1 **Small interfering RNA therapy against carbohydrate sulfotransferase 15 inhibits**
2 **cardiac remodeling in rats with dilated cardiomyopathy**

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22 **Running title:** Effects of CHST15 siRNA in rats with dilated cardiomyopathy

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1 **Abstract**

2 Carbohydrate sulfotransferase 15 (CHST15) is a sulfotransferase responsible for biosynthesis
3 of chondroitin sulfate E (CS-E), which plays important roles in numerous biological events
4 such as biosynthesis of proinflammatory cytokines. However, the effects of CHST15 siRNA
5 in rats with chronic heart failure (CHF) after experimental autoimmune myocarditis (EAM)
6 have not yet been investigated. CHF was elicited in Lewis rats by immunization with cardiac
7 myosin, and after immunization, the rats were divided into two groups and treated with either
8 CHST15 siRNA (2 µg per week) or vehicle. Age matched normal rats without immunizations
9 were also included in this study. After 7 weeks of treatment, we investigated the effects of
10 CHST15 siRNA on cardiac function, proinflammatory cytokines, and cardiac remodeling in
11 EAM rats. Myocardial functional parameters measured by hemodynamic and
12 echocardiographic studies were significantly improved by CHST15 siRNA treatment in rats
13 with CHF compared with that of vehicle-treated CHF rats. CHST15 siRNA significantly
14 reduced cardiac fibrosis, and hypertrophy and its marker molecules (left ventricular (LV)
15 mRNA expressions of transforming growth factor beta1, collagen I and III, and atrial
16 natriuretic peptide) compared with vehicle-treated CHF rats. CHF-induced increased
17 myocardial mRNA expressions of proinflammatory cytokines [interleukin (IL)-6, IL-1β],
18 monocyte chemoattractant protein-1, and matrix metalloproteinases (MMP-2 and -9), and
19 CHST15 were also suppressed by the treatment with CHST15 siRNA. Western blotting study
20 has confirmed the results obtained from mRNA analysis as CHST15 siRNA treated rats
21 expressed reduced levels of inflammatory and cardiac remodeling marker proteins. Our
22 results demonstrate for the first time, that CHST15 siRNA treatment significantly improved
23 LV function and ameliorated the progression of cardiac remodeling in rats with CHF after
24 EAM.

1 **Keywords:** Experimental autoimmune myocarditis; chronic heart failure; cardiac
2 remodeling; CHST15 siRNA.

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1 **1. Introduction**

2 Dilated cardiomyopathy (DCM) is a set of heterogeneous diseases of left ventricular (LV)
3 dysfunction due to unknown etiology [1]. DCM is considered to be sequel to myocarditis [2],
4 and there by two mechanisms by which myocarditis develop into DCM have been proposed:
5 one is persistent viral infection and the other is progressive autoimmune myocardial injury
6 [3].

7 Glycan chains comprising proteoglycans, called glycosaminoglycans (GAG), are variably
8 sulfated by specific sulfotransferases [4, 5]. Chondroitin sulfate (CS) is a typical GAG
9 subtype, and the negatively charged nature of GAG is implicated in several biological
10 processes. In particular, CS-E, which is composed of repeating GlcA β 1-3GalNAc (4, 6-bis-
11 SO₄) disaccharide units with a β 1-4 linkage and were originally identified in squid cartilage
12 [6], has been reported to play diverse roles in studies in vitro.

13 The RNA interference (RNAi) system has been used extensively for silencing the translation
14 of an active gene and has recently been applied to mammalian cells using small interfering
15 RNA (siRNA) technology [7, 8]. The siRNA mediating RNAi against the coxsackievirus–
16 adenovirus–receptor (CAR) has been shown to inhibit CoxB3 infections [9]. Recently, Ito et
17 al. purified N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase, now known as
18 carbohydrate sulfotransferase 15 (CHST15), protein from squid cartilage [10] and
19 subsequently cloned its human orthologue [11]. CHST15 is responsible for the biosynthesis
20 of CS-E from CS-A by transferring a sulfate group to the position C6 of CHST15 (4SO₄) in
21 CS-A. Since the specific antibody directed to CS-E has not been available, analyses of
22 CHST15 gene are helpful to understand the biological roles of CS-E. CHST15 siRNA is a
23 siRNA specifically designed to inhibit the expression of the glycogene, CHST15. It is a
24 synthetic siRNA containing sequences that are complementary to a region of the mRNA
25 sequences of CHST15 gene. The antisense strand of CHST15 siRNA serves as a guide

1 sequence to the RNAi mechanism to specifically recognize and cleave CHST15 mRNAs,
2 which blocks the expression of the CHST15 protein.

3 To date, the effects of CHST15 siRNA in the rat model of myosin-induced chronic heart
4 failure (CHF) are unknown. Cardiac myosin induced EAM is characterized by extensive
5 myocardial necrosis, congestive heart failure and appearance of multinucleated giant cells
6 and reflective to human giant cell myocarditis. Giant cell myocarditis is a fatal disorder, often
7 leads to heart failure or arrhythmias [12]. EAM was demonstrated to progress into the
8 clinicopathological state similar to DCM in the chronic phase, and was characterized by the
9 enlargement of the heart, dilatation of ventricles, diffuse and extensive myocardial fibrosis,
10 and hypertrophic and atrophic changes of myocardial fibers, resembling human
11 cardiomyopathy [13]. Thus we plan to investigate the effects of CHST15 siRNA on cardiac
12 function, proinflammatory cytokines and cardiac remodeling in a rat model of CHF after
13 EAM.

14

1 **2. Materials and Methods**

2 *2.1. Materials*

3 CHST15 siRNA was obtained from STELIC INSTITUTE & CO., INC (Tokyo, Japan) [14],
4 and Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc.,
5 Kanagawa, Japan. All the reagents used were of analytical grade and purchased from Sigma,
6 Tokyo, Japan, until mentioned otherwise.

7 *2.2. Experimental design*

8 All experiments were carried out using 8-weeks-old male Lewis rats and were performed in
9 accordance with the guidelines of our institute [15]. Lewis rats were injected in the footpads
10 with antigen-adjuvant emulsion according to the procedure described previously [15, 16]. In
11 brief, porcine cardiac myosin was dissolved in phosphate-buffered saline at 5 mg/ml and
12 emulsified with an equal volume of complete freund's adjuvant with 11 mg/ml
13 Mycobacterium tuberculosis H37RA (Difco Lab., Detroit, MI, USA). CHF in rats was
14 induced by immunization with 0.1 ml of emulsion once by subcutaneous injection into their
15 rear footpads (0.1 ml to each footpad). The morbidity of EAM was 100% in rats immunized
16 by this procedure [15, 16]. After immunization, the Lewis rats were divided into two groups
17 and received vehicle (group V) or intraperitoneal administration of CHST15 siRNA (group
18 CHST15 siRNA, 2 µg per week) for 7 weeks. Age matched Lewis rats without immunization
19 was used as normal controls (group N).

20 *2.3. Hemodynamic and Echocardiographic Studies*

21 To obtain hemodynamic data, rats were anesthetized with 2% halothane in oxygen during the
22 surgical procedures. A catheter-tip transducer (Miller SPR 249; Miller Instruments, Houston,
23 TX) was introduced into the left ventricle through the right carotid artery for the
24 determination of peak left ventricular pressure (LVP) and left ventricular end-diastolic
25 pressure (LVEDP), and the rates of intraventricular pressure rise (+ dP/dt) and decline (–

1 dP/dt) were recorded as described previously [15]. After instrumentation the concentration of
2 halothane was reduced to 0.5% to minimize the anesthetic effect on hemodynamic
3 parameters. Echocardiographic studies were carried out with a 7.5-MHz transducer (Aloka
4 Inc., Tokyo, Japan). The left ventricular dimensions in diastole (LVDd) and systole (LVDs),
5 percent fractional shortening (FS) and percent ejection fraction (EF) were estimated by using
6 the M-mode measurements.

7 *2.4. Histopathology*

8 The body weight (BW) of rats was noted just before surgical procedure. After the
9 hemodynamic and echocardiographic analyses, the rats were sacrificed, and their
10 myocardium was isolated and weighed to calculate the ratio of heart weight (HW) to BW.
11 The excised myocardium was kept in formalin and the midventricle sections were then
12 embedded with paraffin to prepare the slides. The de-paraffinized midventricle sections were
13 stained with Masson's trichrome dye and using a color image analyzer (CIA-102; Olympus,
14 Tokyo, Japan) the area of myocardial fibrosis (blue fibrotic area opposed to red myocardium)
15 were quantified. The results are presented as the ratio of the fibrotic area to the area of whole
16 myocardium.

17 Using hematoxylin and eosin (H&E) stained sections, myocyte diameter measurements were
18 performed in 10 myocytes selected per field, in 400-fold magnification by light microscopy.
19 Short axis diameters of each myocyte were measured from the hearts of all groups of rats.
20 Each average value was obtained based on the data from 10 myocytes and was used as an
21 independent sampling data. In addition, inflammatory cell infiltrations were identified by
22 using H&E stained sections at 100-fold magnification by light microscopy.

23 *2.5. Mast Cell Staining and Quantitation*

24 Histochemical staining with toluidine blue was performed to identify mast cells. Briefly,
25 slides of paraffinized midventricle sections were dewaxed, rehydrated, incubated with 0.05%

1 (w/v) toluidine blue for 30 min, and subsequently counterstained with 0.01% (w/v) eosin for
2 1 min. Metachromatic staining of mast cell granules was useful in identifying these cells.
3 Mast cell density (MCD) was quantified by counting the number of toluidine blue-positive
4 mast cells per field (x100). At least 15 fields were included in each slide for counting.

5 *2.6. RNA Extraction*

6 Heart tissues were preserved by immersion in RNAlater (Ambion Inc., Austin, TX)
7 immediately after sampling. The extraction of total RNA was performed after
8 homogenization by using Ultra TurraxT8 (IKA Labortechnik, Staufen, Germany) in TRIzol
9 reagent (Invitrogen Corp., CA) according to the standard protocol. Synthesis of cDNA was
10 performed by reverse transcription using total RNA (2 µg) as a template (Super Script II;
11 Invitrogen Corporation, Carlsbad, CA).

12 *2.7. Gene expression analysis by real time RT-PCR*

13 Gene expression analysis was performed by real time reverse transcription polymerase chain
14 reaction (RT-PCR) (Smart cycler; Cepheid, Sunnyvale, CA) using cDNA synthesized from
15 the CHF specimens. Real time RT-PCR by monitoring with TaqMan probe (TaqMan Gene
16 expression assays; Applied Biosystems, Foster City, CA) was performed according to the
17 following protocol: 600 seconds at 95 °C, followed by thermal cycles of 15 seconds at 95 °C,
18 and 60 seconds at 60 °C for extension. Relative standard curves representing several 10 fold
19 dilutions (1:10:100:1000:10,000:100,000) of cDNA from CHF tissue samples were used for
20 linear regression analysis of other samples. Results were normalized to glyceraldehyde-3-
21 phosphate dehydrogenase (GAPDH) mRNA as an internal control and are thus shown as
22 relative mRNA levels.

23 *2.8. Western immunoblotting analysis*

24 Protein lysate was prepared from heart tissue as described previously [17]. The total protein
25 concentration in samples was measured by the bicinchonic acid method. For Western blotting

1 experiments, 50 mg of total protein was loaded and proteins were separated by SDS-PAGE
2 (200 V for 40 min) and electrophoretically transferred to nitrocellulose filters (semidry
3 transfer at 10 V for 30 min). Filters were blocked with 5% non-fat dry milk in Tris-buffered
4 saline (20 mM Tris, pH 7.6, 137 mM NaCl) with 0.1% Tween 20, washed, and then
5 incubated with primary antibody. Primary antibodies employed included: mouse monoclonal
6 anti-interferon-gamma (IFN- γ), goat polyclonal anti-interleukin-6 (IL-6), goat polyclonal
7 anti-monocyte chemo attractant protein-1 (MCP-1), goat polyclonal anti-connective tissue
8 growth factor (CTGF), rabbit monoclonal anti-nuclear factor kappa-B p65 (p-NF κ B), rabbit
9 polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Purchased from Santa
10 Cruz Biotechnology Inc., CA, USA), rabbit polyclonal transforming growth factor-beta1
11 (TGF- β 1) (Purchased from Promega, Madison, WI, USA) and rabbit monoclonal NF κ B (Cell
12 Signaling Technology Inc., MA, USA). After incubation with the primary antibody, the
13 bound antibody was visualized with horseradish peroxidase-coupled secondary antibodies
14 (Santa Cruz Biotechnology) using LI-COR C-DiGit blot scanner (LI-COR Biotechnology,
15 Lincoln, NE, USA). For Western blotting analysis, all primary antibodies were used at a
16 dilution of 1:1000 and secondary antibodies were used at a dilution of 1:5000-10000.

17 *2.8. Statistical analysis*

18 All values are expressed as means $\pm$ S.E. Statistical analysis of differences between the
19 groups was performed by one-way ANOVA, followed by Tukey's test or two tailed *t*-test
20 when appropriate. Survival analysis was performed by Log-rank (Montel-Cox) test. A value
21 of $P < 0.05$ was considered as statistically significant.

22 **3. Results**

23 *3.1. Survival rate*

24 The effects of CHST15 siRNA on survival rate are shown in Fig. 1 and Table 1. Four (50%)
25 and three (30 %) of 8, 10 rats in group V and CHST15 siRNA, respectively, died between

1 days 21 and 56. None of the rats died in group N (n=4). The survival rates were higher in
2 group CHST15 siRNA compared with group V.

3 3.2. *Effects of CHST15 siRNA on myocardial functions*

4 Although heart rate was not different among the three groups of rats, central venous pressure
5 (CVP) and LVEDP were significantly higher and mean blood pressure (MBP), LVP and
6 $\pm dP/dt$ were significantly lower in group V in comparison to group N indicating systolic and
7 diastolic dysfunction in vehicle-treated rats (Table 1). CVP and LVEDP were significantly
8 decreased in treatment CHST15 siRNA group compared to those in group V. Myocardial
9 contractility parameters $\pm dP/dt$ were slightly improved in CHF rats treated with CHST15
10 siRNA. However, MBP and LVP were slightly improved by the treatment.
11 Echocardiographic studies in group V rats showed evidence of LV remodeling, with
12 increased LVDd and LVDs ($P < 0.01$), reduced FS and EF ($P < 0.01$), indicating impaired
13 systolic function compared with that in group N rats (Table 1). Treatment with CHST15
14 siRNA significantly decreased LVDs and increased FS and EF compared with those in group
15 V (Table 1).

16 3.4. *Histopathology*

17 HW and HW/BW were significantly larger in group V than in group N rats (Table 1).
18 CHST15 siRNA significantly reduced HW and HW/BW, when compared with group V. The
19 hearts from group V rats showed massive fibrosis compared to those from group N rats (Figs.
20 2A and A1). The percent area of fibrosis was significantly lower in the CHST15 siRNA
21 treated rats than in vehicle-treated rats (Figs. 2A and A1). The myocyte size in group V was
22 significantly larger than that in group N (Figs. 2B and B1). CHST15 siRNA treatment
23 significantly reduced the myocyte size compared to those in group V (Figs. 2B and B1).
24 Focal accumulations of infiltrated inflammatory cells were detected in the hearts of group V

1 rats (Fig 2C). However, the proportion of cell accumulation was less in the CHST15 siRNA
2 treated rats (Fig 2C).

3 3.5. Cardiac mast cell density

4 Cardiac MCD was significantly greater in the group V samples than in the group N samples
5 (Fig. 2D). Treatment with the CHST15 siRNA decreased the cardiac MCD in comparison to
6 that in vehicle-treated rats (Fig. 2D).

7 3.6. Myocardial mRNA expressions of collagen-I and III, transforming growth factor-beta 1 8 (TGF- β 1) and atrial natriuretic peptide (ANP)

9 RT-PCR analysis showed that collagen-I and III, TGF- β 1 and ANP mRNA expression levels
10 were significantly increased in vehicle-treated rats, compared to group N rats (Figs. 3A-D). In
11 contrast, treatment with CHST15 siRNA significantly decreased the myocardial mRNA
12 expressions of collagen-I and III, TGF- β 1 and ANP compared to group V rats (Figs. 3A-D).

13 3.7. Myocardial mRNA expressions of inflammatory cytokines and matrix metalloproteinases 14 (MMPs)

15 RT-PCR data showed that interleukin (IL)-6, IL-1 β and monocyte chemoattractant protein-1
16 (MCP-1) mRNA expression levels were significantly increased in vehicle-treated rats,
17 compared to group N rats (Figs. 4A-C). In contrast, treatment with CHST15 siRNA
18 significantly decreased the myocardial mRNA expressions of IL-6, IL-1 β and MCP-1
19 compared to group V rats (Figs. 4A-C). Moreover, mRNA expressions of MMP-2 and 9 were
20 significantly increased in vehicle-treated rats, compared to group N rats (Figs. 4D and 4E).
21 But treatment with CHST15 siRNA significantly reduced the myocardial mRNA expressions
22 of MMP-2 and -9 compared to group V rats (Figs. 4D and 4E).

23 3.8. Effects of CHST15 siRNA on CHST15 expressions

24 RT-PCR analysis showed that CHST15 mRNA expression levels were significantly increased
25 in vehicle-treated rats, compared to group N rats (Fig. 5). In contrast, treatment with CHST15

1 siRNA significantly decreased the myocardial mRNA expressions of CHST15 compared to
2 group V rats (Fig. 5).

3 *3.9. Effect of CHST15 siRNA on inflammatory mediators protein expression levels*

4 Western blotting analysis has confirmed the results obtained from RTPCR studies as the
5 myocardial protein expression levels of inflammatory cytokines and mediators such as IFN γ ,
6 IL-6 and MCP-1 were significantly suppressed by CHST15 siRNA treatment (Fig. 5 B-D). In
7 addition CHST15 siRNA treatment also significantly attenuated the activation of NF κ B and
8 prevented the loss of I κ B (Fig. 6 A and B).

9 *3.10. Effect of CHST15 siRNA on cardiac remodeling marker proteins*

10 Western blotting protein expression analysis showed that the cardiac remodeling marker
11 proteins such as CTGF and TGF- β were significantly suppressed in DCM rats treated with
12 CHST15 siRNA (Fig. 6 C and D) in addition to the reduced expression of TAK-1, an
13 upstream MAPK signaling protein (Fig. 6E).

14

1 **4. Discussion**

2 Cardiac myosin-induced autoimmune myocarditis, which was exclusively not related to viral
3 infection, was demonstrated to progress into the clinicopathological state similar to DCM in
4 the chronic phase, and was characterized by enlargement of the heart, dilatation of both
5 ventricles, diffuse and extensive myocardial fibrosis, and hypertrophic and atrophic changes
6 of myocardial fibers, resembled human DCM.

7 CHST15 is a key sulfotransferase functioning in the biosynthesis of CS-E, a highly sulfated
8 form of CS. CHST15 was originally purified from squid cartilage [10], and cDNA encoding
9 human CHST15 has been cloned [11]. Although CS-E plays important roles in various
10 biological events [18], the expression profile of CHST15 in CHF has not been reported. Here
11 for the first time, we investigate the effects of CHST15 siRNA on cardiac function,
12 proinflammatory cytokines and cardiac remodeling in EAM rats. Results of our study reveal
13 that CHST15 siRNA treatment improved both systolic (+dP/dt, EF and FS) and diastolic
14 (−dP/dt and LVEDP) functions, and as well as ameliorated myocardial remodeling
15 (hypertrophy & fibrosis) and its marker molecules.

16 Myocardial fibrosis probably plays an important role in both diastolic and systolic
17 dysfunction [19] and has adverse clinical consequences that results in increased mortality
18 caused by progressive heart failure. It has been proposed that increase in myocardial fibrosis
19 during heart failure is due to both increased collagen synthesis by fibroblasts and unchanged
20 or decreased fibrillar collagen degradation [20]. In addition, it has been reported that TGF-β1
21 enhances the synthesis of proteoglycans such as decorin and biglycan by cultured fibroblasts
22 from the LV myocardium, which leads to intensive matrix deposition [21]. Decorin, a small
23 chondroitin/dermatan sulfate (CS/DS) proteoglycan, is one of the best characterized
24 proteoglycans and reported to be elevated in the myocardium of failing heart [22, 23, 24].
25 Previous studies have shown that decorin and biglycan are able to bind with TGF-β1 [25, 26],

1 which is known to induce the expression of these glycoproteins in cardiac fibroblasts [21] and
2 therefore may act as a negative regulatory element during myocardial fibrosis. Myocardial
3 fibrosis, the hallmark of DCM, is observed in DCM hearts as indicated by Azan–Mallory
4 staining and increased marker molecules (TGF- β 1, collagen-I and collagen-III) (Figs. 2 and
5 3A-C). We have also observed the increased expressions of TGF- β 1 and collagen-III mRNA
6 in group V, and these increased mRNA was suppressed by CHST15 siRNA treatment (Figs.
7 3A-C). In addition the protein expression levels of cardiac remodeling markers TGF- β 1 and
8 CTGF were suppressed along with the reduced expression of TAK-1, an upstream MAPK
9 signaling protein, by CHST15 siRNA treatment. Taken together, the above reports suggest
10 that CS-E has an important role in cardiac remodeling and that CHST15 siRNA may
11 contribute to the inhibition of cardiac remodeling. However, more evidence is needed to
12 support this concept.

13 The resulting imbalance in homeostasis of the ECM is believed to result in the diastolic
14 stiffness of the failing heart. This fibrotic remodeling is characterized by an excessive
15 deposition of newly synthesized ECM molecules [27]. During this process proteoglycans,
16 especially decorin and biglycan, are the prime molecules inducing the subsequent
17 fibrillogenesis of the collagens, which form up to 85% of the ECM of the heart and have a
18 supportive function as a scaffold [28, 29, 30]. Consequently, the elevation of the
19 proteoglycan biosynthesis requires increased amounts of the glycosyltransferases involved in
20 the biosynthesis of these glycoproteins. Proteoglycans with their highly diverse GAG chain
21 glycosylation are a major part of the ECM and exhibit multiple functions, *e.g.* modulation of
22 growth factor activities, regulation of collagen fibrillogenesis, or tensile strength [31, 32, 33].
23 Interestingly, we could observe an increase in myocardial mRNA levels of MMP-2 and -9 in
24 rats with DCM (Fig. 4D and 4E). CHST15 siRNA treatment significantly decreased

1 myocardial mRNA levels of MMP-2 and -9, results in improved myocardial function which
2 in turn supports the notion that CS-E is involved in the ECM remodeling.

3 Plasma ANP concentration is a useful prognostic indicator in patients with CHF, and is
4 documented to be elevated in cardiac hypertrophy or failure [34]. It has reported that ANP
5 and decorin genes were highly expressed in DCM and hypertrophic cardiomyopathy [35, 36].
6 Authors reported that imbalance in sulfation of CS molecules and subsequent accumulation
7 of chondroitin-6-sulfate in cardiomyocytes contribute to the development of the hypertrophic
8 cardiomyopathy of costello syndrome [37]. Vehicle-treated rats had developed cardiac
9 hypertrophy and LV dilatation, evidenced by an increase in mRNA levels of ANP (Fig. 3D),
10 myocyte size, HW, HW/BW, LV diastolic and systolic dimensions, and decrease in FS (Fig.
11 2 and Table 1). A significant reduction in those parameters and increased FS were seen in the
12 CHST15 siRNA treated rats. Taken together, all these results indicate that CHST15 siRNA
13 improved LV function and ameliorated the progression of cardiac remodeling.

14 Mast cells are essential elements in allergic inflammation. Mediators produced by mast cells
15 are packaged within secretory granules and, on activation, are released into the extracellular
16 environment within minutes. Principal granule constituents include histamine, serine
17 proteases, carboxypeptidase A and GAGs (CS and heparin). Histamine in granules is found in
18 ionic association with acidic residues of the side chains of CS and heparin, and dissociates in
19 extracellular fluids by exchanging with sodium ions [38]. It has been reported that close
20 association between histamine and CS-E proteoglycan release was demonstrated in human
21 colonic mucosa [39]. We have reported that mast cell stabilization with disodium
22 cromoglycate and IL-10 gene therapy attenuated the myocardial remodeling after EAM [40,
23 41]. A significant reduction in MCD was seen in the CHST15 siRNA treated rats compared
24 to vehicle-treated rats (Figure 2D). Taken together, the above result suggests that CHST15
25 siRNA has a significant anti-inflammatory effect.

1 Several clinical studies have described the participation of proinflammatory cytokines in the
2 pathogenesis of cardiac diseases. The levels of circulating proinflammatory cytokines such as
3 tumor necrosis factor (TNF)- α and IL-1 and IL-6 are elevated in patients with myocarditis
4 [42]. The oversulfated CS/DS chains containing GlcA β 1/IdoA α 1–3GalNAc (4,6-*O*-disulfate)
5 bind to proinflammatory molecules, such as selectins and chemokines, and regulate
6 chemokine activity, further indicate the versatility of these oversulfated chains in regulating
7 biological responses [43]. Interestingly, we could observe an increase in myocardial mRNA
8 levels of IL-6, IL-1 β and MCP-1 in rats with DCM and these changes in mRNA levels were
9 significantly decreased by CHST15 siRNA treatment (Fig. 4A-C). These results were also
10 confirmed by the protein expression analysis using Western blotting for IFN- γ , IL-6 and
11 MCP-1. Further, CHST15 siRNA treatment might be a promising strategy for new therapy
12 for chronic myocarditis, particularly for LV remodeling where ongoing autoimmune
13 processes may play a role in the disease development. Moreover, we demonstrate for the first
14 time that CHST15 siRNA suppressed cytotoxic myocardial damage in heart failure after
15 EAM.

16 This the first report demonstrating the effect of siRNA-based CHST15-blockade in cardiac
17 remodeling *in vivo*. In conclusion, we developed a novel cardiac-directed approach for the
18 treatment of myosin induced DCM, and to mediate therapeutic RNA interference in the heart.
19 The first data from a rat cardiomyopathy model suggest that treatment with the synthetic
20 siRNA (CHST15 siRNA) results in functional protection of the heart against myosin induced
21 CHF.

22

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3

1 **Figure legends**

2 **Figure 1.** The effects of CHST15 siRNA on survival rate in rats with CHF induced by
3 autoimmune myocarditis. Four (50%) and three (30 %) of 8, 10 rats in groups V and CHST15
4 siRNA, respectively, died between days 28 and 56. None of the rats (n=4) died in group N.
5 The survival rates were higher in group CHST15 siRNA than in group V, but there was no
6 significant (ns) difference observed between the survival curves (Log-rank (Mantel-Cox)
7 test). Group N, age-matched untreated rats; group V, CHF rats administered with vehicle;
8 group CHST15 siRNA, CHF rats treated with CHST15 siRNA (2 µg/week).

9 **Figure 2.** Effects of CHST15 siRNA on myocardial remodeling in rats with CHF induced by
10 autoimmune myocarditis. [A] Azan–Mallory staining for fibrosis of the cross-sectional tissue
11 slices of hearts. Fibrosis is indicated by blue area as opposed to the red myocardium (×200).
12 [B] Hematoxylin and eosin staining of the cross-sectional tissue slices of hearts depicting
13 cardiomyocyte hypertrophy (×400). [C] Extensive inflammatory cell infiltration was
14 observed in the hearts of myosin-immunized rats (hematoxylin-eosin staining; ×100). [D]
15 Effects of CHST15 siRNA on mast cell density (MCD). Toluidine blue–positive mast cells
16 are higher in group V than in group N. Treatment with CHST15 siRNA significantly reduced
17 the cardiac MCD. Toluidine blue–positive (bluish or violet) mast cells are shown by black
18 arrows (x100). Bar graph shows quantitative analysis of fibrosis [A1], myocyte diameter [B1]
19 and MCD [D1] in groups N, V and CHST15 siRNA. Group N, age-matched untreated rats;
20 group V, CHF rats administered with vehicle; group CHST15 siRNA, CHF rats treated with
21 CHST15 siRNA (2 µg/week). The values are mean ± SE. * $P<0.05$ and *** $P<0.001$ vs group
22 N; ## $P<0.01$ and ### $P<0.001$ vs group V.

23 **Figure 3.** Myocardial messenger RNA expression levels of collagen-I [A], collagen-III [B],
24 TGF-β1 [C] and ANP [D] in rats with CHF were determined by quantitative RT-PCR. The
25 expression level of each sample was expressed relative to the expression level of GAPDH

1 gene. Data are mean $\pm$ SEM of 3 to 4 rats. Group N, age-matched untreated rats; group V,
2 CHF rats administered with vehicle; group CHST15 siRNA, CHF rats treated with CHST15
3 siRNA (2 μ g/week). The values are mean $\pm$ SE. ** P <0.01 and *** P <0.001 vs group N;
4 # P <0.05 and ## P <0.01 vs group V.

5 **Figure 4.** Myocardial messenger RNA expression levels of [A] MCP-1, [B] IL-1 β , [C] IL-6,
6 [D] MMP-2 and [E] MMP-9 in rats with CHF were determined by quantitative RT-PCR. The
7 expression level of each sample was expressed relative to the expression level of GAPDH
8 gene. Data are mean $\pm$ SEM of 3 to 4 rats. Group N, age-matched untreated rats; group V,
9 CHF rats administered with vehicle; group CHST15 siRNA, CHF rats treated with CHST15
10 siRNA (2 μ g/week). The values are mean $\pm$ SE. * P <0.05, ** P <0.01 and *** P <0.001 vs group
11 N; # P <0.05, ## P <0.01 and ### P <0.001 vs group V.

12 **Figure 5.** A, Myocardial messenger RNA expression level of CHST15 in rats with CHF were
13 determined by quantitative RT-PCR. The CHST15 expression level was expressed relative to
14 the expression level of GAPDH gene. B-D, Western blotting analysis for determining the
15 myocardial protein levels of interferon (IFN)- γ , interleukin (IL)-6 and monocyte chemo
16 attractant protein (MCP)-1. Graphs depict the mean density values of each protein expressed
17 as ratios relative to their glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content. Data
18 are mean $\pm$ SEM of 3 to 4 rats. Group N, age-matched untreated rats; group V, CHF rats
19 administered with vehicle; group CHST15 siRNA, CHF rats treated with CHST15 siRNA (2
20 μ g/week) respectively. The values are mean $\pm$ SE. * p <0.05 and *** p <0.001 vs group N,
21 # P <0.05 and ## p <0.01 vs group V.

22 **Figure 6.** Western blotting analysis for determining the myocardial protein levels of A,
23 phospho-nuclear factor kappa B (p-NF κ B) and total NF κ B; B, NF κ B inhibitor (I κ B- α); C,
24 connective tissue growth factor (CTGF); D, transforming growth factor (TGF)- β ; TGF- β
25 activated kinase (TAK)-1. Graphs depict the mean density values of phospho proteins

1 expressed as ratios relative to their unphosphorylated forms and others relative to their
2 GAPDH content. Data are mean $\pm$ SEM of 3 to 4 rats. Group N, age-matched untreated rats;
3 group V, CHF rats administered with vehicle; group CHST15 siRNA, CHF rats treated with
4 CHST15 siRNA (2 μ g/week) respectively. The values are mean $\pm$ SE. * p <0.05, ** p <0.01 and
5 *** p <0.001 vs group N, [#] P <0.05 and ^{##} p <0.01 vs group V.

6

7

Table 1. Changes in hemodynamic, echocardiographic and histopathological parameters after 7 weeks of treatment with CHST15 siRNA in rats with CHF.

	Group N (n=4)	Group V (n=8)	Group CHST15 siRNA (n=10)
Number of rats died	0	4	3
Survival rate (%)	100	50	70
BW (g)	361±7.5	295±5.6 ^{**}	294±6.2 ^{**}
HW (g)	0.91±0.02	1.30±0.07 ^{**}	1.08±0.03 [#]
H/B (g/kg)	2.35±0.06	4.49±0.18 ^{**}	3.8±0.13 ^{**#}
Area of fibrosis (%)	2.25±0.2	30±2.04 ^{**}	15±2.0 ^{***}
CVP (mmHg)	0.09±0.6	4.7±1.2 ^{**}	1.94±0.22 ^{***}
Peak LVP (mmHg)	114.66±5.2	66.7±7.2 ^{**}	77.8±2.68 ^{**}
LVEDP (mmHg)	15.02±1.5	33.00±3.2 [*]	21.52±2.2 [*]
+ dP/dt (mmHg/s)	6393±562	2267±201 ^{**}	3215±233 ^{**}
– dP/dt (mmHg/s)	5954±463	1825±193 ^{**}	2607±160 ^{**}
HR (beats/min)	336±18	279±13	325±8.4
LVDd (mm)	7.1±0.08	8.7±0.4 ^{**}	7.9±0.28 ^{*,#}
LVDs (mm)	3.9±0.19	7.9±0.5 ^{**}	6.1±0.1 ^{***}
FS (%)	45.7±2.6	9.5±2.6 ^{**}	24.5±1.6 ^{***}
EF (%)	81.8±2.3	18±3.98 ^{**}	57.7±2.8 ^{***}

Results are presented as the mean ± S.E. n, no. of rats. BW, body weight; HW, heart weight; HW/BW, ratio of heart weight to body weight; CVP, central venous pressure; MBP, mean blood pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; ± dP/dt, rate of intra-ventricular pressure rise and decline; HR, heart rate; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction; group N, aged matched untreated rats; group V, rats with heart failure treated with vehicle; group CHST15 siRNA rats with heart failure treated CHST15 siRNA (2 µg/week), respectively; ^{*}*P* < 0.05 and ^{**}*P* < 0.01 vs. group N; [#]*P* < 0.05 and ^{##}*P* < 0.01 vs. group V.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6