nature portfolio

Peer Review File

FGF21 Protects Against HFpEF by Improving Cardiac Mitochondrial Bioenergetics in Mice

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors show that upregulation of FGF21, particularly in adipose tissue, plays an important role in protecting the heart from HFpEF. The effect of FGF21 is mediated through stimulation of adiponectin and PI-3K-Akt and downregulation of PDK4.
The study extends the authors' past publication in Cell Metabolism in 2013 (showing that adiponectin mediates the effect of FGF21) to show its significance in the pathogenesis of HFpEF.

General:
1. The authors may want to clarify the underlying mechanism through which HFpEF inactivates PI3K-Akt and upregulates PDK4 despite the upregulation of FGF21 (Fig. 6), which appears more fundamental as a pathogenesis of HFpEF.

2. The authors should clarify the level of FGF21 at various organs in HFpEF mice in the presence of AAV-FGF21.

3. The authors should show how the downregulation of PDK4 and adiponectin affects systolic cardiac function in HFpEF mice. The mechanism may not be specific in HFpEF.

4. The authors should show the level of PDK4 in Figure 5K.

5. This reviewer wished to see more data to support that FGF21 improves energetics, including fatty acid oxidation.

Specific:
1. The authors should show the effect of AAV-FGF21 alone in the control heart in many experiments, such as Fig. 3J-L and PDK4shRNA alone in the control heart in the entire Fig. 5.

2. Many morphological studies, including EM and oxidative stress, lack quantification or statistical analyses.

3. Regarding the data presentation, the Y axis in many bar graphs does not use full range (not starting from zero), which is misleading.

Reviewer #2

(Remarks to the Author)
The authors attempted to examine the role of FGF21 in heart failure with preserved ejection fraction (HFpEF). The authors found that circulating FGF21 levels are increased and negatively associated with cardiac diastolic function in patients with HFpEF. Whole body or adipose FGF21 deficiency can exacerbate cardiac diastolic dysfunction and damage in high-fat diet (HFD)+L-NAME-induced HFpEF mice. Treatment of HFpEF mice with AAV-FGF21 improves cardiac diastolic dysfunction and damage. FGF21 directly acts on cardiomyocytes and negatively regulates PDK4 production by activating PI3K/AKT signals, thereby mitigating HFpEF-induced cardiac damage. The protective actions of FGF21 on cardiac diastolic dysfunction are medicated partly by its ability to increase production of adiponectin. The authors concluded that FGF21 protects against HFpEF via fine-tuning the multiorgan crosstalk among the adipose, liver, and heart.

This reviewer has several specific comments.
1. The authors seemed to inject AAV9-FGF21 into mice via tail vein. What is the main tissue that produces circulating FGF21 after AAV9-FGF21 treatment (FigureS1D)? AAV9 typically targets heart, liver and muscle. Is cardiomyocyte-derived FGF21 protective against cardiac diastolic dysfunction in HFpEF mice after AAV9-FGF21 treatment? The authors mentioned that liver and adipose tissues may be served as the primary sites for production of endogenous FGF21 in response to HFpEF. These points should be clarified.
2. The authors should examine whether AAV9-FGF21 can rescue the cardiac phenotype of FGF21 KO mice or adipose FGF21 KO mice with HFpEF.
3. The authors used high fat diet (HFD)+L-NAME-induced HFpEF mice in this study. Both FGF21 and adiponectin are metabolic regulators. It is possible that FGF21 and adiponectin could prevent cardiac diastolic dysfunction and damage by modulating glucose and lipid metabolism. The authors should measure metabolic parameters in these models.
4. Adiponectin is known to promote cardiac AMPK signaling pathway which typically antagonizes Akt cascade. The points should be clarified.
5. PI3-Akt signal pathway induces cardiac hypertrophy and growth. FGF21-KO mice with HFpEF showed increased cardiomyocyte size and decreased phosphorylation levels of PI3 and Akt. Please comment.
6. It is better to present the same blots in data from co-culture experiments (NRVMs with wt-SAT and ko-SAT) (i.e. Figures 8A and S9A).
7. In Figure S8B, AAV9-FGF21 appears to decrease adiponectin levels.

Reviewer #3

(Remarks to the Author)
These authors have explored the role of FGF21 in the pathogenesis of HFpEF. In a series of detailed analyses, they point to a role of HFpEF-induced increases in FGF21 as a putatively compensatory response, impacting mitochondrial energetics among other things. In the end, they posit an APN-PI3K/AKT-PDK4 axis in this biology. Overall, I find the data to be strong but novelty to be limited.

FGF21 has been shown previously to confer benefit in the context of a variety of cardiovascular disorders acting through glucose and lipid metabolism and insulin sensitivity. The novelty in the present report is limited to its focus on HFpEF. The authors report that FGF21 levels are increased in HFpEF, an observation reported previously. Similarly, the role of PI3K/AKT in the actions of FGF21 have been reported previously. There are other aspects of the paper with limited novelty.

In the end, it remains unclear to me the relative effects of APN per se versus its action downstream impacting PDK4.

To claim a statistical association based on Figure 1C (r=-.204) is a considerable stretch.

Overall, the manuscript is well written. However, there are a fair number of grammatical mistakes. An incomplete list includes the following:

Variant cardiac disorders

Mechanically should be mechanistically

Multi-organic crosstalk

These manners were also observed

Expressional site

Pharmaceutic inhibition

Lowed PDH activities

Upper animal results

The sentence starting at line 393 is incomplete.

Have been showed

Reviewer #4

(Remarks to the Author)
In this manuscript, the authors identified PDK4 as a potential downstream target of FGF21. While other experiments were beyond my expertise, the experimental design and data interpretation of the Mass Spectrometry analysis and proteomic part, from a MS filed reviewer's point of view, is well designed and performed. I believe this is a very strong manuscript, I recommend publication in its current form.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Lines 164-167: Although the authors claim that AAV-Fgf21 increases FGF21 levels in the heart, the data shows no increase in the heart (Supplementary Fig. 1D and I).
Lines 167-168: In the two-hit mouse model of HEpEF, the development of HFpEF relies on high blood pressure. It would be hard to exclude the possibility that FGF21 alleviates HFpEF just because it lowers blood pressure.

Reviewer #2

(Remarks to the Author)
The authors adequately addressed my concerns. As the only remaining minor comment, the authors should mention the comments for the questions #4 (adiponectin/AMPK/Akt) and #5 (PI3-Akt and cardiac hypertrophy) in the Discussion section in the revised manuscript.

Reviewer #3

(Remarks to the Author)
Whereas I do not disagree at all with the comments raised by the other reviewers, my concerns, then and now, center around the relative lack of novelty in this report.

Point-by-point responses to the reviewer's criticisms

Re: NCOMMS-24-21529
FGF21 Protects Against HFpEF by Improving Cardiac Mitochondrial Bioenergetics via Induction of Adiponectin and Suppression of PDK4 in Mice

We thank the editor of *Nature Communications* and the four external peer reviewers for their constructive suggestions and encouraging comments. We have carefully reviewed both the editor's issues and the reviewers' criticisms and have made our best effort to address them one by one. Several new experiments have been conducted. We have updated the text, main, and Supplemental Figures accordingly. We believe our findings have been confirmed and extended with these added data and clarifications, and the manuscript has been significantly improved. Here, please find detailed, point-by-point responses to all the comments. For ease of re-evaluation, we stated changes and included new data (marked in red) in this response-to-reviewers letter. We hope that you will find our work in the current version makes this manuscript a strong candidate for *Nature Communications*.

Reviewer #1:

Q1: The authors may want to clarify the underlying mechanism through which HFpEF inactivates PI3K-Akt and upregulates PDK4 despite the upregulation of FGF21 (Fig. 6), which appears more fundamental as a pathogenesis of HFpEF.

Answer: Thank you for your comments. PDK4, a member of the pyruvate dehydrogenase (PDH) family that negatively regulates the activity of the PHD complex by phosphorylating its subunits, plays a critical role in mitochondrial bioenergetics. In this study, our data indicated that cardiac PDK4 was significantly increased in the mitochondria but not the nucleus of mice with HFpEF (Figure 4), and overexpression of PDK4 significantly exacerbated HFpEF and its related cardiac damage, followed by lowered PDH activity, impaired pyruvate oxidation, decreased Acetyl-CoA and ATP production, alleviated mitochondrial bioenergetics, and finally aggravated cardiac damage (Figure S7). In contrast, genetic knockdown of PDK4 profoundly reversed these adverse effects (Figure 5), suggesting that PDK4 is a critical target in negatively regulating the mitochondrial bioenergetics in the pathological process of HFpEF.

Our previous studies show that FGF21 is upregulated as a compensatory response in various metabolic diseases such as type 2 diabetes mellitus, coronary heart disease, atherosclerosis, and so on, and is known to activate the PI3K-Akt signaling pathway, which plays a vital role in glucose and lipid metabolism as well as insulin sensitivity[1, 2]. In this study, our *in vivo* data showed that FGF21 protects against HFpEF by attenuating PDK4-related mitochondrial dysfunction (Figures 5 and S7). Moreover, our *in-vitro* results demonstrated that FGF21 promotes mitochondrial bioenergetics by activating the PI3K-Akt signaling pathway, which in turn suppresses mitochondrial PDK4 expression in primary neonatal rat ventricular myocytes (NRVMs), followed by inhibited PDH phosphorylation, elevated PDH activities, Acetyl-CoA, and ATP production (Figure 6). Considering that cardiac energy metabolism dysfunction is one of the critical events in the pathogenesis of HFpEF[3, 4]. Taken together, these findings indicated that HFpEF-induced upregulation of PDK4 may be more fundamental as a pathogenesis of HFpEF. FGF21, as an upstream endocrinal protective molecule in response to metabolic stress, plays a vital role in maintaining the myocardial energy metabolism homeostasis during the pathogenesis of HFpEF, and upregulation of FGF21 may be a compensatory response during the pathological process of HFpEF.

Q2: The authors should clarify the level of FGF21 at various organs in HFpEF mice in the presence of AAV-*Fgf21*.

Answer: To clarify the level of FGF21 in various organs of HFpEF mice treated with AAV-*Fgf21*, the abundance of FGF21 in multiple organs was tested by immunoblotting. Our new data showed that the elevated FGF21 contents were seen in white adipose tissue (WAT), liver, and heart but not in brown adipose tissues (BAT) and muscle after treatment with AAV-*Fgf21* (*Figures S1D–S1H*). These data are now included in the revised manuscript (*see page 4, lines 164 – 167*).

Q3: The authors should show how the downregulation of PDK4 and adiponectin affects systolic cardiac function in HFpEF mice. The mechanism may not be specific in HFpEF.

Answer: Thank you for your comments. In this study, our primary aim is to explore the relationship between FGF21 and HFpEF. Therefore, we used HFD plus L-NAME to build an HFpEF mouse model, as previously reported[5]. Interestingly, echocardiographic results showed no significant difference in left ventricular systolic function (LVEF and LVFS) between adiponectin null mice and WT controls with HFpEF (*Figure R1A*). However, a significant decrease in left ventricular diastolic function (E/A and E/E'ratios) was seen in adiponectin null mice compared with WT controls (Figure 7C), suggesting that adiponectin deficiency did not impact cardiac systolic function but exacerbated cardiac diastolic function in HFD plus L-NAME-induced HFpEF mice. Consistently, cardiac-specific knockdown of PDK4 by treatment with *Pdk4*-shRNA did not change cardiac systolic function but significantly improved cardiac diastolic function between *Fgf21* KO mice and controls with HFpEF induced by HFD plus L-NAME (*Figure R1B and 5*). These findings suggest that downregulation of PDK4 or adiponectin deficiency does not influence cardiac systolic function but not diastolic function in this HFD plus L-NAME-induced HFpEF model.

We agree with the reviewer's concern that the impact of adiponectin and PDK4 on cardiac function may not be specific in HFpEF. Previous studies have demonstrated that adiponectin and PDK4 are crucial in modulating cardiac systolic function across various cardiovascular disease models. Adiponectin has been shown to enhance systolic function in various cardiac pathologies, including post-infarction remodeling and diabetic cardiomyopathy[6, 7]. Similarly, the influence of PDK4 on cardiac function is not limited to the context of HFpEF. In models of post-myocardial infarction, downregulation of PDK4 has been associated with significant improvements in cardiac remodeling, systolic function, and cardiomyocyte metabolism[8]. These data indicated that PDK4 and adiponectin are likely involved in the pathogenesis of various cardiovascular diseases. Their exact roles and mechanisms in the context of different cardiovascular disease models require further investigation.

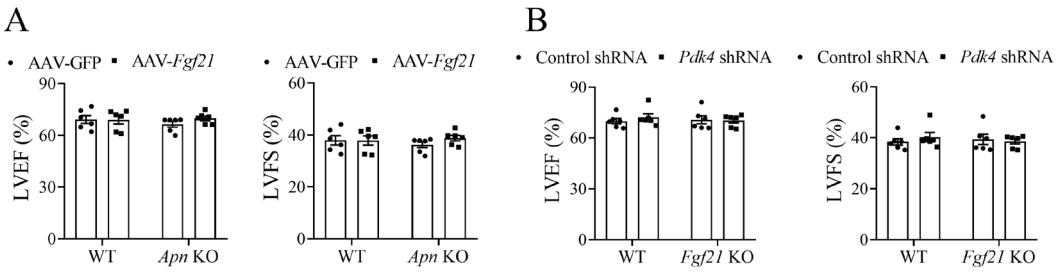

Figure R1. The downregulation of PDK4 and adiponectin does not affect systolic cardiac function in mice with HFpEF. (A) Left ventricular systolic function of *Apn* KO and WT mice with HFpEF after treatment with AAV-*Fgf21* or AAV-GFP, respectively. (B) Left ventricular systolic function of *Fgf21* KO and WT mice with HFpEF after treatment with or without *Pdk4* shRNA. Statistical significance was determined by the two-way ANOVA, followed by Tukey's multiple comparison test. Data are presented as mean ± SEM. N=6 per group.

Q4: The authors should show the level of PDK4 in Figure 5K.

Answer: We now included the data based on the reviewer's suggestion (*Figure 5L*).

Q5: This reviewer wished to see more data to support that FGF21 improves energetics, including fatty acid oxidation.

Answer: Thank you for your advice. To clarify the influence of FGF21 in mitochondrial bioenergetics, glucose and lipid uptake, transport, and oxidation were tested in FGF21 null mice with HFpEF and WT controls. Our new data demonstrated that FGF21 deficiency exacerbated glucose and insulin intolerance in mice with HFpEF (Figures S5A–S5B). Notably, *Fgf21* KO HFpEF mice exhibited a significant decrease in cardiac mRNA levels of *Glut1* but not *Glut4*, compared to WT controls, while other glycolysis-related enzymes, including *Hk1, Hk2,* and *Pkm2,* were comparable (Figure S5C). Furthermore, treatment with rmFGF21 markedly restored the palmitic acid (PA)-induced decrease in glucose uptake in primary neonatal rat ventricular myocytes (NRVMs), as detected by 2-NBDG fluorescence (Figure S5D). In addition, cardiac phosphorylated PDH protein levels were significantly increased in *Fgf21* KO mice with HFpEF compared to WT controls (Figure S5E). These findings show that the protective effects of FGF21 against HFpEF may be related to promoting glucose uptake and oxidation in mice.

Regarding lipid metabolism, FGF21 deficiency significantly elevated plasma FFA and TG levels and their contents in the heart of mice with HFpEF compared with WT controls (Figures S5F–S5G). Moreover, Oil Red O staining revealed a substantial increase in cardiac lipid accumulation in *Fgf21* KO mice with HFpEF compared to WT controls (Figure S5H). In addition, treatment with recombinant mouse FGF21 (rmFGF21) also significantly decreased PA-induced lipid uptake in primary NRVMs (Figure S5I). Consistently, cardiac mRNA expression level of fatty acid transporter *Slc27a1* but not *CD36, Fabp4, and Lpl*, was increased between *Fgf21* KO mice and WT controls with HFpEF. Accordingly, no significant difference in the cardiac mRNA expression levels of fatty acid oxidation-related enzymes, including *Cpt1b, Acadm, Acadl, Acadvl*, and *Echs1*, were seen between *Fgf21* KO and WT controls with HFpEF (Figure S5J). Collectively, these data suggest that the beneficial effects of FGF21 against HFpEF may be related to enhancing lipid transport and lowering lipid accumulation in the heart of mice. We also included these new data in our manuscript (*see page 5, lines 184 – 207*).

Specific:

Q1: The authors should show the effect of AAV-*Fgf21* alone in the control heart in many experiments, such as Fig. 3J-L and PDK4shRNA alone in the control heart in the entire Fig. 5.

Answer: Thank you for your suggestion. We have now included relevant AAV-*Fgf21* and *Pdk4* shRNA control groups in corresponding Figures 3 and 5, respectively, and these revisions did not influence our conclusion (*Figures 3J-L, S3, and Figure 5, see pages 6-7, lines 260-284*).

Q2: Many morphological studies, including EM and oxidative stress, lack quantification or statistical analyses.

Answer: We have now included relevant quantification analysis results in corresponding morphological studies and updated all relevant figures in our manuscript (*Figures 3B-3C, 3M-3N, 6H-6I, and 8F-8G*).

Q3: Regarding the data presentation, the Y axis in many bar graphs does not use the full range (not starting from zero), which is misleading.

Answer: We have revised the Y-axis by a full scale (commencing from zero) respectively.

Reviewer #2:

Q1. The authors seemed to inject AAV9-*Fgf21* into mice via tail vein. What is the main tissue that produces circulating FGF21 after AAV9-*Fgf21* treatment (Figure S1D)? AAV9 typically targets heart, liver and muscle. Is cardiomyocyte-derived FGF21 protective against cardiac diastolic dysfunction in HFpEF mice after AAV9-*Fgf21* treatment? The authors mentioned that the liver and adipose tissues may serve as the primary sites for producing endogenous FGF21 in response to HFpEF. These points should be clarified.

Answer: Thank you for your comment. Just like the response to Reviewer #1 Q2, our new data showed that the elevated FGF21 contents were seen in white adipose tissue (WAT), liver, and heart, but not in brown adipose tissues (BAT) and muscle after treatment with AAV9-*Fgf21* (*Figures S1D–S1H*). These data are now included in the revised manuscript (*see page 4, lines 164 - 167*).

To confirm if cardiomyocyte-derived FGF21 protects against HFpEF in mice after AAV9-*Fgf21* treatment, all mice used for the HFpEF model or control were pre-treated with AAV9-*Fgf21* or AAV9-GFP by intramyocardial injection, respectively, before two weeks of HFD+L-NAME treatment (*Figure R2A*). The transfected effects were confirmed by immunoblotting after two weeks of AAV9-*Fgf21* treatment (*Figure R2B*). Our new data revealed that AAV9-mediated cardiac FGF21 expression significantly increased the E/A and decreased the E/E' (E/A: 1.40 ± 0.01 vs. 1.29 ± 0.01, $p < 0.05$; E/E': 23.79 ± 0.33 vs. 26.04 ± 0.71, $p < 0.01$, *Figure R2C*), suggesting that cardiomyocytes-derived FGF21 plays a mildly protective effect against HFpEF in mice after treatment with AAV9-*Fgf21*.

Notably, our data in the first version revealed that administration of AAV-*Fgf21* by the tail vein injection resulted in more pronounced improvements in diastolic function compared to intramyocardial injection (E/A: 1.56 ± 0.04 vs. 1.31 ± 0.03, p < 0.05; E/E': 21.85 ± 0.48 vs. 26.14 ± 0.59, p < 0.01, *Figure S3D*). These results indicate that even though cardiac-derived FGF21 partially ameliorates HFpEF-induced cardiac diastolic dysfunction in AAV9-*Fgf21*-treated mice, adipose tissue may be one of the primary expressive sites for producing FGF21 against HFpEF. To support this opinion, adipose-specific knockdown of FGF21 significantly exacerbated the pathogenesis of HFpEF (*Figure S2*), and no significant difference in HFpEF-related cardiac systolic dysfunction and cardiac damage was seen between general knockout and adipose-specific knockdown FGF21 mice (*Figures 2 and S2*).

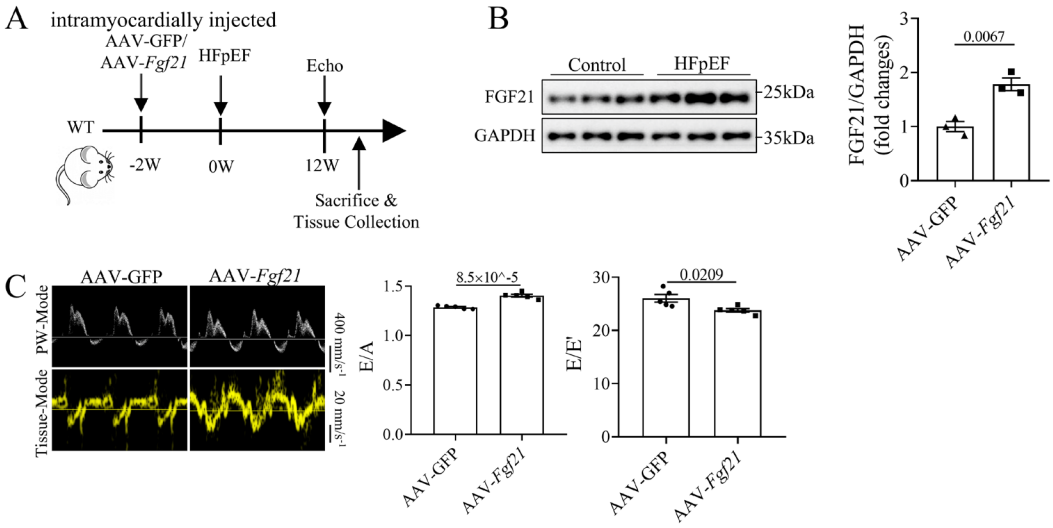

Figure R2. Cardiomyocyte-derived FGF21 improves HFpEF-induced cardiac diastolic dysfunction. (A) Schematic diagram illustrating the effects of intramyocardial injection of AAV-*Fgf21* in mice with HFpEF. (B) Immunoblot analysis of cardiac FGF21 content. (C) Left ventricular diastolic function in mice with HFpEF after intramyocardial injection with AAV-*Fgf21* or AAV-GFP. Left, pulsed-wave Doppler (top) and tissue Doppler (bottom) tracings of mice; Right, quantification of E/A and E/E'. Statistical analysis was

performed by using the two-tailed unpaired Student's t-test, and p-values < 0.05 were considered statistically significant. Data are presented as mean ± SEM. N⩾3 per group.

Q2. The authors should examine whether AAV9-*Fgf21* can rescue the cardiac phenotype of *Fgf21* KO mice or adipose *Fgf21* KO mice with HFpEF.

Answer: Thank you for your comment. To clarify the reviewer's comment, *Fgf21* KO mice with HFpEF were used to evaluate the rescue effect of AAV9-*Fgf21*. As expected, administration of AAV9-*Fgf21* significantly ameliorated HFpEF-induced cardiac diastolic dysfunction, pulmonary congestion, and exercise intolerance in both *Fgf21* KO and WT mice compared to AAV-GFP-treated controls, followed by reduced HW/TL ratios, decreased heart size and cardiomyocyte area, and downregulated the mRNA expressional levels of hypertrophic markers including *Anp, Bnp*, and *Myh7* in both *Fgf21* KO and WT mice with HFpEF. Moreover, AAV9-*Fgf21* administration also significantly attenuated HFpEF-induced ROS production and cardiac fibrosis in *Fgf21* KO and WT mice compared with AAV-GFP controls (*Figure S4*). These results collectively indicate that AAV9-*Fgf21* can effectively rescue the cardiac phenotype of *Fgf21* KO mice with HFpEF. These data are now included in the revised manuscript (*see page 4, lines 178 - 182*).

Q3. The authors used high-fat diet (HFD)+L-NAME-induced HFpEF mice in this study. Both FGF21 and adiponectin are metabolic regulators. It is possible that FGF21 and adiponectin could prevent cardiac diastolic dysfunction and damage by modulating glucose and lipid metabolism. The authors should measure metabolic parameters in these models.

Answer: Thank you for your comment. To clarify the reviewer's comment, we measured relevant metabolic parameters in these models. Our new data demonstrated that FGF21 deficiency exacerbated glucose and insulin intolerance in mice with HFpEF (Figures S5A–S5B). Notably, *Fgf21* KO HFpEF mice exhibited a significant decrease in cardiac mRNA levels of *Glut1* but not *Glut4*, compared to WT controls, while other glycolysis-related enzymes, including *Hk1, Hk2,* and *Pkm2* remained comparable (Figure S5C). Moreover, cardiac phosphorylated PDH protein levels were significantly increased in *Fgf21* KO mice with HFpEF compared to WT controls (Figure S5E). Regarding lipid metabolism, FGF21 deficiency significantly elevated plasma FFA and TG levels and their contents in the heart of mice with HFpEF compared with WT controls (Figures S5F-S5G). Moreover, Oil Red O staining revealed a substantial increase in cardiac lipid accumulation in *Fgf21* KO HFpEF mice compared with WT controls (Figure S5H). Consistently, the mRNA expression level of fatty acid transporter *Slc27a1* but not *CD36, Fabp4, and Lpl*, as well as fatty acid oxidation-related enzymes including *Cpt1b, Acadm, Acadl, Acadvl, and Echs1*, was increased in *Fgf21* KO HFpEF mice compared to WT controls (Figure S5J). Collectively, these data suggest that the beneficial effects of FGF21 against HFpEF may be related to enhanced glucose uptake and lipid transport in mice. We also included these new findings in our manuscript (*see page 5, lines 184 – 207*).

Considering that adiponectin is closely related to glucose and lipid metabolism, glucose and insulin resistance, as well as plasma FFA levels and its abundance in the heart of mice, were examined respectively. Our new data indicated that treatment with AAV-*Fgf21* significantly lowered glucose and insulin tolerance in WT mice with HFpEF. Interestingly, these effects were markedly attenuated in *adiponectin* (*Apn*) KO mice with HFpEF (Figure R3A), suggesting that adiponectin deficiency impacts the beneficial effects of FGF21 lowering glucose and insulin-sensitivity in HFD plus L-NAME-induced HFpEF mice. In addition, administration of AAV-*Fgf21* profoundly decreased plasma FFA and TG levels, as well as cardiac FFA and TG contents in the heart of WT mice with HFpEF. However, these effects were strikingly alleviated in *Apn* KO mice (Figures R3C-R3D). Taken together, our findings

indicated that adiponectin may partly mediate the beneficial effects of FGF21 on regulating glucose and lipid metabolism dysfunction in the pathogenesis of HFpEF.

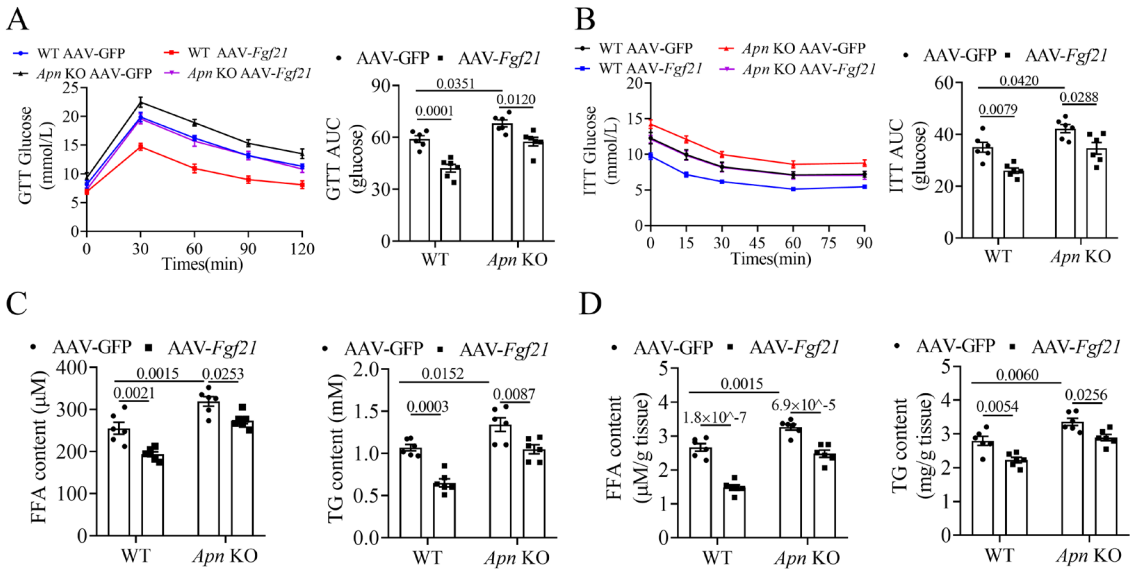

Figure R3. Adiponectin deficiency alleviates the protective effects of FGF21 against HFpEF-induced glucose and lipid metabolism disorders in mice. (A) Glucose tolerance with incorporated AUC of *Apn* KO and WT mice with HFpEF after treatment with AAV-*Fgf21* or AAV-GFP, respectively. (B) Insulin tolerance with incorporated AUC of mice mentioned above. (C) Serum FFA and TG levels of mice mentioned above. (D) Cardiac FFA and TG levels of mice mentioned above. Statistical analysis was performed by using two-way ANOVA, followed by Tukey's multiple comparison test, and p-values < 0.05 were considered statistically significant. Data are presented as mean ± SEM. N=6 per group.

Q4. Adiponectin is known to promote cardiac AMPK signaling pathway which typically antagonizes Akt cascade. The points should be clarified.

Answer: Thank you for your comment. As you pointed out, it is well-established that adiponectin activates cardiac AMPK signaling, which in turn antagonizes the Akt cascade in specific contexts. However, recent studies have demonstrated that adiponectin can also promote cardiac Akt signaling[9]. Notably, adiponectin and its agonists have been shown to concurrently activate both the Akt and AMPK signaling pathways in specific pathological states. For example, treatment of db/db mice with AdipoRon, a synthetic, orally available adiponectin receptor agonist, resulted in the upregulation of both Akt and AMPK signaling pathways in the heart and kidney[10, 11]. Additionally, adiponectin has been reported to exert protective effects against myocardial ischemia-reperfusion injury through simultaneous activation of AMPK, Akt, and nitric oxide pathways[12].

In this study, adipose-secreted adiponectin was found to activate the PI3K-Akt signaling pathway in primary NRVMs exposed to palmitic acid. Crucially, when exposed to the PI3K inhibitor LY294002, the protective effects of adiponectin against PA-induced mitochondrial dysfunction were significantly attenuated (Figure 8A), suggesting that the protective effects of adiponectin against PA-induced mitochondrial dysfunction may be mediated by activating the PI3K-Akt signaling pathway. These findings demonstrate that the interplay between AMPK and Akt signaling pathways is more complex than previously recognized and is highly context-dependent. While adiponectin-induced activation of AMPK can oppose Akt signaling in some cases, in certain disease states such as diabetes myocardiopathy, adiponectin can promote Akt activation despite concurrent AMPK signaling. This complexity presents the diverse biological functions of adiponectin observed under

different pathological conditions, and the relationship between adiponectin, AMPK, and Akt requires further investigation.

Q5. PI3-Akt signal pathway induces cardiac hypertrophy and growth. *Fgf21*-KO mice with HFpEF showed increased cardiomyocyte size and decreased phosphorylation levels of PI3 and Akt. Please comment.

Answer: Thank you for your comment. As you commented, our data indicated that *Fgf21* KO mice with HFpEF showed increased cardiomyocyte size, followed by decreased phosphorylation levels of PI3 and Akt. Given that the PI3K-AKT signaling pathway is pivotal for cell survival and cardiac growth, this seems paradoxical in this context.

Growing evidence indicated that the fundamental causes of cardiomyocyte hypertrophy observed in the HFpEF model could be attributed to several interrelated factors, which include insulin resistance and hyperinsulinemia, hypertension, high-fat diets- and hypertension-related chronic inflammation and oxidative stress, and so on. All these factors work together and increase systemic metabolic disorders, activate the SNS, activate RAAS, prompt oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress, and impair calcium homeostasis, finally resulting in cardiac hypertrophy[13, 14, 15]. Moreover, in the context of HFpEF, cardiomyocytes may struggle to effectively oxidize fatty acids due to mitochondrial dysfunction or impaired oxidative phosphorylation. This inefficiency can result in insufficient ATP production, prompting cardiomyocytes to undergo hypertrophy as a compensatory mechanism to meet increased energy demands[16]. Therefore, we speculate that even though the phosphorylation of the PI3K-Akt signaling pathway is attenuated in *Fgf21* KO mice with HFpEF, there are some other potential mechanisms rather than PI3K-Akt that may contribute to cardiac hypertrophy in *Fgf21* KO mice with HFpEF, its exact mechanism required further investigation. To support this opinion, our data showed that FGF21 deficiency caused a significant increase in cardiac PDK4 expression (Figure 4D), a crucial enzyme that negatively regulates the activity of the pyruvate dehydrogenase (PDH) complex, which has been showed to promote mitochondrial dysfunction. Moreover, our data also indicated that cardiac-specific silencing of PDK4 significantly improved cardiac hypertrophy in mice with HFpEF (Figure 5).

Q6. It is better to present the same blots in data from co-culture experiments (NRVMs with wt-SAT and ko-SAT) (i.e. Figures 8A and S9A).

Answer: Thank you for your comment. We understand your suggestion to present the same blots in the data from the co-culture experiments, including NRVMs with wt-SAT and ko-SAT in Figures 8A and S9A. However, these revisions to present all experimental groups (>13 samples) in the same blot pose a great challenge due to the complexity and redundancy of the experimental groups, which we believe may not enhance the clarity of the presentation.

Considering that SAT from adiponectin KO mice lose the function of adiponectin production, an additional experimental group, NRVMs co-cultured with ko-SAT exposed to PA, was included in our additional experiment (*Figure 8A)* as an adiponectin-deficiency control. This additional experiment enables us to present the same blots within the context of the co-cultured experiments, directly comparing NRVMs with wt-SAT and ko-SAT, as you recommended. Therefore, we updated our results to encompass pyruvate oxidation and mitochondrial bioenergetics (*Figures 8B-8H*), and this revision did not change our conclusion.

Q7. In Figure S8B, AAV9-*Fgf21* appears to decrease adiponectin levels.

Answer: Thank you for your comment. We apologize for the confusion caused by the error in the original Figure S8B. We have now corrected this issue in the revised *Figure S9B*. The updated figure now accurately demonstrates

that circulating APN levels were markedly decreased in mice with HFpEF, and these levels were significantly restored following intervention with AAV-*Fgf21*.

Reviewer #3

Q1. These authors have explored the role of FGF21 in the pathogenesis of HFpEF. In a series of detailed analyses, they point to a role of HFpEF-induced increases in FGF21 as a putatively compensatory response, impacting mitochondrial energetics among other things. In the end, they posit an APN-PI3K/AKT-PDK4 axis in this biology. Overall, I find the data to be strong but novelty to be limited.

FGF21 has been shown previously to confer benefits in the context of various cardiovascular disorders, acting through glucose and lipid metabolism and insulin sensitivity. The novelty in the present report is limited to its focus on HFpEF. The authors report that FGF21 levels are increased in HFpEF, an observation reported previously. Similarly, the role of PI3K/AKT in the actions of FGF21 has been reported previously. There are other aspects of the paper with limited novelty.

Answer: Thank you for your comment. As you mentioned, FGF21 has been shown to positively affect various cardiovascular conditions, mainly by regulating glucose and lipid metabolism as well as insulin sensitivity. There seem to be no significant novel findings.

However, to date, there have been no relevant reports regarding the role of FGF21 in the pathogenesis of HFpEF, a type of heart failure that is significantly different from heart failure with reduced ejection fraction (HFrEF). In this study, we initially provided evidence of both *gain-of-function* and *loss-of-function* to support its potential cardioprotective role against HFpEF in mice (Figures 2 and S3). Moreover, our data, for the first time, demonstrate that the cardioprotective effects of FGF21 against HFpEF-induced cardiac dysfunction and damage are mediated through the regulation of PDK4 signaling, which is the crucial enzyme that negatively regulates mitochondrial bioenergetics (Figures 5 and S7). In addition, we also provide *in vivo* and *in vitro* data to confirm the role of PDK4 in the pathological process of HFpEF and then testify that PDK4 is a downstream target molecule of FGF21 to negatively regulate mitochondrial function and improve HFpEF and its related cardiac damage (Figures 5, 6 and S7). Finally, our findings also indicate that genetic inhibition of PDK4 significantly reverses HFpEF-induced cardiac diastolic dysfunction and myocardial injury, as evidenced by improvements in diastolic function parameters and reductions in cardiac fibrosis and oxidative stress (Figure 5). These findings not only identify the FGF21-PDK4 axis as a novel therapeutic target with potential applications for treating this challenging condition but also open up an intriguing avenue for developing targeted therapies for HFpEF management.

Q2. In the end, it remains unclear to me the relative effects of APN per se versus its action downstream impacting PDK4.

Answer: Thank you for your comment. Adiponectin (APN), a cytokine mainly expressed in adipocytes, plays a crucial role in various biological processes, including metabolic regulation and anti-inflammatory and anti-apoptotic activity. APN deficiency exacerbates the development of obesity-related cardiovascular disease, including hypertension, adverse cardiac remodeling, myocardial infarction, and heart failure with preserved ejection fraction (HFpEF)[17, 18], and overexpression of adiponectin or activation of APN receptor by its agonist adipoRon significantly ameliorates HFpEF by promoting myocardial fatty acid oxidation, decreasing fatty acid transport, and inhibiting fibrosis[17]. These findings suggest that APN's protective effects against HFpEF may be related to regulating cardiac energy metabolism.

In this study, our new data indicated that APN deficiency markedly exacerbated HFD+L-NAME-induced HFpEF-related diastolic dysfunction in mice, and suppression of PDK4 significantly reversed HFpEF-induced systolic dysfunction in APN null mice (*Figure R4*), suggesting that the beneficial effects of adiponectin against HFpEF may be mediated by partly inhibiting PDK4 expression. Moreover, our *in vitro* data showed that adipose-derived

adiponectin significantly attenuated palmitic acid-induced mitochondrial dysfunction by activating the PI3K-Akt signaling pathway, which in turn inhibited PDK4 expression, then elevating PDH activity, lowering pyruvate accumulation, and finally improving mitochondrial bioenergetics (*Figure 8*). Taken together, these findings suggest that PDK4 is a downstream target of adiponectin that negatively regulates the pathological process of HFpEF.

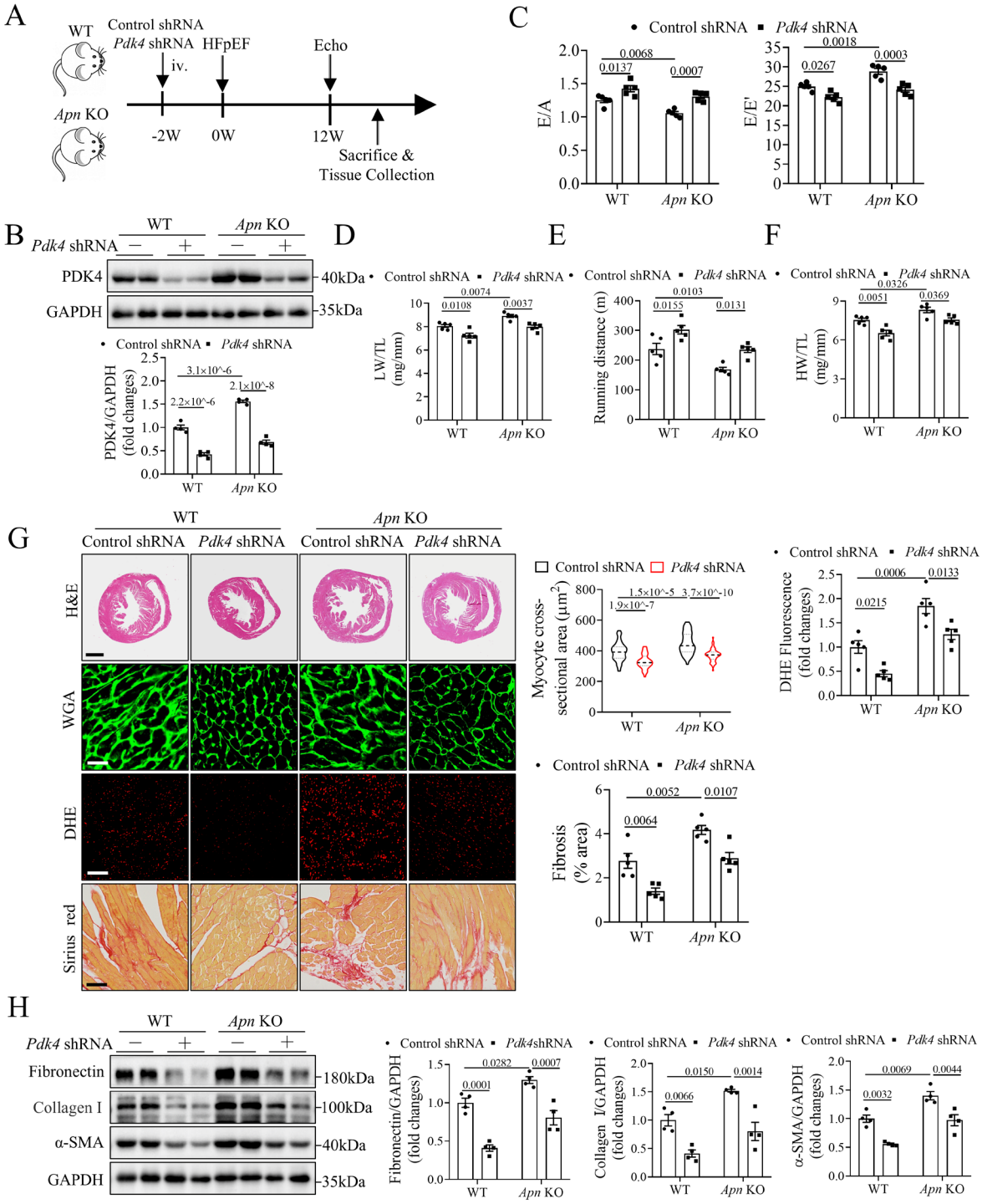

Figure R4. Knockdown of cardiac Pdk4 mitigates HFpEF-induced cardiac diastolic dysfunction in Apn KO mice. (A) Schematic diagram illustrating the effects of cardiac PDK4 knockdown in *Apn* KO mice and WT controls with HFpEF. (B) Immunoblot analysis of cardiac PDK4 content. (C) Left ventricular diastolic function. Left, quantification of E/A. Right, quantification of E/E'. (D) The lung weight to tibia length ratio of mice mentioned above. (E, F) Running distance during exercise exhaustion test (E) and mice's heart weight to tibia length ratio (F) mentioned above. (G) Representative H&E staining, WGA staining, DHE staining, and Sirius red staining of cardiac

sections (left). Quantifying cardiomyocyte cross-sectional area, DHE fluorescence, and fibrotic areas (right). Scale bars, 1 mm (H&E), 50 μm (WGA and Sirius red), and 100 μm (DHE). (H) Cardiac contents of fibrotic factors, including fibronectin, Collagen I, and α-SMA tested by immunoblot. Data are presented as mean ± SEM. Statistical analysis was performed by using two-way ANOVA, followed by Tukey's multiple comparison test, and p-values < 0.05 were considered statistically significant. Data are presented as mean ± SEM. N=4 or 5 per group.

Q3. To claim a statistical association based on Figure 1C (r=-.204) is a considerable stretch.

Answer: We appreciate the reviewer's insightful observation of the statistical association in Figure 1C. We agree that the correlation between FGF21 and LVEF (r = –0.204) does not represent a strong statistical relationship. However, we want to clarify that Figure 1C primarily presents the association between FGF21 and left ventricular systolic function (LVEF) but not the left ventricular diastolic function, such as E/A or E/E' ratios, in patients with HFpEF. Given that the left ventricular diastolic function of patients with HFpEF is defined by more than 50%. Therefore, the relationship between FGF21 and systolic function in patients with HFpEF is not powerful; a low r-value (correlation coefficient value) between FGF21 and LVEF is normal and reasonable; it just represents the characteristics between FGF21 and the left ventricular systolic function but not diastolic function in patients with HFpEF.

Q4. Overall, the manuscript is well written. However, there are a fair number of grammatical mistakes. An incomplete list includes the following:

Variant cardiac disorders

Mechanically should be mechanistically

Multi-organic crosstalk

These manners were also observed

Expressional site

Pharmaceutic inhibition

Lowed PDH activities

Upper animal results

The sentence starting at line 393 is incomplete.

Have been showed

Answer: Thank you for pointing out our manuscript's typos and grammatical errors. We appreciate your attention to detail, which has helped improve our work's clarity and accuracy. We have carefully reviewed the manuscript and corrected the errors as follows:

Line 25: We have corrected " variant cardiac disorders" to "various cardiac disorders".

Line 31: We have corrected " Mechanically" to " Mechanistically".

Line 56: We have corrected "multi-organic crosstalk" to "multi-organ crosstalk".

Line 222: We have corrected "these manners were also observed" to "these patterns were also observed".

Line 251: We have corrected " expressional site" to "expression site".

Line 333: We have corrected "lowed PDH activities" to "decreased PDH activities".

Line 321: We have corrected "upper animal results" to "aforementioned animal results".

Line 422: We have corrected " Have been showed" to "have been shown".

Moreover, we have revised the incomplete sentence starting at line 393 as follows: "Because the therapeutic effects of APN against HFpEF are found to be associated with improving myocardial oxidative stress and mitochondrial function in mice, and our above data indicated that PDK4 is a negative regulator in mitochondrial bioenergetics during the pathogenesis of HFpEF (*Figures 5 and S7*), we next explore whether APN mediates the beneficial effects of FGF21 against HFpEF by regulating PDK4-related mitochondrial bioenergetics." (See page 9,

lines 373–377). We also thoroughly reviewed the manuscript to identify and correct any typos or grammatical errors, ensuring the text's clarity and accuracy.

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Point-by-point responses to the reviewer's criticisms

Re: NCOMMS-24-21529B
FGF21 Protects Against HFpEF by Improving Cardiac Mitochondrial Bioenergetics in Mice

Reviewer #1:
Q1: Lines 164-167: Although the authors claim that AAV-Fgf21 increases FGF21 levels in the heart, the data shows no increase in the heart (Supplementary Fig. 1D and I).
Answer: Thank you for your comment. In this study, AAV9-FGF21 with a CMV promoter was used to perform the FGF21 overexpression experiment. Our current data indicate that treatment with AAV-FGF21 did not alter the FGF21 levels in the gastrocnemius (*Supplementary Fig. 1D*) and brown adipose tissue (BAT) (*Supplementary Fig. 1G*). However, there was a significant increase in FGF21 levels in the liver, white adipose tissue (WAT), and the mice's heart (*Supplementary Figs. 1E, 1F, and 1H*), as determined by immunoblotting, respectively.

On the other hand, our initial data indicated that treatment with AAV-FGF21 did not affect cardiac FGF21 expression in mice when all upper organ tissues were tested together through immunoblotting with a short exposure time (*Supplementary Fig. 1I and Fig.R1A*). However, a slight but significant increase in FGF21 levels was noted in the heart tissue of HFpEF mice when tested with a longer exposure time during immunoblotting, even though its abundance of FGF21 was prominently low compared with other tissues such as liver and WAT (*Figure R1B*). Consistently, our immunoblot data from the heart tissue of mice indicated that cardiac FGF21 contents were significantly increased in mice after treatment with AAV-FGF21 (*Supplementary Fig.1H*). Taken together, these results suggest that cardiac FGF21 contents are slightly but significantly increased in HFpEF mice after treatment with AAV-FGF21. Therefore, we will update Supplementary Fig.1I in our manuscript, and this revision does not influence our conclusion of this manuscript.

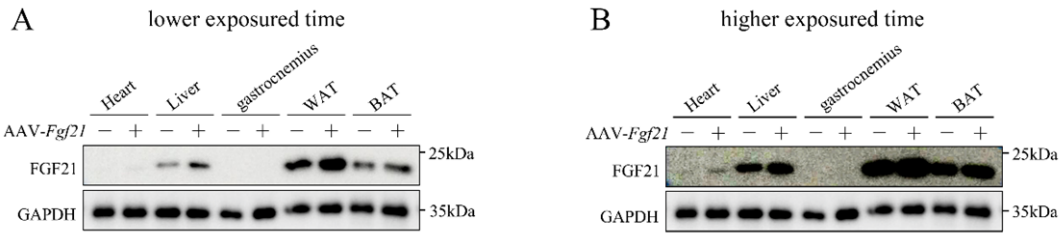

Figure R1. Immunoblotting with lower and higher exposure times was used in supplementary Figure 1I. A: lower exposure time; B: higher exposure time.

Q2. Lines 167-168: In the two-hit mouse model of HEpEF, the development of HFpEF relies on high blood pressure. It would be hard to exclude the possibility that FGF21 alleviates HFpEF just because it lowers blood pressure.
Answer: Thank you for your comment. Regarding FGF21's beneficial effects against HFpEF, we agree with the reviewer that it would be hard to exclude the possibility that FGF21 alleviates HFpEF just because it lowers blood pressure.

In this study, our current data indicated that loss of FGF21 increased blood pressure in L-NAME+HFD-fed mice, but these effects were significantly reversed when treated with AAV-FGF21 (*Fig. 2 and Supplementary Fig.3*), suggesting that the protective effects of FGF21 against HFpEF may be related to lowering blood pressure. Moreover, our data also demonstrated that FGF21 deficiency exacerbates glucose and lipid

metabolism disorders and aggravates mitochondrial dysfunction in L-NAME+HFD-induced HFpEF mice (*Supplementary Fig. 5*), suggesting that FGF21's protective effects in response to HFpEF may be mediated by improving cardiac metabolic disorders and mitochondrial dysfunction.

Considering that mitochondrial dysfunction is the principal pathologic molecular event of HFpEF, and PDK4 is a critical enzyme that regulates pyruvate oxidation in mitochondria. To assess the impact of blood pressure on the pathological progression of HFpEF, cardiac PDK4 expression of mice was evaluated after administration of L-NAME stimulation alone before performing this project. Our unpublished data revealed that treatment with L-NAME alone did not alter cardiac PDK4 expression in mice (*Figure R2*), suggesting that hypertension alone is insufficient to replicate the full spectrum of HFpEF pathology.

Taken together, our data demonstrated that FGF21's protective effects on HFpEF may depend on its ability to lower blood pressure and improve cardiac metabolic disorders and mitochondrial dysfunction.

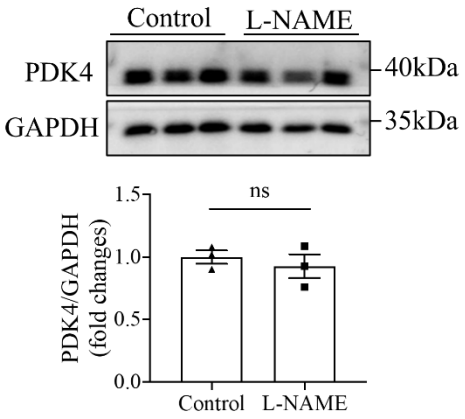

Figure R2. The expressional difference of cardiac PDK4 in the L-NAME-treated mice and controls. Statistical analysis was performed by using the two-tailed unpaired Student's t-test. Data are presented as mean ± SEM. N = 3 per group.