Peer Review File

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Reviewer A

Comment 1: Abstract

There is little description of methods in the Abstract, and we recommend that the authors report the main methods of this study. For example, immunofluorescence staining, western blot and scratch test were used to investigate the anti-fibrotic action of miR-29b-mediated activation of M_3 receptor in cardiac fibroblasts.

Reply 1: We have modified our text as advised and present the revisions below .

Methods: Proliferation of cardiac fibroblasts were induced by transforming growth factor (TGF)-β1 in vitro. The expression of miR-29b in cardiac fibroblasts was detected by qRT-PCR. Protein levels of collagens I, connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA) and beta-site app cleaving enzyme 1 (BACE1) were determined by Western blot analysis. Fibroblast-myofibroblast transition were identified by immunofluorescence staining. Proliferation and migration of cardiac fibroblasts as indicated by transwell and scratch assays.

Changes in the text: See Page 3, line 32-38

Comment 2: Highlight box

We are glad to see that the authors have filled the Highlight box. Would the authors further add directions for future research in the highlight box?

Reply 2: In fact, we have a lot of ideas for future research directions. Firstly, whether the cardioprotective effect produced by the activation of M_3 receptor can be translated into clinical practice, and secondly, whether miR-29b can be used as a prognostic marker for myocardial fibrosis, which is very important for our research. But this requires us to work with clinical trials, validated over a long period of time. Finally, we experimentally identified a target called BACE1, which plays a role in myocardial fibrosis, and it also plays an important role in amyloid deposition. This provides insight into the role of BACE1 in heart failure and myocardial fibrosis. Since myocardial fibrosis is usually a concomitant change in the heart, perhaps BACE1 plays some role in this process. This is also worth further consideration. We have also added this point in the highlight box as advised.

What is the implication, and what should change now?

This further illustrates the role of activating M_3 subtype of muscarinic acetylcholine receptor in exerting anti-fibrotic effects and provides ideas for clinical treatment.

Further explore the role of Beta-site app cleaving enzyme 1, the target identified in this study, in the progression of myocardial fibrosis and heart failure.

Changes in the text: See Page 5

Comment 3 (1) : Introduction

We suggest the authors clarify the study gap between previous work and this work in the Introduction, which was mentioned in Results: “However, how M3 receptor activation leads to fibrosis inhibition remains elusive.”

Reply 3 (1): We have modified our text as advised.

Our pilot study revealed that M_3 receptor was expressed in cardiac fibroblasts in almost the same abundance as cardiomyocytes under basal conditions, and activation of M_3 receptor inhibited cardiac fibrosis and reduces collagen secretion in mice by modulating the classical pathways of fibrosis, TGF-β1/Smad2/3 and p38 MAPK signaling pathways. M_3 receptor belongs to the class of G protein-coupled receptors that mediate many physiological responses in eukaryotes, and it is not clear whether new molecules are involved in this anti-fibrotic process after the activation of M_3 receptor, nor what its upstream and downstream factors are. We explored new regulatory factors and targets of this process in the present study.

Changes in the text: See Page 7, line 93-97

Comment 3 (2): Introduction

Please explain how the animal species used address the scientific objectives and, where appropriate, the relevance to human biology.

Reply 3 (2): The mice we used were characterized by easy rearing, high reproduction rate, easy accessibility, small inter-individual differences, and high disease resistance and adaptability, which improved the efficiency of our experiments and facilitated the observation of the results of parallel experiments. Meanwhile they're close to humans in terms of biological evolution, with similar tissue organs and cellular functions and good genetic homology. Cells in primary culture have just been separated from living tissues and are closer to the morphology, structure and functional activities of the original tissues in the organism, and are able to represent signal transduction in vivo very closely, thus allowing for better cellular disease models to be established through primary cells.

Comment 3 (3): Introduction

"... with a focus on miR-29b that is known to be a regulator of cardiac fibrosis". Since miR-29b is the focus of the study, we suggest the authors give a brief introduction to it (Some of the Discussion could be put in the Introduction).

Reply 3 (3): We have modified our text as advised, adding more introduction about miR-29b and restructuring the discussion.

Micro-RNAs belong to a highly conserved, endogenous non-coding RNA family with a short length of approximately 22 nucleotides, and these molecules post-transcriptionally regulate gene expression by binding to the 3' -untranslated region of targeted messenger RNAs by partial complementarity. MiR-29 forms a miRNA seed family, which consists of several members including miR-29a, miR-29b and miR-29c. Mature miR-29 is highly conserved in human, mouse and rat. MiR-29b is involved in a variety of pathways that regulate cardiovascular disease, and it may be useful for treatment or diagnostic of diseases. While a part of roles of miR-29b remain poorly understood, recent studies have provided important insights into the relevance of miR-29b to fibrotic diseases, such as the miR-29b plays a protective role in renal fibrosis, liver fibrosis, and pulmonary fibrosis. Moreover, miR-29b plays an important role in myocardial fibrosis.

Changes in the text: See Page 8, line 110-121

Comment 3 (4): Introduction
Please provide the specific hypotheses of this study.
Reply 3 (4): We have modified our text as advised, adding the hypotheses in the end of introduction. "We assumed that the expression of miR-29b may be raised after activating the M_3 receptor, and the downstream target gene is controlled to affect fibrosis."
Changes in the text: See Page 9, line 138-140

Comment 4 (1): Materials and methods
Please provide the body weight and sex of the Neonatal Kunming mice and further relevant information on the health/immune status.
Reply 4 (1): Neonatal Kunming mice (1-3days old) were selected for this experiment, with a weight of 3-5g. We did not limit the sex of the mice, and randomly selected them for two reasons: first, it was difficult to distinguish the sex of the mice for one day years old, and second, the sex differences did not lead to bias in this experiment. The mice were provided by the Animal Center of the Second Affiliated Hospital of Harbin Medical University, and were quarantined as laboratory animals. We were able to obtain the neonatal kunming mice only after it was bred by the staff of the Animal Center of the Second Hospital of Harbin Medical University. The license number is SCXK(HeilongJiang)2019-001. We have added the license number to the materials and methods so that readers can access it.
The license number is SCXK(HeilongJiang)2019-001. The animal study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University, in compliance with "Laboratory animal—Guideline for ethical review of animal welfare" (GB/T 35892-2018) national guidelines for the care and use of animals. Approval number: Ky2016-103. Approval time: 9 March 2016.
Changes in the text: See Page 9-10, line 145-151

Comment 4 (2): Materials and methods
Were there any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress?
Reply 4 (2): We strive to reduce the animals required by experiments by improving experimental methods, and obtain more data with the minimum quantity of animals. Moreover, we painlessly execute animals and then take their hearts for in vitro studies, which is a relative substitute for live animals. Throughout the experiment, we are kindly treate with experimental animals, respect animal life, and thank animals for the effort for our human scientific research. We have modified our text as advised.
Changes in the text: See Page 10, line 151-157

Comment 4 (3): Materials and methods
Since this article is all vitro cell tests without any vivo verifications, the authors are strongly recommended to also carry out animal studies. Afterwards, please further revise the manuscript following the ARRIVE checklist (https://arriveguidelines.org/sites/arrive/files/documents/Author%20Checklist%20-%20Full.pdf).

Reply 4 (3): Thank you very much for pointing out this important issue, as this paper focuses on the molecular mechanism of anti-fibrotic effects of M_3 receptor activated by choline, which is a supplement to previous studies, only cell experiments were used. The related animal experiments are indeed in progress as well. But this part of the content will be published as data for another article.

Comment 5 (1): Results
Aside from the fact that it is unclear how the M3 receptor upregulates miR-29b expression, what are the other limitations? Such as potential sources of bias and imprecision associated with the results, etc.
Reply 5 (1): For statistical analysis applied to western blot, calculating the average of all data from different batches of experiments represents a suitable and more powerful analysis. However, in this study, western blot experiments showed too much variability across different batches of experiments, so we normalized control the data for each western blot gel and analyzed normalized data from different batches. This may be a limitation of this study that needs to be validated in future studies.

Comment 5 (2): Results
“whereas the opposite change or upregulation of miR-29b following choline stimulation”. Please clarify what is the opposite change.
Reply 5 (2): Because the expression of miR-29b has increased after choline stimulation, "the opposite change" is in contrast to a reduction in miR-29b caused by TGF-β1, and we have corrected our expression so that readers can understand.
The results showed the level of miR-29b was downregulated in cardiac fibroblasts induced by TGF-β1, whereas upregulated after further addition of choline, and 4-DAMP, a competitive antagonist of M3 receptor reversed the effect of choline (Figure 1A).
Changes in the text: See Page 16, line 277-280

Comment 5 (3): Results
"Transfection efficiency of miR-29b mimics was shown in supplementary data". Since there are two figures in supplementary data, we suggest the authors make it clear that it is Supplementary Figure 1. Also, please revise the similar situations together.
Reply 5 (3): We have modified our text as advised
Transfection efficiency of miR-29b mimics (supplementary Figure 1A) and miR-29b inhibitor (supplementary Figure 1B) was shown in supplements.
Changes in the text: See Page 16, line 287-288

Comment 6: Discussion
“However, pharmacological studies suggest that a high-choline diet aggravates cardiac dysfunction, inflammation, and fibrosis in a mouse model of heart failure.” Please add references.
Reply 6: Thank you for your careful reading of our manuscript. We have modified our text as advised.
However, pharmacological studies suggest that a high-choline diet aggravates cardiac

dysfunction, inflammation, and fibrosis in a mouse model of heart failure (26).
26. Shuai W, Wen J, Li X, Wang D, Li Y, Xiang J. High-Choline Diet Exacerbates Cardiac Dysfunction, Fibrosis, and Inflammation in a Mouse Model of Heart Failure With Preserved Ejection Fraction. *J Card Fail.* 2020 Aug;26(8):694-702. doi: 10.1016/j.cardfail.2020.04.017. Epub 2020 May 15. PMID: 32417378.
Changes in the text: See Page 23, line 421-423 and reference no.26

Comment 7: Would the authors describe who was aware of the group allocation at the different stages of the experiment (during the conduct of the experiment, the outcome assessment, and the data analysis)?
Reply 7: The different stages of the experiment (allocation period, experiment Implementation) were handled by different person. Specifically, WEN LI was responsible for the acquisition of cells as well as the grouping of administration and labeled with numbers, but subsequent experiments such as western blot, immunofluorescence, transwell, etc. were carried out by Jie Yu, Yilian Yang and Jia Wang, who went through the experiments only by the labeled numbers, without knowing what kind of treatment each group received. Results the evaluation and data analysis results were entered according to the corresponding numbers of each group by another person. The authors' contributions section also partially reflects the relevant content. Throughout the process, we tried to exclude experimental errors brought about by the subjectivity of the experimenters.

Comment 8: Please clearly define all outcome measures assessed (e.g. cell death or molecular markers).
Reply 8: We have defined the outcome measures in the text as advised.
We then overexpressed or inhibited the expression of miR-29b in cardiac fibroblasts by transfecting the cells with miR-29b mimics or inhibitor and detected the expression of cardiac fibrosis markers such as collagen Ⅰ, α-SMA and CTGF.

Changes in the text: See Page 16, line 283-285

Comment 9: The full text lacks specific values, so we suggest that the authors add specific data in the text or figure legends. If appropriate, please also report specific values for key results in the Abstract, rather than simply “inhibit” or “increase”. In addition, if p>0.01, please report the specific P value in the body text with two decimal places, for example, “P =0.01” “P =0.06” “P =0.10” “P =0.90”.
Reply 9: We have modified the figures and our text as advised and reported specific values for key results in the Abstract.
Results: The expression of miR-29b decreased when treated with TGF-β1 (P=0.0389) and increased after choline stimulated (P=0.0001). Overexpression of miR-29b could reverse the high expression of collagen Ⅰ (P<0.0001), α-SMA (P=0.0007), and CTGF (P=0.0038) induced by TGF-β1, whereas inhibition of miR-29b had a tendency to even further increase the expression of fibrosis markers. Meanwhile, inhibition of miR-29b could reverse the anti-fibrotic effect of choline, increasing the expression of collagen Ⅰ (P=0.0040), α-SMA

(P=0.0001), and CTGF (P=0.0185), and promoting the fibroblast proliferation and migration. Moreover, BACE1 protein level, increased after TGF-β1 treatment (P=0.0037) and reversed by overexpression of miR-29b (P=0.0493). Choline could reduce the increase of BACE1 induced by TGF-β1 (P=0.0264), and 4-DAMP increased the expression of BACE1 (P=0.0060). Furthermore, overexpression of BACE1 can reverse the protective effect of miR-29b in cardiac fibrosis, increasing the protein level of collagen Ⅰ (P=0.0404).

Changes in the text: See Page 3-4, line 39-51

Comment 10 (1): Western Blot
To further improve the reporting transparency of Western Blot figures when Western Blot experiments are used in a study, we strongly recommend that its report adopt several recommendations as follows.

· Ensure that the raw data is accessible to readers. The authors could provide the original images without cropping or any other processing in the Supplement.

· Crop as few as possible. The presence of non-specific bands is very common and the reader needs to be aware of these.

· Better to present with dot plots, which can inform readers about sample size and spread of data.
Reply 10 (1): Thanks for your suggestion, we have uploaded all the original data of western blot with the revision. Other original data will also be uploaded as needed.

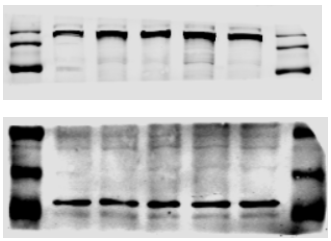
The figures in our text, we adopted a combination of dot plots and column bar charts so that readers can visualize the expression of each sample.

Comment 10 (2): Western Blot

In Figure 2A, the protein bands of Collagen Ⅰ in lane 3 and Lane 5 are different in depth, but the difference in the expression of histogram is not obvious. The same is true for lane 5 in Figure 2B. We suggest the authors give the original figure.
Reply 10 (2): Thank you for your rigorous consideration. We have uploaded all the original data of western blot with the revision. We suggest this is due to the different batches of samples we used. Regarding the statistical analysis applied to western blots, calculating the average of all data from different batches of experiments represents an appropriate and more powerful analysis. However, in our study, western blot experiments presented too much variability in batches of experiments, so we normalized the data to control in each western blot gel firstly, then analyzed the normalized data from different batches. This might represent a limitation of the study, which should be validated in future study.

Figure 2A (Collagen Ⅰ 129kda; GAPDH 36kda)

1.



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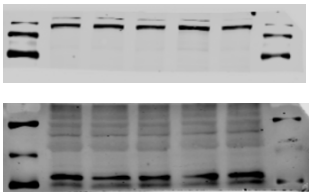

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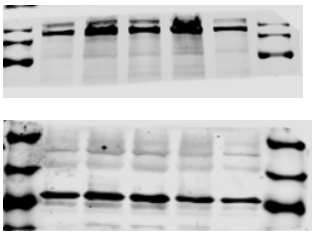

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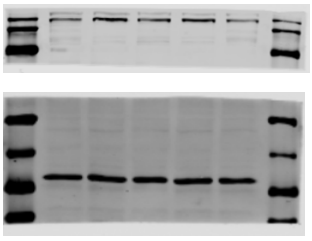

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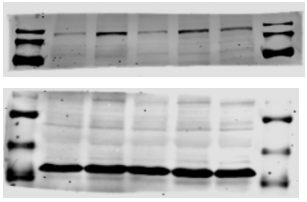

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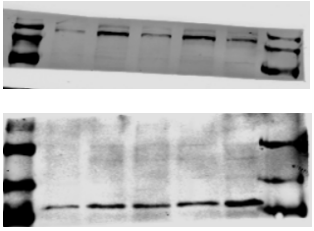
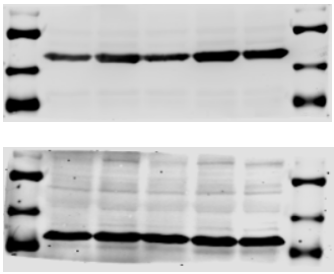
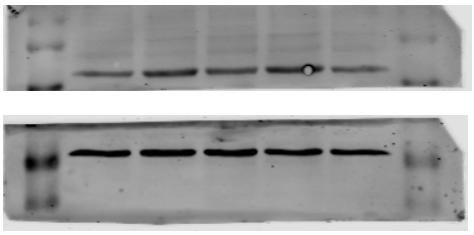


Figure 2B (α-SMA 43kda; GAPDH 36kda)

1



2



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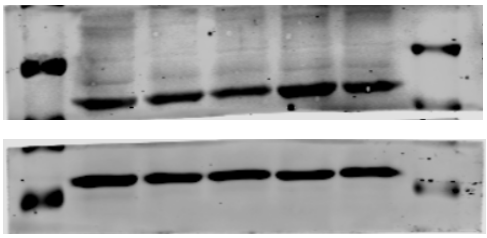
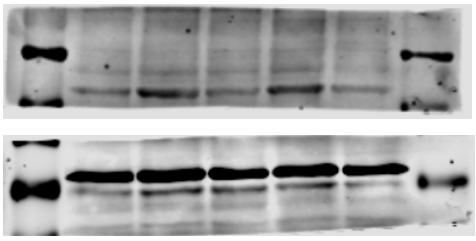


4



Comment 11: In section 3.2, the “Figure 4A” mentioned should be Figure 4A-B and “Figure 4B” should be “Figure 4C-D”.

Reply 11: We were really sorry for our careless mistakes. Thank you for your reminder. We have modified our text as advised.

We found that TGF-β1 promoted the migration of cardiac fibroblasts, which was inhibited by choline, indicating the anti-fibrotic effect of the M_3 receptor. Transfection of miR-29b inhibitor eliminated the protective effect of choline and aggravated the TGF-β1-induced migration of cardiac fibroblasts (Figure 4A-B). In the second set of experiments, we used the transwell assay to measure the migratory and invasive ability of cardiac fibroblasts. The results showed that the migration/invasion suppressing effects of choline were abolished following a knockdown of miR-29b by its antisense inhibitor (Figure 4C-D).

Changes in the text: See Page 18, line 320 and line 324

Comment 12: We suggest the authors simplify the figure legends, for example, Figure 1

(B-D) can be simplified to "Relative protein level of collagen Ⅰ (B, n=6), α-SMA (C, n=6), and CTGF (D, n=7) in cardiac fibroblasts detected by western blot. **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05".

Reply 12: Thank you for your suggestion. We have modified the figure legends as advised.

Figure 1. (B-G) Relative protein level of collagen Ⅰ (B, n=6; E, n=6), α-smooth muscle actin (α-SMA) (C, n=6; F, n=4), and connective tissue growth factor (CTGF) (D, n=7; G, n=6) in cardiac fibroblasts detected by western blot.

Figure 3. (A,E) Relative collagen Ⅰ protein level in cardiac fibroblasts detected by immunofluorescence. (B,F) Statistical results of immunofluorescence of collagen Ⅰ (B, n=6; F, n=3). (C,G) Relative α-SMA protein level in cardiac fibroblasts detected by immunofluorescence. (D,H) Statistical results of immunofluorescence of α-SMA (D, n=6; H, n=3).

Figure 5. (C,D,F,G) Relative protein level of BACE1 in cardiac fibroblasts detected by western blot (C, n=4; D, n=4; F, n=5; G, n=4).

Changes in the text: See the figure legends highlighted.

Comment 13 (1): Abbreviations

We suggest that the abbreviations in the title, keywords and Highlight box be changed to the full name, such as miR-29b and BACE1.

Reply 13 (1): Thank you for your suggestion. We have modified the figure legends as advised.

Title: M3 subtype of muscarinic acetylcholine receptor inhibits cardiac fibrosis via targeting micro RNA-29b/beta-site app cleaving enzyme 1 axis

Keywords: Cardiac fibrosis, M3 subtype of muscarinic acetylcholine receptor, Micro RNA-29b, Beta-site app cleaving enzyme 1

Highlight box:

Key findings

□Choline produces the anti-fibrotic effects through activating M3 subtype of muscarinic acetylcholine receptor to upregulate micro RNA-29b in cardiac.

□Beta-site app cleaving enzyme 1, a downstream target gene of micro RNA-29b, involved in the anti-fibrotic effects of activing of M3 subtype of muscarinic acetylcholine receptor.

What is known and what is new?

□Anti-fibrotic effects of micro RNA-29b are known.

□Whether the M3 subtype of muscarinic acetylcholine receptor can influence micro RNA-29b/Beta-site app cleaving enzyme 1 expression and thus exert an antifibrotic effect is unknown.

What is the implication, and what should change now?

□This further illustrates the role of activating M3 subtype of muscarinic acetylcholine receptor in exerting anti-fibrotic effects and provides ideas for clinical treatment.

□Further explore the role of Beta-site app cleaving enzyme 1, the target identified in this study, in the progression of myocardial fibrosis and heart failure.

Changes in the text: See the changes highlighted in the title, keywords and Highlight box.

Comment 13 (2): Abbreviations

Please give the full name of the abbreviations in the legends of Figure 1-5, such as GAPDH, CTGF, α-SMA, etc.

Reply 13 (2): Thank you for your valuable suggestion. We have modified the figure legends as advised.

Figure 1: The anti-fibrotic effects of microRNA-29b (miR-29b) and it participate in the role of activating M3 subtype of muscarinic acetylcholine receptor (M3 receptor) in cardiac fibroblasts. (A) qRT-PCR was performed to detecte the relative expression of miR-29b in transforming growth factor beta 1 (TGF-β1)-induced cardiac fibroblasts when choline and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) stimulation. n = 6. (B-G) Relative protein level of collagen Ⅰ (B, n=6; E, n=6), α-smooth muscle actin (α-SMA) (C, n=6; F, n=4), and connective tissue growth factor (CTGF) (D, n=7; G, n=6) in cardiac fibroblasts detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. The data are expressed as the mean ± SEM. NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values are shown in the figure.

Figure 2: Inhibition of microRNA-29b (miR-29b) after M3 subtype of muscarinic acetylcholine receptor (M3 receptor) activation increases the protein level of cardiac fibrosis markers. Relative protein level of collagen Ⅰ (A, n=6), α-smooth muscle actin (α-SMA) (B, n=4), and connective tissue growth factor (CTGF) (C, n=7) in cardiac fibroblasts detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values are shown in the figure.

Figure 3: Inhibition of microRNA-29b (miR-29b) reverses the anti-fibrotic effects of activating M3 subtype of muscarinic acetylcholine receptor (M3 receptor) and promotes the expression of collagen Ⅰ and α-SMA. (A,E) Relative collagen Ⅰ protein level in cardiac fibroblasts detected by immunofluorescence. (B,F) Statistical results of immunofluorescence of collagen Ⅰ (B, n=6; F, n=3). (C,G) Relative α-SMA protein level in cardiac fibroblasts detected by immunofluorescence. (D,H) Statistical results of immunofluorescence of α-SMA (D, n=6; H, n=3).The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor; 4-DAMP: 4-diphenylacetoxy-N-methylpiperidine methiodide. The P values are shown in the figure.

Figure 4: Inhibition of microRNA-29b (miR-29b) after M3 subtype of muscarinic acetylcholine receptor (M3 receptor) activation promotes the migration ability of cardiac fibroblasts. (A) The migration rate of cardiac fibroblasts detected by scratch test. (B) Statistical results about the migration rate of cardiac fibroblasts. n = 4. (C) The migration ability of cardiac fibroblasts was detected by transwell. (D) Statistical results about migration ability of cardiac fibroblasts. n = 4. The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b

inhibitor. The P values are shown in the figure.

Figure 5: MicroRNA-29b (miR-29b) participates in the anti-fibrotic effects after M3 subtype of muscarinic acetylcholine receptor (M3 receptor) activation through targeting beta-site app cleaving enzyme 1 (BACE1). (A) Screening target genes of miR-29b by online prediction websites: miRDB, miRTarBase, TargetScan, and PicTar. (B) The binding sites of miR-29b and BACE1 in different species. (C,D,F,G) Relative protein level of BACE1 in cardiac fibroblasts detected by western blot (C, n=4; D, n=4; F, n=5; G, n=4). (E) qRT-PCR assay was used to examine the expression of miR-29b in the presence of BACE1 overexpression. n=8. (H) Relative protein level of Collagen Ⅰ in cardiac fibroblasts detected by western blot. n = 6. The data are expressed as the mean ± SEM. Student's t-test was used for two-group comparisons. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values are shown in the figure.

Changes in the text: See the figure legends highlighted.

Comment 14: Please provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.

Reply 14: Thank you for your suggestion, we designed the research protocol before the formal experiment, but we apologize that we did not register it. We reported it within the school as well as reviewed it. There are paper copies of the materials in the school archives to prove this and we are ready to provide them when you need them.

Comment 15: The fund 81673424 has matured (2017-2020).

Reply 15: Thanks for your careful checks. Although the fund 81673424 has matured, according to the relevant regulations of the National Natural Science Foundation of China, within three years of the expiration date, additions can still be made to the original results, which is why we have listed this fund.

Comment 16: Based on the Author Instruction (https://cdt.amegroups.org/pages/view/guidelines-for-authors#content-2-1), the structure of the Abstract, Introduction and Discussion can be organized following the structure template (https://cdn.amegroups.cn/static/public/2.1-Structure%20of%20Original%20Articles-template-V2022.11.4.docx?v=1690872634463).

Reply 16: We organized the structure of the Abstract, Introduction and Discussion following the structure template, modified our text as advised.

Changes in the text: See the Abstract, Introduction and Discussion

Reviewer B

The present manuscript by Li et al. deals with the anti-fibrotic effect of choline on cardiac fibroblasts and proposes a M3/miR-29b/BACE1 dependent mechanism. The manuscript is well written and most of the presented data are reasonable. However, there are a few points which are not clear yet and need to be addressed in the revised version of the

manuscript. Thus, this reviewer recommends the paper for publication after some minor corrections:

Comment 1: The abstract is not well written. The reader is getting confused whether miRNA-29b is beneficial or detrimental to cardiac fibrosis. Please make the summary of the results clearer and give a short introduction to the measured factors regarding their role in cardiac fibrosis. Furthermore, the used model should be introduced quickly.

Reply 1: We gratefully appreciate for your valuable suggestion. We have modified our text as advised and have attached the revised abstract below.

Backgroud: Previous studies have confirmed that choline exerts anti-fibrotic effect in the heart by activating the M_3 subtype of muscarinic acetylcholine receptor (M_3 receptor), but the mechanism remains to be clarified. Micro RNA-29b (miR-29b) plays an important role in the fibrotic process and can directly target collagen to resist myocardial fibrosis. This study investigated whether miR-29b is involved in the anti-fibrotic effect of activating M_3 receptor.

Methods: Proliferation of cardiac fibroblasts were induced by transforming growth factor (TGF)-β1 in vitro. The expression of miR-29b in cardiac fibroblasts was detected by qRT-PCR. Protein levels of collagens I, connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA) and beta-site app cleaving enzyme 1 (BACE1) were determined by Western blot analysis. Fibroblast-myofibroblast transition were identified by immunofluorescence staining. Proliferation and migration of cardiac fibroblasts as indicated by transwell and scratch assays.

Results: The expression of miR-29b decreased when treated with TGF-β1 (P=0.0389) and increased after choline stimulated (P=0.0001). Overexpression of miR-29b could reverse the high expression of collagen Ⅰ (P<0.0001), α-SMA (P=0.0007), and CTGF (P=0.0038) induced by TGF-β1, whereas inhibition of miR-29b had a tendency to even further increase the expression of fibrosis markers. Meanwhile, inhibition of miR-29b could reverse the anti-fibrotic effect of choline, increasing the expression of collagen Ⅰ (P=0.0040), α-SMA (P=0.0001), and CTGF (P=0.0185), and promoting the fibroblast proliferation and migration. Moreover, BACE1 protein level, increased after TGF-β1 treatment (P=0.0037) and reversed by overexpression of miR-29b (P=0.0493). Choline could reduce the increase of BACE1 induced by TGF-β1 (P=0.0264), and 4-DAMP increased the expression of BACE1 (P=0.0060). Furthermore, overexpression of BACE1 can reverse the protective effect of miR-29b in cardiac fibrosis, increasing the protein level of collagen Ⅰ (P=0.0404).

Conclusions: The results suggested that M_3 receptor activation can exert cardioprotective effects in cardiac fibrosis by mediating miR-29b/BACE1 axis.

Changes in the text: See Page 3-4, line 25-53

Comment 2: The sentence in line 26 “Choline and […] of M3 receptor.” Makes no sense to me.

Reply 2: Thank you for your suggestion. We meant to give a brief overview of their purpose, we have modified our text as advised and briefly described them in the main text.

Changes in the text: See Page 3, line 33

Comment 3: In line 27 the authors write “mRNA level of micro-RNA-29b”. This is wrong as miRNA´s are no messenger RNA´s (mRNAs). Rather state “the expression of micro-RNA-29b…”
Reply 3: Thank you very much for pointing out this important issue. We have modified all the statements in our text as advised.
Changes in the text: See the changes highlighted in the text.

Comment 4: Line 33: …fibroblast proliferation and migration...
Reply 4: Thank you for your suggestion. We have modified our text as advised.
Meanwhile, inhibition of miR-29b could reverse the anti-fibrotic effect of choline, increasing the expression of collagen Ⅰ (P=0.0040), α-SMA (P=0.0001), and CTGF (P=0.0185), and promoting the fibroblast proliferation and migration.
Changes in the text: See Page 3, line 45-46

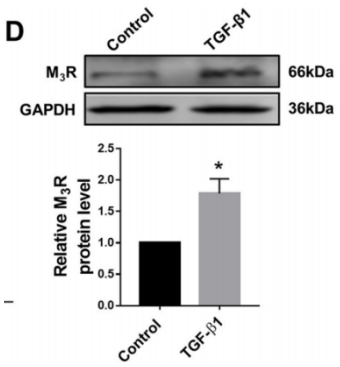
Comment 5: Line 34: ···increased after TGF-ß1 treatment···
Reply 5: Thank you for your suggestion. We have modified our text as advised.
Moreover, BACE1 protein level, increased after TGF-β1 treatment (P=0.0037) and reversed by overexpression of miR-29b (P=0.0493).
Changes in the text: See Page 3-4, line 46-47

Comment 6: The authors often use the term “Antimyocardial fibrosis” which is generally correct, but confusing because it suggests anti-myocardial effects. I rather suggest to just state “anti-fibrotic effects”.
Reply 6: Thank you for your suggestion. We have modified all the statements in our text as advised.
Changes in the text: See the changes highlighted.

Comment 7: Line 64: … is a hallmark change in myocardial fibrosis, characterized by the secretion of extracellular matrix proteins including collagen deposition…
Reply 7: Thanks for your suggestion. We have modified our text as advised.
The fibroblast-myofibroblast transformation is a hallmark change in myocardial fibrosis, characterized by the secretion of extracellular matrix proteins including collagen deposition.
Changes in the text: See Page 6, line 68-69

Comment 8: In lines 85-89 the authors state that M3 receptor is expressed in cardiac fibroblasts and its activation reduces collagen production. I was wondering whether M3 receptor is differentially expressed in fibroblasts versus activated myofibroblasts?
Reply 8: We agree with the reviewer’ s assessment,and verified the expression of M_3 receptors in fibroblasts and activated myofibroblasts in previous study, as demonstrated in the Figure 1D (1). TGF-β1 stimulated the transformation of fibroblasts to myofibroblasts, and the protein level of M_3 receptor was slightly elevated in this condition ($P<0.05$). We

considered that this might be a compensatory elevation in pathological conditions. Specific figure have attached below



(D) Effect of TGF-β1 (20 ng/ml, 48 h) on protein level of M_3R in CF. *p < 0.05 vs. Ctrl, n = 4.

(1) Zhao L, Chen T, Hang P, Li W, Guo J, Pan Y, Du J, Zheng Y, Du Z. Choline Attenuates Cardiac Fibrosis by Inhibiting p38MAPK Signaling Possibly by Acting on M3 Muscarinic Acetylcholine Receptor. Front Pharmacol. 10 (2019): 1386. doi:10.3389/fphar.2019.01386.

Comment 9: Line 211: ".. addition of 100 µl." 100 µl of what?

Reply 9: Thank you for your careful reading of our manuscript. We apologize for the confusion caused. What we were trying to convey is that 100 µl of the cell suspension redispersed in serum-free culture medium was taken and added to the chambers. We have modified our text to make it easier for readers to understand.

The transfected cells were harvested and reconstituted in serum-free buffer at 5×105 cells/mL, and 100 µL of the cell suspension was taken and added to the chambers.

Changes in the text: See Page 14-15, line 252-254

Comment 10: Line 233: The authors state that miR-29b was upregulated by Choline. In Fig 1A there is no statistical comparison of the control vs. the TGF-ß1+choline treated groups. Moreover, Suppl. Fig. 2 shows that treatment with solely choline has no effect on miR-29b expression. Please rephrase the conclusion made in line 233.

Reply 10: Thank you for your comment. Based on our experimental results, We suggest that under physiological conditions, the activation of M_3 receptor has no effect on the expression of miR-29b, which means without TGF-β1 stimulation. These data are displayed in Supplementary Figure 2. However, fibroblasts were transformed into myofibroblasts after TGF-β1 stimulation, at which time administration of choline to activate M_3 receptor increased the expression of miR-29b. There must be other mechanisms involved in this process, which also provides a direction for our subsequent research, and we will explore further. On this basis, our data in Figure 1A focus on comparing the expression of miR-29b with or without choline under TGF-β1 stimulation. And there is a statistical comparison of the TGF-β1 treated groups vs. the TGF-β1+choline treated groups. We have modified our text as advised.

The results showed the level of miR-29b was downregulated in cardiac fibroblasts induced by TGF-β1, whereas upregulated after further addition of choline, and 4-DAMP, a

competitive antagonist of M_3 receptor reversed the effect of choline (Figure 1A).
Changes in the text: See Page 156, line 277-280

Comment 11: Line 278: “had been being activated” – please correct the English spelling here.
Reply 11: We sincerely thank the reviewer for careful reading. As suggested by the reviewer, we have corrected the “had been being activated” into “had been activated”. These consistent results suggested that miR-29b inhibitor promoted cardiac fibrosis even while the M_3 receptor had been activated.
Changes in the text: See Page 18, line 326

Comment 12: Please shortly introduce BACE1 and its role in fibrosis and inflammatory disease. As it also plays a role in amyloid-deposition, it might be interesting to discuss its contribution to cardiac amyloidosis and potential therapeutic implications based on your findings.
Reply 12: At present, the pro-fibrotic role of BACE1 is unclear. It is reported that three types of myocardial fibrosis have been identified: replacement fibrosis from tissue necrosis, reactive fibrosis from myocardial stress, and infiltrative interstitial fibrosis from progressive deposition of non-degradable material such as amyloid (1). This provides a conjecture for the role of BACE1 in myocardial fibrosis. ST β-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) is a type II membrane protein that is commonly localized in the Golgi apparatus, and has been shown to regulate matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) in lung cancer (2). BACE1 can cleaves and releases ST6GAL1 from the trans-Golgi into the secretory pathway to regulate the secretion of inflammatory factors in COPD (3,4). Moreover, we reviewed the literature and found that NKX-COUP-TFII composite elements (NCCE) in regulatory regions of Madcam1 and St6gal1 involved in morphogenesis of the heart (5). Meprin β contributes to collagen deposition in lung fibrosis (6), and meprin β complements the role of BACE1 in Alzheimer's disease (7). These have the potential to play a role in the heart, but follow-up experiments are needed to confirm this. As BACE1 plays a role in amyloid-deposition, this provides insight into the role of BACE1 in heart failure and myocardial fibrosis. Since myocardial fibrosis is usually a concomitant change in the heart, perhaps BACE1 plays some role in this process. This also provides ideas for our next research direction, which deserves further consideration. We added this part to the discussion.
(1) Karur GR, Aneja A, Stojanovska J, et al. Imaging of Cardiac Fibrosis: An Update, From the AJR Special Series on Imaging of Fibrosis [published online ahead of print, 2023 Sep 27. AJR Am J Roentgenol. 2023;10.2214/AJR.23.29870. doi:10.2214/AJR.23.29870.
(2) Yuan Q, Chen X, Han Y, et al. Modification of α2,6-sialylation mediates the invasiveness and tumorigenicity of non-small cell lung cancer cells in vitro and in vivo via Notch1/Hes1/MMPs pathway. *Int J Cancer.* 2018;143(9):2319-2330. doi:10.1002/ijc.31737.
(3) Kitazume S, Oka R, Ogawa K, et al. Molecular insights into beta-galactoside alpha2,6-sialyltransferase secretion in vivo. *Glycobiology.* 2009;19(5):479-487. doi:10.1093/glycob/cwp003.
(4) Krick S, Helton ES, Easter M, et al. ST6GAL1 and α2-6 Sialylation Regulates IL-6

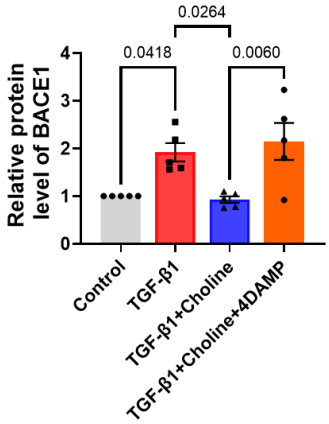
Expression and Secretion in Chronic Obstructive Pulmonary Disease. *Front Immunol.* 2021;12:693149. Published 2021 Jul 5. doi:10.3389/fimmu.2021.693149.
(5) Dinh TT, Xiang M, Rajaraman A, et al. An NKX-COUP-TFII morphogenetic code directs mucosal endothelial addressin expression. *Nat Commun.* 2022;13(1):7448. Published 2022 Dec 2. doi:10.1038/s41467-022-34991-2.
(6) Biasin V, Wygrecka M, Marsh LM, et al. Meprin β contributes to collagen deposition in lung fibrosis. *Sci Rep*. 2017;7:39969. Published 2017 Jan 6. doi:10.1038/srep39969.
(7) Marengo L, Armbrust F, Schoenherr C, et al. Meprin β knockout reduces brain Aβ levels and rescues learning and memory impairments in the APP/lon mouse model for Alzheimer's disease. *Cell Mol Life Sci*. 2022;79(3):168. Published 2022 Mar 2. doi:10.1007/s00018-022-04205-5.

Three types of myocardial fibrosis have been identified: replacement fibrosis from tissue necrosis, reactive fibrosis from myocardial stress, and infiltrative interstitial fibrosis from progressive deposition of non-degradable material such as amyloid. This provides a conjecture for the role of BACE1 in myocardial fibrosis ..... However, the role of BACE1 in cardiac fibrosis remains to be further investigated. ST β-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) is a type II membrane protein that is commonly localized in the Golgi apparatus, and has been shown to regulate matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) in lung cancer. BACE1 can cleaves and releases ST6GAL1 from the trans-Golgi into the secretory pathway to regulate the secretion of inflammatory factors in COPD. Moreover, we reviewed the literature and found that NKX-COUP-TFII composite elements (NCCE) in regulatory regions of Madcam1 and St6gal1 involved in morphogenesis of the heart. Meprin β contributes to collagen deposition in lung fibrosis, and meprin β complements the role of BACE1 in Alzheimer's disease. These have the potential to play a role in the heart, but follow-up experiments are needed to confirm this.

Changes in the text: See Page 23-24, line 430-457

Comment 13: Please indicate in Fig. 5D that the last two columns were simultaneously treated with TGF-ß1.

Reply 13: Thanks for your suggestion, which makes our figure look clearer. We have modified Figure 5D as advised.



Changes in the text: See Figure 5D

Comment 14: It is not clear from Fig. 5F that overexpression of BACE1 increases collagen levels, as indicated in the text. There is no additional effect of BACE1 besides the TGF-ß1-dependent increase in collagen. Please verify that the treatment combination legend is correct.

Reply 14: We feel sorry for our carelessness. The text "Moreover, BACE1 overexpression increased the expression of collagen I, whereas miR-29b mimics decreased it. "was described in the original manuscript used to explain Figure 5D. However, during subsequent revisions, we also realized that this was inappropriate, but omitted this part from the process. Thank you for pointing this out.

Regarding the second question you raised, we made a correction and we think your comment is correct. Overexpression of BACE1 only eliminates the role of miR-29b mimics in cardiac fibroblasts, and as to whether or not it would act directly on collagen I, that's really one of the limitations of this article. Meanwhile, we have plans to further investigate the role of BACE1 in the process of myocardial fibrosis and heart failure, and this part may be published as a new article. But the data available in this paper can confirm that BACE1 is indeed involved in the regulation of M_3 receptor and miR-29bin cardiac fibrosis. We have corrected the description in the text and added some descriptions in the discussion to answer this question.

Results: Moreover, overexpression of BACE1 eliminated the anti-fibrotic effect elicited by miR-29b mimics and restored the expression of collagen I (Figure 5F).

Discussion: Furthermore, BACE1 is involved in the anti-fibrotic effects of increasing the expression of miR-29b caused by the activation of M_3 receptor, and overexpression of BACE1 eliminated the role of miR-29b mimics in cardiac fibrosis. However, it is still unclear whether BACE1 can directly affect the level of collagen I, or whether we can directly act on BACE1 to exert anti-fibrotic effects. This problem is also one of the limitations of this article. We plan to further explore the relationship between BACE1 and cardiac fibrosis in future experiments. Hopefully, it will provide a new strategy for the treatment of cardiac fibrosis.

Changes in the text: See Page 19, line 349-351 and page 21, line 381-388

Comment 15: It remains unclear whether miR-29b levels are altered by overexpression of BACE1 in cardiac fibroblasts. Please show data on that in Fig. 5.

Reply 15: Thank you for your suggestion. Based on your suggestion, we added this part of the experiment and we found out that the expression of miR-29b can be inhibited when overexpressing BACE1, which is something we had not explored before. We think that it may be a negative feedback regulatory effect of BACE1, or caused by other modes of action of BACE1 with miR-29b, or other roles that BACE1 plays in myocardial fibrosis. This is also a new discovery for us and will be explored more deeply in subsequent studies. The relevant data have been displayed in Figure 5E as advised.

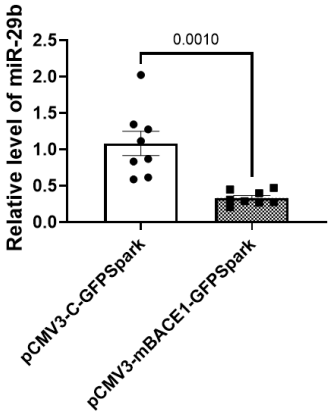


Figure 5. (E) qRT-PCR assay was used to examine the expression of miR-29b in the presence of BACE1 overexpression. n=8.

Comment 16: What about the endogenous expression of BACE1? Please comment on known mechanisms of the induction of pro-fibrotic BACE1.

Reply 16: Regarding the endogenous expression of BACE1, we believe that the first lanes of Figure 5D and Figure 5E can demonstrate the expression of BACE1 in cardiac fibroblasts without any treatment.

At present, the pro-fibrotic role of BACE1 is unclear. However, it is reported that three types of myocardial fibrosis have been identified: replacement fibrosis from tissue necrosis, reactive fibrosis from myocardial stress, and infiltrative interstitial fibrosis from progressive deposition of non-degradable material such as amyloid (1). This provides a conjecture for the role of BACE1 in myocardial fibrosis. This paper also proposed for the first time that BACE1 may play a pro-fibrotic role. In addition to the role of miR-29b on BACE1, in our previous reply, we also speculated whether BACE1 affects the fibrotic process by acting with ST6GAL1 or meprin β. We intend to further investigate the role of BACE1 and this may be published as a new article.

(1) Karur GR, Aneja A, Stojanovska J, et al. Imaging of Cardiac Fibrosis: An Update, From the AJR Special Series on Imaging of Fibrosis [published online ahead of print, 2023 Sep 27]. AJR Am J Roentgenol. 2023;10.2214/AJR.23.29870. doi:10.2214/AJR.23.29870

Comment 17: Figure legends: Please define the abbreviation “NC”; Please define “AMO-miR-29” in figure 2.

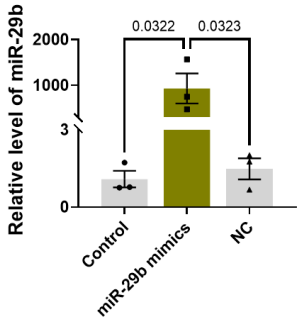
Reply 17: Thank you for your suggestion. We have modified the figure legends.

Figure 2. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor.

Changes in the text: See the legends of Figure 2.

Comment 18: Suppl. Fig. 1: With this display type, the individual measured values in the middle bar are not visible. Please select an axis display that allows a view of all measuring points.

Reply 18: Thank you for your suggestion. We have modified the Supplementary Figure1A as advised.



Changes in the text: See Supplementary Figure 1A.

Reviewer C

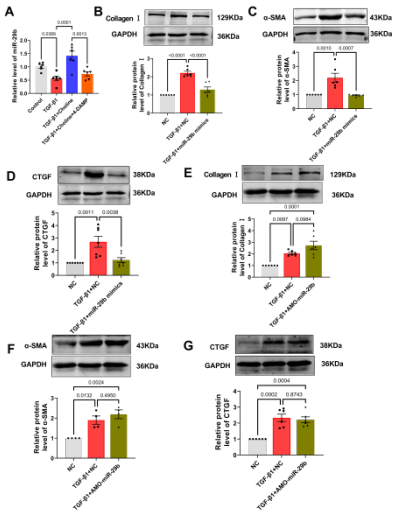
Wen Li has submitted an article titled “M3 subtype of muscarinic acetylcholine receptor inhibits cardiac fibrosis via targeting miR-29b/BACE1 axis. The authors used in vitro system to investigate the association between M3 receptor and miR-29b, a known cardiac fibrosis miR, in cardiac fibrosis. Using M3 receptor activator an inhibitor, choline and 4-DAMP, authors showed the modulation of miR-29b indicated a possible role in cardiac fibrosis. Further, using mimic and inhibitor of miR-29b followed by the treatment with choline and 4-DAMP, authors provided evidence that suggest a link between miR-29b and M3 in cardiac fibrosis. Finally, authors showed that BACE1, a possible target for miR-29b. Overexpression of BACE1 enhance collagen I level and both choline and 4-DAMP regulates the BAEC1 expression. Authors concluded that M3 receptor activation may provide cardioprotective effect by modulating miR-29b-BACE1-axis.

Comments

This a continuing investigation by the group in the line M3 and its role in cardiac fibrosis. The current study included a new player, the miR-29b in their analysis. The study is interesting and has a merit. The data support the authors conclusion. However, there are few stuffs that need to clarify and provide more information before it gets accepted. They are as suggested below:

Comment 1: Please add the data for miR-29b inhibitor in Fig1.

Reply 1: We gratefully appreciate for your comment. We experimentally validated and supplemented the data in Figure 1E,1F,1G. Related figures are listed below.



Changes in the text: See Figure 1E,1F,1G.

Comment 2: Add 4-DAMP treated data in Fig 2. I suggest making swap meaning bring the AMO-miR-29b data in Fig 1. Then add 4-DAMP data in Fig 2.

Reply 2: Thanks for your kind suggestions. Regarding your proposal to add the 4-DAMP treated group in Figure 2, relevant results have been shown in our previous studies(1), see Figure 2C and Figure 4B. These figures were illustrative of the point we are making about the role of 4-DAMP. So we did not repeat the addition of the 4-DAMP treated group. In Figure 1, we added the effects of inhibiting the expression of miR-29b on myocardial fibrosis as advised. And in Figure 2, we would like to further explain the role played by miR-29b in the process of activating M_3 receptor to against fibrosis. Therefore, we have not adjusted the order of the figures at this time, thank you for your suggestion.

(1) Zhao L, Chen T, Hang P, et al. Choline Attenuates Cardiac Fibrosis by Inhibiting p38MAPK Signaling Possibly by Acting on M3 Muscarinic Acetylcholine Receptor. *Front Pharmacol.* (2019): 1386. doi:10.3389/fphar.2019.01386.

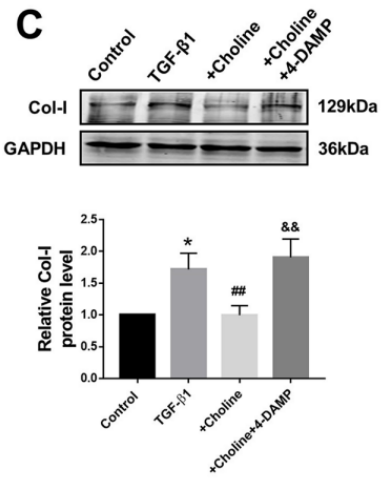


Figure 2. (C) Effect of choline on collagen I protein level in the different experimental groups. *p < 0.05 vs. Ctrl, ##p < 0.01 vs. TGF-β1, &&p < 0.01 vs. choline, n = 7.

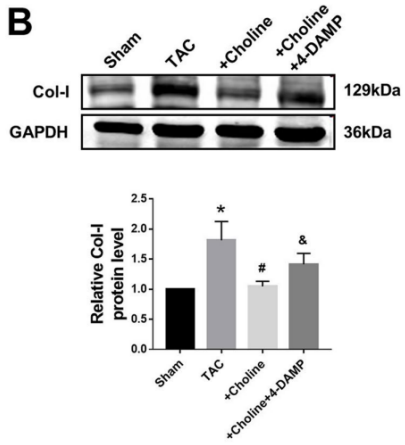


Figure 4. (B) Effects of choline and 4-diphenylacetoxy-Nmethylpiperidine methiodide (4-DAMP) on the protein level of collagen I. *p < 0.05 vs. sham, #p < 0.05 vs. TAC, &p < 0.05 vs. choline, n = 8.

Comment 3: Provide mimic data with 4-DAMP in Fig 3.
Reply 3: Thank you for your suggestion. We performed experiments and supplemented the groups and added the results in Figure 3.
Changes in the text: See Figure 3E,3F,3G,3H.
Changes in the text: See Figure 3.

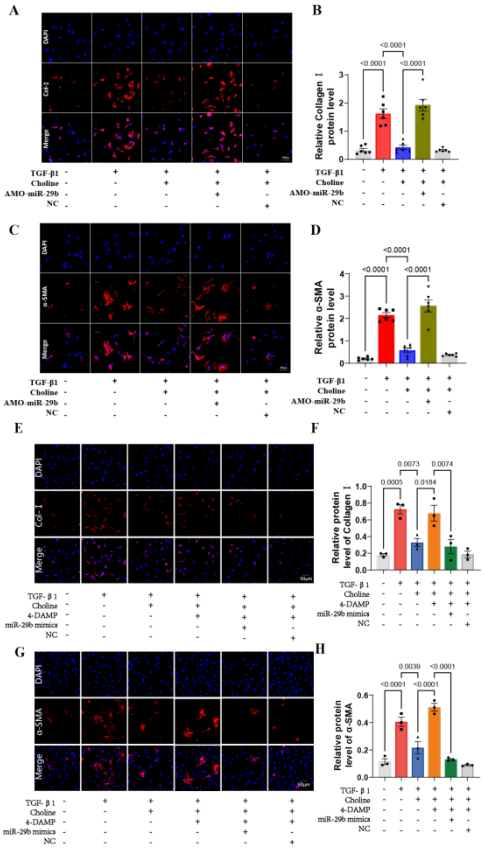
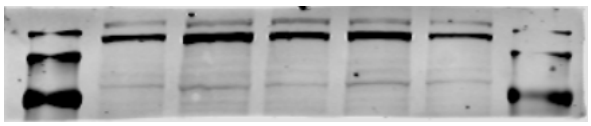


Figure 3: Inhibition of microRNA-29b (miR-29b) reverses the anti-fibrotic effects of

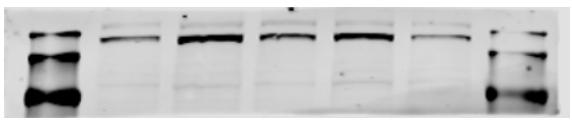
activating M3 subtype of muscarinic acetylcholine receptor (M3 receptor) and promotes the expression of collagen Ⅰ and α-SMA. (A,E) Relative collagen Ⅰ protein level in cardiac fibroblasts detected by immunofluorescence. (B,F) Statistical results of immunofluorescence of collagen Ⅰ (B, n=6; F, n=3). (C,G) Relative α-SMA protein level in cardiac fibroblasts detected by immunofluorescence. (D,H) Statistical results of immunofluorescence of α-SMA (D, n=6; H, n=3).The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor; 4-DAMP: 4-diphenylacetoxy-N-methylpiperidine methiodide. The P values are shown in the figure.

Comment 4: The Fig 5 is pivotal. This section needs few data for conclusion. Please add miR-29b inhibitor data in Fig 5C. The NC lane the BACE1 level is almost depleted, however, in Fig 5D, there is a strong band of BACE1 in control lane. Could authors clarify it? Does NC mean negative control (transfected cells, eight?) Please clarify all these in the legends.
Reply 4: Thanks for your suggestion. We have added the miR-29b inhibitor group and showed in the Figure 5D.
Your question about the different levels of BACE1 between Figure 5C and Figure 5D in the NC lane, this is due to the fact that we used different batches of cells, and we can also find the level of GAPDH is also different in these figures. On the other hand, there may be a difference in the process of adjusting the bands. We have uploaded all the original data of western blot in the Supplementary Data for your easy access at any time.
NC means the negative control (transfected cells n=4), which we have modified the figure legend as advised.
Changes in the text: See Figure 5 and the figure legends.

Comment 5: In Fig 5 F, can authors check whether the 4th bar (magenta color) is significant when compared with second bar? It seems the reduction is minimum.
Reply 5: We thank the reviewer for pointing this out. We think this is related to the fact that we adjusted the gray scale values too deeply, so we readjusted the gray scale values. Adjusted strips were added in Figure 5F, and we have submitted the original data of western blot with the revision. The strips before and after the adjustment are attached below.
before adjustment



after adjustment

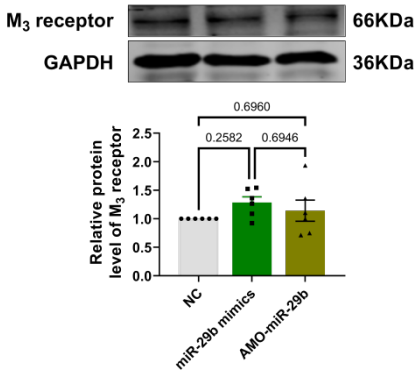


Changes in the text: See Figure 5F.

Comment 6: Can authors provide the data for M3R data (expression/level) in miR-29b mimic and inhibitor transfected cells? This is lacking.

Reply 6: Thank you for your valubale feedback, and we have verified it by supplementary experiments, but the current experimental results found that there is no effect on the protein level of M3 receptor when transfected with miR-29 mimics or miR-29b inhibitor. We suggested that M3 receptor may influence the expression of miR-29b through TGF-β/Smad signaling pathway, perhaps related to the way they interact. However, this still needs to be confirmed by further experiments. The data of the protein level of M3R in miR-29b mimic and inhibitor transfected cells have shown below and added in the Supplementary Figure 3 as advised.

Changes in the text: See Supplementary Figure 3.



Supplementary Figure 3. Relative protein level of M_3 receptor in cardiac fibroblasts transfected with miR-29b mimics and inhibitor detected by western blot. n = 6.

Comment 7: Please edit all legends and provide a description of the experiment performed. All abbreviation must be mentioned in all legends which are lacking.

Reply 7: Thank you for your suggestion. We have revised the legends and added abbreviations to all of them.

Figure 1: The anti-fibrotic effects of microRNA-29b (miR-29b) and it participate in the role of activating M3 subtype of muscarinic acetylcholine receptor (M3 receptor) in cardiac fibroblasts. (A) qRT-PCR was performed to detecte the relative expression of miR-29b in transforming growth factor beta 1 (TGF-β1)-induced cardiac fibroblasts when choline and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) stimulation. n = 6. (B-G) Relative protein level of collagen Ⅰ (B, n=6; E, n=6), α-smooth muscle actin (α-SMA) (C, n=6; F, n=4), and connective tissue growth factor (CTGF) (D, n=7; G, n=6) in cardiac fibroblasts detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. The data are expressed as the mean ± SEM. NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values are shown in the figure.

Figure 2: Inhibition of microRNA-29b (miR-29b) after M3 subtype of muscarinic acetylcholine receptor (M3 receptor) activation increases the protein level of cardiac fibrosis markers. Relative protein level of collagen Ⅰ (A, n=6), α-smooth muscle actin (α -SMA) (B, n=4), and connective tissue growth factor (CTGF) (C, n=7) in cardiac fibroblasts detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values

are shown in the figure.

Figure 3: Inhibition of microRNA-29b (miR-29b) reverses the anti-fibrotic effects of activating M3 subtype of muscarinic acetylcholine receptor (M3 receptor) and promotes the expression of collagen Ⅰ and α-SMA. (A,E) Relative collagen Ⅰ protein level in cardiac fibroblasts detected by immunofluorescence. (B,F) Statistical results of immunofluorescence of collagen Ⅰ (B, n=6; F, n=3). (C,G) Relative α-SMA protein level in cardiac fibroblasts detected by immunofluorescence. (D,H) Statistical results of immunofluorescence of α-SMA (D, n=6; H, n=3).The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor; 4-DAMP: 4-diphenylacetoxy-N-methylpiperidine methiodide. The P values are shown in the figure.

Figure 4: Inhibition of microRNA-29b (miR-29b) after M3 subtype of muscarinic acetylcholine receptor (M3 receptor) activation promotes the migration ability of cardiac fibroblasts. (A) The migration rate of cardiac fibroblasts detected by scratch test. (B) Statistical results about the migration rate of cardiac fibroblasts. n = 4. (C) The migration ability of cardiac fibroblasts was detected by transwell. (D) Statistical results about migration ability of cardiac fibroblasts. n = 4. The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values are shown in the figure.

Figure 5: MicroRNA-29b (miR-29b) participates in the anti-fibrotic effects after M3 subtype of muscarinic acetylcholine receptor (M3 receptor) activation through targeting beta-site app cleaving enzyme 1 (BACE1). (A) Screening target genes of miR-29b by online prediction websites: miRDB, miRTarBase, TargetScan, and PicTar. (B) The binding sites of miR-29b and BACE1 in different species. (C,D,F,G) Relative protein level of BACE1 in cardiac fibroblasts detected by western blot (C, n=4; D, n=4; F, n=5; G, n=4). (E) qRT-PCR assay was used to examine the expression of miR-29b in the presence of BACE1 overexpression. n=8. (H) Relative protein level of Collagen Ⅰ in cardiac fibroblasts detected by western blot. n = 6. The data are expressed as the mean ± SEM. Student's t-test was used for two-group comparisons. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor. The P values are shown in the figure.

Changes in the text: See the legends of Figure1-5.

Comment 8: In conclusion, authors should make a clear statement regarding their observation. The miR-29's role is known in cardiac fibrosis, M3R role in cardiac fibrosis is also known as authors previous have shown. So, I wonder what suggestion the authors are providing to audience for therapeutic use, miR-29b or M3 or BACE1.

Reply 8: We feel great thanks for your professional review work on our article. Indeed, our present article is an addition to the previous data, but we suggest for the first time that miR-29b can be involved in the anti-fibrotic effects of activating M_3 receptor. This part has not

been reported previously. It is also the first time that BACE1 was found to play a role in cardiac fibrosis. And we will further explore the role of BACE1 in the heart. These findings are a new addition to the previous studies and suggest new hypotheses for the mechanism of myocardial fibrosis, which may helpful to improve the process of myocardial fibrosis in the future. We have made a supplement in the discussion to reply to this comment.

In previous studies, we found that M_3 receptor are not only expressed in cardiomyocytes, but also in cardiac fibroblasts, and exert anti-fibrotic effects when activated by choline. In this study, we further confirmed that the role of M_3 receptor in myocardial fibrosis. But on top of that, we discovered a new member involved in this process, which is miR-29b. Activation of the M_3 receptor by choline can promote the expression of miR-29b, and we also found another member, BACE1, a new target of miR-29b in myocardial fibrosis. These results complement our previous research and provide new insights.

Changes in the text: See page 21, line 390-397

Re-review comments

Comment 1: It seems that the authors actually are doing animal experiment while they decide to publish the results in another article. I am wondering the possibility of getting their animal results?

Reply 1: Preliminary results from animal experiments were consistent with the experimental results in this article, in which we used a transverse aortic constriction (TAC) model to induce myocardial fibrosis in mice. Three days after TAC, the mice were divided into five experimental groups: sham, TAC, TAC+choline, TAC+choline+antagomiR-NC and TAC+choline+antagomiR-29b. Choline was administered by intraperitoneal injection at a dose of 14mg/kg. AntagomiR-NC and antagomiR-29b were administered caudally intravenously at a dose of 20 mg/kg once a week for 8 weeks. After eight weeks, heart tissue was taken for subsequent experiments. Due to the period of the model and the size limitations of the mouse heart, our experiments do not have a sufficiently large number of cases at this time and are ongoing. However, we showed a portion of preliminary data, including the level of miR-29b in each group of hearts, as well as masson staining and HE staining. As we would like to further test the role of BACE1 in the heart, we may supplement other animal experimental subgroups. However, related experiments of BACE1 have not been performed, and it remains to be verified whether the results are consistent with the vitro experiments.

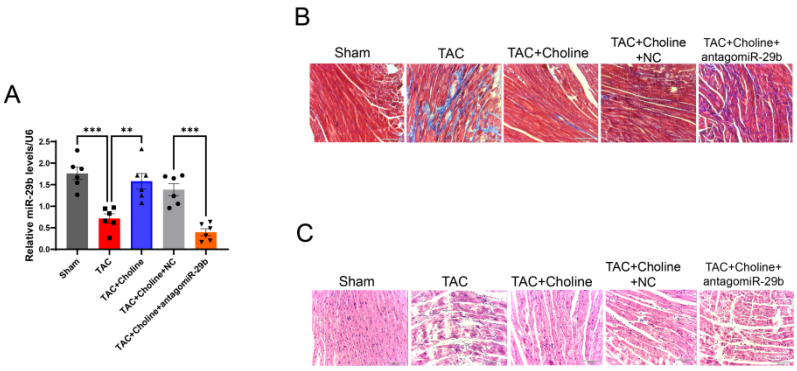


Figure 1. (A) Relative expression of microRNA-29b (miR-29b) in heart tissues detected by qRT-PCR. n=6, **P<0.01,***P<0.001. (B,C) Representative heart section with Masson staining (B) and HE staining (C) in the experimental groups, scale bar: 100 μm.

Comment 2: Format of P values still need revision.

Reply 2: We apologize for the poor formatting of our P values modifications. We have reformatted the format of the P values. We have used "*" instead of specific numbers in the figures, but reported specific P values in the figure legends and showed some of the main P values in the main text. When P>0.01, we reported the specific P value in the body text with two decimal places. We have showed the modifications in the figure legends below. If you think there are still changes that need to be made, we will be pleased to do them again. Thank you for your suggestions and for taking responsibility for the article. We have modified our text as advised.

Changes in the text: See highlights in the text and figure legends.

Figure 1: The anti-fibrotic effects of microRNA-29b (miR-29b) and it participate in the role of activating M_3 subtype of muscarinic acetylcholine receptor (M_3 receptor) in cardiac fibroblasts. (A) qRT-PCR was performed to detecte the relative expression of miR-29b in transforming growth factor beta 1 (TGF-β1)-induced cardiac fibroblasts when choline and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) stimulation. n=6. *** P=0.0001 versus TGF-β1 groups, ** P=0.0013 versus TGF-β1+Choline groups, * P=0.0389 versus Control groups. (B-G) Relative protein level of collagen I (B, n=6,**** P<0.0001 versus NC and TGF-β1+NC groups; E, n=6,*** P=0.0001 versus NC groups, ** P=0.0097 versus NC groups), α-smooth muscle actin (α-SMA) (C, n=6, *** P=0.0007 versus TGF-β1+NC groups, ** P=0.0010 versus NC groups; F, n=4, ** P=0.0024 versus NC groups, * P=0.0132 versus NC groups), and connective tissue growth factor (CTGF) (D, n=7, ** P=0.0011 versus NC groups, ** P=0.0038 versus TGF-β1+NC groups; G, n=6, *** P=0.0002 versus NC groups, *** P=0.0004 versus NC groups) in cardiac fibroblasts detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. The data are expressed as the mean ± SEM. NC: negative control; AMO-miR-29b: miR-29b inhibitor.

Figure 2: Inhibition of microRNA-29b (miR-29b) after M_3 subtype of muscarinic acetylcholine receptor (M_3 receptor) activation increases the protein level of cardiac fibrosis markers. Relative protein level of collagen I (A, n=6, ** P=0.0094 versus Control groups, ** P=0.0040 versus TGF-β1+Choline groups, * P=0.0425 versus TGF-β1 groups), α-smooth muscle actin (α-SMA) (B, n=4, *** P=0.0001 versus TGF-β1+Choline groups, * P=0.0125 versus Control groups, * P=0.0233 versus TGF-β1 groups), and connective tissue growth factor (CTGF) (C, n=7, ** P=0.0023 versus Control groups, * P=0.0123 versus TGF-β1 groups, * P=0.0185 versus TGF-β1+Choline groups) in cardiac fibroblasts detected by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control. The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor.

Figure 3: Inhibition of microRNA-29b (miR-29b) reverses the anti-fibrotic effects of activating M_3 subtype of muscarinic acetylcholine receptor (M_3 receptor) and promotes the expression of collagen I and α-SMA. (A,E) Relative collagen I protein level in cardiac fibroblasts detected by immunofluorescence. Scale bar=50 μm. (B,F) Statistical results of immunofluorescence of collagen I (B, n=6, **** P<0.0001 versus Control groups, TGF-β1 groups and TGF-β1+Choline groups; F, n=3, *** P=0.0007 versus Control groups, ** P=0.0095 versus TGF-β1 groups, ** P=0.0097 versus TGF-β1+Choline+4-DAMP groups, * P=0.0233 versus TGF-β1+Choline groups). (C,G) Relative α-SMA protein level in cardiac fibroblasts detected by immunofluorescence. Scale bar=50 μm. (D,H) Statistical results of immunofluorescence of α-SMA (D, n=6, **** P<0.0001 versus Control groups, TGF-β1 groups and TGF-β1+Choline groups; H, n=3, *** P=0.0001 versus Control groups, *** P=0.0003 versus TGF-

β1+Choline+4-DAMP groups, ** P=0.0016 versus TGF-β1 groups, ** P=0.0026 versus TGF-β1+Choline groups). The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor; 4-DAMP: 4-diphenylacetoxy-N-methylpiperidine methiodide.

Figure 4: Inhibition of microRNA-29b (miR-29b) after M_3 subtype of muscarinic acetylcholine receptor (M_3 receptor) activation promotes the migration ability of cardiac fibroblasts. (A) The migration rate of cardiac fibroblasts detected by scratch test. Scale bar=500 μm. (B) Statistical results about the migration rate of cardiac fibroblasts. n=4, *** P=0.0001 versus Control groups, ** P=0.0094 versus TGF-β1 groups, ** P=0.0095 versus TGF-β1+Choline groups. (C) The migration ability of cardiac fibroblasts was detected by transwell. Scale bar=150 μm. (D) Statistical results about migration ability of cardiac fibroblasts. n=4, **** P<0.0001 versus Control groups and TGF-β1 groups, ** P=0.0062 versus TGF-β1+Choline groups. The data are expressed as the mean ± SEM. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor.

Figure 5: MicroRNA-29b (miR-29b) participates in the anti-fibrotic effects after M_3 subtype of muscarinic acetylcholine receptor (M_3 receptor) activation through targeting beta-site app cleaving enzyme 1 (BACE1). (A) Screening target genes of miR-29b by online prediction websites: miRDB, miRTarBase, TargetScan, and PicTar. (B) The binding sites of miR-29b and BACE1 in different species. (C,D,E,F) Relative protein level of BACE1 in cardiac fibroblasts detected by western blot (C, n=4, ** P=0.0037 versus NC groups, * P=0.0493 versus TGF-β1+NC groups; D, n=4, * P=0.0115 versus NC groups, * P=0.0248 versus NC groups; E, n=5, ** P=0.0060 versus TGF-β1+Choline groups, * P=0.0418 versus Control groups, * P=0.0264 versus TGF-β1 groups; F, n=4, ** P=0.0073 versus Control groups, * P=0.0256 versus pcMV3-mBACE1-GFPSpark.) (G) qRT-PCR assay was used to examine the expression of miR-29b in the presence of BACE1 overexpression. n=8, *** P=0.0010 versus pcMV3-C-GFPSpark. (H) Relative protein level of Collagen I in cardiac fibroblasts detected by western blot. n = 6, *** P=0.0005 versus Control groups, ** P=0.0057 versus TGF-β1 groups, * P=0.0404 versus TGF-β1+miR-29b mimics groups. The data are expressed as the mean ± SEM. Student's t-test was used for two-group comparisons. TGF-β1: transforming growth factor beta 1; NC: negative control; AMO-miR-29b: miR-29b inhibitor.

Comment 3: Original figures of western blots need clearer mark for bands.
Rely 3: Thank you for your suggestion, we have modified the format of original figures to make it easier for you to review more clearly. For your reference, we have included an example figure below.
Changes in the text: See the original figures of western blots(see supplement below).

Supplement:

Original western bolt(Figure 1)

Figure 1B (n=6)

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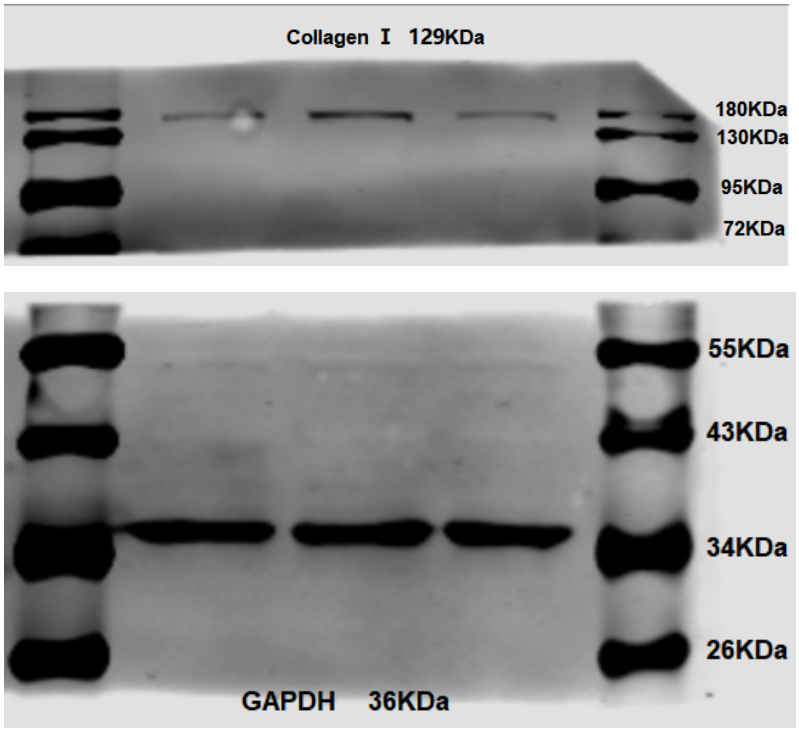

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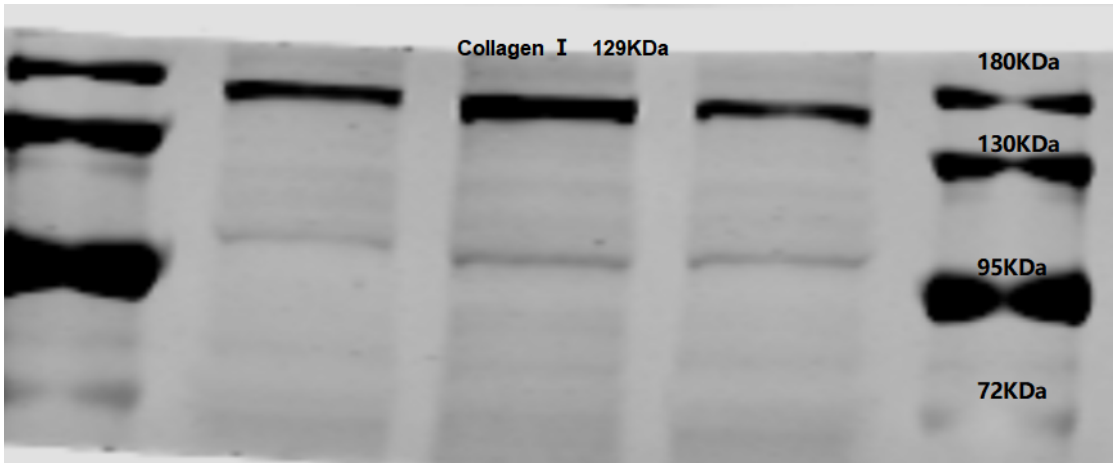

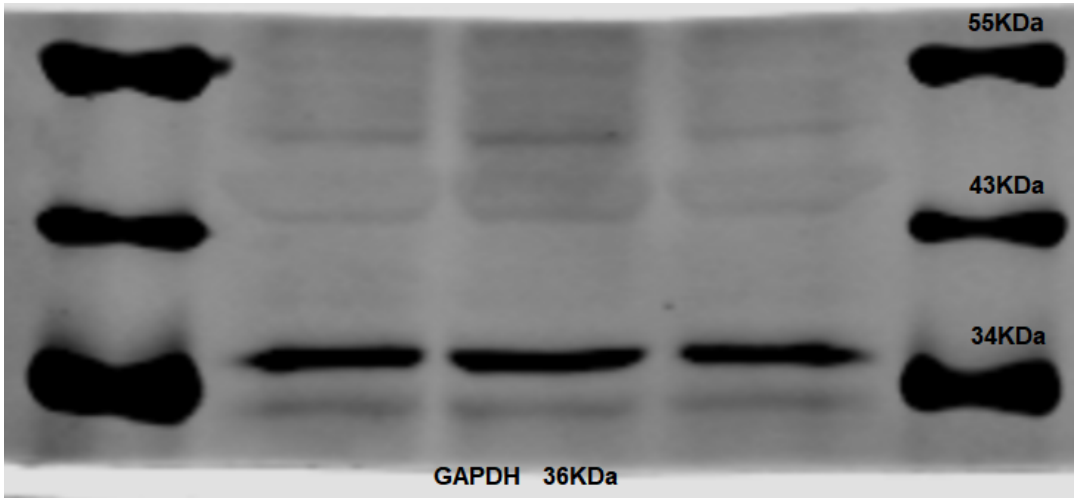
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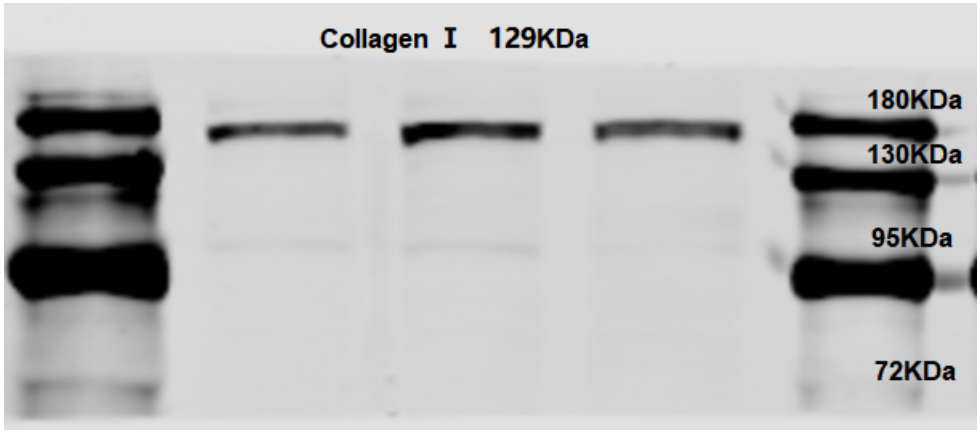
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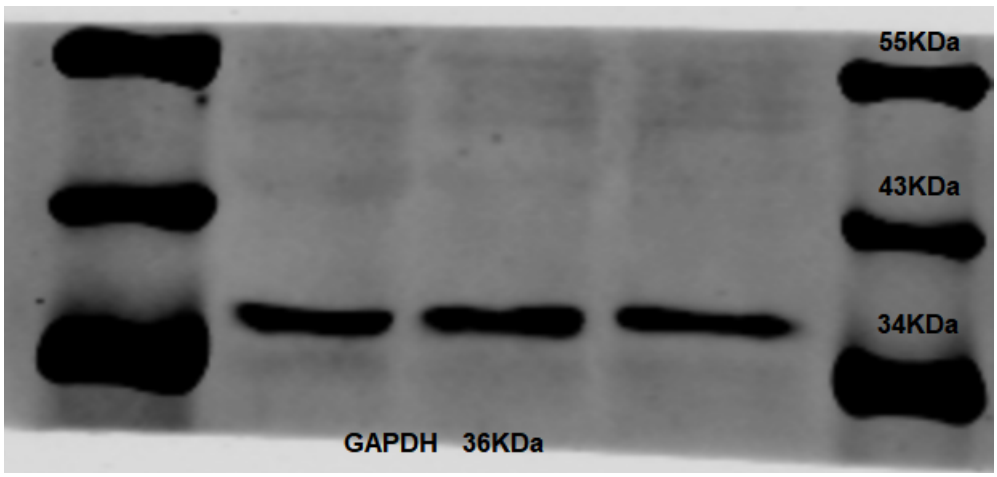
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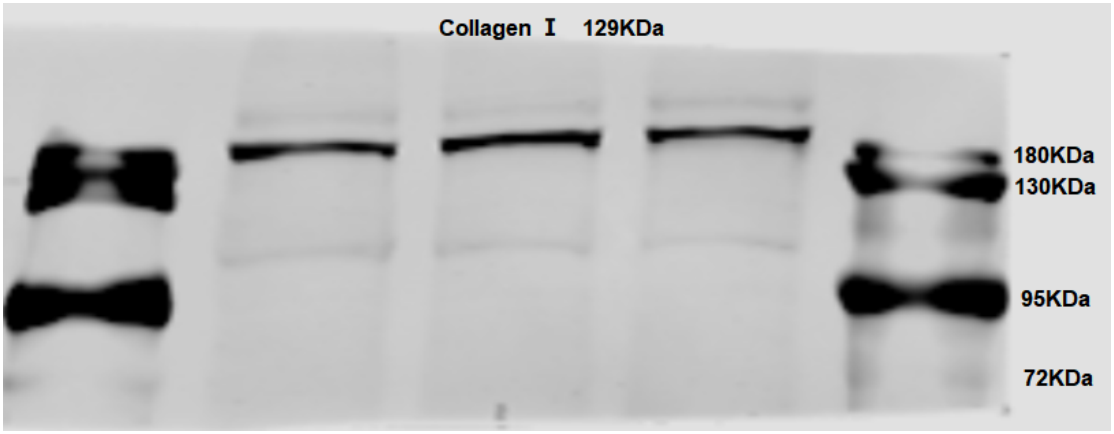
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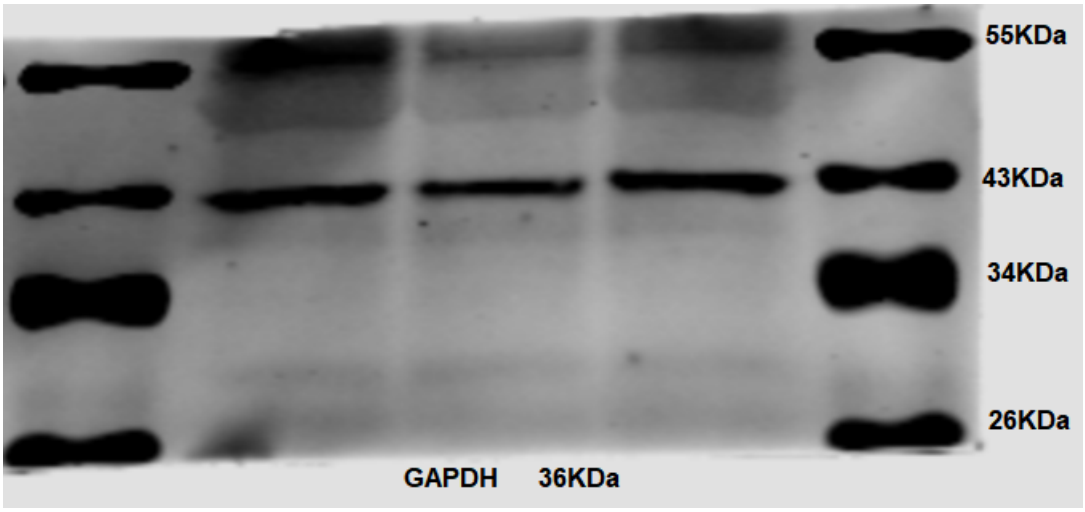
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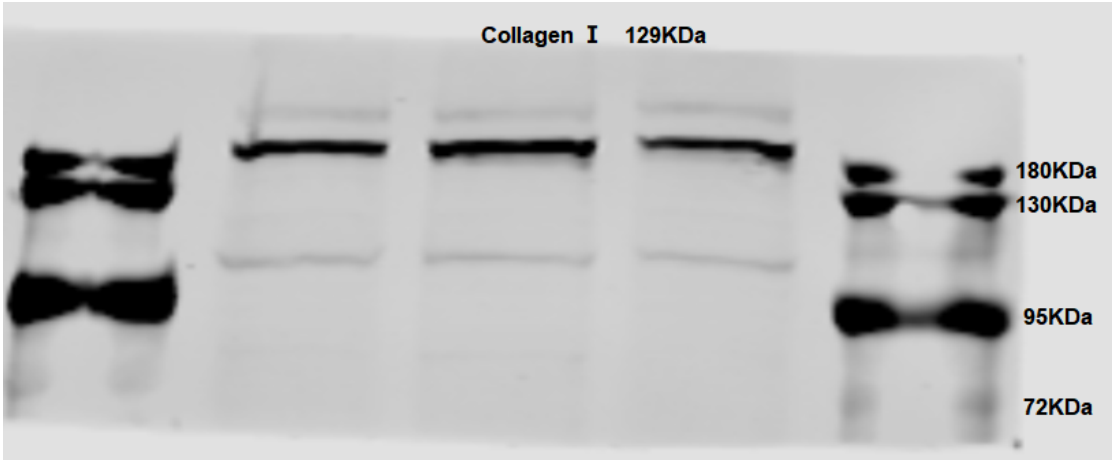
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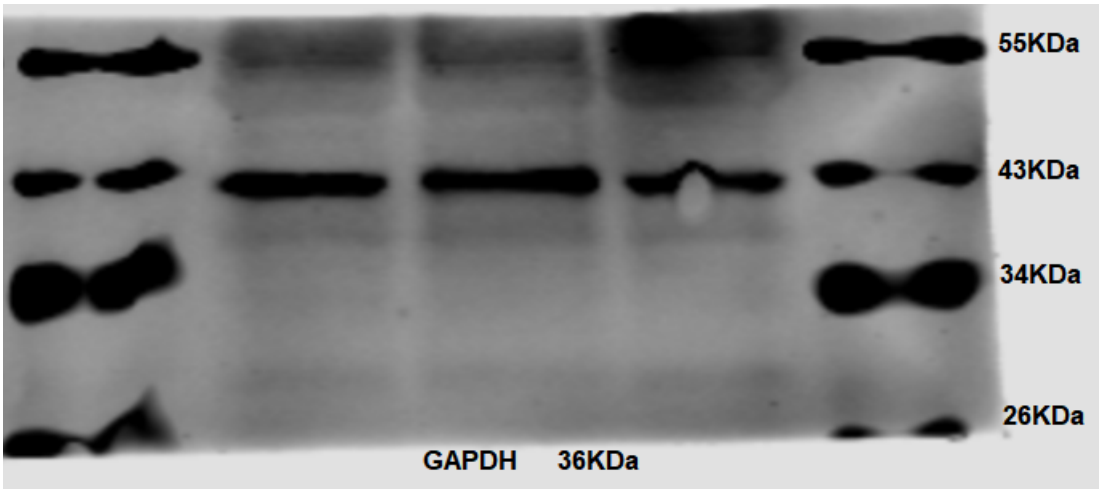

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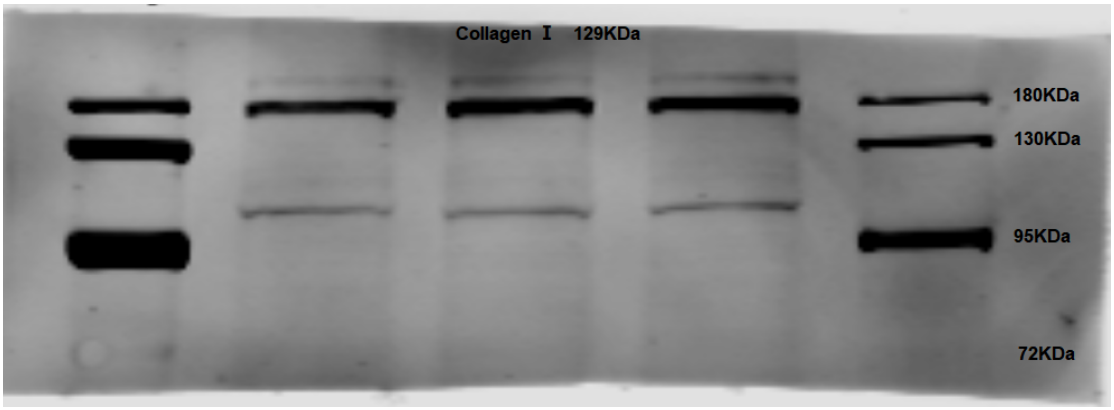

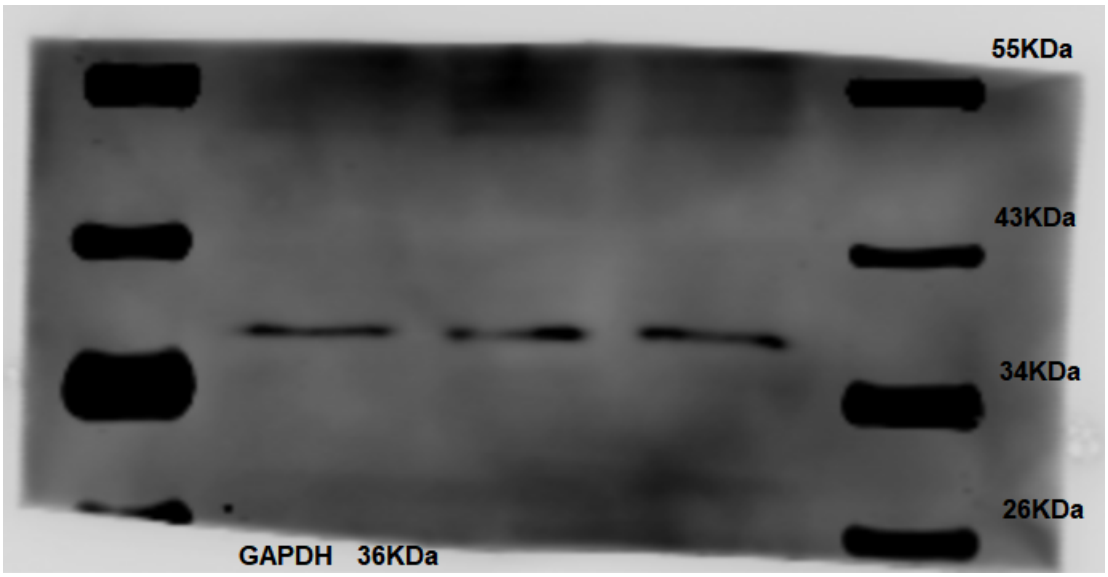

Figure 1C (n=6)

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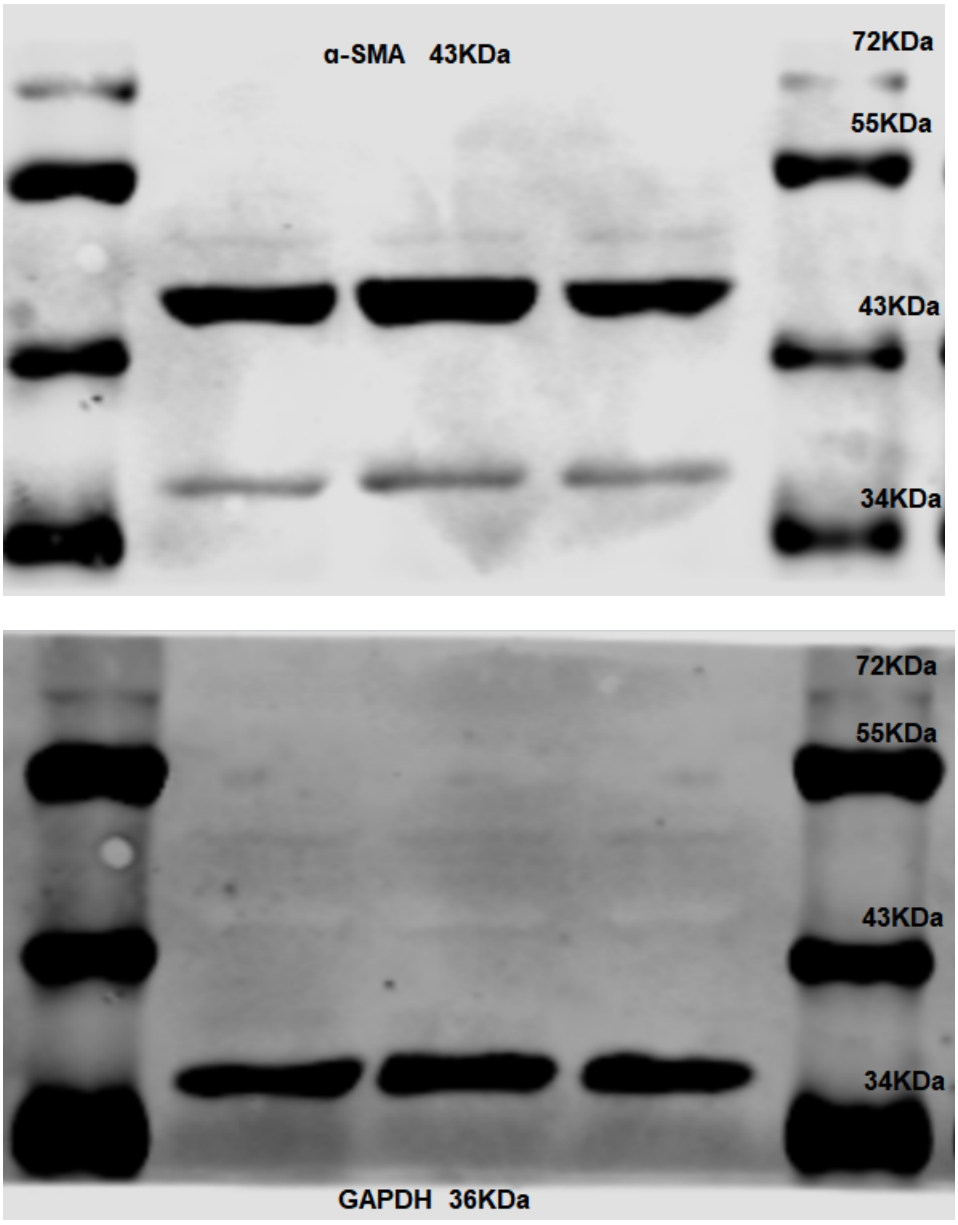
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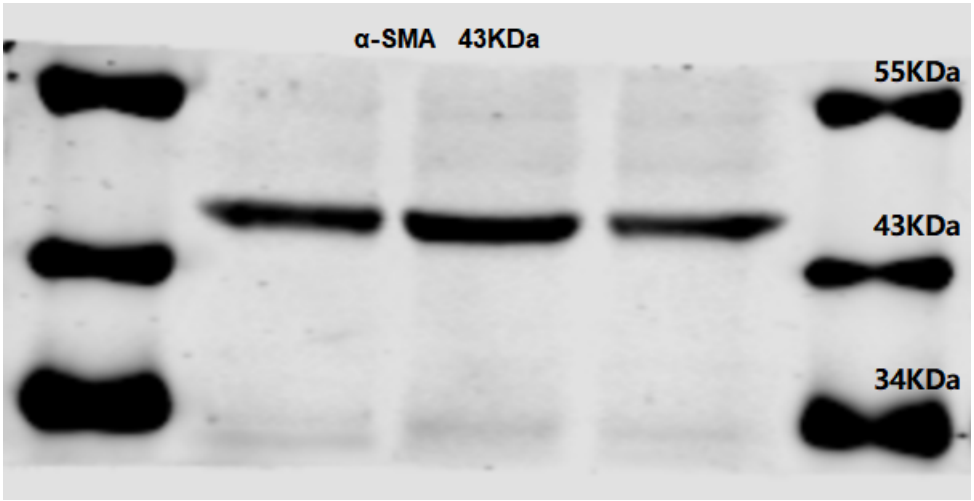
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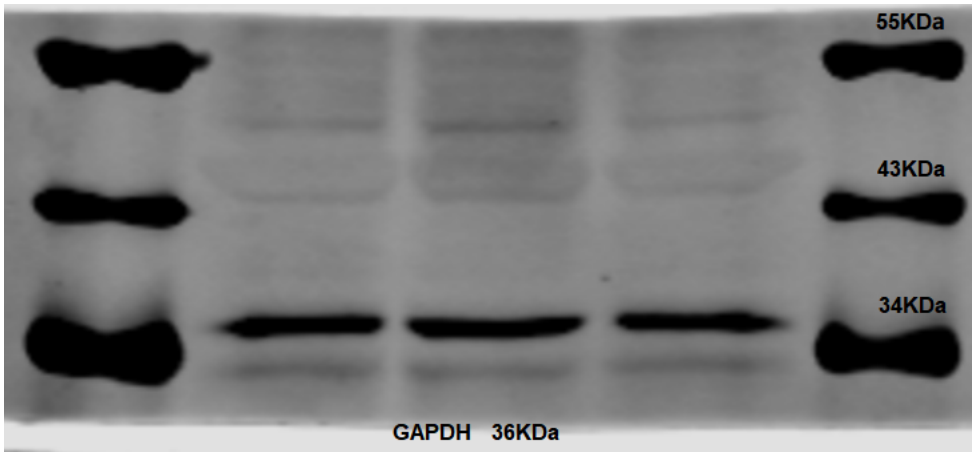
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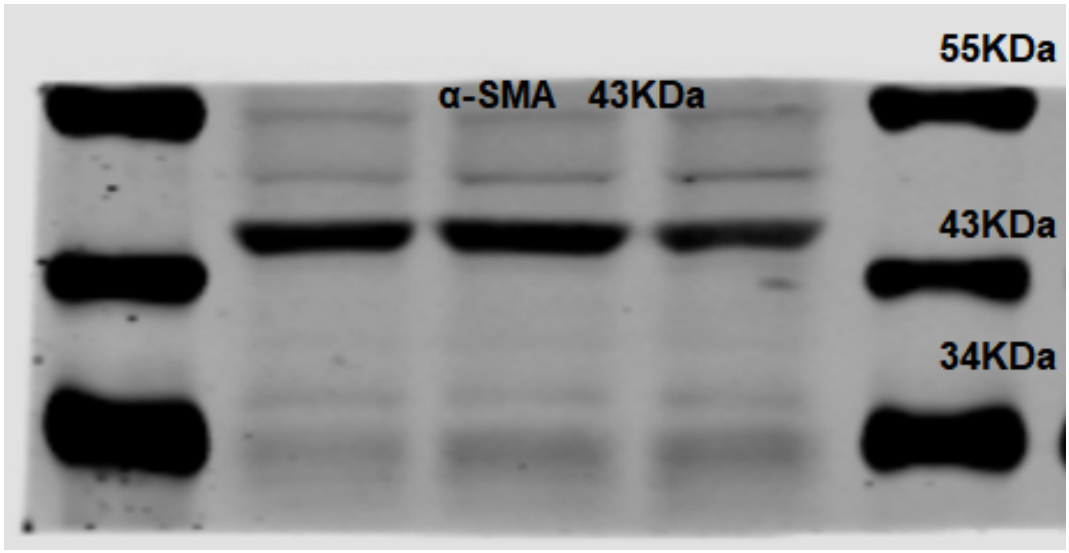
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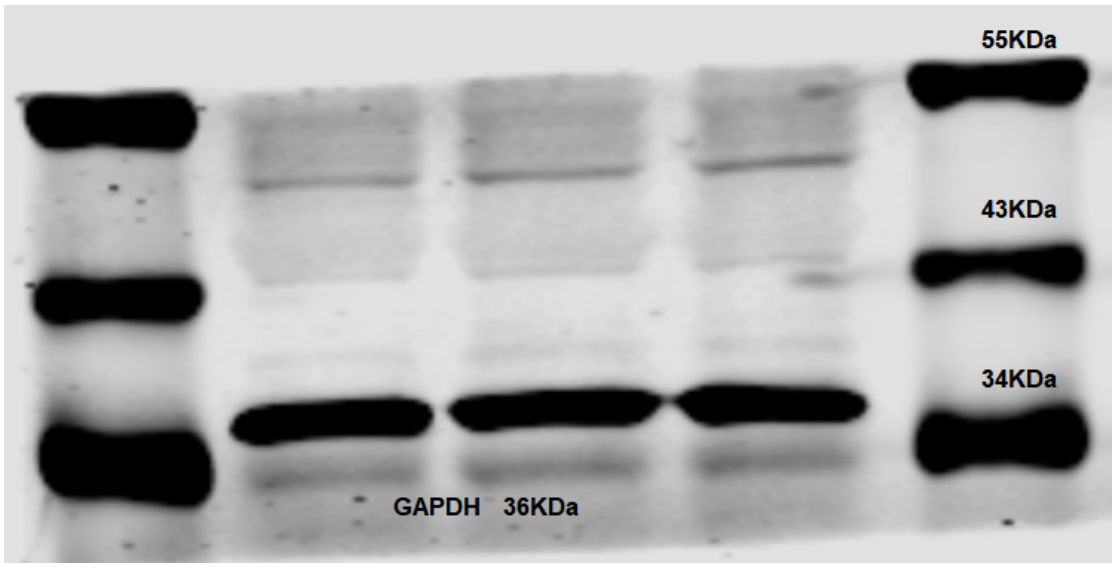
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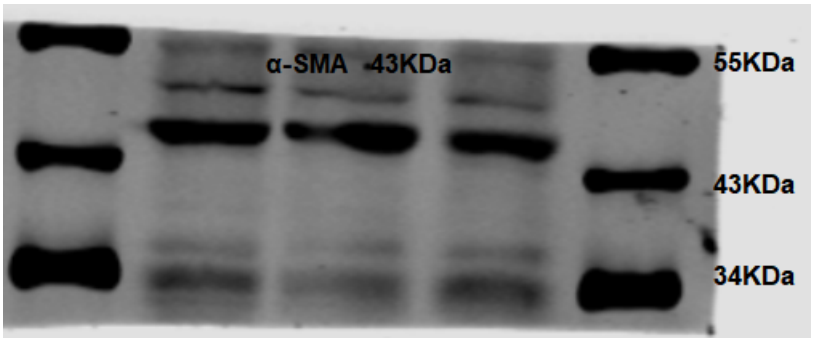
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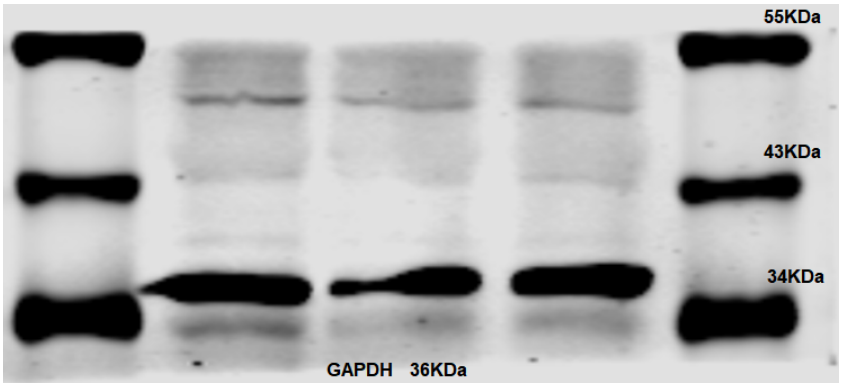
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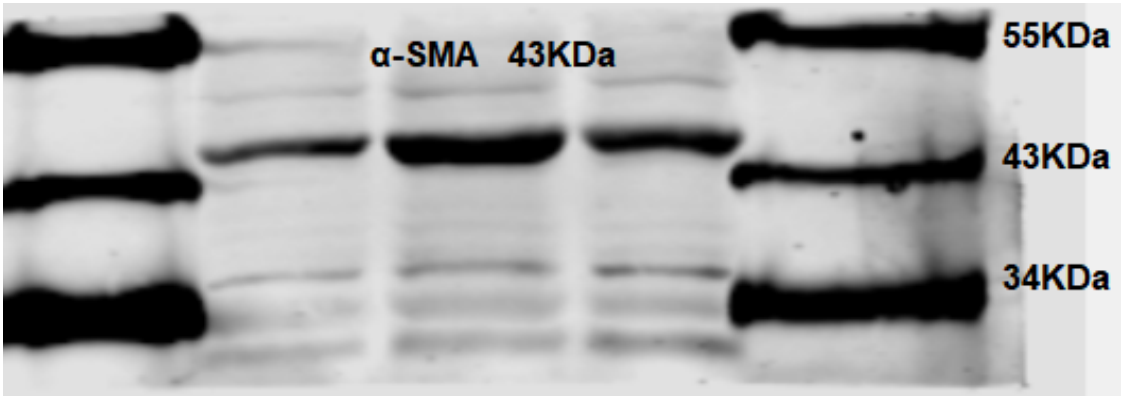
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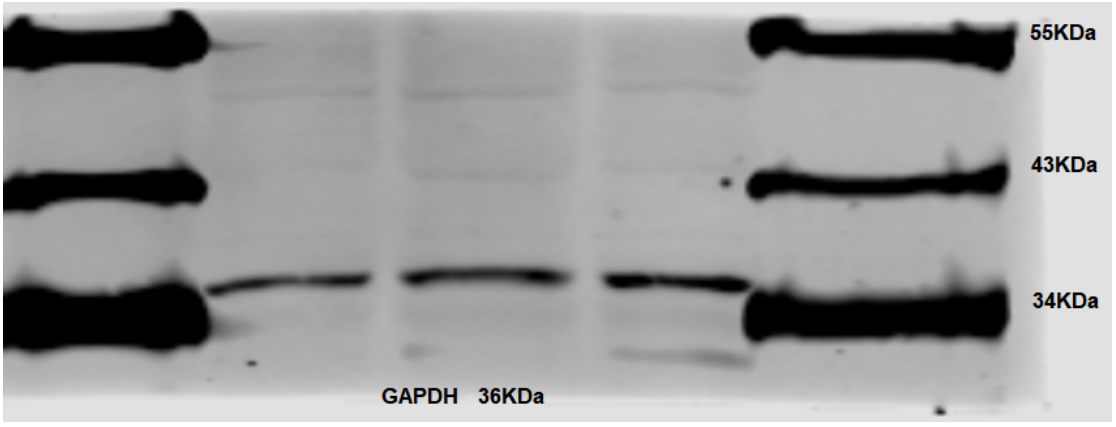
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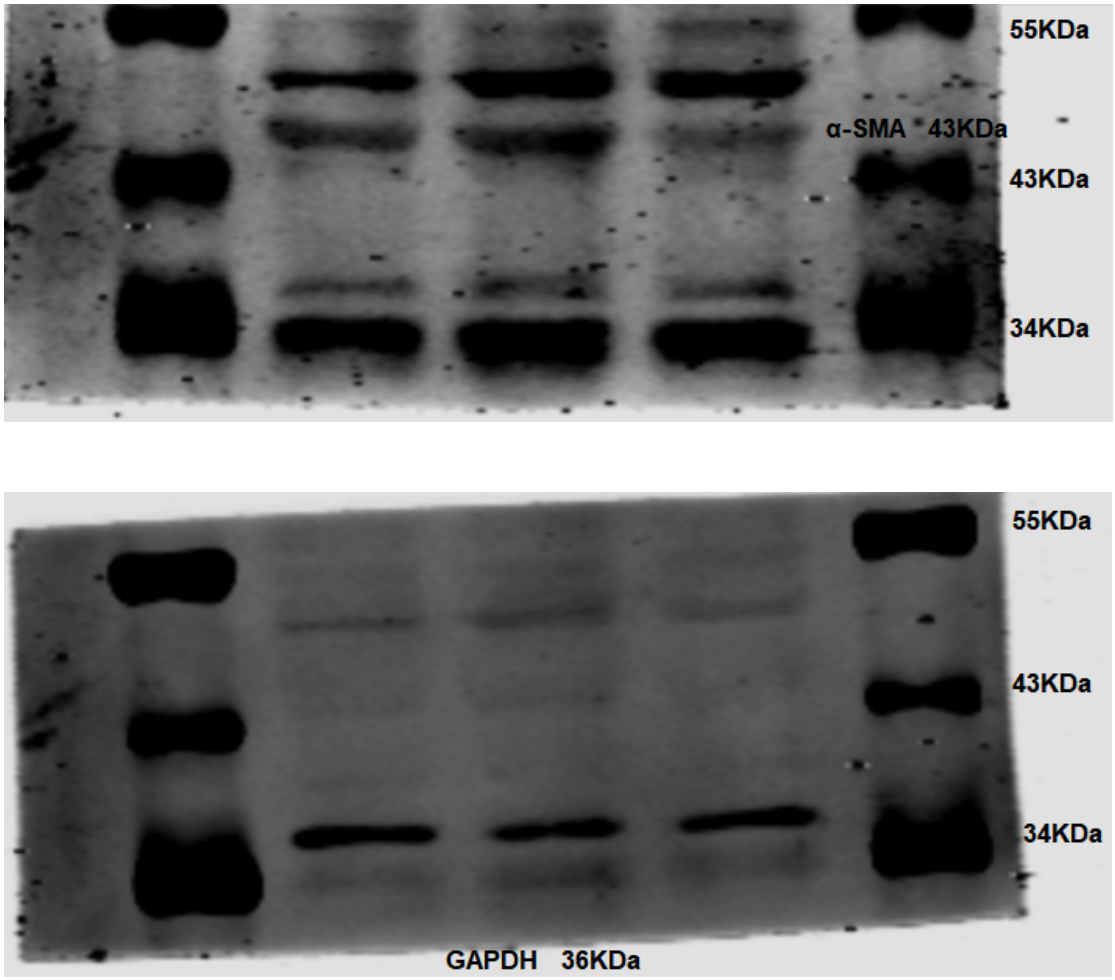

Figure 1D (n=7)

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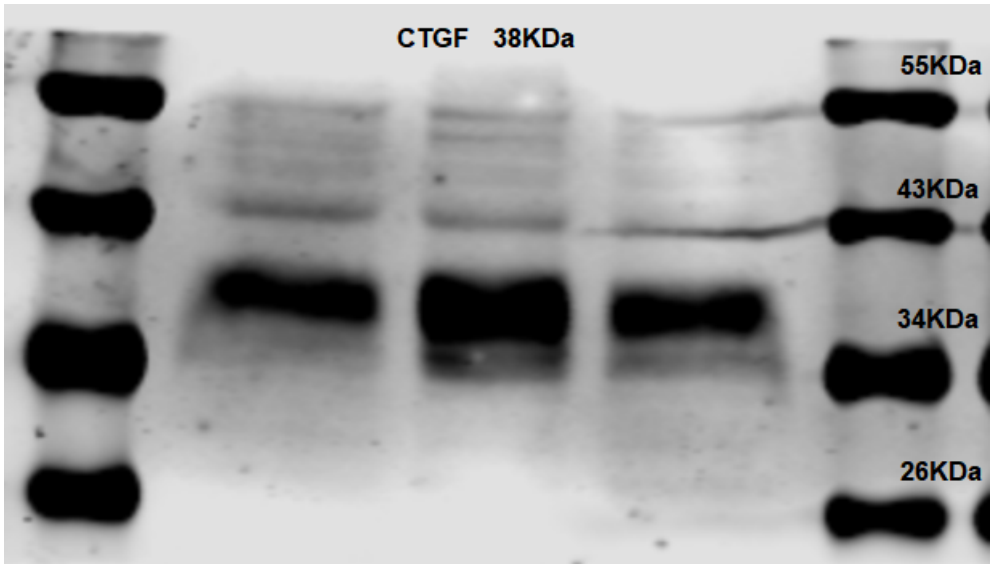

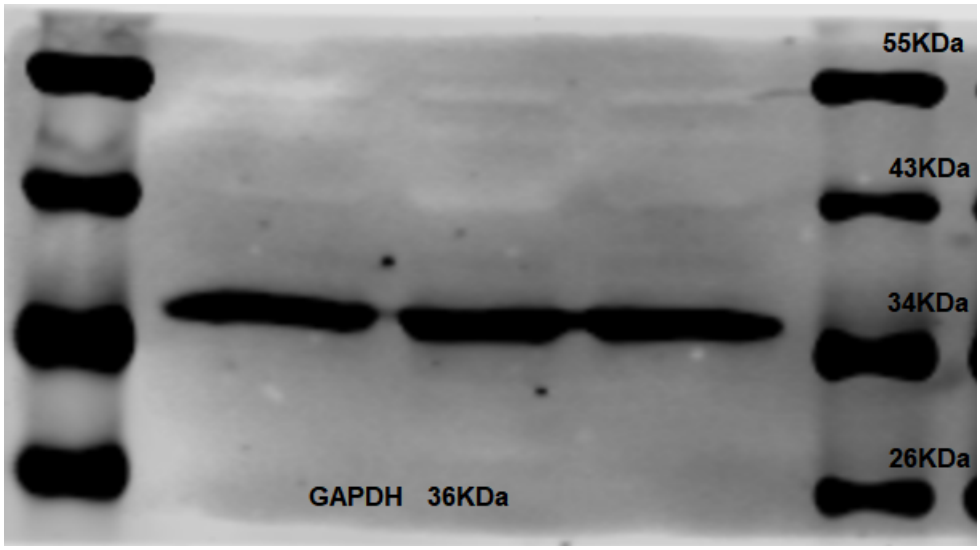
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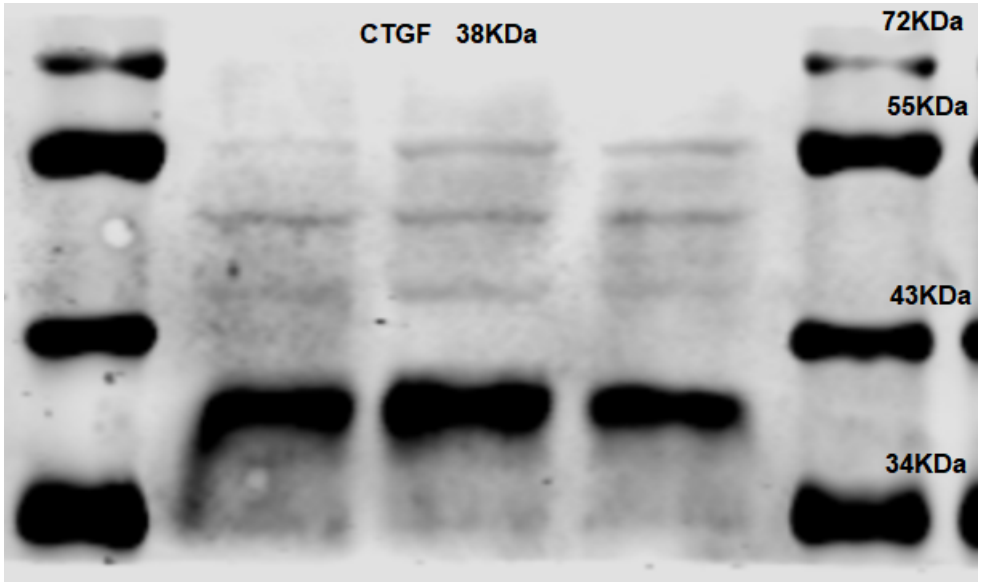
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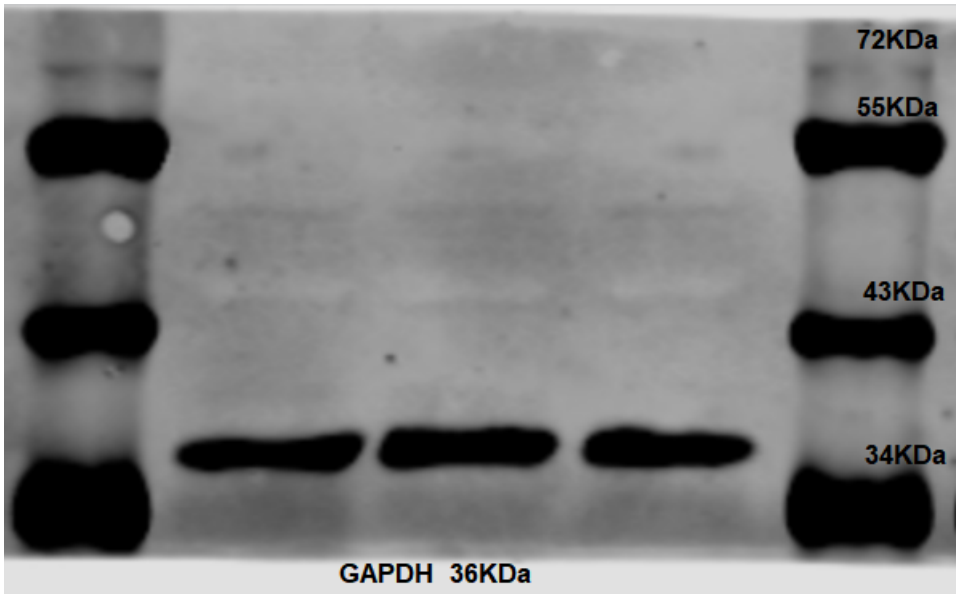
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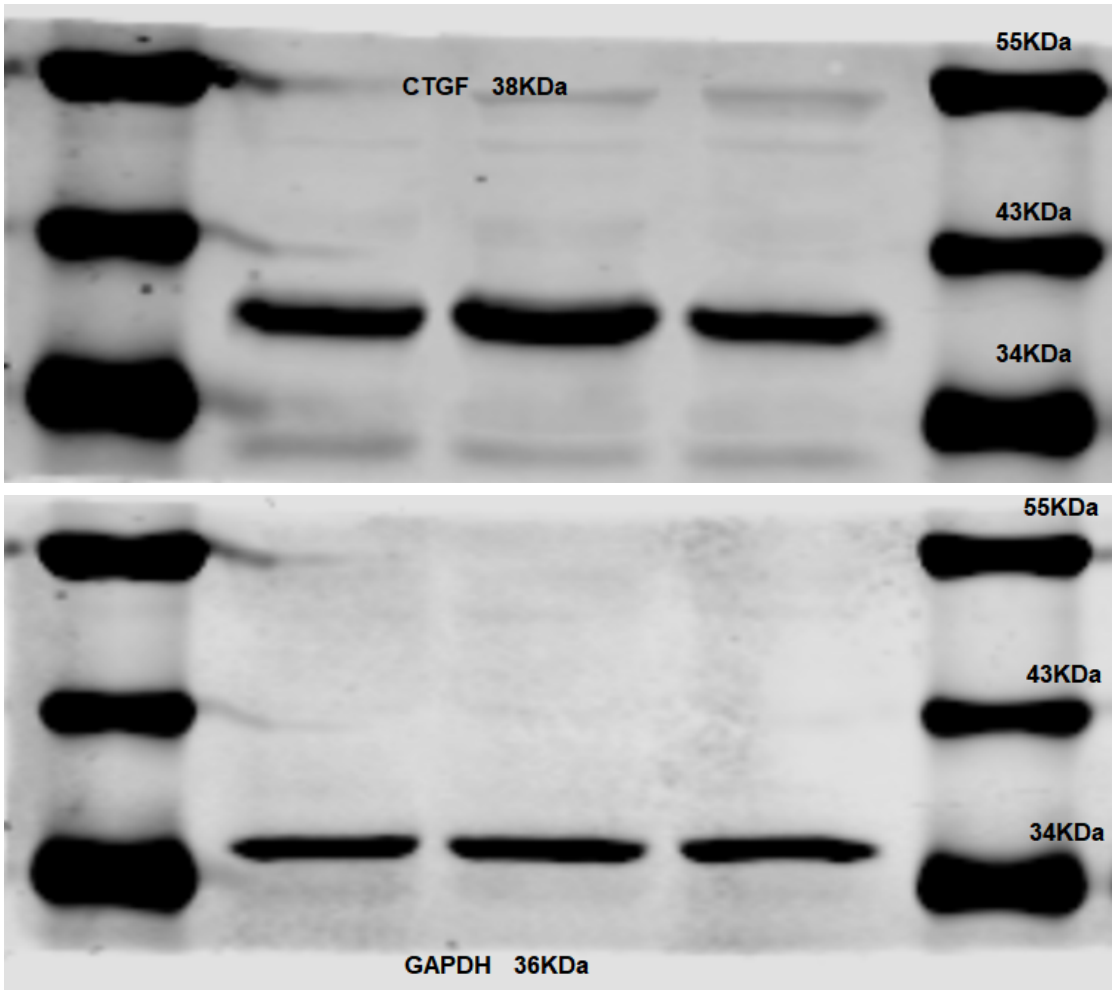
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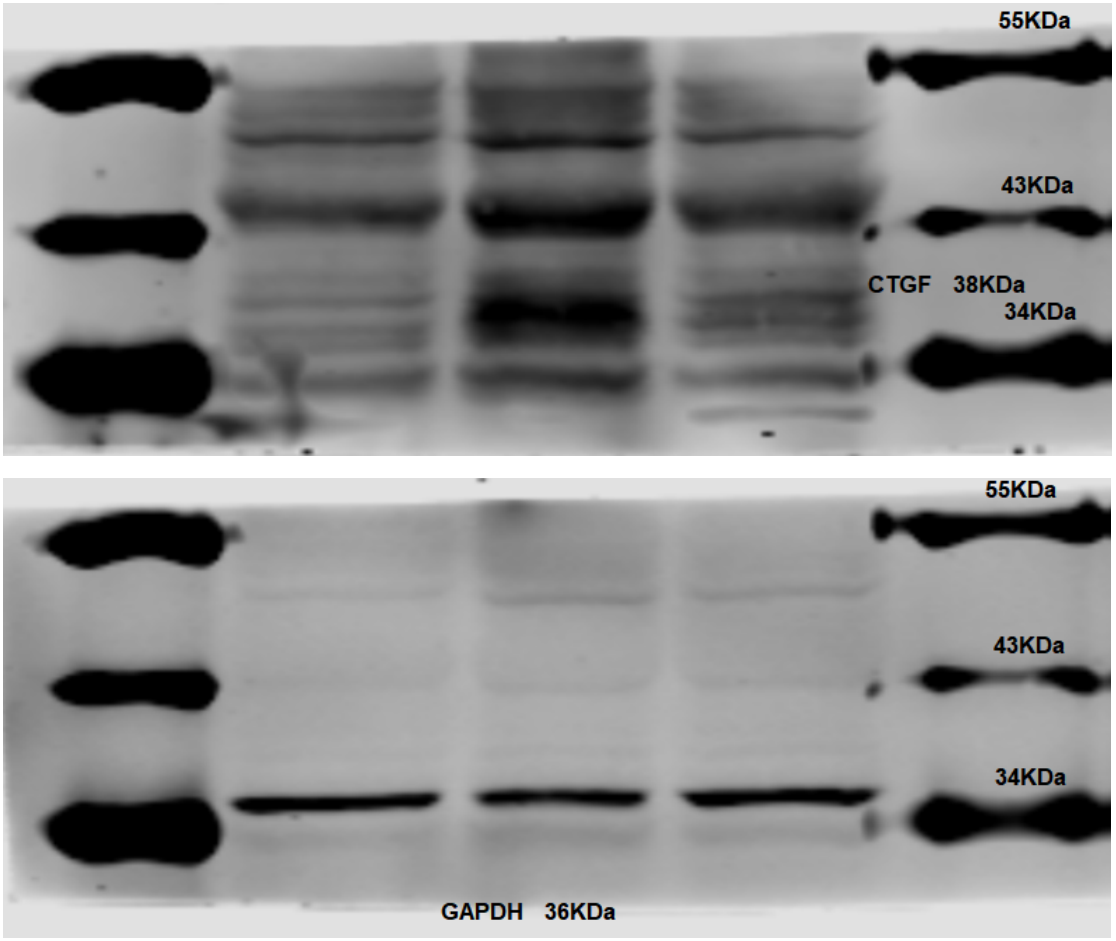
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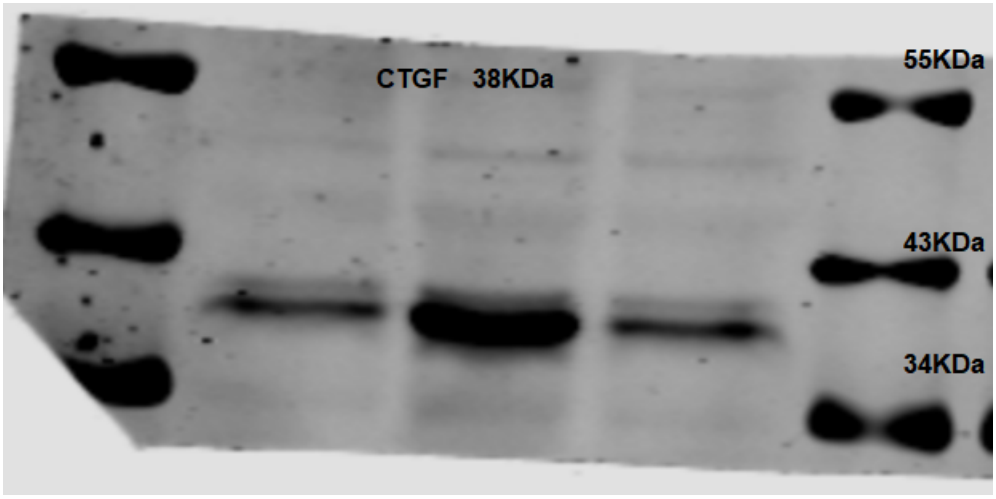
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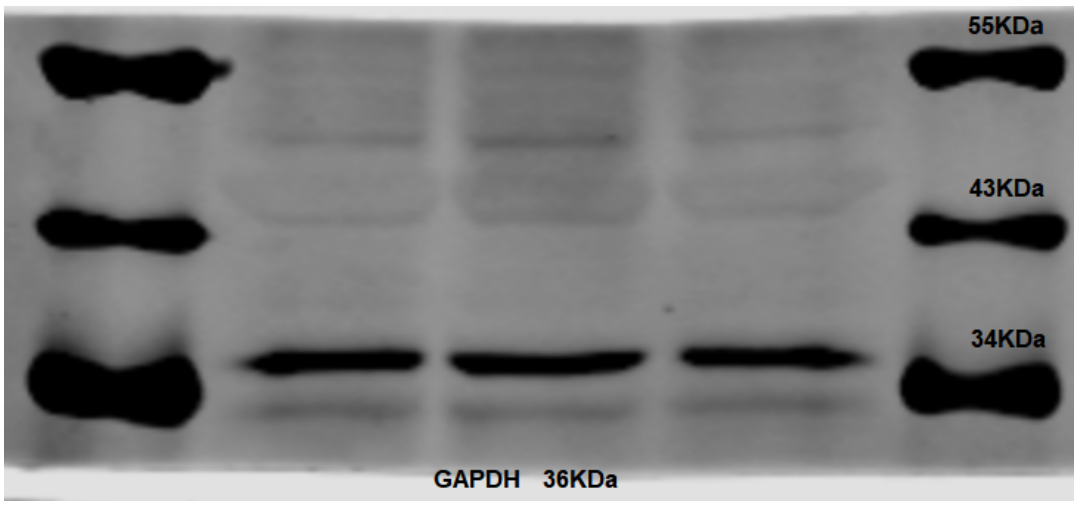
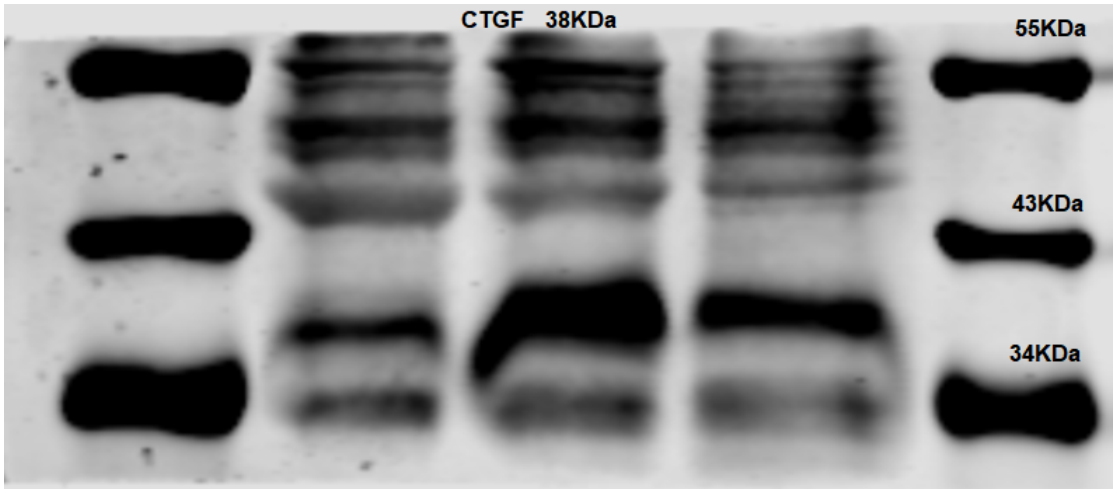
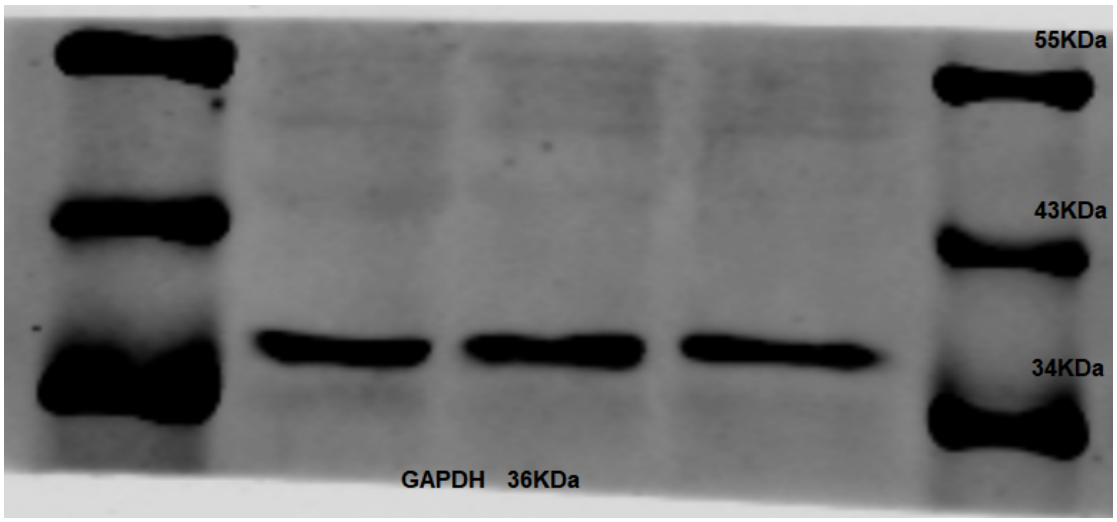
34KDa


55KDa
43KDa
34KDa
GAPDH 36KDa

6


CTGF 38KDa
55KDa
43KDa
34KDa


55KDa
43KDa
34KDa
GAPDH 36KDa

7

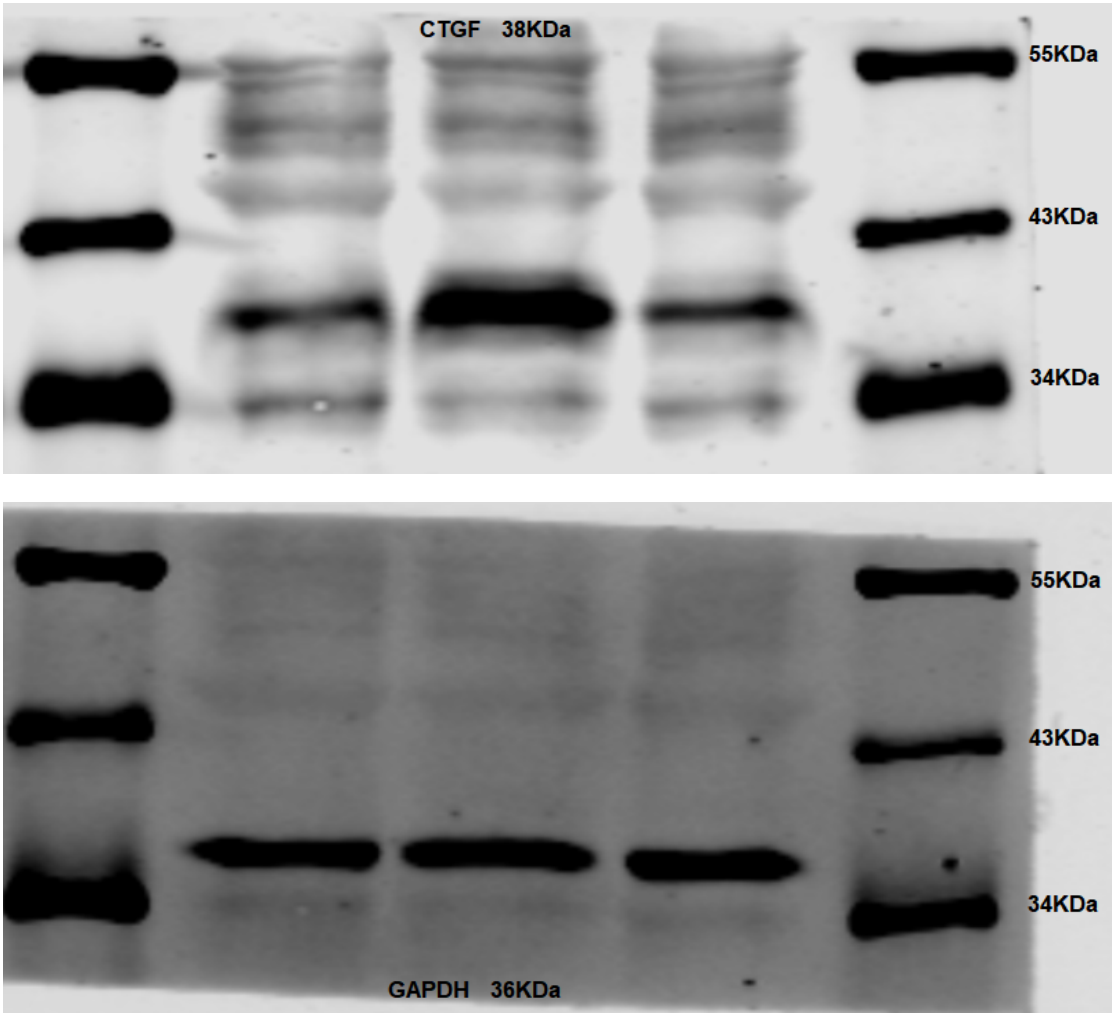
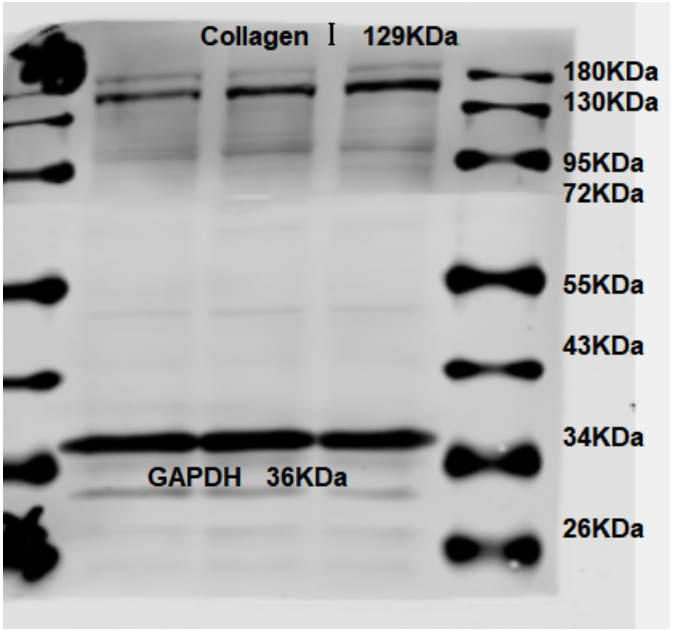
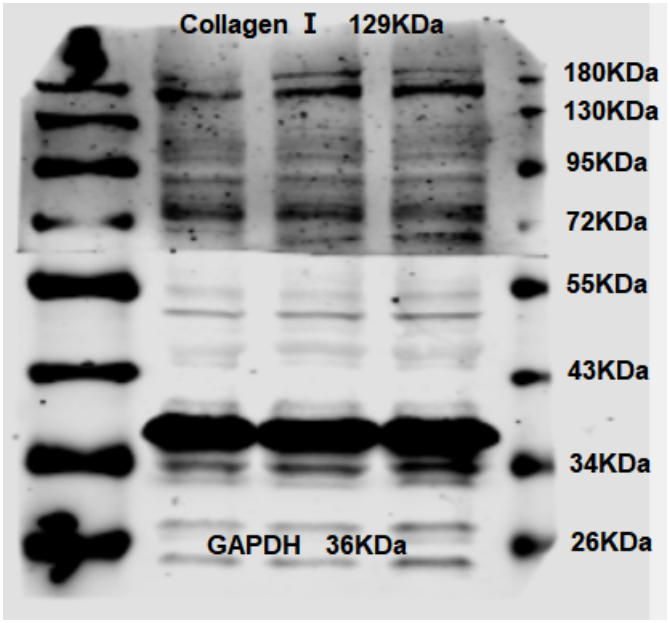


Figure 1E (n=6)

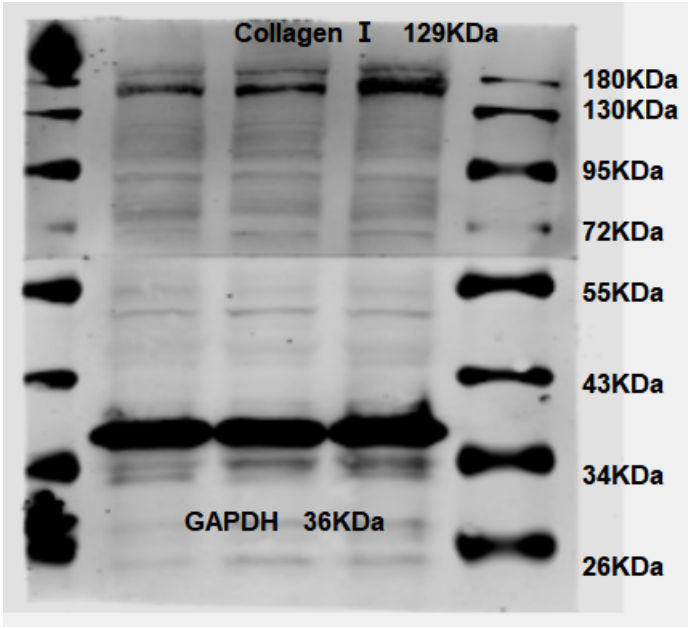
1


Collagen Ⅰ 129KDa
180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

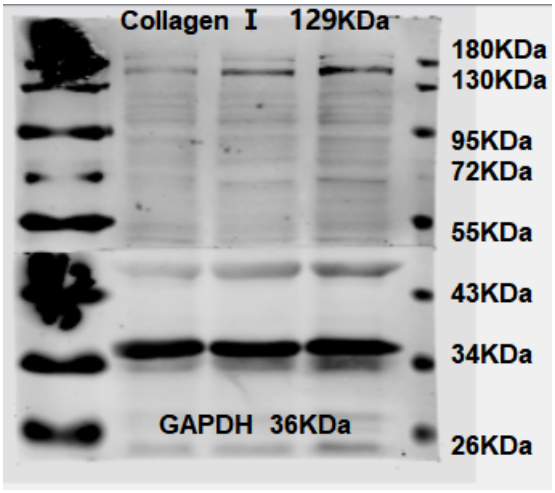
2


Collagen Ⅰ 129KDa
180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

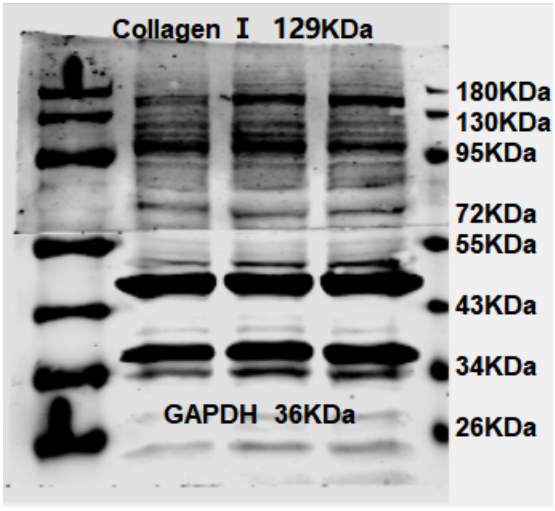
3


Collagen I 129KDa
180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

4


Collagen I 129KDa
180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

5


Collagen I 129KDa
180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

6

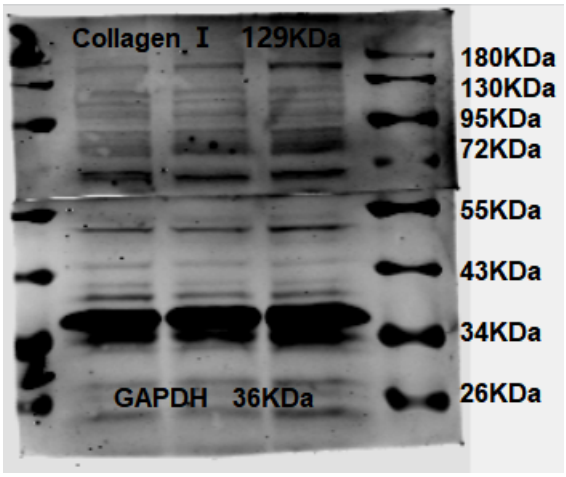
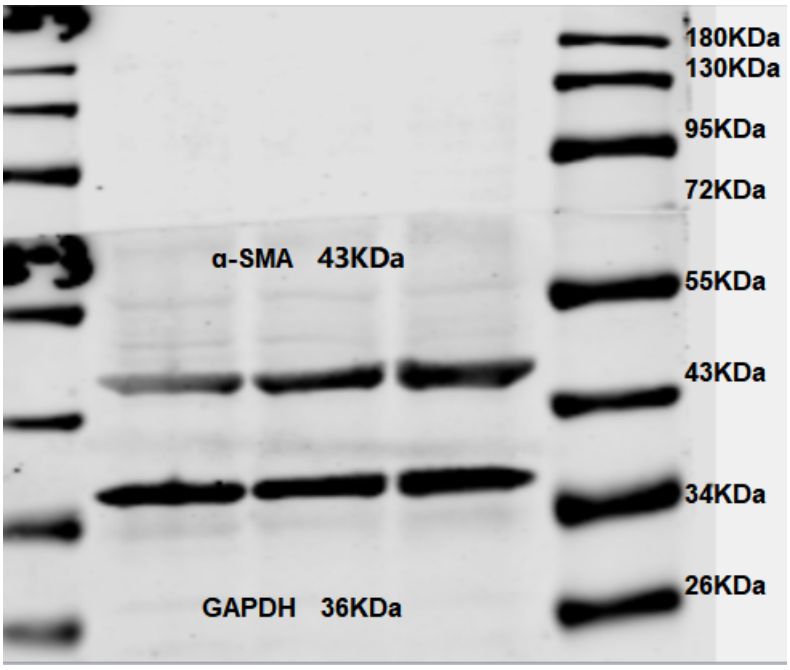
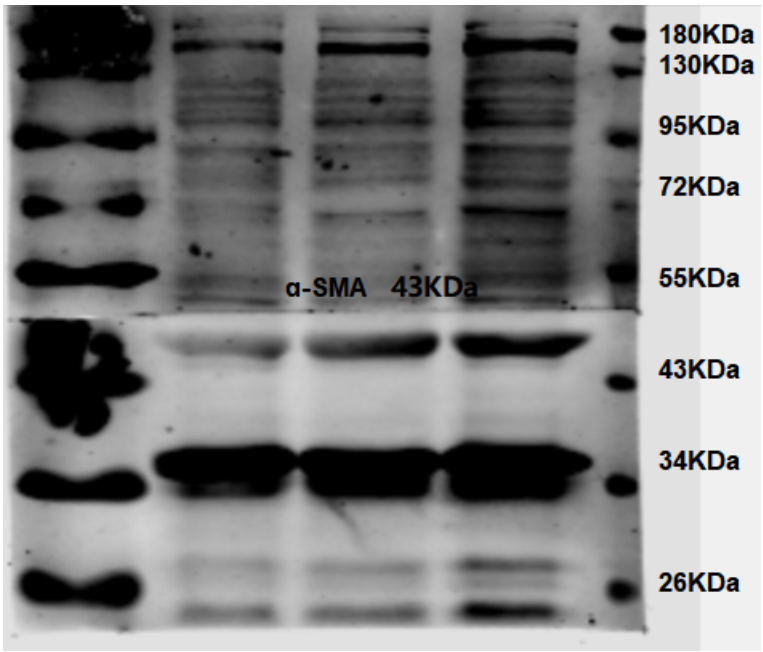


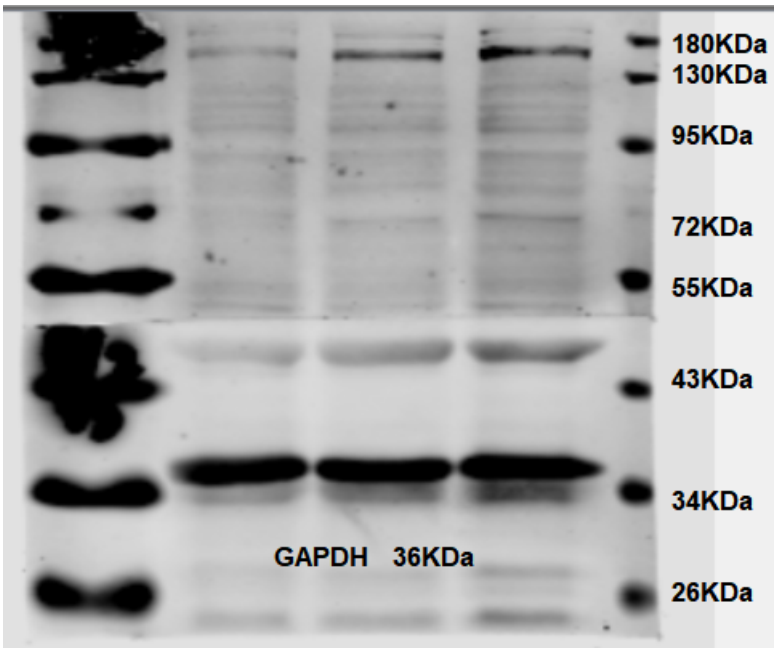
Figure 1F (n=4)

1

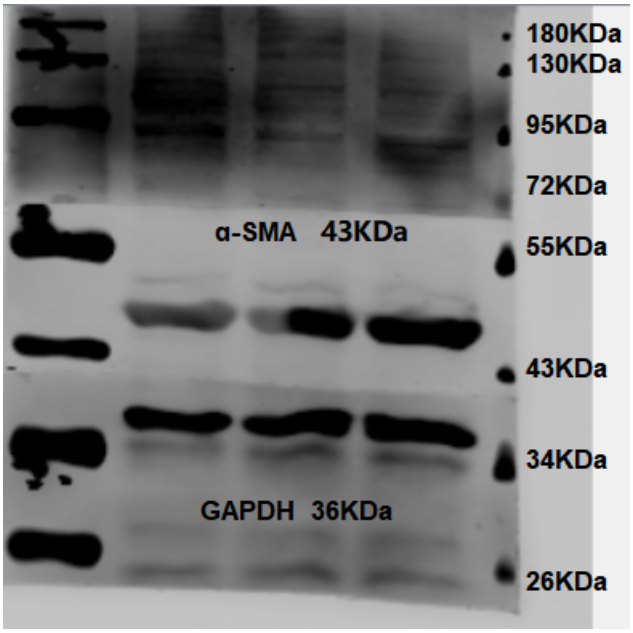


2


180KDa
130KDa
95KDa
72KDa
55KDa
α-SMA 43KDa
43KDa
34KDa
26KDa


180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

3



4

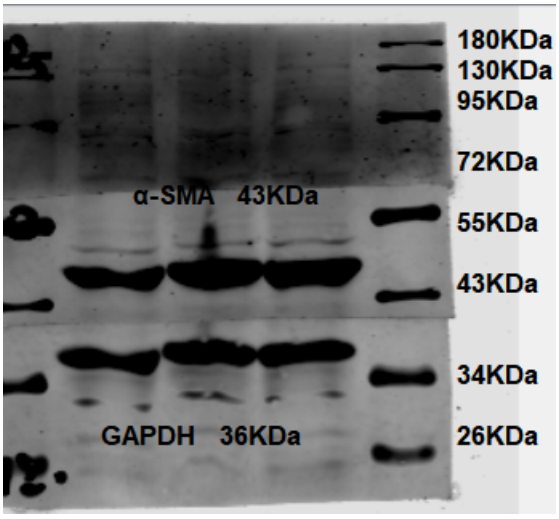
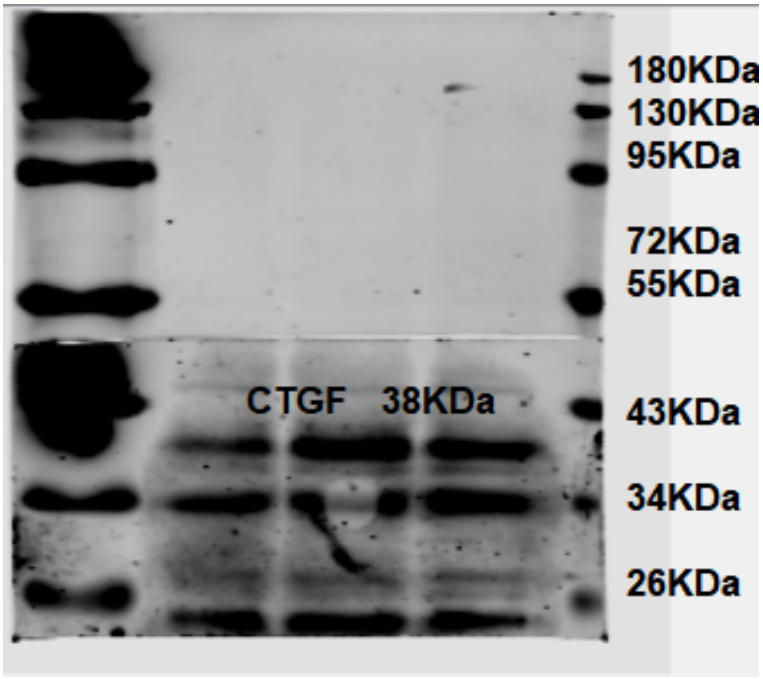
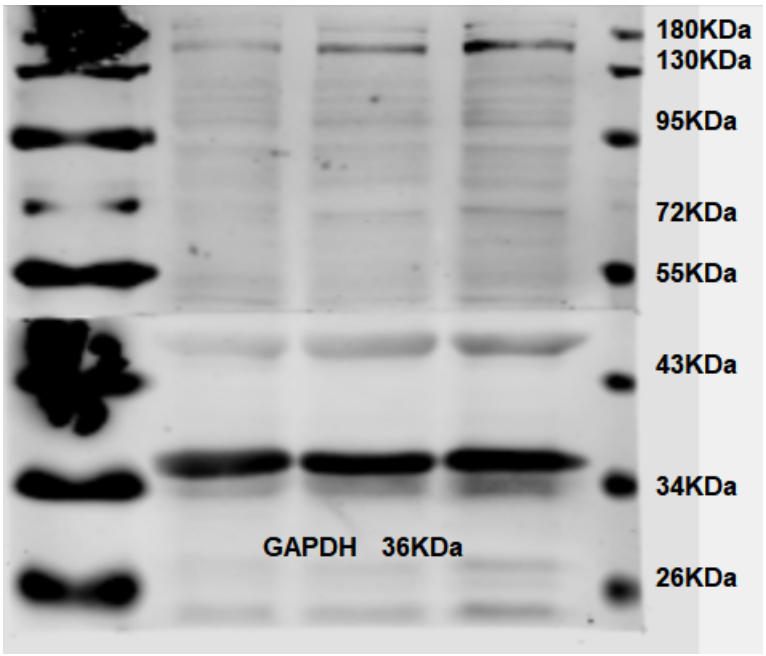


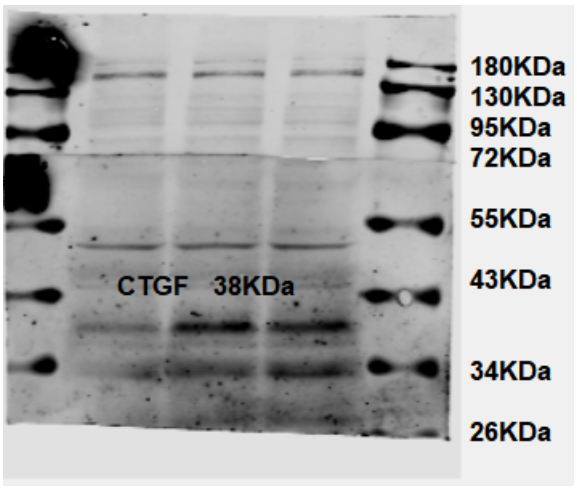
Figure 1G (n=6)

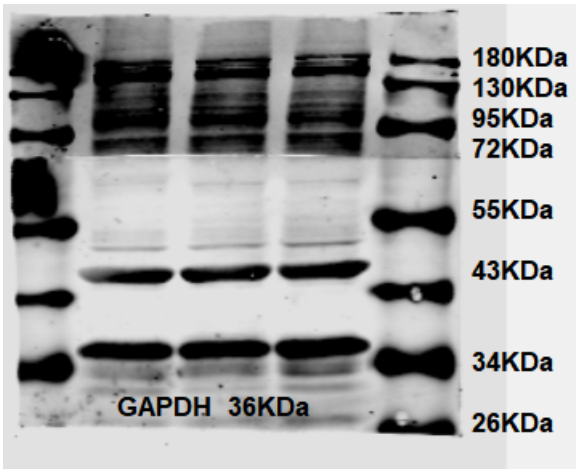
1


180KDa
130KDa
95KDa
72KDa
55KDa
CTGF 38KDa
43KDa
34KDa
26KDa

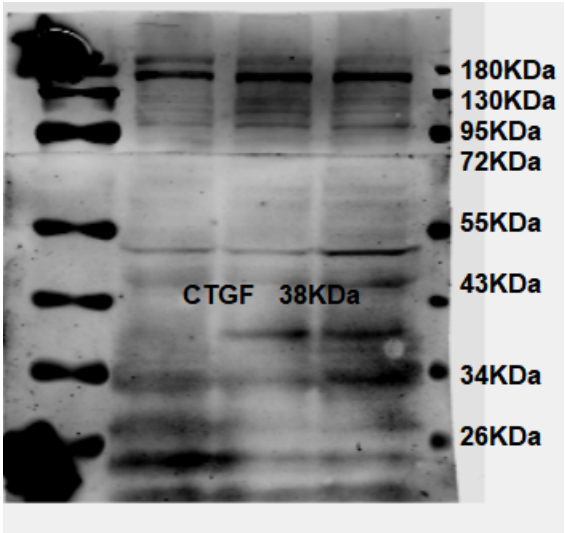

180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

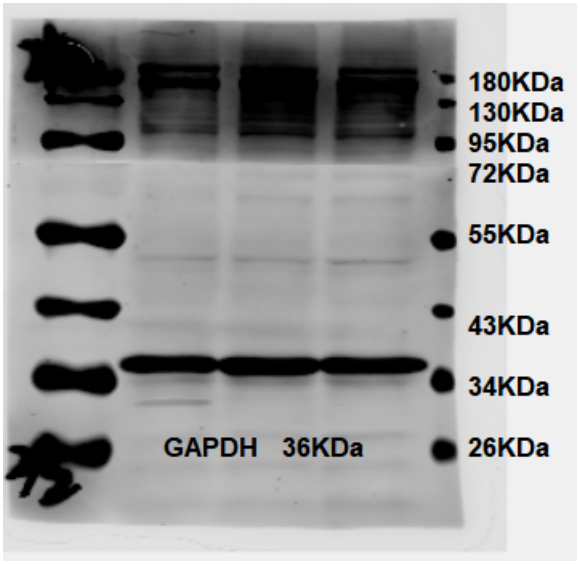
2


180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
26KDa
CTGF 38KDa

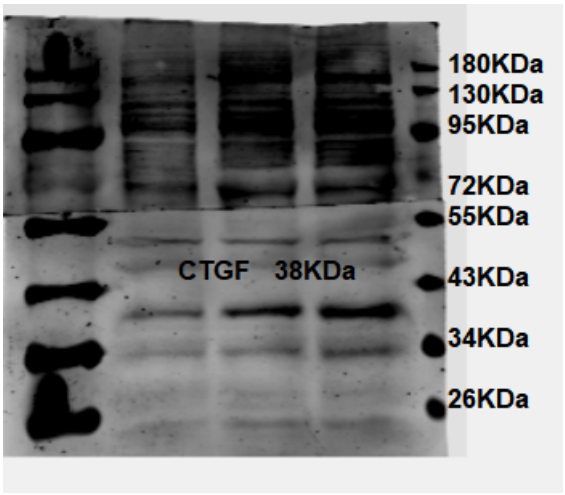

180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
26KDa
GAPDH 36KDa

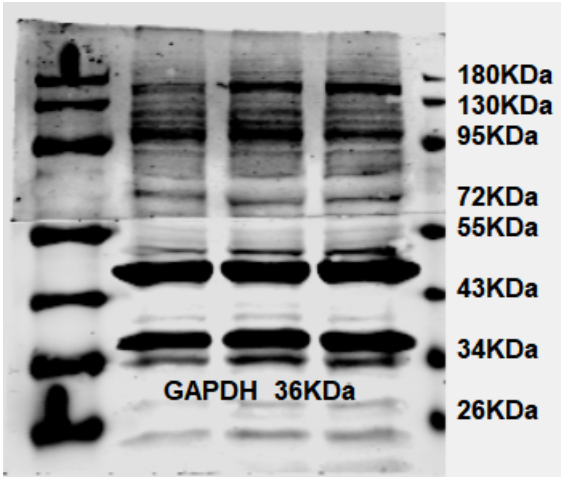
3


180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
26KDa
CTGF 38KDa

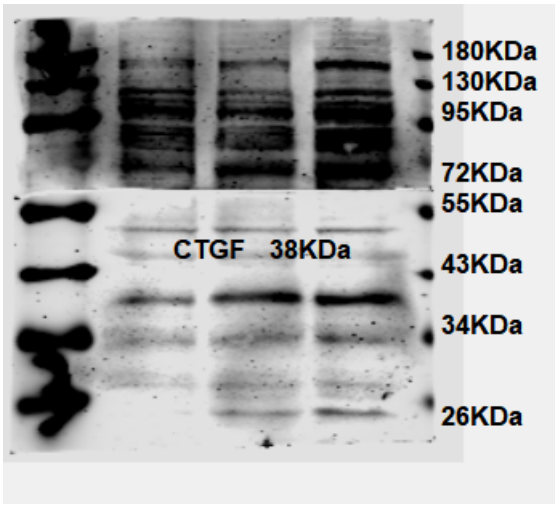

180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

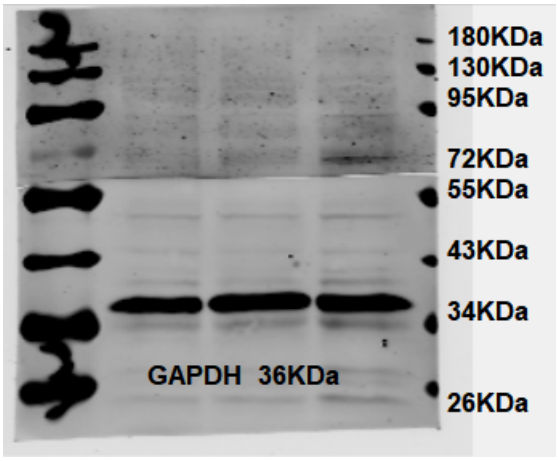
4


180KDa
130KDa
95KDa
72KDa
55KDa
CTGF 38KDa
43KDa
34KDa
26KDa

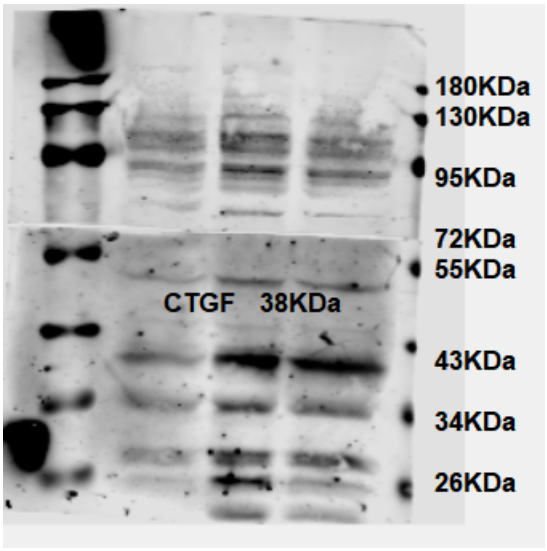

180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

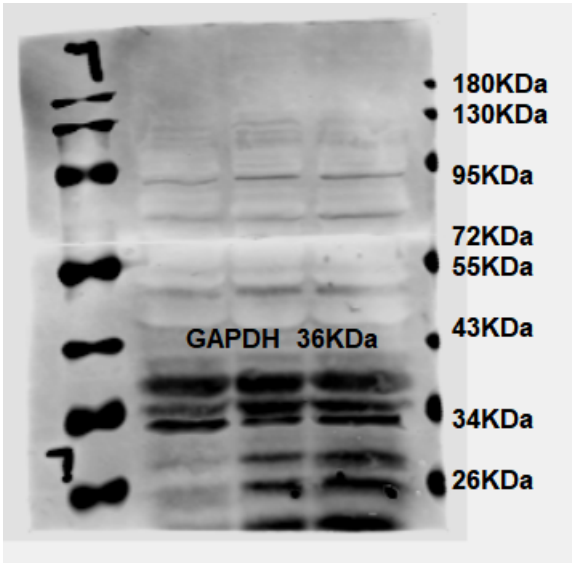
5


180KDa
130KDa
95KDa
72KDa
55KDa
CTGF 38KDa
43KDa
34KDa
26KDa


180KDa
130KDa
95KDa
72KDa
55KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

6

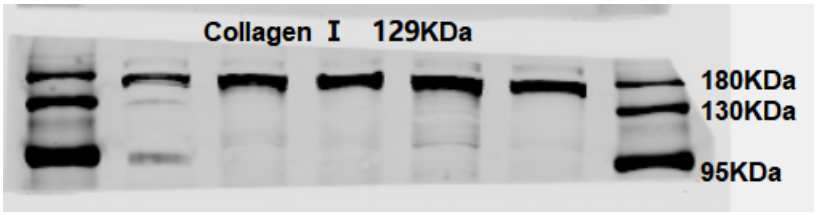

180KDa
130KDa
95KDa
72KDa
55KDa
CTGF 38KDa
43KDa
34KDa
26KDa

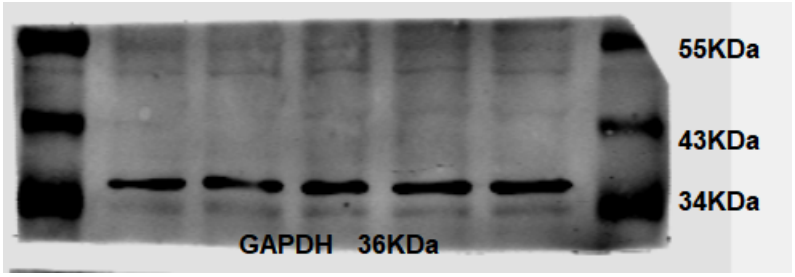


Original western bolt(Figure 2)

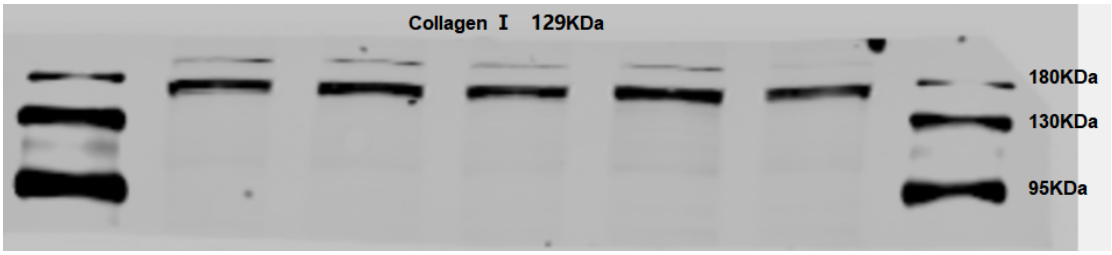
Figure 2A (n=6)

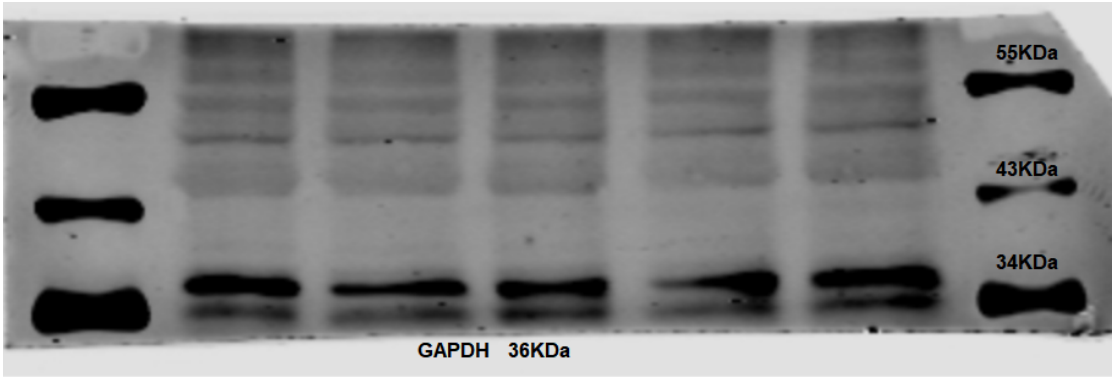
1



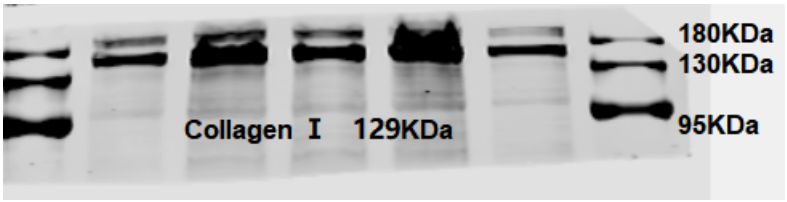


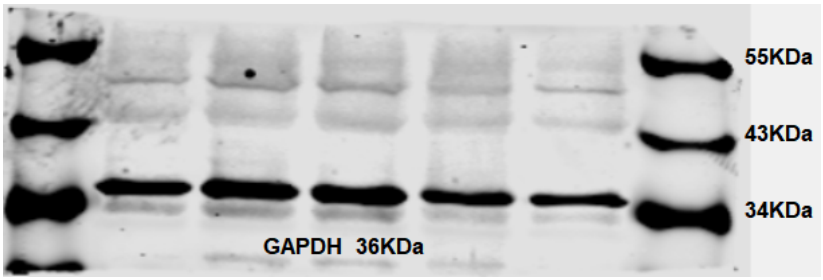
2



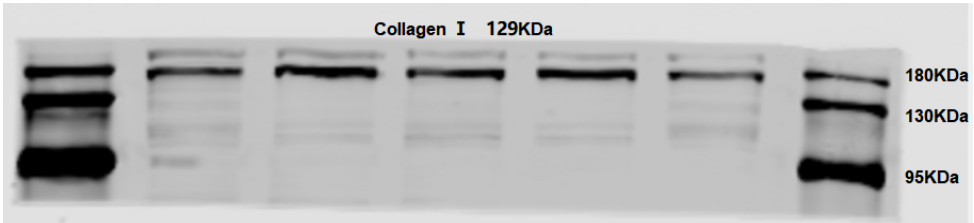

55KDa
43KDa
34KDa
GAPDH 36KDa

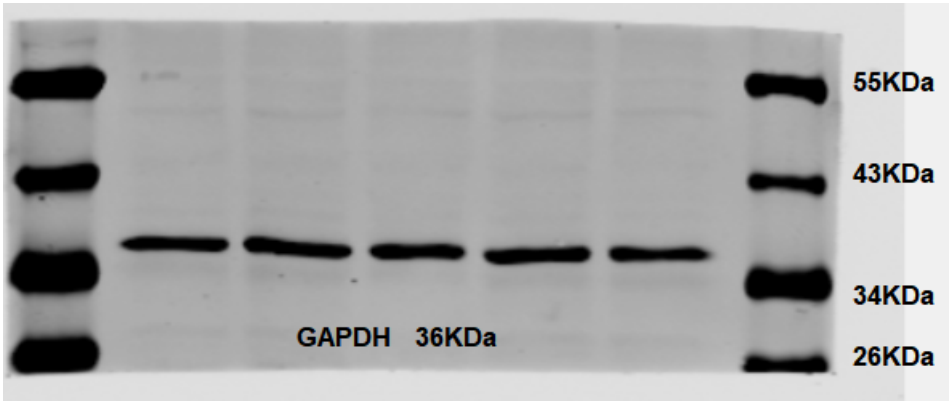
3


180KDa
130KDa
95KDa
Collagen I 129KDa

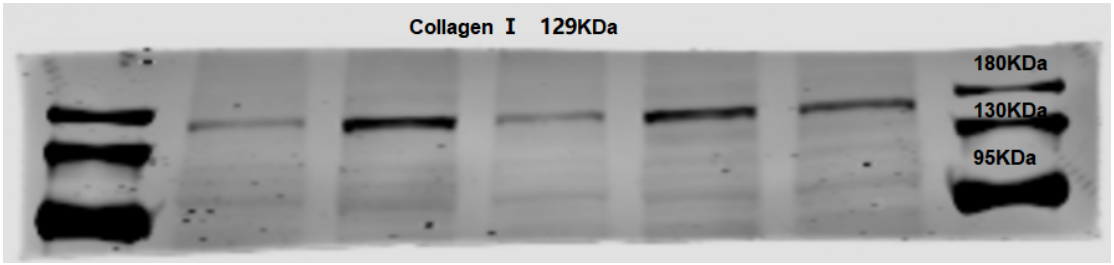

55KDa
43KDa
34KDa
GAPDH 36KDa

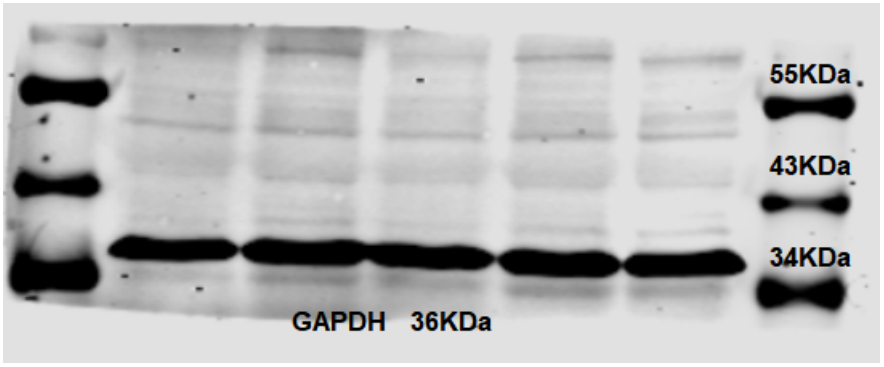
4


Collagen I 129KDa
180KDa
130KDa
95KDa

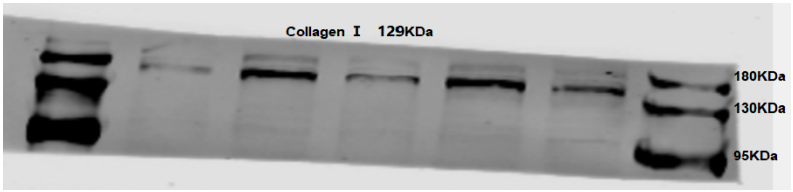

55KDa
43KDa
34KDa
26KDa
GAPDH 36KDa

5





6



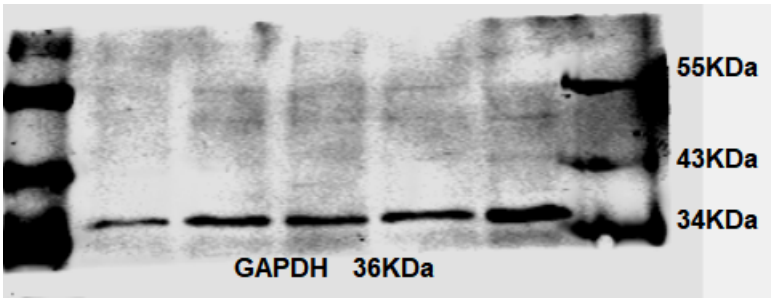
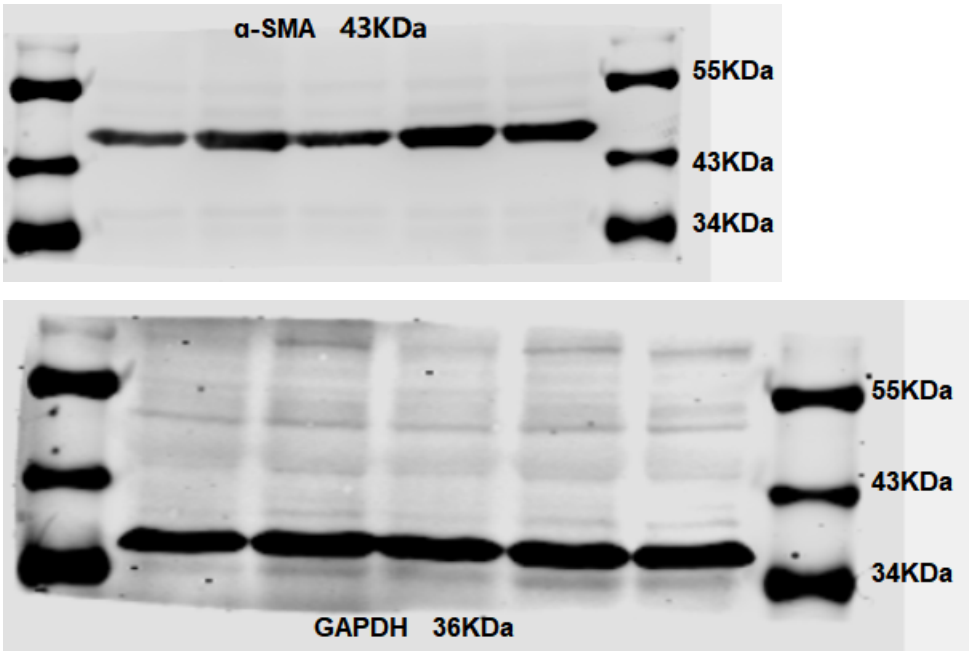
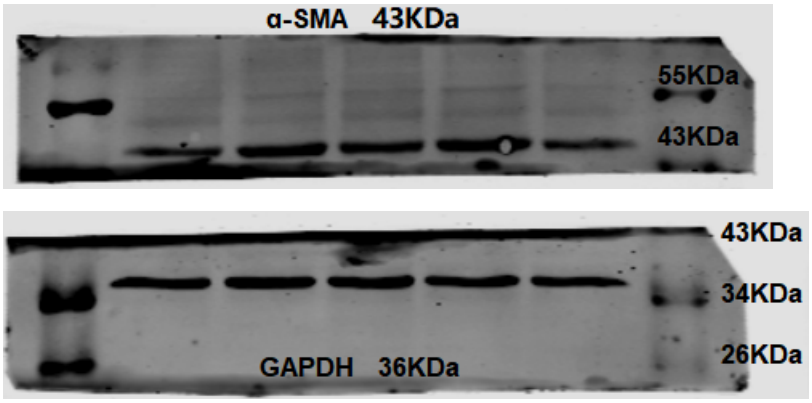


Figure 2B (n=4)

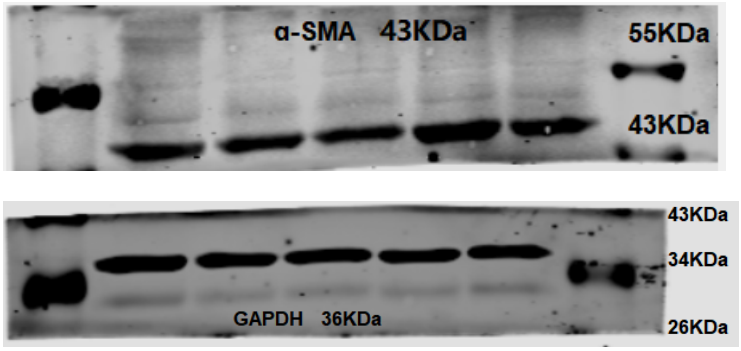
1


α-SMA 43KDa
55KDa
43KDa
34KDa
55KDa
43KDa
34KDa
GAPDH 36KDa

2


α-SMA 43KDa
55KDa
43KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

3


α-SMA 43KDa
55KDa
43KDa
43KDa
34KDa
GAPDH 36KDa
26KDa

4

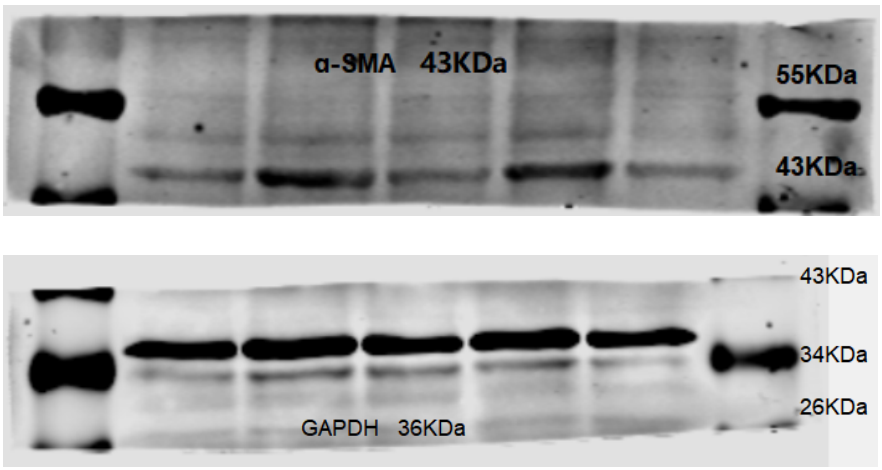
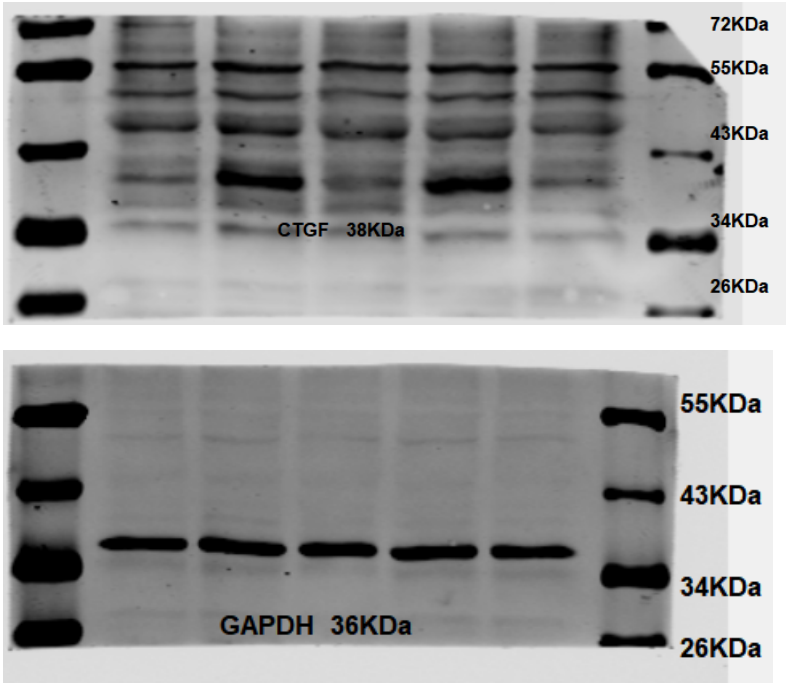
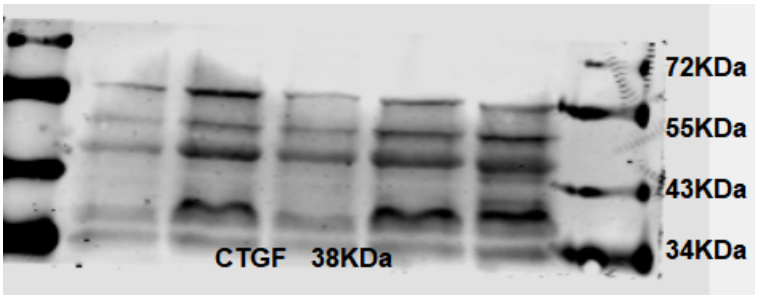


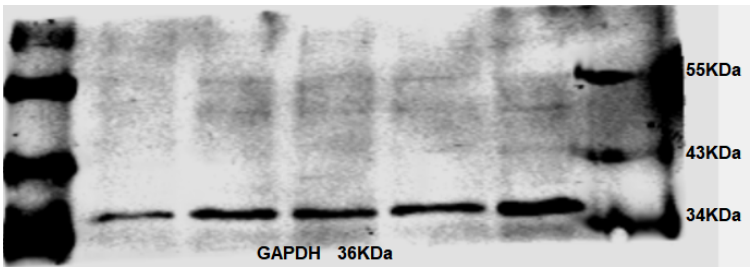
Figure 2C (n=7)

1

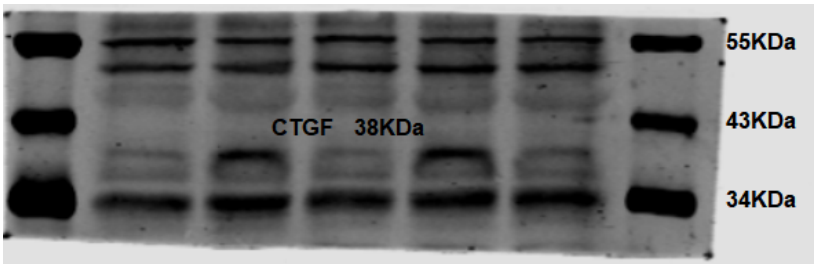


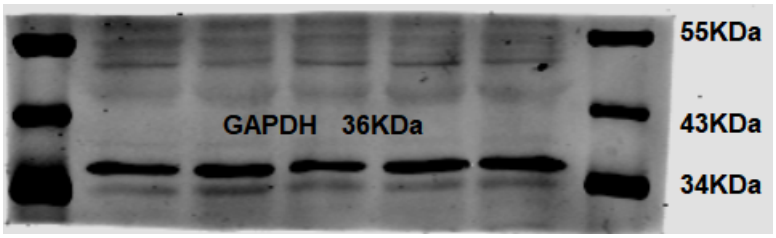
2



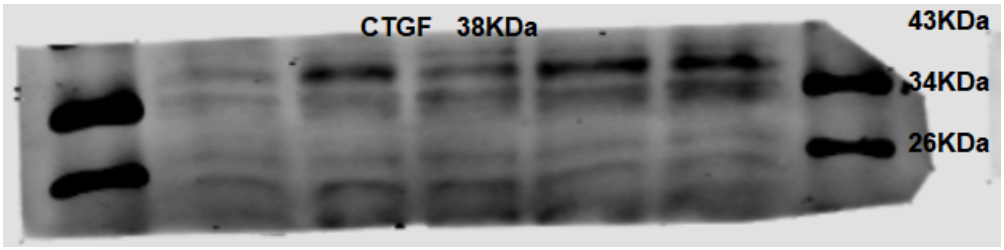

55KDa
43KDa
34KDa
GAPDH 36KDa

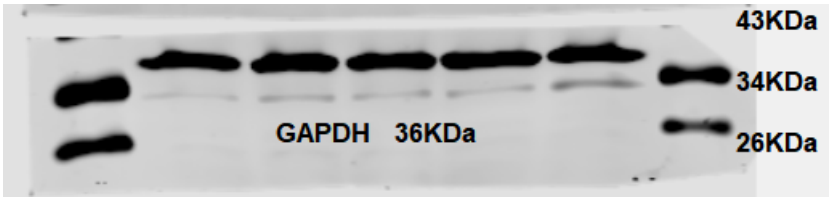
3


55KDa
CTGF 38KDa
43KDa
34KDa

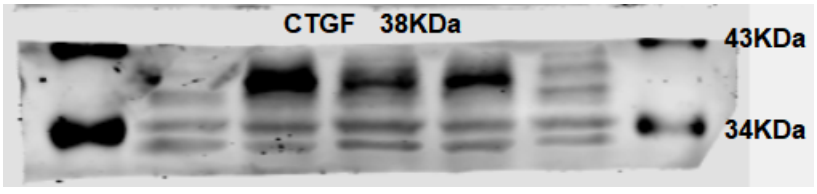

55KDa
GAPDH 36KDa
43KDa
34KDa

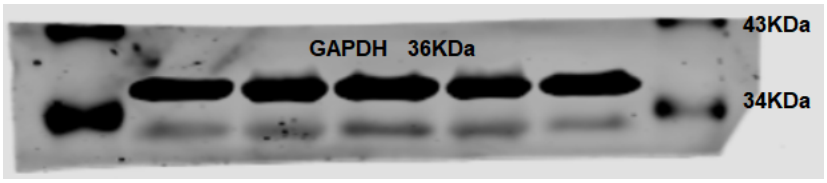
4


CTGF 38KDa
43KDa
34KDa
26KDa

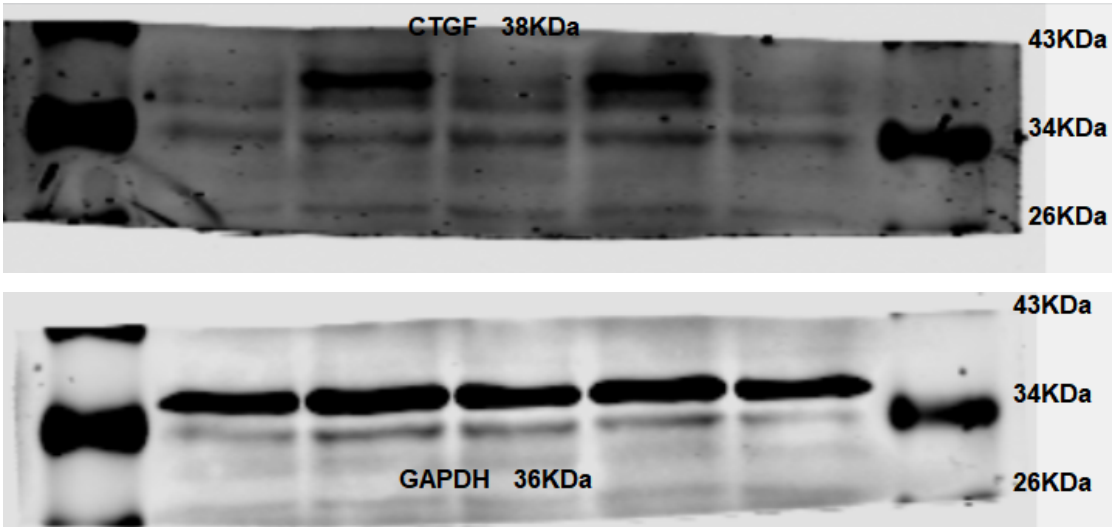

43KDa
34KDa
GAPDH 36KDa
26KDa

5

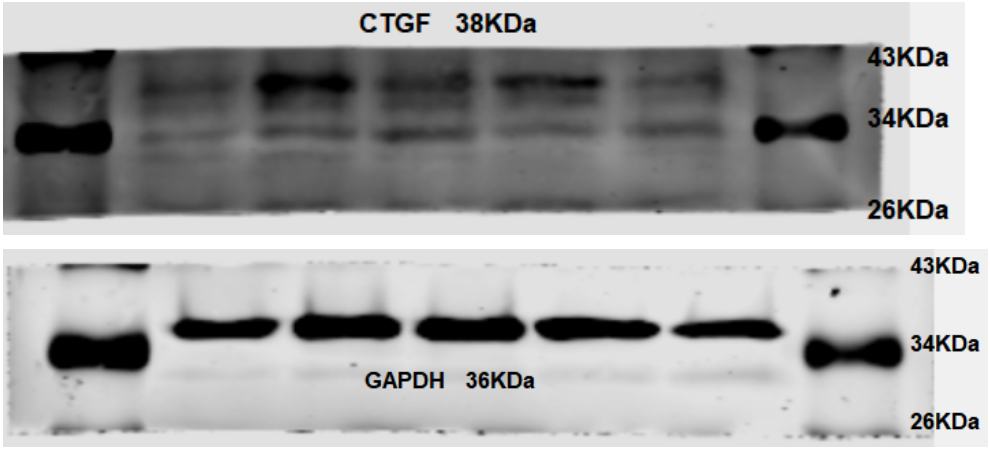

CTGF 38KDa
43KDa
34KDa



6



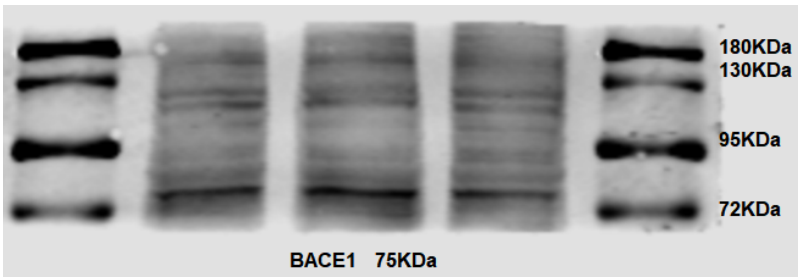
7

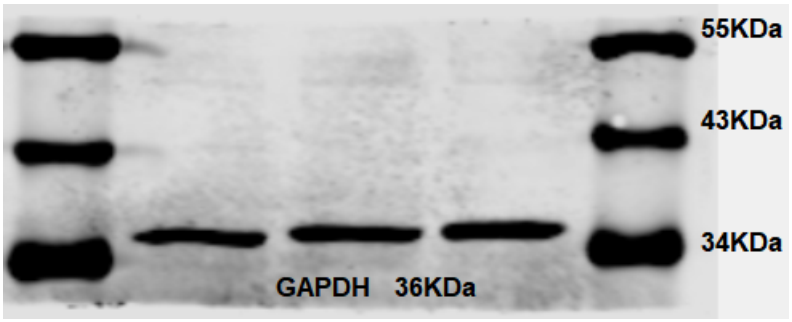


Original western bolt(Figure 5)

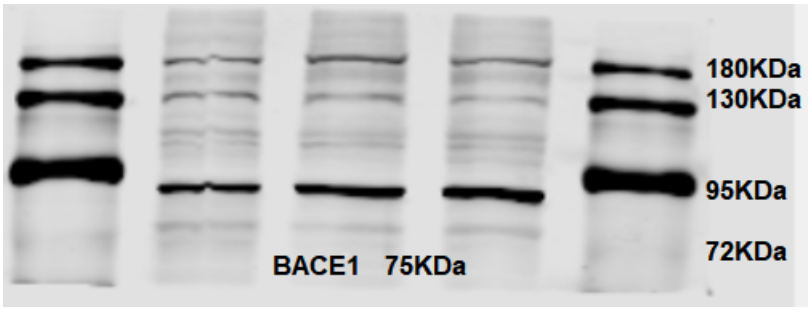
Figure 5C (n=4)

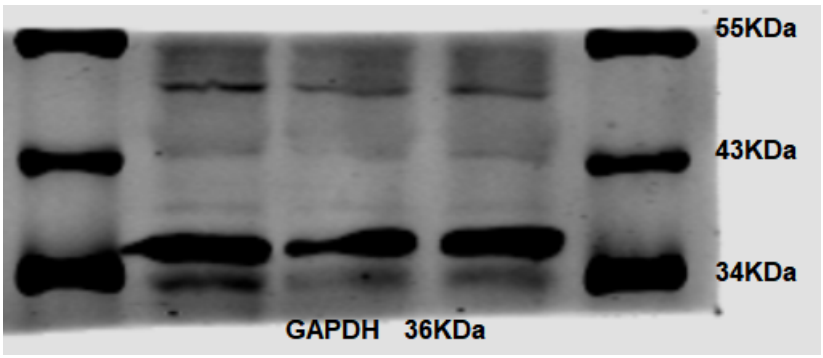
1


180KDa
130KDa
95KDa
72KDa
BACE1 75KDa

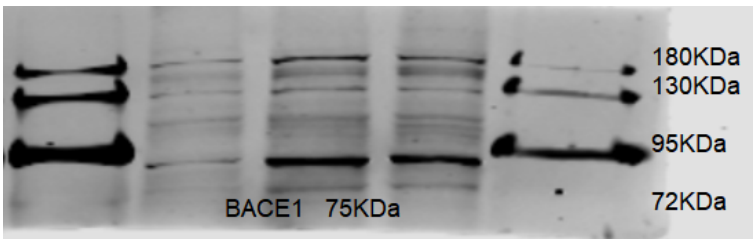

55KDa
43KDa
34KDa
GAPDH 36KDa

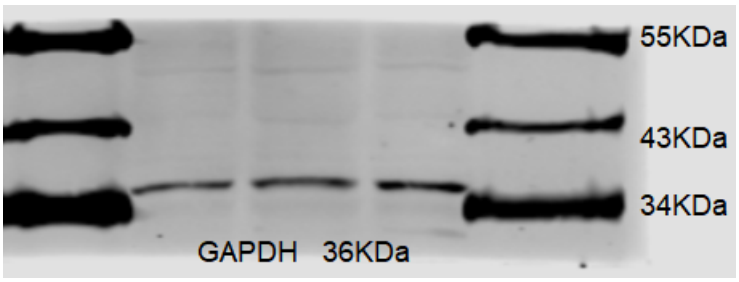
2


180KDa
130KDa
95KDa
72KDa
BACE1 75KDa


55KDa
43KDa
34KDa
GAPDH 36KDa

3


180KDa
130KDa
95KDa
72KDa
BACE1 75KDa



4

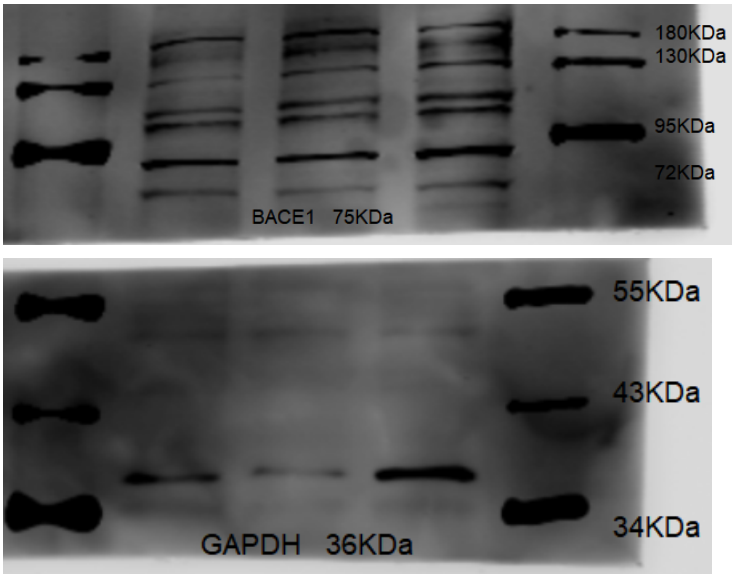
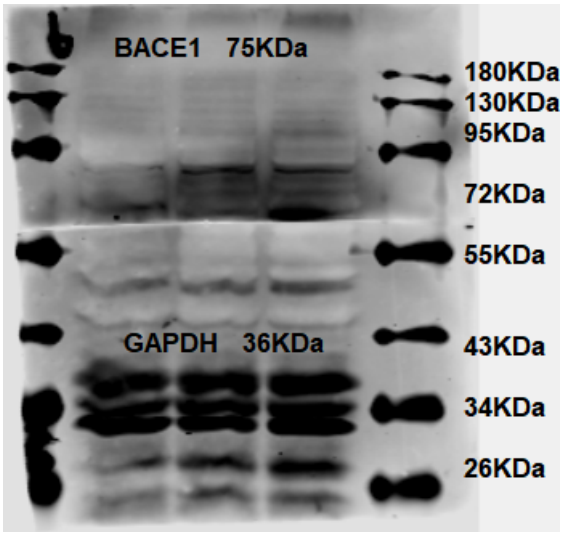
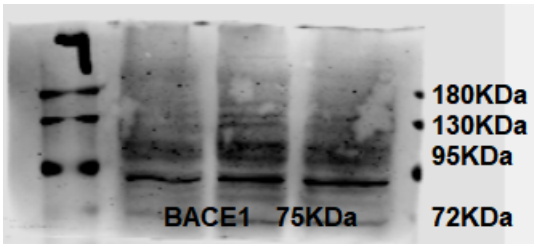


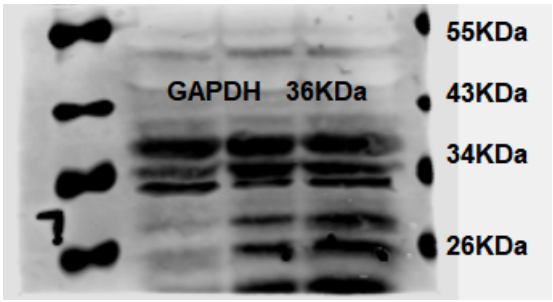
Figure 5D (n=4)

1

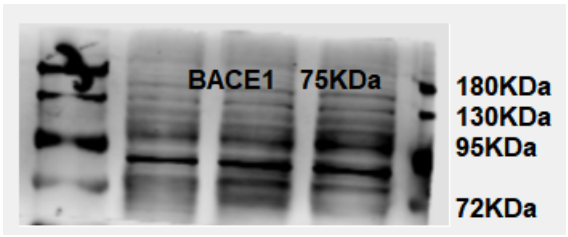


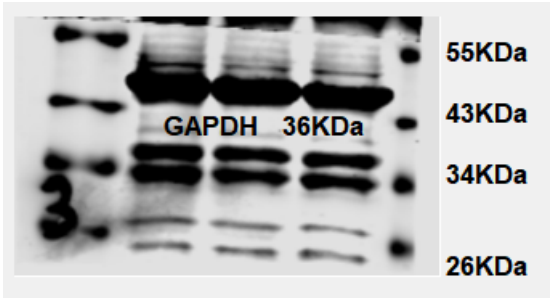
2


180KDa
130KDa
95KDa
BACE1 75KDa
72KDa


55KDa
GAPDH 36KDa
43KDa
34KDa
26KDa

3


BACE1 75KDa
180KDa
130KDa
95KDa
72KDa


55KDa
GAPDH 36KDa
43KDa
34KDa
26KDa

4

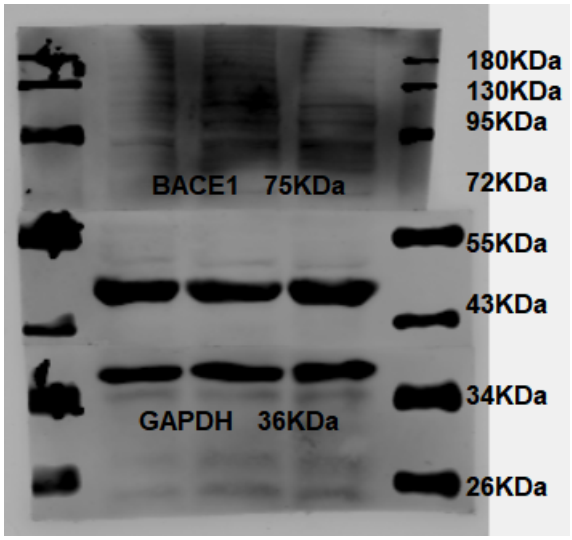
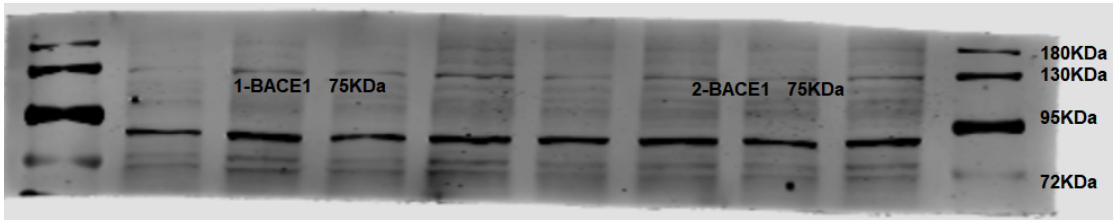
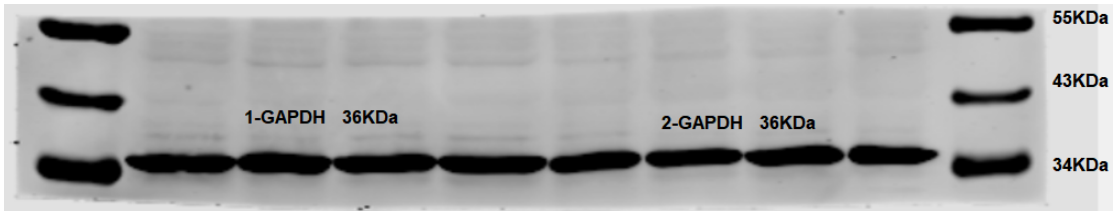


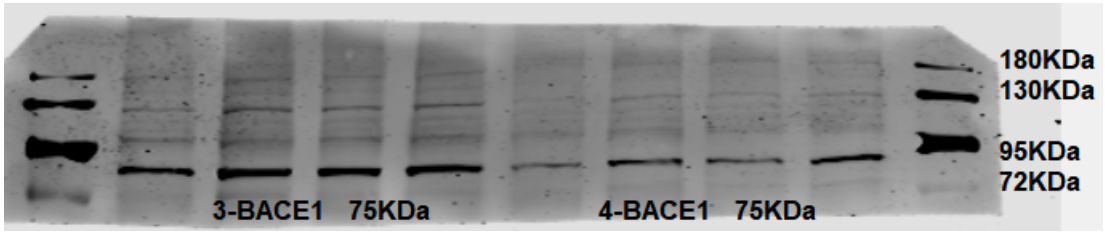
Figure 5E (n=5)

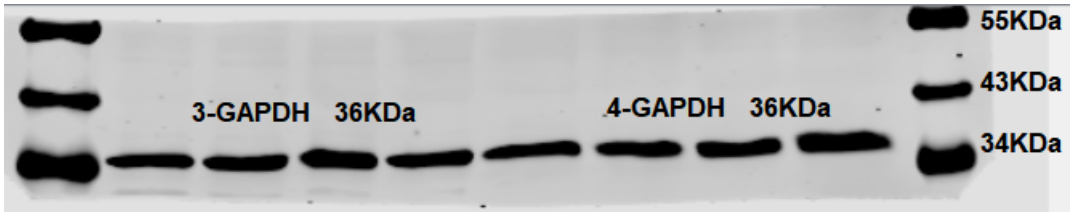
1 and 2





3 and 4





5

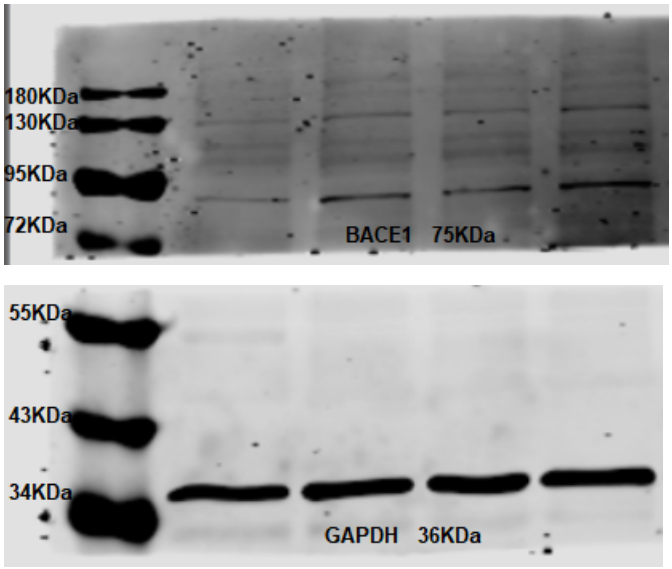
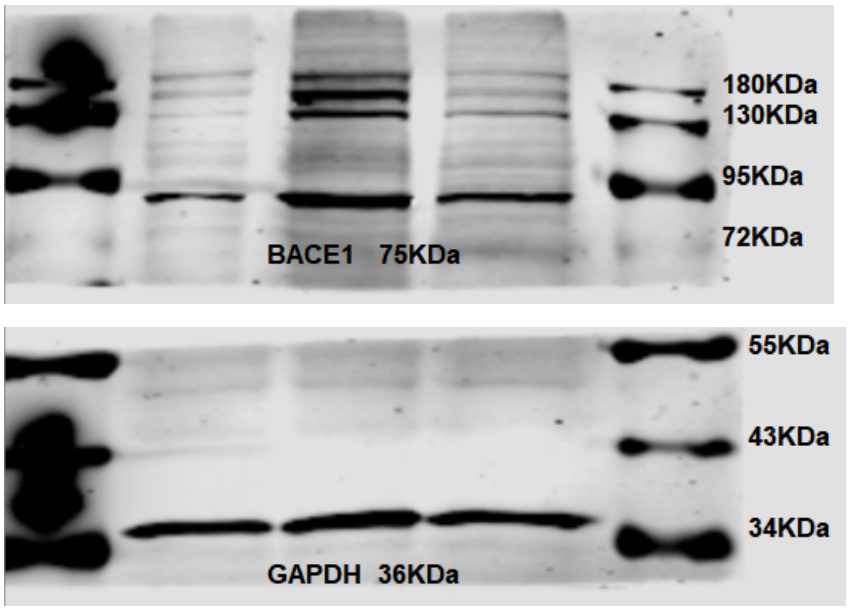
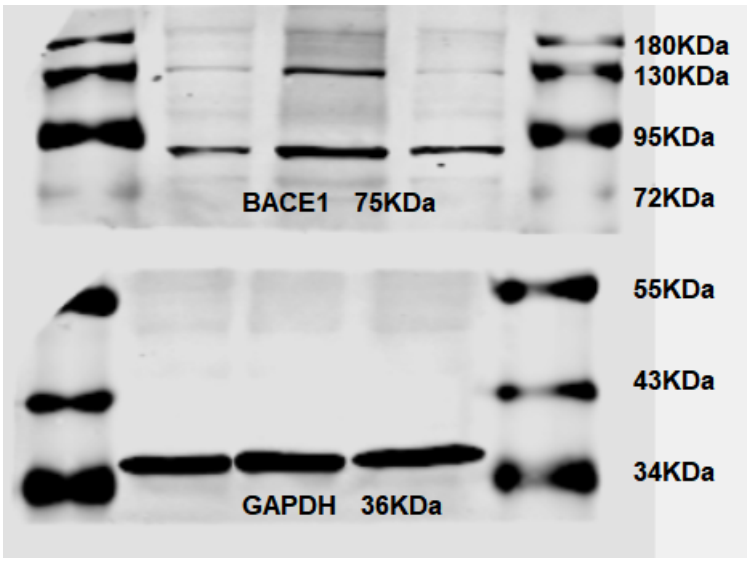


Figure 5F (n=4)

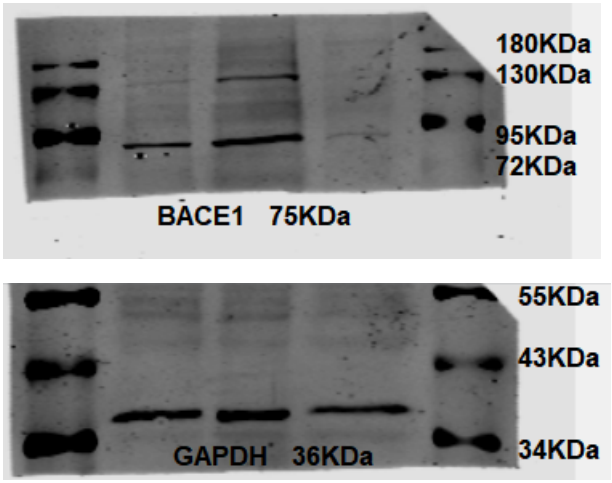
1



2


180KDa
130KDa
95KDa
72KDa
BACE1 75KDa
55KDa
43KDa
34KDa
GAPDH 36KDa

3


180KDa
130KDa
95KDa
72KDa
BACE1 75KDa
55KDa
43KDa
34KDa
GAPDH 36KDa

4

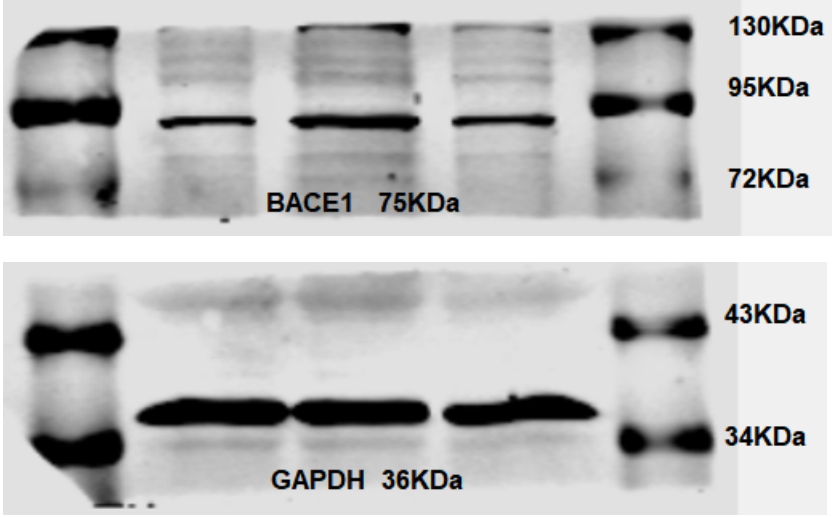
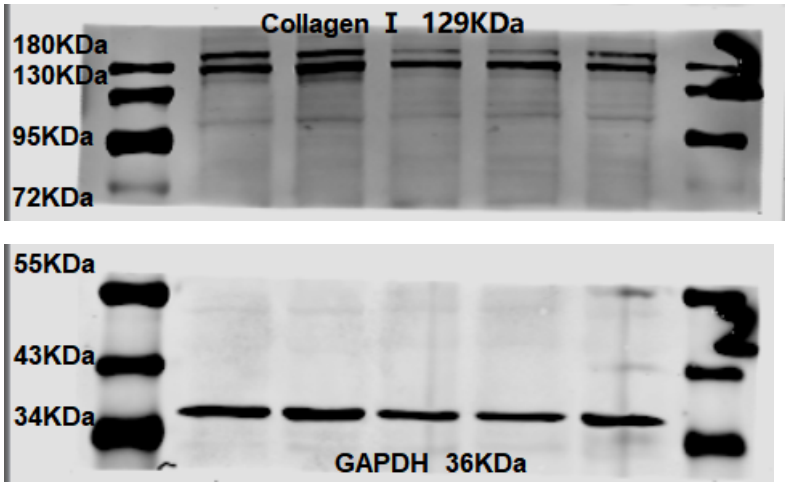
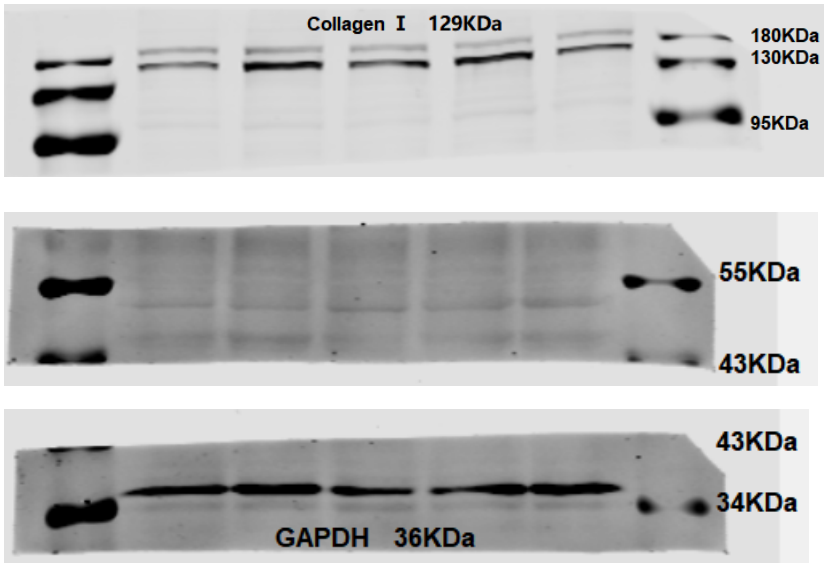

130KDa
95KDa
72KDa
BACE1 75KDa
43KDa
34KDa
GAPDH 36KDa

Figure 5H (n=6)

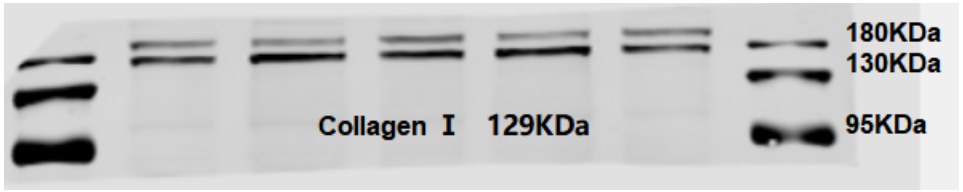
1

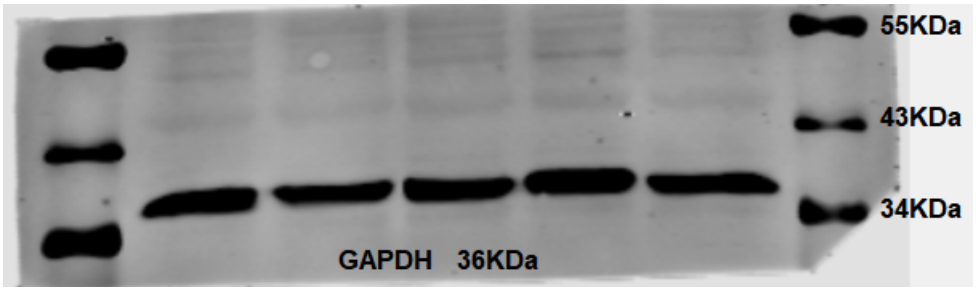


2

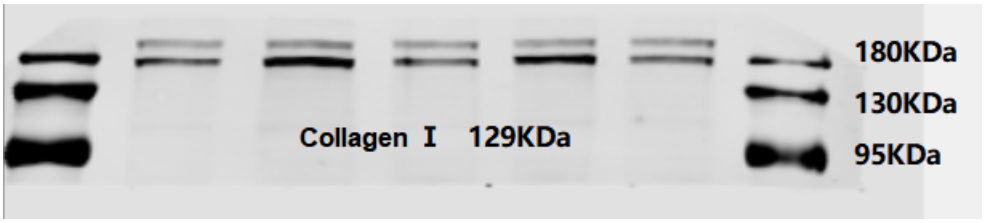


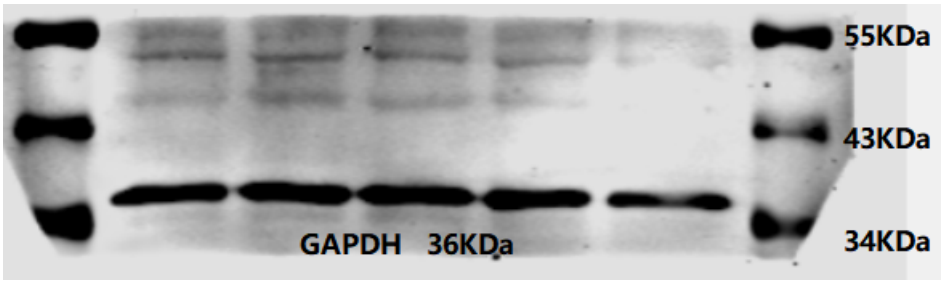
3



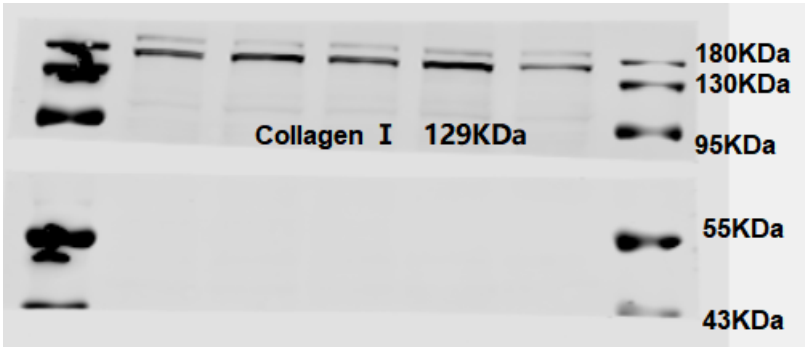

55KDa
43KDa
34KDa
GAPDH 36KDa

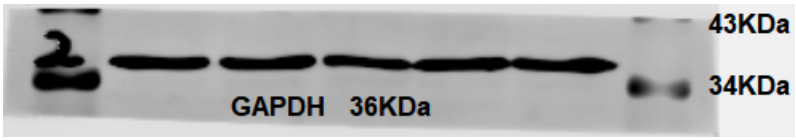
4


180KDa
130KDa
95KDa
Collagen I 129KDa

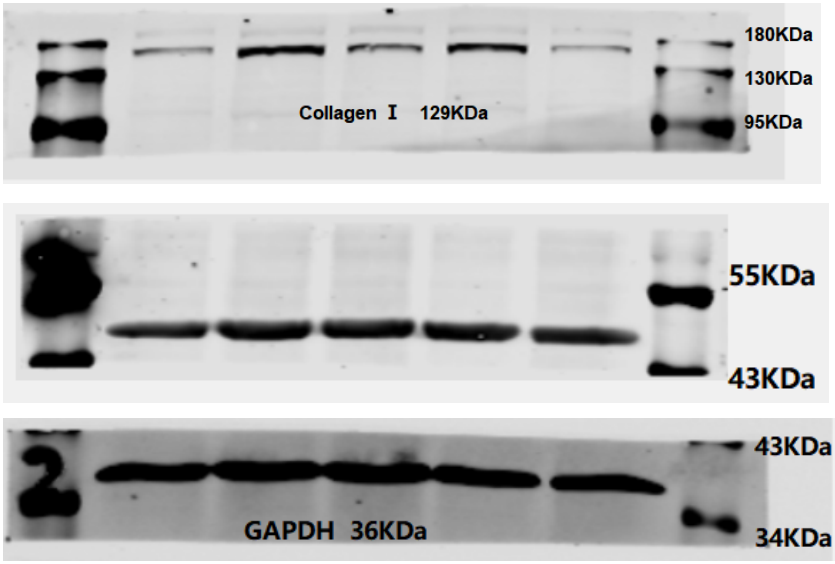

55KDa
43KDa
34KDa
GAPDH 36KDa

5


180KDa
130KDa
95KDa
Collagen I 129KDa
55KDa
43KDa


43KDa
34KDa
GAPDH 36KDa

6


180KDa
130KDa
95KDa
Collagen Ⅰ 129KDa
55KDa
43KDa
43KDa
GAPDH 36KDa
34KDa