig. 1c).

KUS121 preserves ATP levels, reduces ER stress, and suppresses cell death in cultured H9C2 cells

To examine the cell protective effect of KUS121 on cardiomyocytes, H9C2 rat cardiomyoblast cells were treated with tunicamycin or cultured in glucose-free medium to induce cell death. KUS121 suppressed cell death and maintained ATP levels in tunicamycin-treated H9C2 cells in a dose-dependent manner (Fig. 2a–c). KUS121 also preserved ATP levels and protected H9C2 cells against cell death when the cells were cultured in glucose-free medium (Fig. 2d and e). KUS121 reduced ER stress, which was determined by the reduction in C/EBP homologous protein (CHOP) and immunoglobulin heavy chain-binding protein (BiP) levels in these conditions, without changing VCP expression levels (Fig. 2f and g). In addition, KUS121 reduced hydrogen peroxide (H₂O₂)-induced H9C2 cell death (Fig. 2h). KUS121 itself did not affect cell growth, cellular ATP levels, or CHOP, BiP, or VCP expression levels (Supplementary Fig. 1a–c) in normal culture conditions.

KUS121 preserves mitochondrial function after tunicamycin treatment

It is known that ER stress and ischemia affect mitochondrial functions (22–24); therefore,

we examined mitochondrial functions using an XF96 extracellular flux analyzer. H9C2 cells were treated with tunicamycin in the absence or presence of KUS121 for 6 hours, and the oxygen consumption rate (OCR) was measured (Fig. 3a). The numbers of H9C2 cells in these conditions were similar to that in normal culture (Supplementary Fig. 2a). The parameters of mitochondrial respiration, such as basal respiration, ATP production-linked respiration, maximal respiration, spare respiratory capacity, and proton leak were calculated, as shown in Supplementary Fig. 2b. The parameters of mitochondrial respiration were lower in H9C2 cells treated with tunicamycin than in control cells (Fig. 3b–f). However, the parameters of mitochondrial respiration were preserved in tunicamycin and KUS121-treated cells, similar to those in normal cells without tunicamycin treatment. KUS121 also significantly increased mitochondrial respiration in normal culture conditions (Supplementary Fig. 2c–h). Thus, the protective effects of KUS121 against H9C2 cell death are most likely mediated by ATP preservation, ER stress reduction, and conservation of mitochondrial functions.

KUS121 pretreatment attenuates cardiac damage and preserves cardiac function in murine ischemia and reperfusion injury models

Based on the *in vitro* cell protective effects on H9C2 cardiomyoblast cells, we next investigated whether KUS121 had protective effects on ischemic hearts by use of a murine I/R injury model. KUS121 was injected intraperitoneally at a dose of 160 mg/kg, which was followed by LAD coronary artery occlusion for 45 min (Supplementary Fig. 3a). Subsequently, KUS121 was re-injected at the same dose once every 24 hours for 6 days after reperfusion. Using Masson's trichrome staining at 7 days after reperfusion, the infarcted area/left ventricle (LV) area ratios in KUS121-treated mice was significantly lower than those in non-treated mice

(“control mice” hereafter) (Supplementary Fig. 3b and c). We also measured the infarcted area/area at risk (AAR) and AAR/LV by double staining with TTC and Evans blue (Supplementary Fig. 3d). The infarcted area/AAR ratios of KUS121-treated mice were significantly lower than those in control mice, whereas the AAR/LV ratios were indistinguishable, compared with control mice (Supplementary Fig. 3e and f). The expression of CHOP was significantly reduced in the border and remote zone, but not in the ischemic zone (Supplementary Fig. 3g).

By echocardiographic analysis, both ejection fraction (EF) and fractional shortening (FS) in mice with I/R injury without KUS121 were reduced, compared to sham-operated mice, at 7 days after reperfusion. However, the systolic function of KUS121-treated mice was preserved at almost the same level as that in sham-operated mice (Supplementary Fig. 4a–e). We also performed serial echocardiography at 1, 3, 7, 14, and 28 days after reperfusion (Supplementary Fig. 3a). EF, FS, and LV systolic diameters were significantly preserved throughout the time course in KUS121-treated I/R mice, compared with I/R control mice (Supplementary Fig. 4f–i).

KUS121 treatment after reperfusion also attenuates the infarcted size and preserves cardiac function

Next, we examined whether KUS121 could produce therapeutic benefits even when administered after I/R injury. Immediately after reperfusion, 80 mg/kg of KUS121 was injected intravenously, and the same amount was administered intraperitoneally (Fig. 4a). Subsequently, KUS121 was repeatedly injected intraperitoneally at a dose of 160 mg/kg daily until 4 days after reperfusion. As a result, the infarcted area/LV area ratios were significantly reduced in

KUS121-treated mice at 7 days after reperfusion compared with control mice (Fig. 4b and c). The expression of CHOP was significantly reduced in the border zone (Fig. 4d). In addition, to investigate the protective effects of KUS121 on cardiomyocyte apoptosis induced by I/R injury, we performed terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays. The rate of apoptotic cardiomyocytes, defined as TUNEL-positive nuclei surrounded by troponin I, was significantly lower in KUS121-treated mice compared with control mice (Fig. 4e and f).

Echocardiographic analysis at 7 days after reperfusion showed that both the EF and FS of KUS121-treated mice were better preserved than those of control mice (Fig. 5a–e). Similar results were obtained in serial echocardiographic analyses of cardiac functions (Fig. 4a and 5f–i).

KUS121 maintains ATP levels in ischemia and reperfusion injury models

To investigate whether KUS121 preserved ATP levels *in vivo*, we created I/R injury models using GO-ATeam2 mice. In the images, high FRET ratios (i.e. higher ATP levels) were pseudo-colored using warmer colors and low FRET ratios (i.e. lower ATP levels) using cooler colors (Supplementary Fig. 5a). ATP levels in infarcted areas of KUS121-treated mice were significantly higher from 0 to 60 min after reperfusion (Supplementary Fig. 5a and b) than those of control I/R mice, when KUS121 was administered before ischemia, as shown in Supplementary Fig. 3a. In non-infarcted areas of the LV, ATP levels in KUS121-treated mice were also higher than those of control I/R mice (Supplementary Fig. 5c). Although ATP levels in the right ventricle (RV) of KUS121-treated mice were similar to those of control mice (Supplementary Fig. 5d), the relative ATP ratio in the infarcted area to that in the RV was higher

in KUS121-treated mice than in control mice (Supplementary Fig. 5e).

Next, we examined whether KUS121 administration after reperfusion could also preserve ATP levels. As shown in Fig. 4a, we administered the same amount of KUS121 immediately after reperfusion in I/R injury models of GO-ATeam2 mice. ATP levels in infarcted areas of KUS121-treated mice were the same as those of control mice during ischemia. However, ATP levels of KUS121-treated mice recovered immediately and significantly after KUS121 administration compared with control mice (Fig. 6a and b). These data indicate that KUS121 was able to preserve ATP levels in I/R models *in vivo*.

Beneficial effects are observed with a single administration of KUS121 after reperfusion

We further examined whether a single KUS121 administration after reperfusion could benefit the infarcted area or not. As shown in Fig. 7a, 25 mg/kg of KUS121 was injected intravenously and the same amount was injected intraperitoneally immediately after reperfusion. The infarcted area/LV area ratio was significantly reduced in KUS121-treated mice at 7 days after reperfusion compared with control mice (Fig. 7b and c). However, a dose of 16 mg/kg KUS121 (Supplementary Fig. 6a), failed to produce a beneficial effect (Supplementary Fig. 6b and c).

KUS121 attenuates the infarcted area in porcine ischemia and reperfusion injury models

Finally, we examined whether KUS121 could produce therapeutic effects in porcine I/R injury models. Reperfusion was induced after 60 min of endovascular LAD coronary artery occlusion, and KUS121 was administered by intracoronary injection at a dose of 0.64, 2.5, or 5.0 mg/kg for 3 minutes through the wire lumen of a balloon catheter immediately after

reperfusion (Fig. 8a). Using double-staining with TTC and Evans blue, the infarcted area/AAR ratios of KUS121-treated pigs were found to be significantly lower than those of the control pigs in a dose-dependent manner, although the AAR/LV ratios of KUS121-treated pigs were the same as those of control pigs (Fig. 8b–d). In addition, we confirmed that the appearance of infarcted areas, as determined using Masson's trichrome staining, was almost the same as that evaluated using TTC staining (Supplementary Fig. 7a and b). We also evaluated the infarcted area by late gadolinium enhancement with cardiac MRI (Fig. 8e). The infarcted area/LV area ratios in KUS121-treated pigs were also significantly lower than those of control pigs (Fig. 8f). These data demonstrate that KUS121 intracoronary administration provides significant benefits in the presence of cardiac damage in porcine I/R injury models.

Discussion

In this study, we investigated the cardio-protective effects of KUS121 using murine I/R injury models that mimic reperfusion therapy for MI, and we showed that KUS121 administration even after reperfusion was able to maintain ATP levels and attenuate the infarcted area. Echocardiographic evaluation revealed that KUS121 administration preserved cardiac function at levels similar to those of normal mice. Notably, similar beneficial effects of KUS121 were also confirmed in a porcine I/R injury model using a single administration into the coronary artery in a dose-dependent manner.

This study demonstrated that KUS121 had cardio-protective effects *in vivo* by the same mechanisms as in *in vitro* analyses. Additionally, we also confirmed that the acute cardio-protective effects of KUS121 within 24 hours after IR injury were responsible for reducing the infarct size. Using Go-ATeam2 mice, we found that KUS121 treatment even after reperfusion maintained ATP levels in an I/R injury model, most likely due to the inhibition of ATP consumption by VCP and/or preservation of mitochondrial function observed in H9C2 cells. As reported previously (25), we examined the expression of CHOP in I/R injury and demonstrated that KUS121 treatment after reperfusion significantly reduced CHOP expression in border zone. This also suggested that KUS121 reduced ER stress *in vivo* and rescued the injured myocardium from cell death. KUS121 treatment also reduced cardiomyocyte apoptosis induced by I/R injury. In our *in vitro* experiment, KUS121 protected H9C2 cells from H₂O₂-induced cell death. As reported previously (26), H₂O₂ is considered to induce necrotic cell death rather than apoptotic cell death. Moreover, ER stress was also reported to induce necrotic cell death (27). This indicates that KUS 121 may protect cardiomyocytes from necrotic cell death by reducing ER stress. Thus, KUS121 is presumed to attenuate the infarct size by

reducing both apoptotic and necrotic cell death.

KUS121 was developed to selectively inhibit the ATPase activity of VCP without affecting its other cellular functions, and previous studies demonstrated that KUS121 can maintain cellular ATP levels, reduce ER stress, and prevent cell death *in vitro* when challenged with many cell-death-inducing insults in many cell types (16–19). Consistent with the ability of KUS121 to maintain ATP levels and reduce ER stress, KUS121 has been reported to have neuroprotective effects *in vivo*, e.g. in models of murine retinitis pigmentosa, murine glaucoma, rat retinal ischemic injury, and murine Parkinson's disease (16–19). In this study, we further demonstrated the close link between ER stress, decreased ATP levels, and cell death in the heart.

To the best of our knowledge, the cardio-protective mechanisms of KUS121 are quite different from other drugs that have been tested for the treatment of MI (7,28–30), making KUS121 virtually unique in its ability to maintain ATP levels in animal I/R injury models. Recently, LV mechanical support using Impella was reported to reduce infarct size after I/R injury (31). This was possibly due to a reduction in excessive myocardial energy demand relative to supply, which would be similar in principle to KUS121 administration in our *in vivo* models.

It is especially noteworthy that KUS121 is able to reduce infarct size by administration even after reperfusion, especially through intracoronary injection. This can be easily performed after primary PCI in everyday clinical practice. Thus, we concluded that KUS121 is a promising compound that could be used in conjunction with primary PCI for the treatment of MI.

Study limitations

We showed that KUS121 reduced the infarct size in murine and porcine I/R injury models by maintaining ATP levels and reducing ER stress, but the detailed mechanism of the link between ER stress, decreased ATP levels, and cell death remains to be elucidated. Further investigations are needed to fully explore the complex signaling mechanisms that are at play during MI and reperfusion.

In porcine models, we evaluated the infarct size only at 7 days after reperfusion, but we did not evaluate the effect of KUS121 at later time points. Moreover, we did not confirm the detailed safety of KUS121, although no obvious toxicity of KUS121 was observed in our study. We need to perform further studies with long-term follow-up to confirm the cardiac protective efficacy and safety of KUS121.

As previously reported (16,32), KUS121 was confirmed to inhibit the ATPase activity of recombinant VCP *in vitro* with half maximal inhibitory concentration (IC₅₀) value of 330 nM, which is much lower than that of a VCP inhibitor, DBcQ (1 μ M). However, we did not evaluate the specificity and off-target effects of KUS 121 *in vivo*. Further studies are need to elucidate them *in vivo* by analysis of the pharmacokinetics and pharmacodynamics of KUS121.

Conclusions

Here, we have shown that in I/R injury models, KUS121 reduced infarct size and preserved cardiac function by maintaining ATP levels and reducing ER stress. We also showed that this effect can be achieved by the administration of KUS121 only once after reperfusion, which was confirmed in porcine I/R models. Our study indicates that KUS121 is a promising therapeutic agent for MI in conjunction with primary PCI. Progression of studies leading to a clinical trial of KUS121 is expected in the near future.

Perspectives

Competency in Medical Knowledge

Although primary PCI is the only therapy for MI to reduce infarct size and improve outcomes, a larger infarction, even after primary PCI, results in a poorer prognosis. However, there are currently no therapies additional to primary PCI to further reduce infarct size and improve clinical outcomes. Our study demonstrated that KUS121 attenuated the infarct size in murine and porcine I/R injury models. These results indicate that KUS121 may be a novel therapy for MI in conjunction with primary PCI.

Translational Outlook

We expect that KUS121 will one day be used in clinical practice. However, further studies are needed to investigate the pharmacokinetics and pharmacodynamics of KUS121, especially those after intracoronary KUS121 administration. Additionally, we need to verify the detailed safety of KUS121. If these are confirmed, the cardio-protective effects of KUS121 may be evaluated in clinical trials.

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Figure legends

Visual Abstract: KUS121 has cardio-protective effects *in vitro* and in animal ischemic heart disease models.

Figure 1. VCP is expressed in human and murine hearts, especially in cardiomyocytes.

(a, b) Expression levels of VCP in various human organs (a) and mouse organs (n=4; b). The human samples were from various sources, pooled from one or more healthy adults (Human Total RNA Master Panel II, Clontech Laboratories, Inc). Expression of β -actin was used as an internal control. Black bars indicate expression in hearts. (c) Comparison of VCP expression in cardiomyocytes (n=4) with that in fibroblasts (n=4). ***P<0.001, using unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM. BAT, brown adipose tissue; WAT, white adipose tissue.

Figure 2. KUS121 preserves ATP levels, reduces endoplasmic reticulum stress, and suppresses cell death in cultured cells.

(a) Representative images of H9C2 rat cardiomyoblast cells cultured with tunicamycin (Tm, 0.1 μ g/ml) for 48 hours with different concentrations of KUS121 (50, 100, and 200 μ M). (b–e) Number and ATP levels of H9C2 cells, cultured with Tm (0.1 μ g/ml) for 48 hours (b, c), or cultured in glucose-free medium for 48 hours (d, e), with different concentrations of KUS121 (25, 50, 100, and 200 μ M, n=3). **P<0.01 vs Tm (0.1 μ g/ml) without KUS121 (b, c), **P<0.01, ***P<0.001 vs glucose-free without KUS121 (d, e) using one-way ANOVA with Dunnett's post-hoc test. (f, g), Western blotting analysis of H9C2 cells, cultured with Tm (0.1 μ g/ml) for 12 hours (f), or cultured in glucose-free medium for 24 hours (g), with and without KUS121

(200 μ M, n=3). (h) Relative viability of H9C2 cells treated with hydrogen peroxide (H_2O_2 , 750 μ M) for 24 hours with different concentrations of KUS121 (25, 50, and 100 μ M, n=7). Viability of cells in normal culture conditions was the reference, indicated as 1. * P <0.05, ** P <0.01, *** P <0.001 vs H_2O_2 (750 μ M) without KUS121 using one-way ANOVA with Dunnett's post-hoc test. All data are presented as mean $\pm$ SEM.

Figure 3. KUS121 preserves mitochondrial function after tunicamycin treatment.

(a) Oxygen consumption rate (OCR) measured using an XF96 extracellular flux analyzer in H9C2 cells treated with tunicamycin (0.2 μ g/ml) and KUS121 (200 μ M). After basal OCR was measured, oligomycin (2 μ M), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (1 μ M), and a mix of rotenone (1 μ M) and antimycin A (1 μ M), were added sequentially to assess mitochondrial respiration. OCR at each time point was obtained from an average of 10 replicate wells and presented as mean $\pm$ SEM. (b–f) Parameters of mitochondrial respiration: basal respiration (b), ATP production-linked respiration (c), maximal respiration (d), spare respiratory capacity (e), and proton leak (f). ** P <0.01, *** P <0.001, using one-way ANOVA with Sidak's post-hoc test. Data are presented as mean $\pm$ SEM.

Figure 4. KUS121 treatment after reperfusion attenuates infarct size and reduces cardiomyocyte apoptosis.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures and schedules of KUS121 administration. (b) Representative images of Masson's trichrome staining in the short axis of left ventricles (LVs) at 7 days after I/R injury. Black bars indicate 1000 μ m. (c) Quantification of infarcted area in LVs at 7 days after I/R injury (Control, n=7; KUS121, n=10).

*P<0.05, using unpaired two-tailed Student's t-test. (d) Expression levels of C/EBP homologous protein (CHOP) in LVs at 1 hour after I/R injury. LVs were divided into three parts: ischemic zone, border zone, and remote zone (Sham, n=6; Control, n=6; KUS121, n=8). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. *P<0.05, using unpaired two-tailed Student's t-test. (e) Representative images of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining in LVs at 4 hours after reperfusion. White bars indicate 100 μ m. (f) Quantification of apoptotic cardiomyocytes, defined as TUNEL-positive cells surrounded by troponin I (Control, n=6; KUS121, n=6). *P<0.05, using unpaired two-tailed Student's t-test. All data are presented as mean $\pm$ SEM.

Figure 5. KUS121 treatment after reperfusion preserves cardiac function in ischemia and reperfusion injury models.

(a) Representative images of M-mode echocardiogram of Control and KUS121-treated animals at 1 week after ischemia and reperfusion (I/R) injury. (b–e) Echocardiographic data 1 week after I/R injury. Ejection fraction (EF; b), fractional shortening (FS; c), left ventricle (LV) diastolic diameter (LVDd; d), and LV systolic diameter (LVDs; e) were measured (Sham, n=4; Control, n=6; KUS121, n=6). *P<0.05, **P<0.01, ***P<0.001, using one-way ANOVA with Sidak's post-hoc test. (f-i) Echocardiographic data at the indicated time points after I/R injury. *P<0.05, using unpaired two-tailed Student's t-test. All data are presented as mean $\pm$ SEM.

Figure 6. KUS121 treatment after reperfusion also maintains ATP levels in ischemia and reperfusion injury models.

(a) Representative pseudocolor ratiometric fluorescence resonance energy transfer (FRET) images of whole hearts in ischemia and reperfusion injury models of Go-ATeam2 mice. Pseudocolor images were obtained at various time points in ischemia and after reperfusion. In pseudocolor images, warmer colors represent high FRET ratios and cooler colors low FRET ratios. (b) Quantification of ATP levels in the infarcted area by FRET ratio (Control, n=7; KUS121, n=8). *P<0.05, using unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.

Figure 7. Beneficial effect of a single administration of KUS121 after reperfusion.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures and schedules of KUS121 administration. (b) Representative images of Masson's trichrome staining in the short axis of left ventricles (LVs) at 7 days after I/R injury. Black bars indicate 1000 μ m. (c) Quantification of infarcted area in LVs at 7 days after I/R injury (Control, n=14; KUS121, n=15). *P<0.05, using unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.

Figure 8. KUS121 attenuates infarcted areas in porcine ischemia and reperfusion injury models.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures in pigs. (b) Representative images of double-staining with triphenyltetrazolium chloride (TTC) and Evans blue in the short axis of left ventricles (LVs) at 7 days after reperfusion. (c, d) Quantification of infarcted area/area at risk (AAR) and AAR/LV in double-staining with TTC and Evans blue (Control, n=7; KUS121 at 0.64 mg/kg, n=4; at 2.5 mg/kg, n=5; at 5.0 mg/kg, n=5). *P<0.05, **P<0.01 vs Control, using one-way ANOVA with Dunnett's post-hoc test. (e) Representative

images of late gadolinium enhancement on cardiac magnetic resonance imaging (MRI) at 7 days after reperfusion. (f) Quantification of the infarcted area/LV using MRI. * $P < 0.05$ vs Control, using one-way ANOVA with Dunnett's post-hoc test. All data are presented as mean $\pm$ SEM.

Figure 1

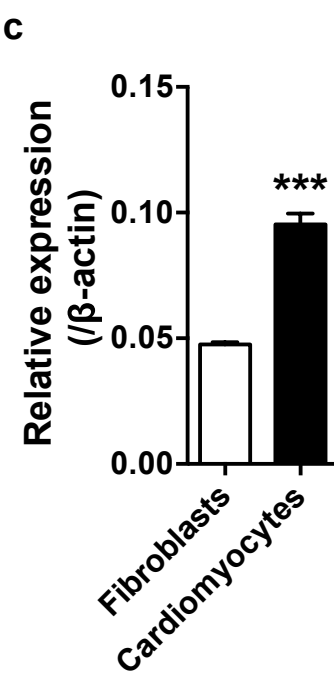
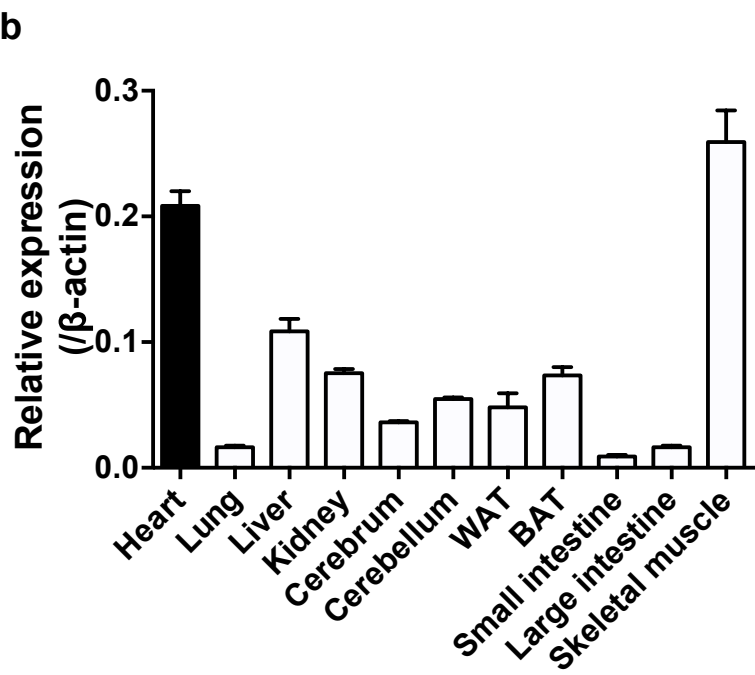
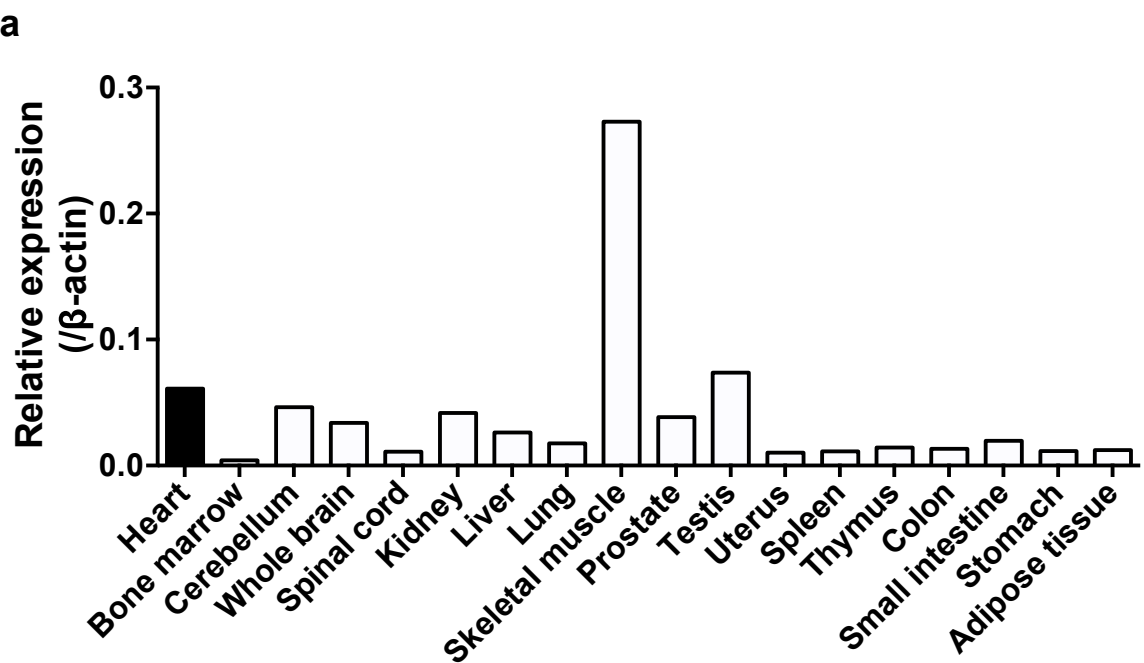
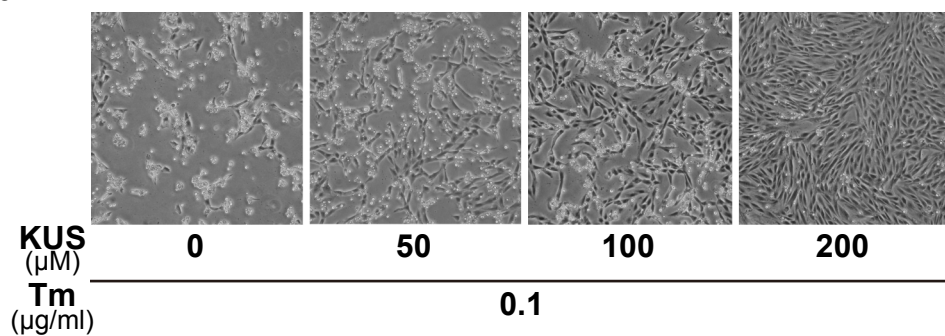
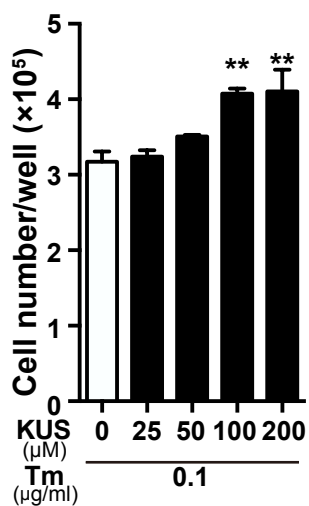


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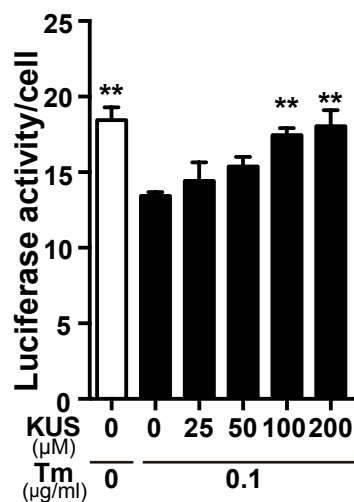
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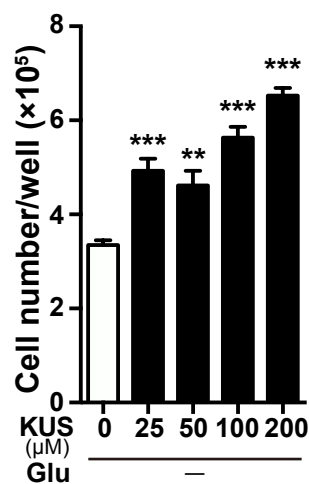
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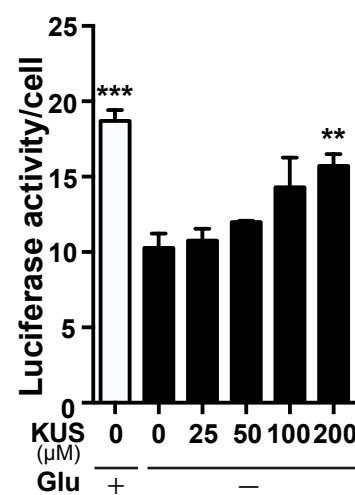
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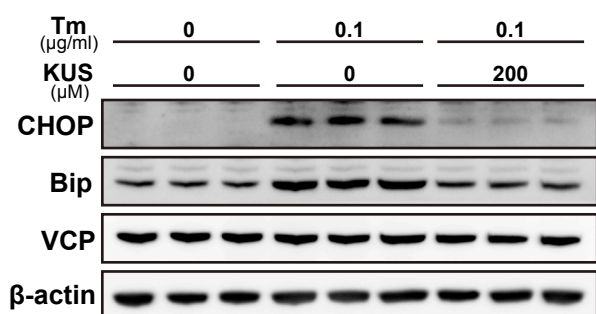
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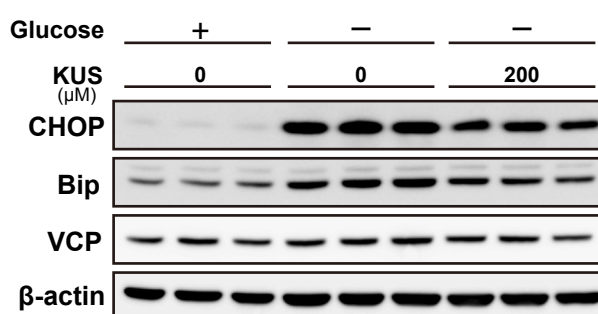
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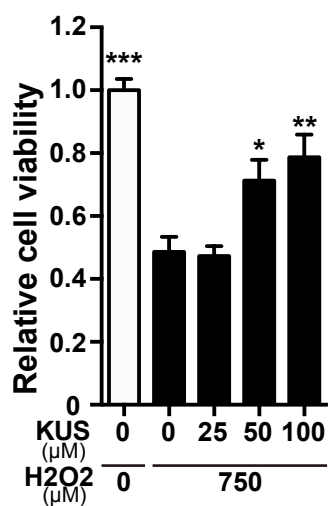


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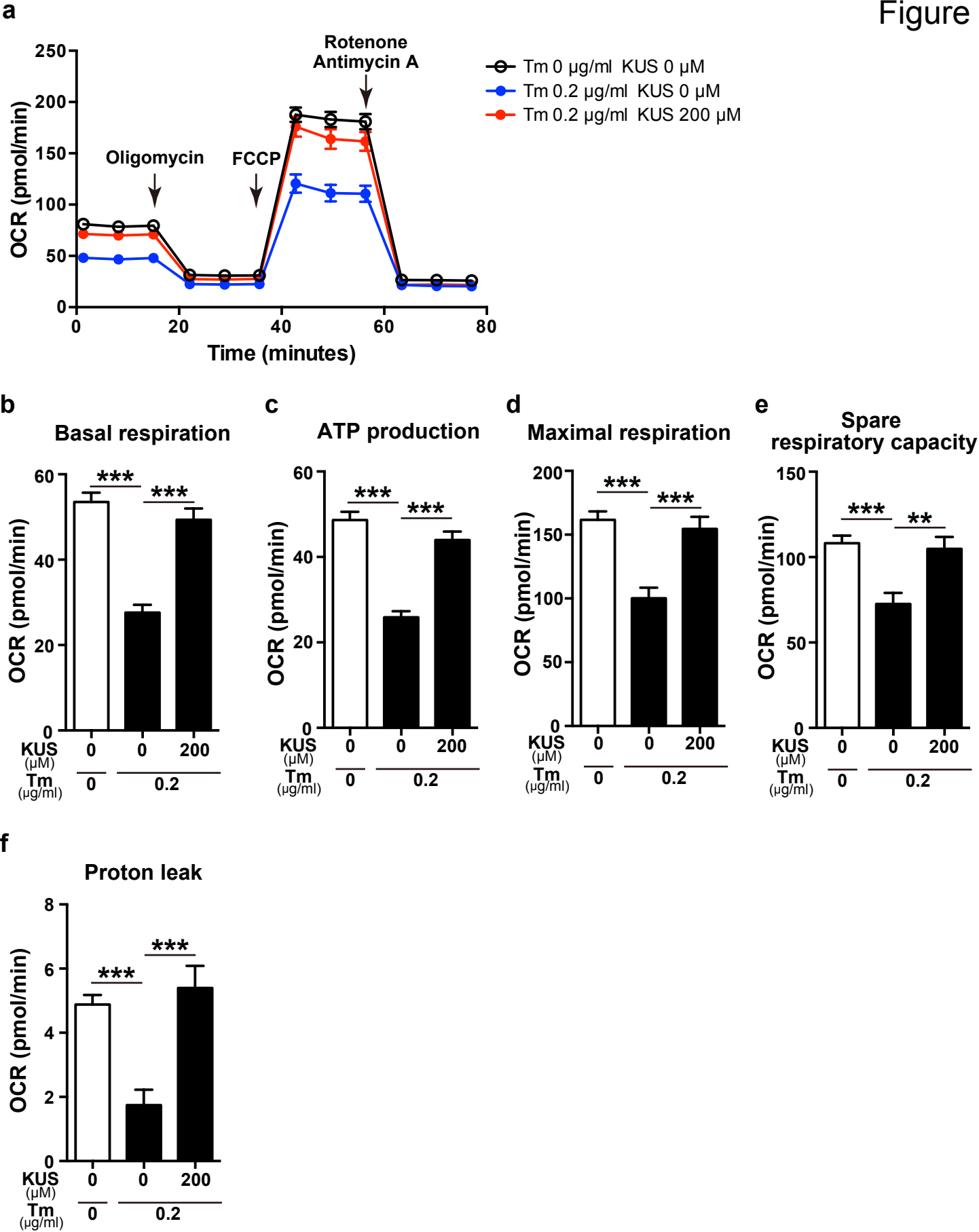


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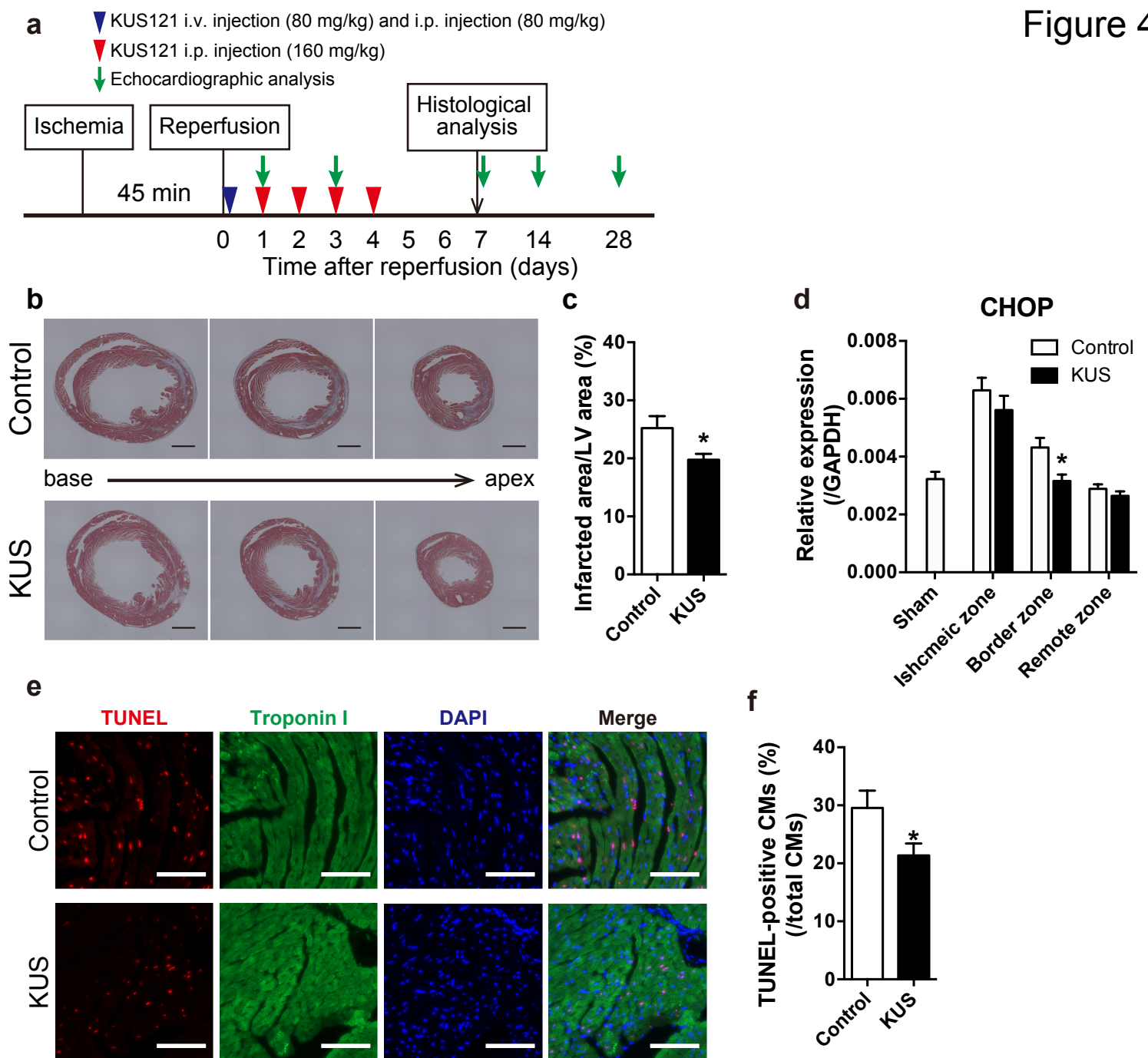


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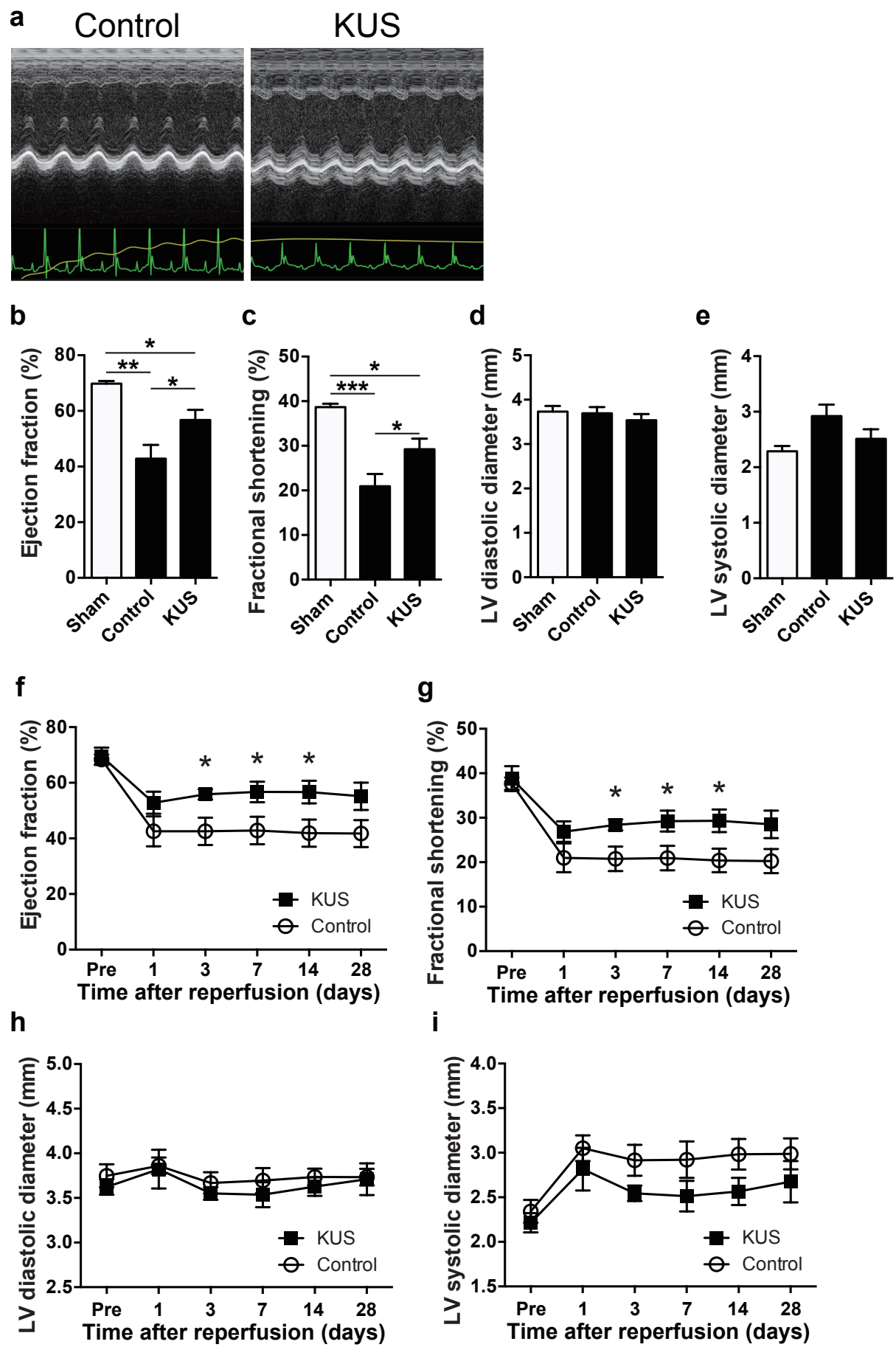


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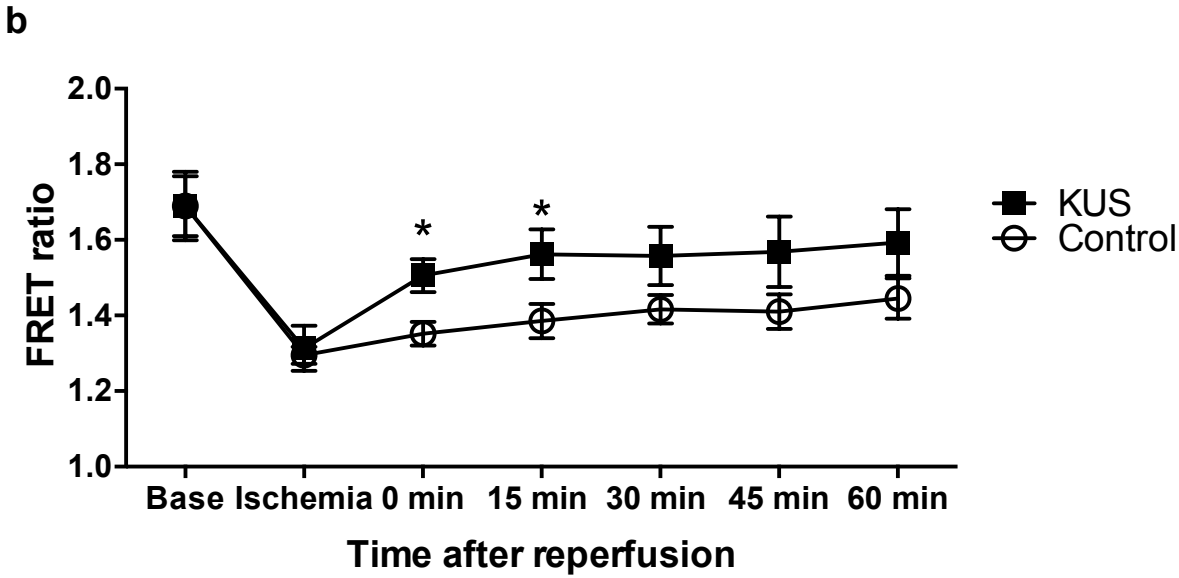
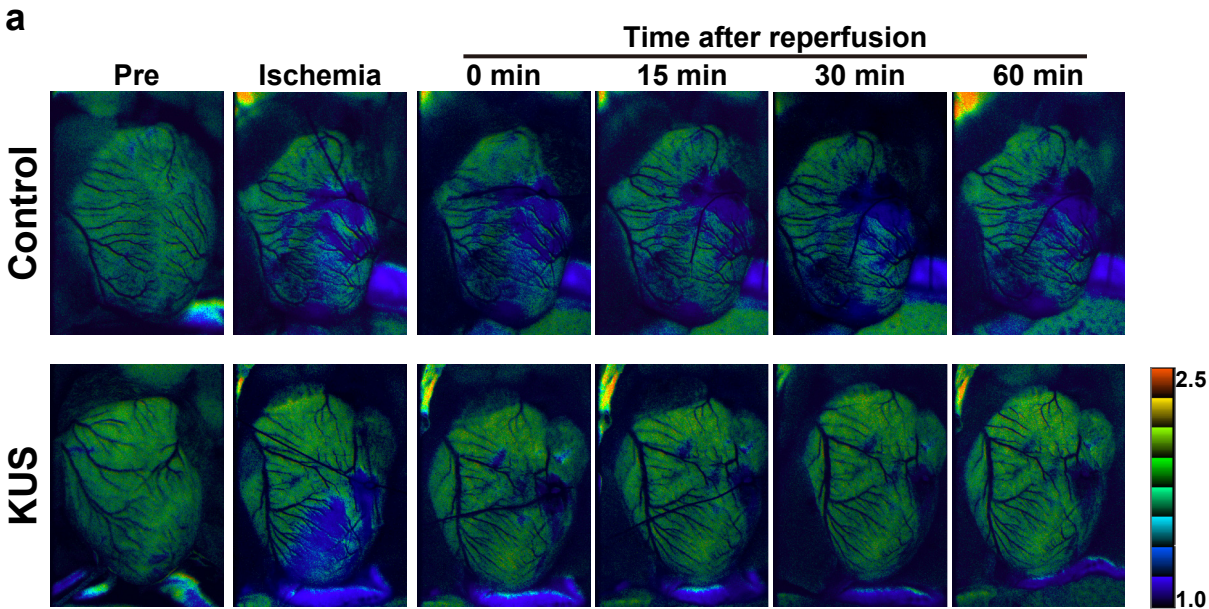


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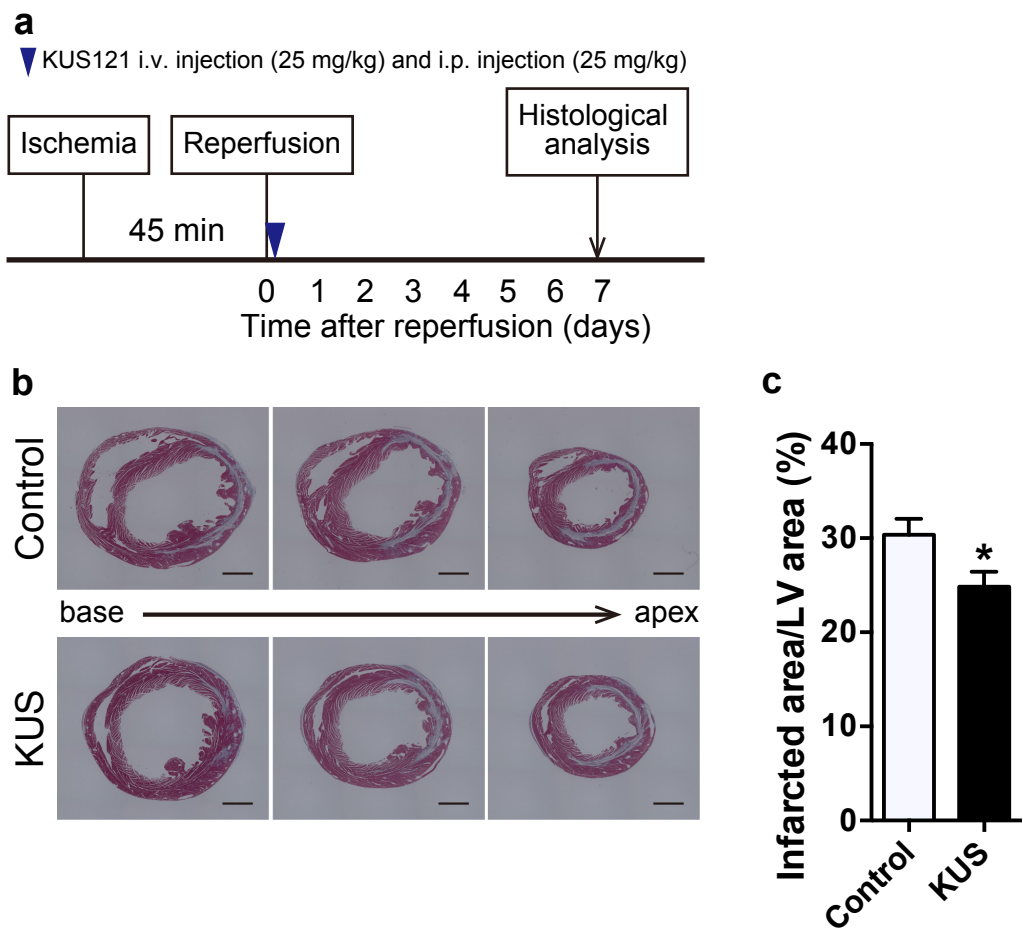
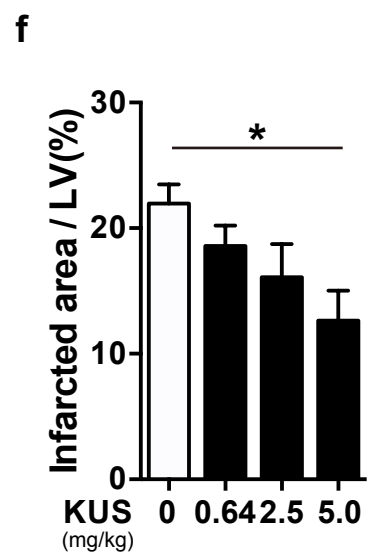
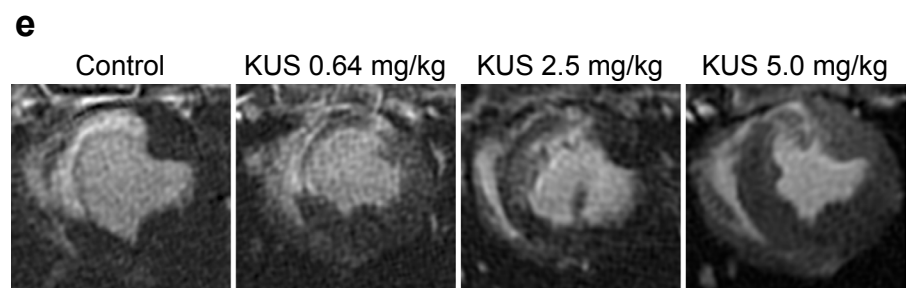
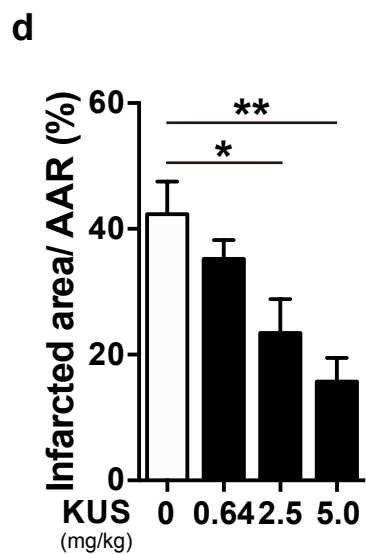
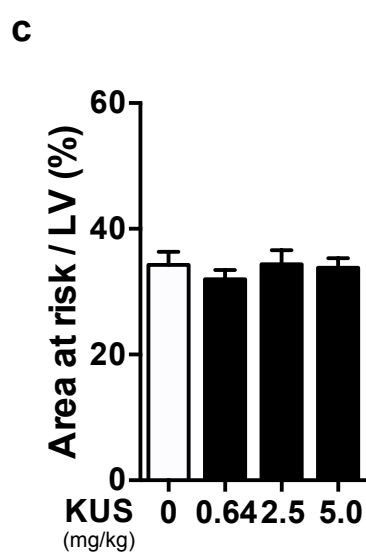
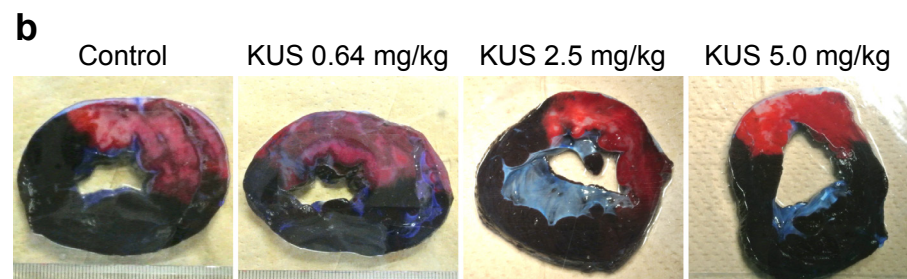
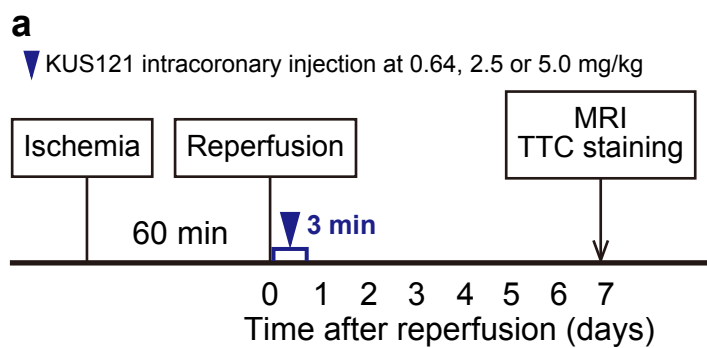


Figure 8



Supplemental Data

Cardio-protective effects of VCP modulator KUS121 in models of myocardial infarction

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Supplemental Methods

Mice

C57BL/6J male mice were purchased from Japan SLC. Mice were maintained in temperature-controlled rooms with a 14:10 h light:dark cycle in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine. This study was approved by the Kyoto University Ethics Review Board.

Cell culture

H9C2 rat cardiomyoblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1% glucose) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco™, 10378016). To induce ER stress, tunicamycin (0.1–0.2 µg/ml, Sigma-Aldrich, T7765) was added to the cultured cells. Cultured cells were counted using Countess II (Thermo Fisher Scientific).

To evaluate cellular ATP levels, an ATP assay reagent for cells (Toyo B-net, Tokyo, Japan) was added to cultured cells, and luciferase activities were measured using a plate reader (ARVO X3, PerkinElmer).

Measurements of mitochondrial respiration using an XF96 Extracellular Flux analyzer

Oxygen consumption rate (OCR) was measured using an XF96 extracellular flux analyzer (Agilent Technologies). First, H9C2 rat cardiomyoblast cells were seeded at a density of 10,000 cells/well and cultured in DMEM supplemented with 5% FBS and antibiotics for 12 hours in XF96-well plates (Agilent Technologies). Then, H9C2 cells were cultured with tunicamycin in the absence or presence of KUS121 for 6 hours. Cells were washed once with assay medium (Agilent Technologies) supplemented with 25 mM glucose (Sigma-Aldrich, G8769) and 1 mM sodium pyruvate (Gibco™, 11360070), and incubated with assay medium for 1 hour in a 37°C in a non-CO₂ incubator. Then, OCR was measured using an XF96 extracellular flux analyzer.

For measurements of mitochondrial respiration, the inhibitors were used as followed: 2 μ M oligomycin (Sigma-Aldrich, 75351), ATP synthase inhibitor; 1 μ M carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich, C2920), uncoupler of mitochondrial oxidative phosphorylation; a mix of 1 μ M rotenone (Sigma-Aldrich, R8875) and 1 μ M antimycin A (Sigma-Aldrich, A8674), complex I and III inhibitor respectively. As shown in Supplementary Fig. 2b, after the basal respiration was measured, oligomycin, FCCP, and a mix of rotenone and antimycin A, were added sequentially to measure ATP production-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Then, proton leak (non-ATP production-linked respiration) and spare respiratory capacity were calculated using the basal respiration and these parameters.

Ischemia and reperfusion injury models in mice

The ischemia and reperfusion (I/R) injury models were as described previously (1).

Briefly, 8-week-old mice were anesthetized with sodium pentobarbital (64.8 mg/kg) administered intraperitoneally. An endotracheal tube was introduced and positive pressure ventilation was provided using a rodent respirator. After mice were fixed in a right lateral position, the thoracic cavity was opened through left thoracotomy in the third intercostal space. A 7-0 prolene suture was passed underneath the left anterior descending (LAD) coronary artery at 2 mm below the tip of the left auricle, and the LAD was ligated with a PE-10 tube. The occlusion of the LAD coronary artery was confirmed by checking for the appearance of a paler color in the anterior wall of the LV within a few seconds after ligation. After 45 min of ischemia, reperfusion was induced by untying the knot and removing the PE-10 tube. Reperfusion was confirmed by checking restoration of a red color in the anterior wall of the LV. The thoracic cavity was closed with 7-0 prolene sutures, and the skin was closed with 4-0 silk sutures.

Double staining with triphenyltetrazolium chloride and Evans blue

To evaluate the ischemic and infarcted area, double-staining using triphenyltetrazolium chloride (TTC) and Evans blue dye was performed in the I/R injury models. After 24 hours of reperfusion, mice were anesthetized, the thoracic cavity was reopened, and the LAD coronary artery was re-ligated at the same site. The heart was then perfused with 1% Evans blue dye (Wako, 054-04062), to distinguish the ischemic area, the area at risk (AAR) that was not stained with dye. The heart was excised and the left ventricle (LV) was cut into 5 transverse slices from the apex to the base. The slices were incubated in 1% TTC solution (Sigma-Aldrich, T8877) at 37°C for 15 min, photographed and weighed. For each slice, the TTC-unstained area (infarcted area), AAR, and LV area were calculated using Image J. Then, the weight of the infarcted area and AAR were calculated from each slice weight. The components of all slices were summed, and the total infarcted area weight was divided by the total AAR weight (infarcted area/AAR) for injury size, and total AAR weight divided by total LV weight (AAR/LV) for ischemic size.

Histology

After administration of an overdose of anesthetics, mice were perfused with 4% paraformaldehyde (PFA) before excising the heart, and tissue samples were further fixed in 4% PFA at 4°C overnight. The following day, the tissue samples were transferred to 70% ethanol for dehydration before embedding in paraffin. The sequential sections of the LVs were obtained at an 800 µm intervals from the point of ligation to the apex (about 4 sections per heart). Then, the sections were deparaffinized and stained with Masson's trichrome staining. Images were acquired using a microscope (BZ-9000, Keyence). We quantified the total infarcted area in LVs by measuring the infarcted area of each sections using ImageJ software.

Histological analysis was performed by an experimenter who was blinded to treatment groups.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL)

Staining

TUNEL staining was performed to investigate the protective effects of KUS121 on cardiomyocyte apoptosis in I/R injury models. As shown in Fig. 4a, KUS121 was administered immediately after reperfusion, and the heart was excised 4 hours after reperfusion. The heart was fixed in 4% PFA at 4°C for 24 hours, transferred to 15% sucrose solution at 4°C for 12 hours and to 30% sucrose solution at 4°C overnight, and then embedded in Tissue-Tek OCT compound (Sakura Finetech). The heart was frozen using dry ice, and cut into 7 µm-thick sections. TUNEL assay was performed using the In-Situ Cell Death Detection Kit, TMR Red (Roche Applied Science), according to the manufacturer's instructions. The sections were co-stained with anti-cardiac troponin I (1:200) (rabbit polyclonal, Abcam, ab47003) followed by Alexa Fluor 488 (1:200) (donkey anti-rabbit, Invitrogen, A21206) to identify cardiomyocytes. Nuclei were counterstained with DAPI (1 µg/mL, Dojindo, D523).

The number of apoptotic cardiomyocytes, defined as TUNEL-positive cells surrounded by troponin I in ischemia region, was counted more than 10 fields in 2 different sections per heart at x 200 magnification, and expressed as a percentage of the total cardiomyocytes.

Echocardiography

To analyze the cardiac function of mice, we performed echocardiography (Vevo[®]2100, VISUALSONICS) at the indicated time points after I/R injury. Mice were kept under inhalation anesthesia with 2.0% isoflurane. LV wall thickness, LV diameter, and LV function (EF and FS) were measured in M mode of the parasternal short-axis view.

Quantitative real-time PCR

To evaluate mRNA expression levels, single-strand cDNA was synthesized from 1 µg of total RNA by means of a reverse transcriptase reaction, and quantitative PCR (qPCR) was performed using a StepOnePlus[™] (Thermo Fisher) with THUNDERBIRD[®] SYBR qPCR Mix

(TOYOBO, QPS-201). Expression levels were normalized using housekeeping genes as indicated. The primer sequences are as follows:

VCP (human) forward, 5'-CCCAGCCCAAGATGGATGAA-3';

VCP (human) reverse, 5'-CGTTTGCCGTACTTCACATCAG-3';

β -actin (human) forward, 5'-AGGCACTCTTCCAGCCTTCC-3';

β -actin (human) reverse, 5'-GCACTGTGTTGGCGTACAGG-3';

VCP (mice) forward, 5'-TTTTGACAAGGCACGGCAAG-3';

VCP (mice) reverse, 5'-GCTCCACCACCATCTCCAATA-3';

CHOP (mice) forward, 5'-AGCTGGAAGCCTGGTATGAGGA-3';

CHOP (mice) reverse, 5'-AGCTAGGGACGCAGGGTCAA-3';

GAPDH (mice) forward, 5'-AAATGGTGAAGGTCGGTGTG-3';

GAPDH (mice) reverse, 5'-AATCTCCACTTTGCCACTGC3'.

Quantification of heart ATP levels in I/R injury models of Go-ATeam2 mice

Go-ATeam2 mice express a FRET-based ATP biosensor similar to the one used for cell culture, except the fluorescent protein reporters were orange fluorescent protein (OFP) and green fluorescent protein (GFP), rather than yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), respectively (2,3) (M.Y. et al., manuscript in preparation). I/R injury models were utilized as described in *Ischemia and reperfusion injury models in mice*, above. Briefly, 8-week-old Go-ATeam2 mice were anesthetized, and an endotracheal tube was introduced for positive pressure ventilation. The thoracic cavity was opened to expose the heart, and LAD coronary artery was ligated with a PE-10 tube for 45 min. Then, reperfusion was induced by untying the knot and removing the PE-10 tube. Observations of the hearts were performed using a Leica M165 FC stereo microscope (Leica) with a 1 $\times$ objective (Leica, Plan Apo 1.0) and the following DualView2 filter sets (INDEC Biosystems, Santa Clara, CA, USA): for dual emission ratio imaging, 470/40 excitation filter - dichroic mirror 540 DCLP - 515/30

for GFP and 575/40 for OFP. Images of fluorescence emission were captured every 5 minutes from pre-ischemia to 60 min after reperfusion, using ORCA-Flash4.0 (Hamamatsu, Japan). The images were obtained at 30 ms intervals for 1 second. Imaging data of OFP/GFP emission ratios were analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA).

Western blotting

Western blotting was performed using standard procedures as described previously (4). *In vitro* cell lysates were collected using cell scrapers in chilled lysis buffer consisting of 100 mM Tris-HCl, pH 7.4, 75 mM NaCl and 1% Triton X-100 (Nacalai Tesque) supplemented with Complete Mini protease inhibitor cocktail (Roche, 11836153001), 0.5 mM NaF, and 10 mM Na₃VO₄ just before use. The protein concentration was determined using a BCA protein assay kit (Bio-Rad, 5000006JA). All samples (15 µg of protein) were suspended in lysis buffer, fractionated using NuPAGE 4–12% Bis-Tris Mini gels (Thermo Fisher Scientific, NP0322BOX) and transferred to Protran nitrocellulose transfer membranes (Whatman). The membrane was blocked using PBS containing 5% non-fat milk for 30 min and incubated with the primary antibody overnight at 4°C. After a washing step in PBS-0.05% Tween-20 (PBS-T), the membrane was incubated with the secondary antibody for 1 h at room temperature. The membrane was then washed with PBS-T and detected using PierceTM Western Blotting Substrate (Thermo Fisher Scientific, NCI3106) and PierceTM Western Blotting Substrate Plus (Thermo Fisher Scientific, NCI32132) using a LAS-4000 Mini system (Fuji Film). The primary antibodies are as follows: anti-VCP (Cell signaling technology, #2649), anti-BiP (Cell signaling technology, #3183), anti-CHOP (Santa Cruz Biotechnology, sc-575), anti-β-actin (Sigma-Aldrich, A5316). The secondary antibodies are as follows: anti-Rabbit IgG (GE Healthcare, NA934V), HRP-Linked, anti-Mouse IgG, HRP-Linked (GE Healthcare, 931V).

Ischemia and reperfusion injury models in pigs

Porcine I/R injury models were performed as described previously (5,6).

Briefly, 3-month-old pigs (35–40 kg) were anesthetized, an endotracheal tube was introduced, and positive pressure ventilation was provided with respirator. A 7Fr vascular sheath was placed in the right femoral artery for vascular access and then heparin (100 IU/kg) and amiodarone (4.3 mg/kg) were administered intravenously. During the operation, potassium (6 mEq/hr) and amiodarone (20 mg/hr) were administered continuously.

First, left ventriculography (LVG) was performed to assess cardiac function before ischemia. Then, the left main coronary artery was engaged with a 7Fr Hockey Stick guiding catheter and left coronary angiography (CAG) was performed with injection of contrast agent to identify the location of the occlusion. A 0.014-inch guidewire was inserted into the LAD coronary artery and a 3.0–20 mm over-the-wire balloon (Terumo, Japan) was advanced distal to the second diagonal branch. The balloon was inflated there and CAG was performed to confirm complete occlusion of the LAD coronary artery. After 60 mins of occlusion, reperfusion was induced by deflation of the balloon, confirmed by CAG. Then, KUS121 at a dose of 0.64, 2.5, or 5.0 mg/kg, or 5% glucose solution (as a control) was administered into the LAD coronary artery through the wire lumen of the balloon catheter. At last, LVG was performed to assess cardiac function after reperfusion, the 7Fr vascular sheath was removed, the pigs were weaned from ventilation and transferred to the animal care unit.

The dose of 0.64 mg/kg was calculated from a twelfth of the murine dose of 160 mg/kg (the same dose as administered after reperfusion in Fig. 4a) based on body surface area (7), and from coronary blood flow, accounting for 4–5% of cardiac output (8). The dose of 2.5 or 5.0 mg/kg was calculated as 4 or 8 times of the dose of 0.64 mg/kg, respectively.

Quantification of infarcted area in pigs

At 7 days after reperfusion, the pigs were anesthetized, an endotracheal tube was introduced, and positive pressure ventilation was provided with a respirator. First, gadolinium enhanced

cardiac magnetic resonance imaging (MRI) was performed to evaluate the infarcted area by late gadolinium enhancement on cardiac MRI.

Next, to evaluate the infarcted area by histology, double staining with TTC and Evans blue was performed. A 7Fr vascular sheath was placed in the right and left femoral artery for vascular access and then heparin (100 IU/kg) was administered intravenously. First, LVG was performed to assess cardiac function, and then the left and right coronary arteries (LCA and RCA) were engaged with 7Fr Hockey Stick guiding catheters and left and right CAG were performed to confirm no occlusion in the LCA and RCA. A 0.014-inch guidewire was inserted into the LAD coronary artery and a 3.0–20 mm over-the-wire balloon (Terumo, Japan) was advanced to the site of the previous occlusion. The balloon was inflated and CAG was performed to confirm complete occlusion of the LAD coronary artery. Then 60 ml of 1% Evans blue dye was injected into the LCA and 30 ml into RCA through the guiding catheters. In addition, 10 ml of 1% TTC was injected into the occluded area of the LAD coronary artery through the wire lumen of the over-the-wire balloon.

Then, the pigs were sacrificed with an intravenous injection of potassium chloride after double staining, and the heart was excised and sliced in 10-mm-thick slices from the apex to the base of the heart. The slices were submerged in 1% TTC for 15 min at 37°C. All slices were then weighed, each side of the slice was photographed and fixed with a 10% formaldehyde solution. For each slice, the TTC unstained area (infarcted area), the Evans blue unstained area (the AAR), and LV area were calculated using Image J. Then, the weight of the infarcted area and AAR were calculated from each slice weight. The components of all slices were summed and the total infarcted area weight was divided by the total AAR weight (infarcted area/AAR) for injury size and the total AAR weight divided by the total LV weight (AAR/LV) for the ischemic size.

Analysis of TTC staining and cardiac MRI was performed by an experimenter who was blinded to treatment groups.

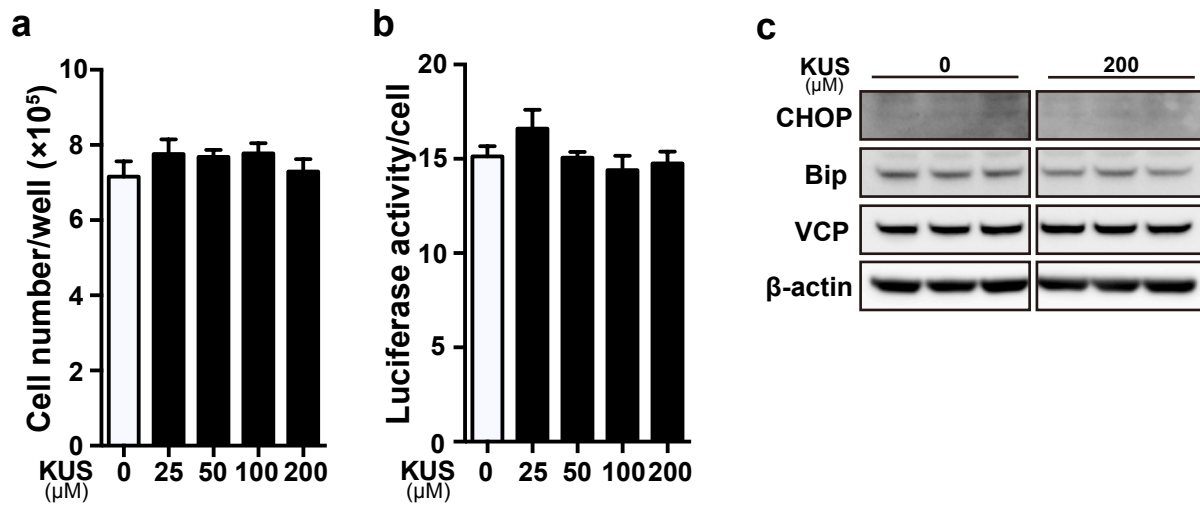
Statistical analysis

Measured data are presented as mean $\pm$ standard error of the mean (SEM). For statistical comparisons between two groups, unpaired Student's t-test was used. For statistical analysis of three or more groups, one-way analysis of variance (ANOVA) was used. In one-way ANOVA, Sidak's post-hoc test was performed to compare all pairs of groups (Fig. 3b–f and 5b–e, and Supplementary Fig. 4b–e) and Dunnett's post-hoc test to compare one group as a control to the other groups (Fig. 2b–e and h, and 8c, d and f). A p-value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

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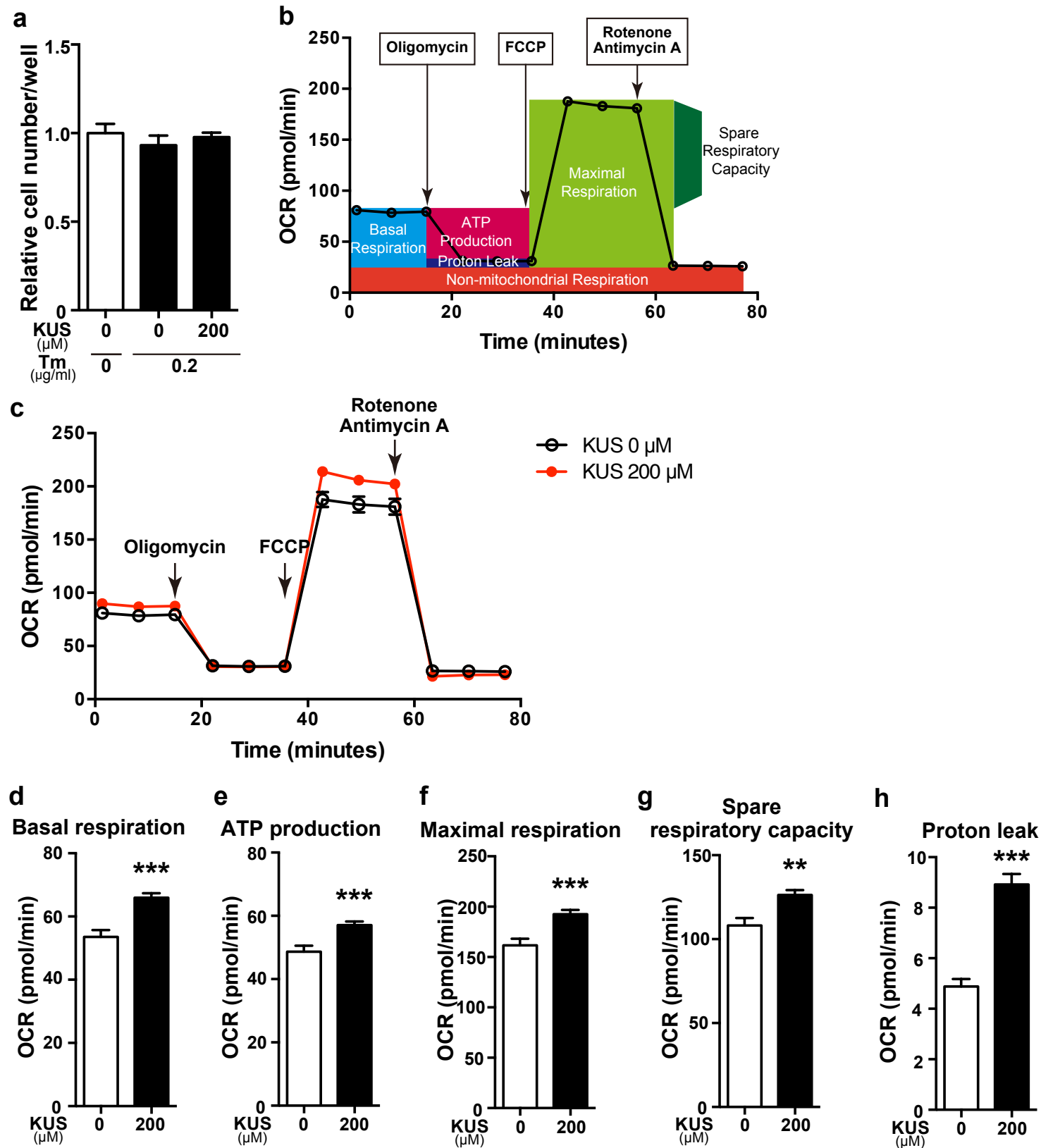
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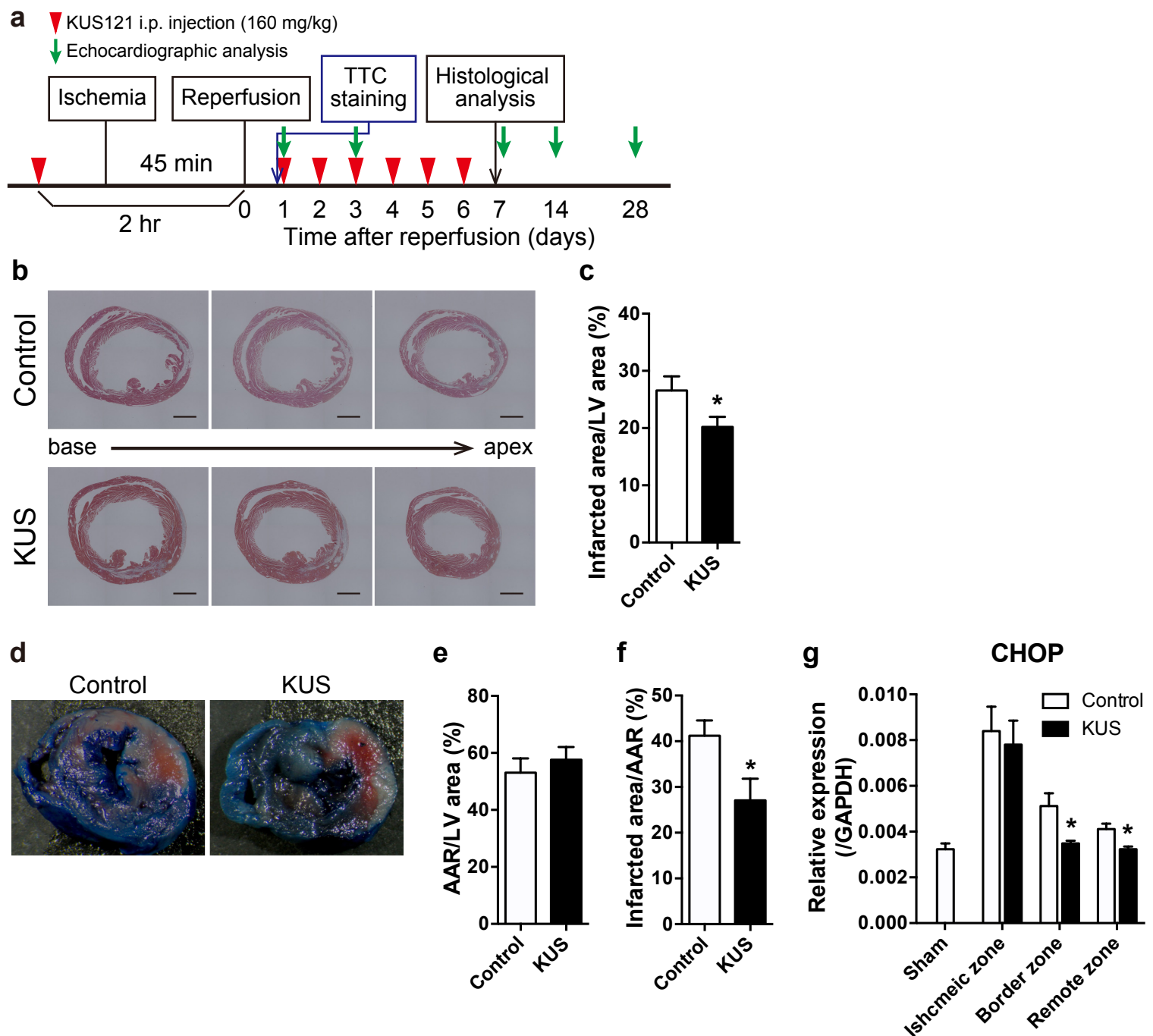
Supplementary Figure 1. KUS121 does not affect cell growth, cellular ATP levels, or protein expression levels in normal culture conditions.

(a, b) Number and ATP levels of H9C2 cells in normal culture with different concentrations of KUS121 (25, 50, 100, and 200 μM , $n=3$). Data are presented as mean $\pm$ SEM. (c) Western blotting analysis of H9C2 cells, cultured with and without KUS121 (200 μM , $n=3$).



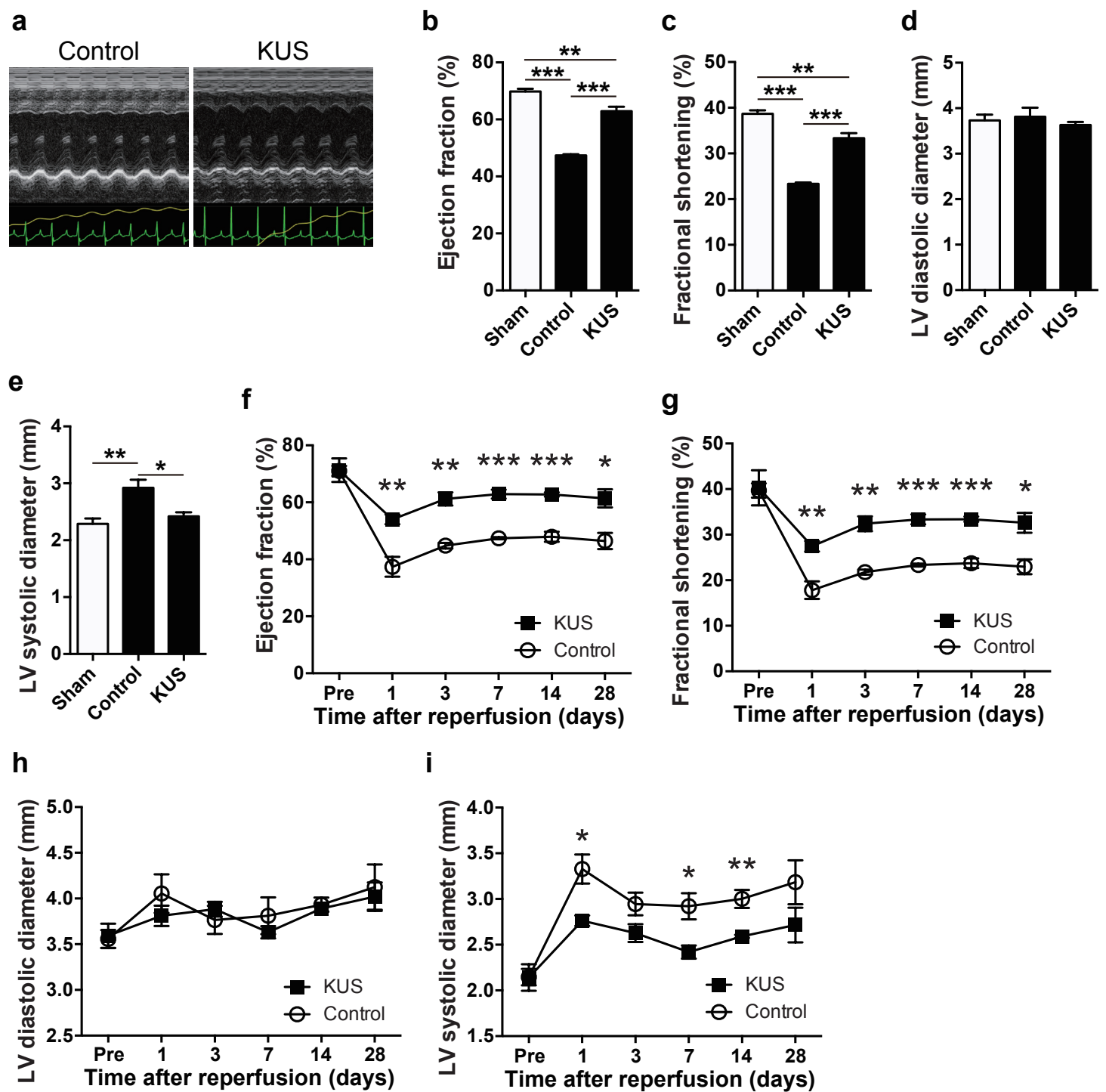
Supplementary Figure 2. KUS121 increases mitochondrial respiration in normal culture conditions.

(a) Relative number of H9C2 cells treated with tunicamycin (0.2 $\mu\text{g/ml}$) for 6 hours, with and without KUS121 (200 μM). The number of cells in normal culture conditions was the reference, indicated as 1. Data are presented as mean $\pm$ SEM. (b) Schematic diagram of oxygen consumption rate (OCR) measured using an XF96 extracellular flux analyzer. After basal OCR was measured, oligomycin (2 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (1 μM), rotenone (1 μM), and antimycin A (1 μM) were added sequentially to assess the parameters of mitochondrial respiration. (c) OCR in normal culture conditions. OCR at each time point was obtained from an average of 10 replicate wells and presented as mean $\pm$ SEM. (d–h) Parameters of mitochondrial respiration: basal respiration (d), ATP production-linked respiration (e), maximal respiration (f), spare respiratory capacity (g), and proton leak (h). ** $P < 0.01$, *** $P < 0.001$, using unpaired two-tailed Student's *t*-test. Data are presented as mean $\pm$ SEM.



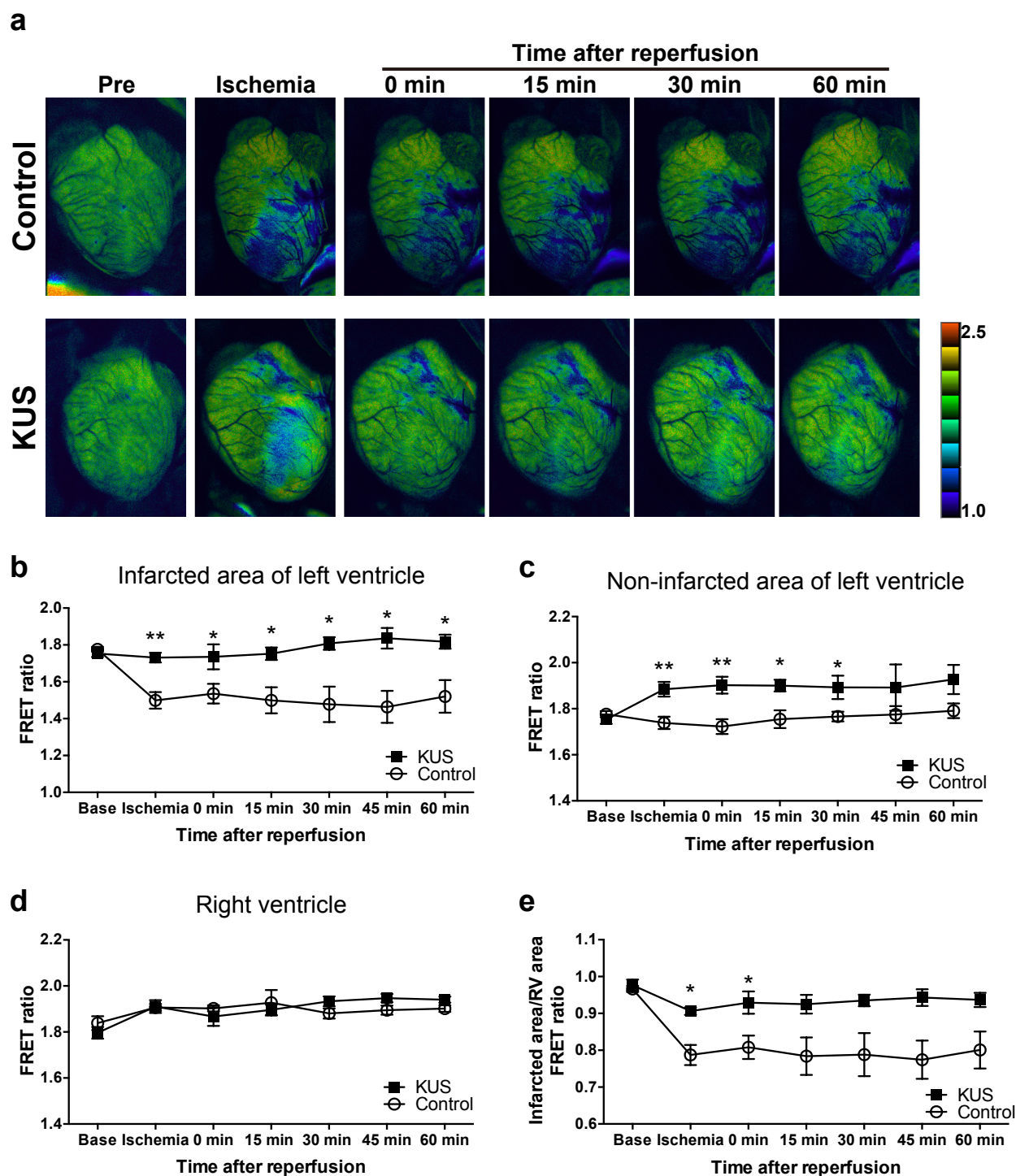
Supplementary Figure 3. KUS121 pretreatment attenuates cardiac damage in murine ischemia and reperfusion injury models.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures and schedules of KUS121 administration. (b) Representative images of Masson's trichrome staining in the short axis of left ventricles (LVs) at 7 days after I/R injury. Black bars indicate 1000 μm . (c) Quantification of infarcted areas in LVs at 7 days after I/R injury (Control, $n=6$; KUS121, $n=10$). * $P<0.05$, using unpaired two-tailed Student's t-test. (d) Representative images of double staining with triphenyltetrazolium chloride (TTC) and Evans blue in the short axis of LVs at 24 hours after I/R injury. (e, f) Quantification of infarcted area/area at risk (AAR) and AAR/LV in double staining with TTC and Evans blue (Control, $n=7$; KUS121, $n=7$). * $P<0.05$, using unpaired two-tailed Student's t-test. (g) Expression levels of C/EBP homologous protein (CHOP) in LVs at 1 hour after I/R injury. LVs were divided into three parts: ischemic zone, border zone, and remote zone (Sham, $n=6$; Control, $n=12$; KUS121, $n=8$). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. * $P<0.05$, using unpaired two-tailed Student's t-test. All data are presented as mean $\pm$ SEM.



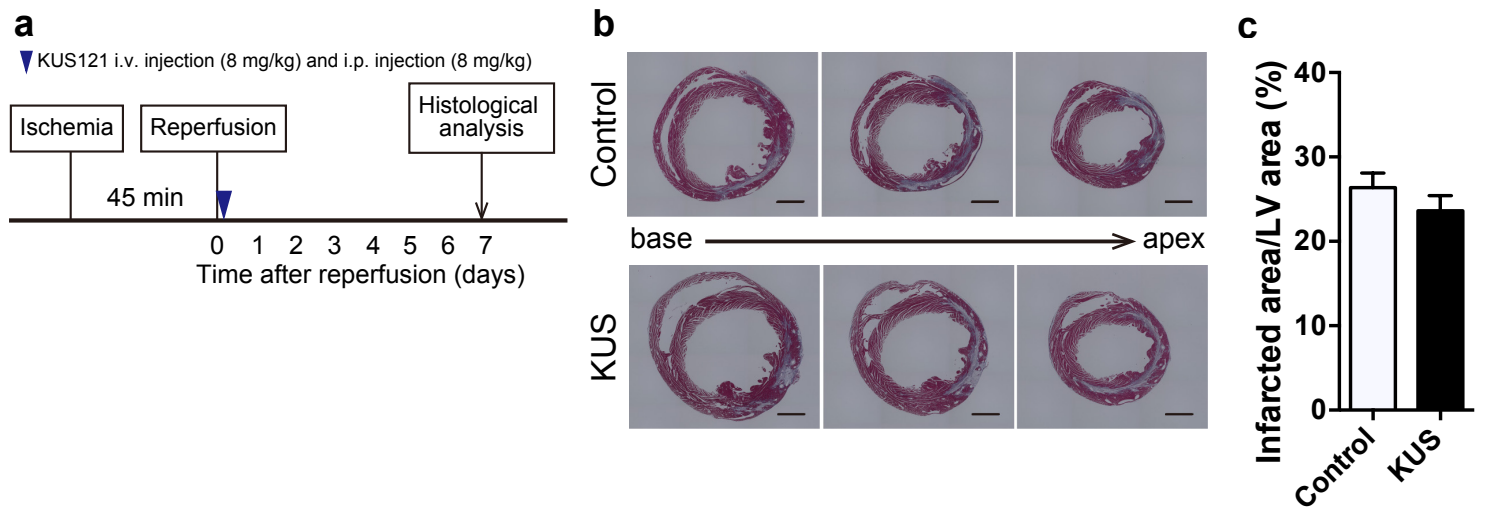
Supplementary Figure 4. KUS121 pretreatment preserves cardiac function in ischemia and reperfusion injury models.

(a) Representative images of M-mode echocardiogram of Control and KUS121-treated animals at 7 days after ischemia and reperfusion (I/R) injury. (b–e) Echocardiographic data 7 days after I/R injury. Ejection fraction (EF; b), fractional shortening (FS; c), left ventricle (LV) diastolic diameter (LVDd; d), and LV systolic diameter (LVDs; e) were measured (Sham, n=4; Control, n=3; KUS121, n=4). *P<0.05, **P<0.01, ***P<0.001, using one-way ANOVA with Sidak's post-hoc test. (f–i) Echocardiographic data at the indicated time points after I/R injury (Supplementary Fig. 3a). *P<0.05, **P<0.01, ***P<0.001, using unpaired two-tailed Student's t-test. All data are presented as mean ± SEM.



Supplementary Figure 5. KUS121 pretreatment maintains ATP levels in ischemia and reperfusion injury models.

(a) Representative pseudocolor ratiometric fluorescence resonance energy transfer (FRET) images of whole hearts in ischemia and reperfusion injury models of Go-ATeam2 mice, when KUS121 was administered before ischemia as shown in Supplementary Fig. 3a. Pseudocolor images were obtained at various time points in ischemia and after reperfusion. In pseudocolor images, warmer colors represent high FRET ratios and cooler colors low FRET ratios. (b–d) Quantification of ATP levels in infarcted areas of left ventricles (LVs) (b), in non-infarcted areas of LVs (c), and in right ventricle (RV) areas (d) by FRET ratio (Control, n=6; KUS121, n=6). *P<0.05, **P<0.01, using unpaired two-tailed Student's t-test. (e) ATP ratio of infarcted area of LV to RV area. *P<0.05, using unpaired two-tailed Student's t-test. All data are presented as mean $\pm$ SEM.



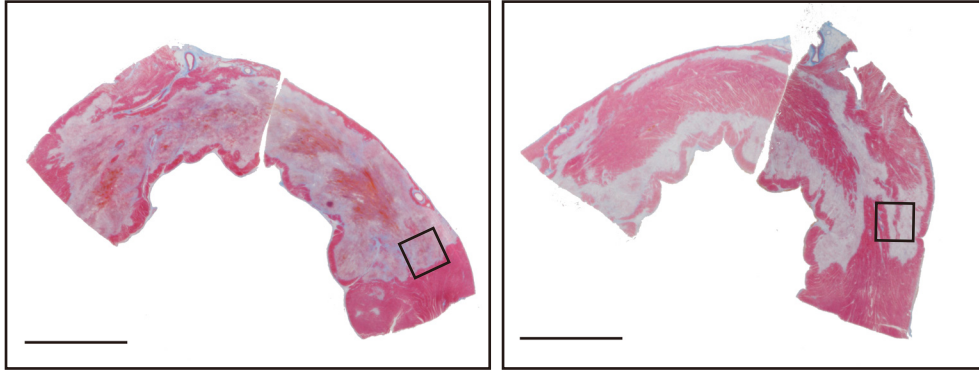
Supplementary Figure 6. Beneficial effect of KUS121 treatment is negligible at a dose of 16 mg/kg.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures and schedules of KUS121 administration. (b) Representative images of Masson's trichrome staining in the short axis of left ventricles (LVs) at 7 days after I/R injury. Black bars indicate 1000 μm . (c) Quantification of infarcted area in LVs at 7 days after I/R injury (Control, n=15; KUS, n=16). Data are presented as mean $\pm$ SEM.

a

Control

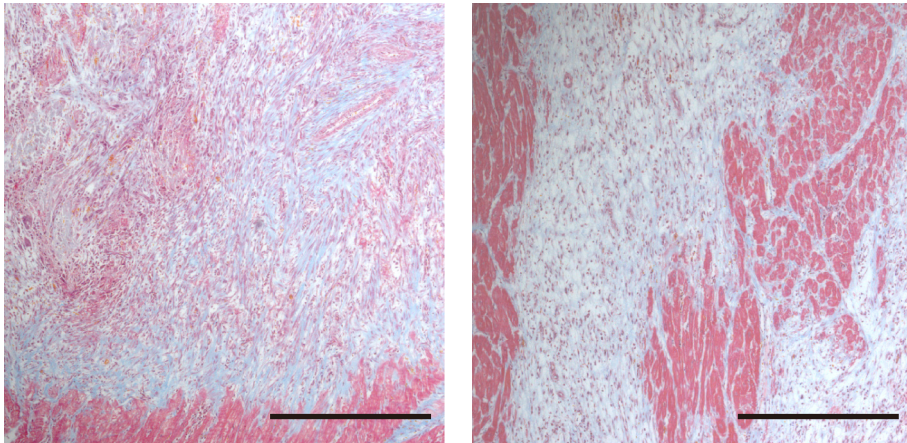
KUS



b

Control

KUS



Supplementary Figure 7. The histology of Masson's trichrome staining is similar to that of triphenyltetrazolium chloride staining in porcine ischemia and reperfusion injury models.

(a) Representative whole images of the infarcted area in the same slices as shown in Fig. 9b. Black bars indicate 1000 μ m. (b) Enlarged images of black squares shown in (a). Black bars indicate 500 μ m.