Original Article

Role of double knockdown of tPA and MMP-9 on regulating the left ventricular function and remodeling followed by transverse aortic constriction-induced hypertrophic cardiomyopathy in mice

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Abstract: This study tested the hypothesis that extracellular matrix accumulation in tPA-/-/MMP-9-/- [double-knockout (DKO)] may be protective against left ventricular (LV) remodeling and dysfunction following transverse aortic constriction (TAC)-induced hypertrophic cardiomyopathy in mice. Wild-type C57BL/6 mice (n = 20) were equally categorized into sham-control (SC1) and TAC1. Similarly, DKO mice (n = 20) were equally divided into two groups (i.e., SC2 and ATC2). By days 28/60 after TAC, LV ejection fraction (LVEF) was significantly higher in TAC2 than TAC1, whereas LV end-systolic/diastolic dimensions displayed an opposite pattern to LVEF between the two groups (all \( P < 0.05 \)). By day 90, LVEF was significantly higher in SC groups than that in TAC1 and TAC2 without notable difference between the latter two groups, whereas LV end-systolic/diastolic dimensions, cardiomyocyte size and right-ventricular systolic pressure showed an opposite pattern compared with LVEF in all groups (all \( P < 0.01 \)). Total heart weight was highest in TAC1 and significantly higher in TAC2 than those in the SC groups (all \( P < 0.01 \)). LV myocardial protein expressions of inflammation (TNF-α/NF-κβ), apoptosis (mitochondrial-Bax/cleaved caspase-3/ PARP), oxidative stress (NOX-1/NOX-2/oxidized protein), fibrosis (Smad3/TGF-β), DNA/mitochondrial damage (γ-H2AX/cytosolic-cytochrome-C) and LV hypertrophy/pressure-overload (β-MHC/BNP) biomarkers were significantly increased in TAC2 compared to TAC1 and SC groups, and significantly increased in TAC1 compared to SC groups (all \( P < 0.001 \)). Histopathology demonstrated that the fibrotic/collagen-deposition areas and sarcomere length exhibited an identical pattern to inflammation among the four groups (all \( P < 0.0001 \)). In conclusion, although tPA-/-/MMP-9-/- seemed to preserve cardiac function in an experimental setting of hypertrophic cardiomyopathy at an early stage, it failed to exert long-term protective effect.

Keywords: Double knockout mice, transverse aortic constriction, hypertrophic cardiomyopathy, heart function

Introduction

Cardiac remodeling refers to molecular, cellular and interstitial changes of the myocardium, as well as alterations in size, shape and function of the heart in response to changing loading conditions [1-4]. Cardiac remodeling represents the final common pathway of different heart diseases and is recognized as a crucial aspect of cardiac and myocardial dysfunction and a well-established determinant of the clinical course of heart failure (HF) [1-6]. Left ventricular (LV) remodeling has been reported to occur and propagate in a variety of heart diseases such as hypertrophic cardiomyopathy (HCM) [4], dilated cardiomyopathy (DCM) [7, 8], myocardial infarction (MI) and chronic ischemic heart disease [5, 6]. Not only is LV remodeling a significant parameter for predicting LV functional deterioration, but it is also an important clini-
tPA-MMP-9 knockdown against TAC-induced HCM

Abundant data have revealed that myocardium of the failing heart commonly undergoes number of structural alterations, most notably hypertrophy of cardiac myocytes and an increase in extracellular matrix (ECM), often seen as primary fibrosis. Studies have further shown that interstitial fibrosis and excessive accumulation of ECM play a crucial role in regulating LV remodeling [12-18]. Additionally, not only has fibrosis in HCM been found to be associated with impaired cardiac function and HF, but it has also been considered a key substrate for ventricular arrhythmias and sudden death [12]. However, the molecular triggers underpinning ECM production are not well established. In addition, although a recent study has demonstrated that elevated levels of matrix metalloproteinases (MMPs) are related to LV remodeling and poor prognosis in patients with HCM [17], the mechanism through which MMPs modulate LV remodeling and their role in the development of HF remain unclear in patients with HCM, DCM and ischemic heart disease. Interestingly, our recent study has demonstrated that the extent of brain infarct was remarkably reduced in MMP-9-/- mice compared with that in wide-type animals in the setting of ischemic stroke [19]. We have also shown that tissue plasminogen activator (tPA) plays a key role in MMP-9 activation which causes degradation of ECM, thereby facilitating the migration of endothelial progenitor cells (EPCs) to the distant zone [20]. Since the Laplace's Law states that wall stress is proportional to radius^4 (r^4), we hypothesized that tPA^/-/MMP-9^/- induced ECM accumulation and LV chamber size reduction may protect the heart from LV remodeling and preserve cardiac function in the setting of TAC-induced hypertrophic cardiomyopathy in a murine model.

Materials and methods

Ethics

All animal procedures were approved by the Institute of Animal Care and Use Committee at Kaohsiung Chang Gung Memorial Hospital (Affidavit of Approval of Animal Use Protocol No. 2016092701) and performed in accordance with the Guide for the Care and Use of Laboratory Animals.
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A 25# needle was placed on the aorta on which a 7-0 prolene ligation was placed between the right and left common carotid arteries. The needle was then carefully removed, and the aortic constriction was created. After the procedure, the wound was closed, and the animal was allowed to recover from anesthesia in a portable animal intensive care unit (ThermoCare®) for 24 hours.

LV functional assessment by echocardiography

The procedure and protocol were based on our previous report [21]. In details, transthoracic echocardiography (Vevo 2100, Visualsonics, Toronto, Ontario, Canada) was performed in animals from each group prior to and at days 28, 60 and 90 after TCA induction by a cardiologist blinded to the experimental design. M-mode standard two-dimensional (2D) left parasternal long axis echocardiographic examination was conducted. Left ventricular internal dimensions [i.e., left ventricular end-systolic diameter (LVESd) and left ventricular end-diatolic diameter (LVEDd)] were measured at mitral valve and papillary levels of the left ventricle, as per the American Society of Echocardiography (Morrisville, NC) leading-edge methodology, using at least three consecutive cardiac cycles. Left ventricular ejection fraction (LVEF) was calculated as follows: LVEF (%) = [(LVEDd³-LVESd³)/LVEDd³] × 100%.

Assessment of right ventricular blood pressure and heart specimen preparation

The procedure and protocol were based on our previous report [20, 22, 23]. In details, on day 90 after TAC induction, the mice were anesthetized with inhalational 2.0% isoflurane. Each animal was endotracheally intubated with positive pressure ventilation using 100% oxygen with a tidal volume of 250 µL at a rate of 120 breaths per minute using a small animal ventilator (SAR-830/A, CWE, Inc., USA). The heart was exposed by left thoracotomy. A sterile 20-gauge, soft plastic needle was inserted into the right ventricle of each mouse to measure the right ventricular systolic pressure (RVSP). The pressure signals were first transmitted to pressure transducers (UFI, model 1050, CA, U.S.A.) and were then exported to a bridge amplifier (ML866 PowerLab 4/30 Data Acquisition Systems, ADInstruments Pty Ltd., Castle Hill, NSW, Australia) where the signals were amplified and digitized. The data were recorded and later analyzed with the Labchart software (ADInstruments). After hemodynamic measurements, the mice were euthanized, and the heart specimens were harvested.

Western blot analysis

The procedure and protocol for Western blot analysis were based on our recent reports [20, 24, 25]. Briefly, equal amounts (50 µg) of protein extracts were loaded and separated by SDS-PAGE using acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (GE, UK). Nonspecific sites were blocked by incubating the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated with the indicated primary antibodies [caspase 3 (1:1000, Cell Signaling), Poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling), nuclear factor (NF)-κB (1:600, Abcam), tumor necrosis factor (TNF)-α (1:1000, Cell Signaling), cytosolic cytochrome C (1:1000, BD), mitochondrial cytochrome C (1:1000, BD), phosphorylated (p)-Smad3 (1:1000, Cell Signaling), transforming growth factor (TGF)-β (1:500, Abcam), NOX-1 (1:1500, Sigma), NOX-2 (1:750, Sigma), γ-H2AX (1:1000, Cell Signaling), matrix metalloproteinase (MMP)-2 (1:1000, Abcam), MMP-9 (1:1000, Abcam), tissue inhibitors of metalloproteinase (TIMP)-2 (1:1000, Abcam), α-MHC (1:300, Santa Cruz), β-MHC (1:1000, Santa Cruz), COX-IV (1:3000, Abcam), and Actin (1:5000, Millipore)] for 1 hour at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Cell Signaling, Danvers, MA, USA) was used as a secondary antibody for one-hour incubation at room temperature. The washing procedure was repeated eight times within one hour. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Amersham, UK) and exposed to Biomax L film (Kodak, Rochester, NY, USA). For the purpose of quantification, ECL signals were digitized using Labwork software (UVP, Waltham, MA, USA).

Immunohistochemical (IHC) and immunofluorescent (IF) staining

The procedure and protocol of IHC and IF staining have been described in detail in our previ-
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Table 1. Time courses of transthoracic echocardiographic finding among the four groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>SC1 (n = 10)</th>
<th>SC2 (n = 10)</th>
<th>TAC1 (n = 10)</th>
<th>TAC2 (n = 10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At day 0</td>
<td></td>
<td>At day 28</td>
<td>At day 60</td>
<td>At day 90</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>61.4 ± 2.4</td>
<td>63.0 ± 2.4</td>
<td>62.7 ± 3.8</td>
<td>63.3 ± 2.0</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>FS (%)</td>
<td>32.5 ± 1.7</td>
<td>33.5 ± 1.7</td>
<td>33.5 ± 2.7</td>
<td>34.2 ± 1.4</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>3.93 ± 0.24</td>
<td>3.89 ± 0.31</td>
<td>4.03 ± 0.22</td>
<td>3.95 ± 0.30</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>LVESd (mm)</td>
<td>2.65 ± 0.16</td>
<td>2.64 ± 0.17</td>
<td>2.68 ± 0.08</td>
<td>2.68 ± 0.31</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>IVS thickness (mm)</td>
<td>0.59 ± 0.02</td>
<td>0.62 ± 0.07</td>
<td>0.59 ± 0.07</td>
<td>0.60 ± 0.08</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>PW thickness (mm)</td>
<td>0.57 ± 0.03</td>
<td>0.59 ± 0.04</td>
<td>0.59 ± 0.04</td>
<td>0.58 ± 0.05</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>At day 28</td>
<td></td>
<td>At day 60</td>
<td>At day 90</td>
<td></td>
</tr>
<tr>
<td>LVEF (%)*</td>
<td>61.4 ± 2.4a</td>
<td>63.0 ± 2.4a</td>
<td>34.90 ± 6.02b</td>
<td>54.16 ± 9.50c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>FS (%)*</td>
<td>32.5 ± 1.7a</td>
<td>33.5 ± 1.7a</td>
<td>17.9 ± 5.3a</td>
<td>23.3 ± 4.1b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LVEDd (mm)*</td>
<td>3.93 ± 0.24a</td>
<td>3.89 ± 0.31a</td>
<td>4.59 ± 0.59b</td>
<td>4.08 ± 0.53c</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>LVESd (mm)*</td>
<td>2.65 ± 0.16a</td>
<td>2.64 ± 0.17a</td>
<td>3.80 ± 0.76b</td>
<td>3.14 ± 0.53c</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IVS thickness (mm)*</td>
<td>0.59 ± 0.02a</td>
<td>0.62 ± 0.07a</td>
<td>0.77 ± 0.14b</td>
<td>0.80 ± 0.11b</td>
<td>&lt; 0.01</td>
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<tr>
<td>PW thickness (mm)*</td>
<td>0.57 ± 0.03a</td>
<td>0.59 ± 0.04a</td>
<td>0.76 ± 0.11b</td>
<td>0.71 ± 0.06b</td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td>At day 60</td>
<td></td>
<td>At day 90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEF (%)*</td>
<td>61.4 ± 2.4a</td>
<td>63.0 ± 2.4a</td>
<td>42.6 ± 11.0b</td>
<td>43.3 ± 13.4b</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>FS (%)*</td>
<td>32.5 ± 1.7a</td>
<td>33.5 ± 1.7a</td>
<td>21.0 ± 5.7b</td>
<td>21.5 ± 7.1c</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LVEDd (mm)*</td>
<td>3.93 ± 0.24a</td>
<td>3.89 ± 0.31a</td>
<td>4.37 ± 0.52b</td>
<td>4.21 ± 0.44a</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>LVESd (mm)*</td>
<td>2.65 ± 0.16a</td>
<td>2.64 ± 0.17a</td>
<td>3.48 ± 0.70b</td>
<td>3.27 ± 0.51b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>IVS thickness (mm)*</td>
<td>0.59 ± 0.02a</td>
<td>0.62 ± 0.07a</td>
<td>1.0 ± 0.10b</td>
<td>0.92 ± 0.10b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>PW thickness (mm)*</td>
<td>0.57 ± 0.03a</td>
<td>0.59 ± 0.04a</td>
<td>0.96 ± 0.09b</td>
<td>0.83 ± 0.10b</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Data was expressed as mean ± standard deviation (SD). Abbreviations: IVS = interventricular septum; LVEDd = Left ventricular end-diastolic internal dimension; LVESd = Left ventricular end-systolic internal dimension; LVEF = Left ventricular ejection fraction; FS = Fractional shortening. Independent t test was performed for comparison of continuous variables between two groups. SC1 = sham control in wild-type B6 mice; SC2 = tPA-/-/MMP-9-/- [i.e., double-knockout-mice (DKO)]; TAC1 = transverse aortic constriction in wild-type B6 mice; TAC2 = transverse aortic constriction in DKO mice. *Indicated the data were expressed as the baseline variables. Letters (a, b, c) indicate significant difference (at 0.05 level) by Tukey multiple comparison procedure.

Assessment of oxidative stress

The procedure and protocol for evaluating the protein expression of oxidative stress have been described in details in our previous reports [20, 24, 25]. The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon, Billerica, MA, USA (S7150). DNPH derivatization was carried out on 6 μg of protein for 15 minutes according to the manufacturer’s instructions. One-dimensional electrophoresis was carried out on 12% SDS/polyacrylamide gel after DNPH derivatization. Proteins were transferred to nitrocellulose membranes that were then incubated in the primary antibody solution (anti-DNP 1:150) for 2 hours, followed by repeated washes.
by incubation in secondary antibody solution (1:300) for 1 hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Amersham, UK) which was then exposed to Biomax L film (Kodak, Rochester, NY, USA). For quantification, ECL signals were digitized using Labwork software (UVP, Waltham, MA, USA). For oxyblot protein analysis, a standard control was loaded on each gel.

**Statistical analysis**

Quantitative data are expressed as means ± SD. Statistical analysis was adequately performed by ANOVA, followed by Bonferroni multiple-comparison post hoc test. Independent t test was performed for comparison of continuous variables between two groups. SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC) was utilized. A P value of less than 0.05 was considered statistically significant.

**Results**

**Time courses of transthoracic echocardiographic findings (Table 1)**

The baseline LVEF, LV fractional shortening (FS), interventricular septal thickness, posterior wall thickness, LVEDd and LVESd did not differ among the four groups. However, by days 28, 60 after TAC procedure, the LVEF and LV fractional shortening were significantly higher in SC and SC2 (i.e., baseline data) than those in TAC1 and TAC2 and significantly higher in TAC2 than those in TAC1, but there were no significant differences between SC1 and SC2. Additionally, by day 90 after TAC procedure, these two parameters were still significantly higher in SC1 and SC2 than those in TAC1 and TAC2 but there were no notable differences between the former two groups and between the latter two groups.

The interventricular septal thickness and posterior wall thickness were significantly lower in SC1 and SC2 than those in TAC1 and TAC2 by days 28, 60 and 90 after TAC procedure, but these two parameters did not differ between the former and later two groups.

Furthermore, the LVEDd and LVESd were significantly lower in SC1 and SC2 than those in TAC1 and TAC2 by days 28, 60 and 90 after TAC procedure. Moreover, these two parameters were significantly higher in TAC2 than those in TAC2 by days 28 and 60 after TAC procedure. However, there were no significant differences in the two parameters between TAC1 and TAC2 by day 90 after TAC induction, suggesting that DKO did not persistently inhibit LV remodeling compared to that in the wide-type animals in the setting of TAC-induced hypertrophic cardiomyopathy.
By day 90 after the TAC procedure, the ratio of total lung weight to the body was significantly higher in TAC1 than that in SC1 but it did not differ among the SC1, SC2 and TAC2 or between TAC1 and TAC2. However, the ratio of total heart weight to the body weight was significantly lower in SC1 and SC2 than that in TAC1 and TAC2 and significantly higher in TAC1 than that in TAC2 but no notable difference was noted between SC1 and SC2. Additionally, by day 90 after the TAC procedure, the right ventricular systolic blood pressure (RVSBP), an indirect indicator of pulmonary arterial systolic blood pressure, was significantly lower in SC1 and SC2 than that in TAC1 and TAC2 but there was no significant difference between the former two groups or the latter two groups.

**Figure 2.** The cardiomyocyte size and LV chamber area by day 90 after TAC procedure. A-D. Illustrating the H.E stain (400 ×) of left ventricular (LV) cross-section for determining cardiomyocyte size. E. Analytical result of cardiomyocyte size, **indicated P < 0.001. F-I. illustrating the pathological finding of cross-section of whole heart (40 ×) for identification of LV chamber area of each group at the same cross-section level. J. Analytical result of LV chamber area, *indicated P < 0.05. n = 4 for each group. SC1 = sham control of wild-type C57BL/6 (B6) mice; TAC1 = SC1 + transverse aortic constriction; SC2 = tPA-/-/MMP-9-/- double knockout (DKO) mice; TAC2 = SC2 + transverse aortic constriction.
Cardiomyocyte size and LV chamber area by day 90 after TAC procedure (Figure 2)

The cardiomyocyte size was significantly larger in TAC1 than that in SC1 and in TAC2 than that of SC2. Additionally, LV chamber size was significantly larger in SC1 and than in TAC1 and SC2 than that in TAC2.

Sarcomere length and condensed collagen-deposition area in LV myocardium by day 90 after TAC procedure (Figure 3)

The sarcomere length of LV myocardium was significantly increased in TAC2 than that in SC1, SC2 and TAC1 and significantly increased in TAC1 than that in SC1 and SC2 but no significant difference was noted between the latter two groups, suggesting that by day 90 after TAC procedure DKO did not attenuate the distension of LV myocardium in the present experimental setting of TAC-induced hypertrophic cardiomyopathy.

Sirius red staining demonstrated that the condensed collagen-deposition area, an indicator of accumulation of ECM, was significantly increased in TAC2 than that in SC1, SC2 and TAC1 and significantly increased in TAC1 than that in SC1 and SC2 but there was no notable difference between the latter two groups, indicating an increase in collagen deposition area in LV myocardium as a result of double gene knockout.
Masson’s trichrome staining revealed that the fibrotic area of LV myocardium was significantly increased in TAC² than that in SC¹, SC² and TAC¹, and significantly increased in TAC² than that in SC¹ and SC² but no difference was found between the latter two groups. Besides, IF microscopic finding showed that the cellular expression of γ-H2AX, an indicator of DNA damage, exhibited a pattern identical to that of fibrosis among the four groups.

**Figure 4.** Fibrosis and DNA-damaged biomarker in LV myocardium by day 90 after TAC procedure. A-D. Illustrating the microscopic finding (400 ×) of Masson’s trichrome stain for identification of fibrotic area (blue color). E. Analytical result of fibrotic area, * vs. other groups with different symbols (‡, †), P < 0.0001. F-I. Showing the immunofluorescent microscopic findings (400 ×) for identifying the cellular expression of γ-H2AX (pink color). J. Analytical result of number of γ-H2AX+ cells, * vs. other groups with different symbols (‡, †), P < 0.0001. Scale bars in right lower corner represent 20 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). SC¹ = sham control of wild-type C57BL/6 (B6) mice; TAC¹ = SC¹ + transverse aortic constriction; SC² = tPA⁻/⁻/MMP-9⁻⁻ double knockout (DKO) mice; TAC² = SC² + transverse aortic constriction.

**Expressions of fibrosis and DNA damage biomarker in LV myocardium by day 90 after TAC procedure (Figure 4)**

Masson’s trichrome staining revealed that the fibrotic area of LV myocardium was significantly increased in TAC² than that in SC¹, SC² and TAC¹, and significantly increased in TAC² than that in SC¹ and SC² but no difference was found between the latter two groups. Besides, IF microscopic finding showed that the cellular expression of γ-H2AX, an indicator of DNA damage, exhibited a pattern identical to that of fibrosis among the four groups.
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The protein expression of oxidative stress in LV myocardium by day 90 after TAC procedure (Figure 5)

The protein expressions of NOX-1, NOX-2 and oxidize protein, three indicators of oxidative stress, were significantly increased in TAC\(^2\) than those in SC\(^1\), SC\(^2\) and TAC\(^1\) and significantly increased in TAC\(^1\) than those in SC\(^1\) and SC\(^2\) without notable difference between the latter two groups. Additionally, the protein expressions of Smad3 and TGF-β, two indicators of fibrosis, showed an identical pattern compared to that of apoptosis among the four groups.

The protein expressions of MMPs, inhibitor of TIMPs and inflammatory biomarkers in LV myocardium by day 90 after TAC procedure (Figure 7)

The protein expression of MMP-2 was significantly higher in TAC\(^2\) than that in SC\(^1\), SC\(^2\) and TAC\(^1\) and significantly higher in TAC\(^1\) than that in SC\(^1\) and SC\(^2\) but it showed no difference between the latter two groups. In addition, the protein expression of MMP-9 was significantly higher in TAC\(^2\) than in SC\(^1\) but it was not detected in SC\(^2\) and TAC\(^2\), suggesting that this is result of double gene knockout. On the other hand, the protein expression of TIMP-2 was significantly higher in SC\(^1\) and SC\(^2\) than that in TAC\(^1\) and TAC\(^2\), and significantly higher in TAC\(^1\) than that in TAC\(^2\) but there was no notable difference between TAC\(^3\) and TAC\(^5\). Furthermore, the protein expressions of TNF-α and NF-κB, two indicators of inflammation, exhibited an opposite pattern compared to that of MMP-2 in all groups of animals.

The protein expressions of DNA/mitochondrial damage, pressure/volume overloaded biomarkers in LV myocardium by day 90 after TAC procedure (Figure 8)

The protein expression of γ-H2AX, a DNA damage biomarker, was significantly higher in TAC\(^3\) and TAC\(^5\) than that in SC\(^1\) and SC\(^2\), and significantly higher in TAC\(^2\) than that in TAC\(^3\) but there was no remarkable difference between SC\(^1\) and SC\(^2\).
Figure 6. Protein expression of apoptotic and fibrotic biomarkers in LV myocardium by day 90 after TAC procedure. A. Protein expression of mitochondrial Bax (mit-Bax), * vs. other groups with different symbols (†, ‡), P < 0.0001. B. Protein expression of cleaved PARP and cleaved poly (ADP-ribose) polymerase (c-PARP), * vs. other groups with different symbols (†, ‡), P < 0.0001. C. Protein expression of cleaved caspase 3 (c-Csp3), * vs. other groups with different symbols (†, ‡), P < 0.0001. D. Protein expression of phosphorylated (p)-Smad3, * vs. other groups with different symbols (†, ‡), P < 0.0001. E. Protein expression of transforming growth factor (TGF)-β, * vs. other groups with different symbols (†, ‡), P < 0.0001. F. Protein expression of p-Smad1/5, * vs. other groups with different symbols (†, ‡, §), P < 0.0001. G. Protein expression of bone morphogenetic protein (BMP)-2, * vs. other groups with different symbols (†, ‡, §), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡, §) indicate significance (at 0.05 level). SC1 = sham control of wild-type C57BL/6 (B6) mice; TAC1 = SC1 + transverse aortic constriction; SC2 = tPA-/-/MMP-9-/- double knockout (DKO) mice; TAC2 = SC2 + transverse aortic constriction.
The protein expression of cytosolic cytochrome C, an indicator of mitochondrial damage, showed a pattern identical to that of γ-H2AX among the four groups. On the other hand, the protein expression of mitochondrial cytochrome C, an index of integrity of mitochondria, exhibited an opposite pattern compared to that of cytosolic cytochrome C in all groups.

It is well known that cardiac hypertrophy is characterized by a switch from α- to β-MHC protein expression (i.e., reactivation of fetal gene program). In the present study, we found that the protein expressions of BNP and β-MHC, two indicators of pressure-volume overload biomarkers, showed a pattern identical to that of γ-H2AX among the four groups. On the other hand, the protein expression of α-MHC was significantly higher in SC1 and SC2 than those of TAC1 and TAC2, and significantly higher in TAC1 than in TAC2 but it showed no difference between SC1 and SC2.

**Discussion**

This study, which investigated the role of an increase in ECM in myocardium (i.e., resulted from tPA-/MMP-9-/- in mice), yielded several striking implications. First, double gene knock-out could offer additional benefits in ameliorating the LV remodeling and preservation of LV function in the present experimental setting at the chosen time points (i.e., by days 28 and 60). Second, by day 90 after TAC procedure, LV function and chamber size (i.e., measured by echocardiography) were found to be identical between TAC1 (i.e., wild-type mice) and TAC2 (i.e., DKO mice) groups but the ratio of heart...
weight to body weight was significantly higher in TAC\textsuperscript{1} than that in TAC\textsuperscript{2}, suggesting that the capacity for intrinsic LV hypertrophy in response to late stage of TAC-induced pressure overload was better in the former than that in the latter. Third, by day 90 after TAC procedure, the molecular-cellular perturbations of LV myocardium were significantly aggravated in TAC\textsuperscript{2} compared to those in TAC\textsuperscript{1}.

Currently, there has been no investigation addressing whether the presence of accumulated ECM in LV myocardium would offer a positive or negative impact on heart function and LV remodeling in the setting of hypertrophic cardiomyopathy. The most important finding in the present study is that, by days 28 and 60 after TAC induction, LV function was preserved and LV chamber dilatation (i.e., index of LV remodeling) was significantly inhibited in TAC\textsuperscript{2} animals compared to those in the TAC\textsuperscript{1} group, suggesting that DKO offered short-term (i.e., by day-28 after TAC) and intermediate-term (i.e., by day-60 after TAC) protection against TAC-induced hypertrophic cardiomyopathy. Surprisingly, long-term (i.e., day-90 after TAC) LV function and LV chamber dimension were identical between TAC\textsuperscript{2} and TAC\textsuperscript{1}, implying that the protective effect of DKO would disappear after a prolonged period in the present experimental setting.

**Figure 8.** Protein expressions of DNA/mitochondrial damaged, pressure/volume overloaded biomarkers in LV myocardium by day 90 after TAC procedure. A. Protein expressions of γ-H2AX, * vs. other groups with different symbols (‡, ‡), P < 0.0001. B. Protein expression of cytosolic cytochrome C (cyto CytC), * vs. other groups with different symbols (‡, ‡), P < 0.0001. C. Protein expression of mitochondrial cytochrome C (mito CytC), * vs. other groups with different symbols (‡, ‡), P < 0.0001. D. Protein expression of alpha myosin heavy chain (α-MHC), * vs. other groups with different symbols (‡, ‡), P < 0.0001. E. Protein expression of ß-MHC, * vs. other groups with different symbols (‡, ‡), P < 0.0001. F. Protein expression of brain natriuretic peptide (BNP), * vs. other groups with different symbols (‡, ‡), P < 0.0001. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n = 6 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). SC\textsuperscript{1} = sham control of wild-type C57BL/6 (B6) mice; TAC\textsuperscript{1} = SC\textsuperscript{1} + transverse aortic constriction; SC\textsuperscript{2} = tPA\textsuperscript{-}/MMP-9\textsuperscript{-} double knockout (DKO) mice; TAC\textsuperscript{2} = SC\textsuperscript{2} + transverse aortic constriction.
An association among sarcomere length, LV chamber size, and heart weight has been clearly identified in our previous studies [26, 27]. An essential finding in the present study is that the ratio of total heart weight to body weight was significantly lower in TAC\textsuperscript{2} than that in TAC\textsuperscript{1} by day 90 after TAC procedure. On the other hand, the sarcomere was significantly longer in TAC\textsuperscript{2} than that in TAC\textsuperscript{1}. The results, in addition to being consistent with the findings of our previous studies [26, 27], may partially explain the deterioration of LVEF and increase of LVEDd/LVESd in TAC\textsuperscript{2} and a further increased LVEF and reduced LVEDd/LVESd in TAC\textsuperscript{1} at day 90 compared with those at day 60 after TAC induction (Table 1).

In the present study, the ratio of lung weight to body weight was similar among the four groups. Additionally, RVSBP, which was less than 30 mmHg in TAC\textsuperscript{1} and TAC\textsuperscript{2}, did not differ between the two groups. These findings imply that we only created a mild pressure overload which may help account for the zero mortality for all animals by day 90 after the TAC procedure.

The association of deterioration in heart function with fibrosis and apoptosis of LV myocardium has been well reported in previous experimental studies [21, 26, 27]. A principal finding in the present study is the remarkably aggravated fibrosis and condensed collagen deposition in TAC\textsuperscript{2} compared to those in TAC\textsuperscript{1}. Additionally, the protein expressions of inflammation, apoptosis, oxidative stress, DNA/mitochondrial damage, and pressure-volume overload biomarkers were notably and consistently up-regulated in TAC\textsuperscript{2} compared to those in TAC\textsuperscript{1}. These findings, in addition to corroborating those of previous studies [21, 26, 27], showed that although tPA\textsuperscript{-/-}/MMP-9\textsuperscript{-/-} seemed to preserve cardiac function at an early stage (i.e., post-TAC 28 and 60 days) in the present experimental setting of hypertrophic cardiomyopathy, it failed to exert long-term protective effect as reflected in a further reduction in LVEF and an increase in LVEDd/LVESd in TAC\textsuperscript{2} animals as well as a preservation of LVEF and a decrease in LVEDd/LVESd in TAC\textsuperscript{1} by day 90 compared with those by day 60 after TAC procedure.

**Study limitations**

This study has limitations. First, since the study period was only 90 days, whether further deterioration of LV function and progression of LV remodeling would develop in TAC\textsuperscript{2} animals remain unknown. Second, the mechanisms underlying the rapid deterioration of LVEF and aggravation of remodeling in the TAC\textsuperscript{2} animals compared to those in the TAC\textsuperscript{1} group at the late stage (i.e., day 90 after TAC) is still unclear.

In conclusion, the results of the present study demonstrated that although genetic knockout of tPA and MMP-9 could offer early protection against hypertrophic cardiomyopathy-induced impairment of cardiac function, it failed to produce long-term protective effects in the present experimental setting.

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**Disclosure of conflict of interest**

None.

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