Genetic and Pharmacological Inhibition of Galectin-3 Prevents Cardiac Remodeling by Interfering With Myocardial Fibrogenesis

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Background—Galectin-3 has been implicated in the development of organ fibrosis. It is unknown whether it is a relevant therapeutic target in cardiac remodeling and heart failure.

Methods and Results—Galectin-3 knock-out and wild-type mice were subjected to angiotensin II infusion (2.5 µg/kg for 14 days) or transverse aortic constriction for 28 days to provoke cardiac remodeling. The efficacy of the galectin-3 inhibitor N-acetyllactosamine was evaluated in TGR(mREN2)27 (REN2) rats and in wild-type mice with the aim of reversing established cardiac remodeling after transverse aortic constriction. In wild-type mice, angiotensin II and transverse aortic constriction perturbations caused left-ventricular (LV) hypertrophy, decreased fractional shortening, and increased LV end-diastolic pressure and fibrosis (P<0.05 versus control wild type). Galectin-3 knock-out mice also developed LV hypertrophy but without LV dysfunction and fibrosis (P=NS). In REN2 rats, pharmacological inhibition of galectin-3 attenuated LV dysfunction and fibrosis. To elucidate the beneficial effects of galectin-3 inhibition on myocardial fibrogenesis, cultured fibroblasts were treated with galectin-3 in the absence or presence of galectin-3 inhibitor. Inhibition of galectin-3 was associated with a downregulation in collagen production (collagen I and III), collagen processing, cleavage, cross-linking, and deposition. Similar results were observed in REN2 rats. Inhibition of galectin-3 also attenuated the progression of cardiac remodeling in a long-term transverse aortic constriction mouse model.

Conclusions— Genetic disruption and pharmacological inhibition of galectin-3 attenuates cardiac fibrosis, LV dysfunction, and subsequent heart failure development. Drugs binding to galectin-3 may be potential therapeutic candidates for the prevention or reversal of heart failure with extensive fibrosis. (Circ Heart Fail. 2013;6:107-117.)

Key Words: cardiac remodeling • fibrosis • galectin-3 • heart failure • renin-angiotensin system

Galectin-3 belongs to the galectin family of mammalian lectins and is characterized by a carbohydrate recognition domain (CRD) that has affinity for β-galactosides. Galectin-3 mediates cell–cell and cell–matrix interactions by binding to lactosamine-containing cell surface glycoconjugates via its CRD. There is mounting evidence demonstrating its key role in inflammatory and fibrotic processes.¹

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In experimental models, galectin-3 was found to be involved in the development of liver and kidney fibrosis.² ³ It has also been associated with cardiac fibrosis in TGR(mREN2)27 (REN2) rats with experimental heart failure (HF).⁴ A continuous intrapericardial infusion of low-dose galectin-3 resulted in cardiac fibrosis and left-ventricular (LV) dysfunction in both failure-prone hypertrophic REN2 and healthy Sprague-Dawley (SD) rats.⁵ ⁶ The upregulation of galectin-3 has also been demonstrated in failing human hearts,⁴ and circulating levels are a powerful predictor of outcome in both acute and chronic HF. This observation has led to the approval of galectin-3 by the Food and Drug Administration as a novel biomarker in HF that may help categorize patients in the remodeling and nonremodeling stages of HF.⁵ ⁷

Despite evidence suggesting the involvement of galectin-3 in the pathophysiology of HF, it remains unknown whether it contributes actively to the disease. In the present study, we

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determined whether the disruption of the galectin-3 gene, shown to attenuate hepatic and renal fibrosis in galectin-3 knock-out (Gal3-KO) mice, would prevent the development of HF in animals with active cardiac remodeling. Furthermore, because the beneficial effects of targeted CRD inhibition of galectin-3 have been demonstrated in a model of kidney injury, we also determined the effects of a galectin-3 inhibitor (Gal3i), N-acetyllactosamine (N-Lac), which has a high affinity for galectin-3 CRD, in TGR(mREN2)27 (REN2) rats with HF and in mice subjected to transverse aortic constriction (TAC).

Methods
For detailed description of the methods, please refer to the online-only Data Supplement.

Animals
Male Gal3-KO mice were generated and bred at the Jackson Laboratory (Bar Harbor, ME) as described previously. Age-matched male transgene–negative wild-type (WT) littermates were used as controls. Figure IA in the online-only Data Supplement depicts the generation of Gal3-KO mice. Male homozygous REN2 (Max Delbrück Center for Molecular Medicine, Berlin, Germany) and age-matched male SD rats (controls) were used as described previously. All experiments were approved by the Animal Ethical Committee of the University of Groningen (the Netherlands) and conducted in accordance with existing guidelines on the care and use of laboratory animals.

Mouse Experiments
Six- to 10-week-old Gal3-KO and WT mice were subjected to an infusion of angiotensin II (AngII) (2.5 µg/kg per day) for 14 days or LV pressure overload by TAC for 28 days (prevention experiment). In another series of experiments (reversal experiment), 6- to 8-week-old male C56Bl/6J mice (Harlan, the Netherlands) underwent TAC for 28 days and were then treated with N-Lac (5 mg/kg per day, IP injections) 3× a week for 28 days (starting day 28 until day 56).

Rat Experiments
SD and REN2 rats were treated with N-Lac (5 mg/kg per day, IP) 3× a week for 6 weeks.

Other Experiments
Cardiac function was studied with echocardiography and hemodynamic measurement as described previously. Immunohistochemical analyses were performed, and collagen digestibility was determined. Cell cultures of human adult dermal fibroblasts were used to study galectin-3–mediated fibrogenesis and the effects of N-Lac. Cardiac and fibroblast gene expression was measured using reverse transcription-quantitative polymerase chain reaction as described previously. The lists of primers are presented in Tables I, II and III in the online-only Data Supplement.

Statistical Analyses
Results are reported as means±SEM. Mice were analyzed in 2 separate subgroups comparing genotype differences (WT versus KO) and model differences (control versus AngII or sham versus TAC). Subgroup A consisted of WT-control, Gal3-KO-control, WT-AngII, Gal3-KO-AngII, and subgroup B consisted of WT-sham, Gal3-KO-sham, WT-TAC, and Gal3-KO-TAC. Levene test was used to test homogeneity of variances within parameters. If there was equality of variances, statistical analyses were performed by 1-way ANOVA with Bonferroni post hoc tests (mice group A, M=6 tests; mice group B, M=6 tests; rats M=3 tests). If there was inequality of variances, statistical analyses were performed by Welch ANOVA with Games-Howell post hoc test. Cell experiments were analyzed with Kruskall–Wallis test (N=3 per group). For the TAC reversal experiment, differences between saline-treated mice and N-Lac treated mice were analyzed at the 8-week time point using an unpaired t test (N=7–9). Baseline TAC reversal is depicted as a dotted line. In all figures, only relevant comparisons are shown by the symbols for reasons of clarity. All P values are 2-tailed, and a value <0.05 was considered significant. All analyses were performed using SPSS version 20.0 software (SPSS, Chicago, IL).

Results
Galectin-3 Knock-Out Mice Are Protected Against LV Dysfunction
The intervention and treatment schemes of the WT and Gal3-KO mice are presented in Figure 1A, and the baseline characteristics and hemodynamic data at euthanasia are presented in Table 1. When compared with control or sham, LV galectin-3 expression was increased almost 2-fold in WT mice treated with AngII or TAC, whereas galectin-3 expression was absent in Gal3-KO mice (mRNA and protein, Figure IB–ID in the online-only Data Supplement). In the WT mice, both interventions caused LV hypertrophy as evidenced by the increases in LV weight (normalized to tibia length, Table 1), wall thicknesses (Table 1), and LV atrial natriuretic peptide expression (Figure 1B) along with a decrease in contractile function (fractional shortening, Figure 1C). Hemodynamic measurements revealed LV relaxation impairment in the WT groups (Figure 1D–1F) with increases in LV end-diastolic pressure (LVEDP) and Tau (an isovolumetric relaxation constant measured according to the Glantz method) along with decreases in dP/dtmin (corrected for peak systolic pressure).

As shown in Table 1, both Gal3-KO and WT mice subjected to AngII infusion or TAC had a similar degree of LV hypertrophy. However, and irrespective of the perturbation, Gal3-KO mice had preserved fractional shortening (Figure 1C). Hemodynamic measurements revealed that Gal3-KO mice were protected against LV relaxation impairment after AngII infusion or TAC (Figure 1D–1F), which did not result in changes in LVEDP and Tau (P=NS versus respective controls). The only exception was the corrected dP/dtmin, which was significantly decreased in the Gal3–KO–AngII group.

Inhibition of Galectin-3 With N-Lac Prevents LV Dysfunction in Failure-Prone REN2 Rats
The intervention and treatment schemes of the SD and REN2 rats are presented in Figure 1A. Table 2 shows the baseline characteristics and hemodynamic data at euthanasia. As expected, LV weight (adjusted for tibia length) was significantly increased in the untreated REN2 rats. Treatment with N-Lac did not prevent the development of LV hypertrophy or decrease LV atrial natriuretic peptide levels (Table 2, Figure 1G). Fractional shortening progressively declined in the untreated REN2 rats (Figure 1I), but was preserved in the Gal3i-treated rats (Figure 1H). Hemodynamic measurements revealed an increased LVEDP in the untreated REN2 rats as compared with the SD rats (Figure 1J) as well as Tau (Figure 1K), associated with increased lung weights (Table 2), all suggestive of developing HF. Treatment with Gal3i reduced LVEDP in REN2, and lung weight, but not Tau. Finally, accelerated cardiac remodeling in untreated REN2 rats was associated with poorer survival than Gal3i-treated REN2 rats (Figure 2 in the online-only Data Supplement).
Galectin-3 Disruption or Inhibition Attenuates the Formation of Fibrosis in the Heart

To determine whether galectin-3 is actively involved in the formation of fibrosis, we analyzed the presence of myocardial fibrosis. Figure 2A and 2B show representative pictures of fibrotic tissue and the fibrosis score in mouse hearts. The hearts of control and sham-operated WT mice had very little fibrosis (≈2%). A significantly higher percentage of fibrosis was evident in the WT-AngII and WT-TAC mice (Figure 2B). However, neither AngII infusion nor TAC resulted in increased fibrosis in Gal3-KO animals. Similar results were also observed in rat hearts. Compared with SD rats, REN2 rats exhibited a high percentage of fibrotic tissue, and treatment with Gal3i significantly reduced the percentage of fibrosis in the REN2 rats (Figure 2C and 2D).

Figure 1. Hemodynamic data in mice and rats at euthanasia. A, Outline of the experimental protocol of the prevention studies in mice and rats. B, Expression of atrial natriuretic peptide (ANP) mRNA in mouse hearts. C, Fractional shortening in mouse hearts assessed with echocardiography. D, Left-ventricular end-diastolic pressure (LVEDP) in mouse hearts. E, Isovolumetric relaxation constant Tau in mouse hearts. F, dP/dtmin corrected for peak systolic pressure in mouse hearts. G, Expression of ANP mRNA in rat hearts. H, Fractional shortening in rat hearts assessed with echocardiography. I, Change in fractional shortening in rat hearts assessed with echocardiography at baseline and before euthanasia. J, Left-ventricular end-diastolic pressure (LVEDP) in rat hearts. K, Tau in rat hearts. *P<0.05 vs wild type (WT)-con, †P<0.05 vs Gal3-KO-con, ‡P<0.05 vs WT-AngII, §P<0.05 vs WT-TAC, ††P<0.05 vs WT-sham, ‡‡P<0.05 vs Gal3-KO-sham, §§P<0.05 vs WT-TAC, †††P<0.05 vs Sprague-Dawley (SD)-con vs REN2-con, †††P<0.05 REN2-con versus REN2-Gal3i, §§§P<0.05 SD-con vs REN2-Gal3i, §§§P<0.05 vs all other groups at euthanasia. AngII indicates angiotensin II; con, control; Gal3i, galectin-3 inhibitor (N-Lac, N-acetyllactosamine); Gal3-KO, galectin-3 knock-out; TAC, transverse aortic constriction; and Wk, week (N=5–12 per group).
Table 1. Baseline Characteristics and Hemodynamic Data at Euthanasia of Mice

<table>
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<tr>
<th></th>
<th>WT-con (N=10)</th>
<th>Gal3-KO-con (N=10)</th>
<th>WT-AngII (N=12)</th>
<th>Gal3-KO-AngII (N=10)</th>
<th>WT-sham (N=7)</th>
<th>Gal3-KO-sham (N=11)</th>
<th>WT-TAC (N=12)</th>
<th>Gal3-KO-TAC (N=14)</th>
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<td>BW, g</td>
<td>28.5±0.4</td>
<td>28.5±0.3</td>
<td>24.6±0.7*</td>
<td>24.3±0.4†</td>
<td>27.8±0.7</td>
<td>27.1±1.3</td>
<td>27.9±0.7</td>
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<td>LV weight</td>
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<td>6.60±0.2</td>
<td>7.75±0.5*</td>
<td>8.28±0.4†</td>
<td>6.27±0.2</td>
<td>7.39±0.3</td>
<td>9.85±0.8§</td>
<td>8.55±0.8</td>
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<td>MAP, mm Hg</td>
<td>71±2</td>
<td>67±2</td>
<td>97±1*</td>
<td>97±2†</td>
<td>75±2</td>
<td>77±2</td>
<td>82±6</td>
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<td>IVSd, mm</td>
<td>0.64±0.01</td>
<td>0.65±0.01</td>
<td>0.88±0.03*</td>
<td>0.85±0.02†</td>
<td>0.69±0.03</td>
<td>0.69±0.02</td>
<td>0.99±0.04§</td>
<td>0.96±0.02§</td>
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<td>LVEDD, mm</td>
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<td>4.18±0.17</td>
<td>4.02±0.11</td>
<td>3.70±0.13</td>
<td>3.73±0.05</td>
<td>3.83±0.10</td>
<td>4.38±1.12§</td>
<td>4.15±0.04</td>
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<td>LVESD, mm</td>
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<td>1.95±0.04</td>
<td>2.21±0.06</td>
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<tr>
<td>LVPWd, mm</td>
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<td>Corrected dPdtmax</td>
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<td>94±7</td>
<td>74±6*</td>
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<td>90±7</td>
<td>60±4§</td>
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<td>−57±2†</td>
<td>−62±4</td>
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AngII indicates angiotensin II; BW, body weight; dPdtmax, index of maximal contraction of the left ventricle and is corrected for peak systolic pressure; Gal3-KO, galectin-3 knock-out; IVSd, thickness of the interventricular septum in diastole; LV, left ventricular; LVEDD, left-ventricular end-diastolic diameter; LVESD, left-ventricular end-systolic diameter; LVPWd, thickness of the left-ventricular posterior wall in diastole; LV weight, corrected for tibia length (mg/mm); MAP, mean arterial pressure; TAC, transverse aortic constriction; and WT, wild type.

We also evaluated collagen digestibility (percentage of collagen released by proteolytic enzymes) as a measure of the extent of collagen cross-linking. In our assay, the higher the numbers of cross-links, the lower the amount of released collagen. Compared with SD rats, collagen digestibility was significantly reduced in the hearts of REN2 rats (Figure 2E). As collagen fibers in fibrotic lesions display a higher level of pyridinoline cross-links, making them more resistant to the enzymatic action of proteinases, our results indicate the presence of more cross-linked collagen in the hearts from SD rats. We also measured the plasma levels of procollagen type I N-terminal propeptide (PINP), a marker of collagen cleavage, with an ELISA assay. In REN2 rats, PINP was significantly increased compared with SD rats, and inhibition of galectin-3 resulted in a significant reduction of PINP concentration (Figure 2F). Collectively, these results suggest that Gal3i can reduce pathological fibrosis through a reduction of collagen deposition and synthesis along with increased collagen digestibility.

Galecitin-3 Inhibition Reduces the Number of α-Smooth Muscle Actin Positive Cells in the Heart

Figure 3 shows typical examples of Gomori, α-smooth muscle actin (α-SMA), CD68 (a macrophage marker), and proliferating cell nuclear antigen (PCNA) staining in rat hearts with their respective quantification. Cardiomyocyte size and α-SMA expression were significantly increased in REN2 rats. Treatment with Gal3i did not decrease cardiomyocyte size (Figure 3A and 3B) but it normalized α-SMA expression, indicating a lower number of myofibroblasts in these groups (Figure 3A and 3C). The number of macrophages was also increased in both REN2 groups (Figure 3A and 3D), as was the number of proliferating cells, although this was only significant in the untreated REN2 group (Figure 3A and 3E). Immunohistochemical analysis revealed that galectin-3 immunoreactivity was predominantly observed in the interstitial space, but not in cardiomyocytes (Figure III in the online-only Data Supplement). In an effort to determine the localization of cardiac galectin-3 and its source, we conducted further studies. We observed that Gal3i colocalized with macrophages (Figure IVA–IVD in the online-only Data Supplement) and at sites of collagen deposition (Figure IVE–IVH in the online-only Data Supplement). Furthermore, galectin-3 protein expression was the highest in macrophages. Galectin-3 protein expression was also clearly detectable in fibroblast, but was not detectable in cardiomyocytes (Figure V in the online-only Data Supplement).

Galecitin-3 Inhibition Prevents Galectin-3–Mediated Effects in Human Dermal Fibroblasts

Stimulation of human dermal fibroblasts with recombinant galectin-3, with or without Gal3i, did not lead to visible differences in fibroblast appearance (Figure 4A). Fibroblast...
phenotype was confirmed by staining for the mesenchymal marker vimentin (Figure 4B). Because our preliminary results demonstrated that the mRNA level of Collagen 1A1 (COL1A1) in the presence of galectin-3 peaked 72 hours post-stimulation (data not shown), the expression of various fibrillar collagens and proteins involved in their processing was measured at the 72 hours time point with a low density array (overview in Table IV in the online-only Data Supplement; results are shown in Figure 4). The expression of COL1A1, COL1A2, COL3A1, and the collagen-modifying proteins encoded by prolyl-4-hydroxylase, heat shock protein (HSP) 47, procollagen C-endopeptidase enhancer, and lysyl oxidase-like 2 (LOXL2) were all upregulated by galectin-3 (Figure 4D–4J) and inhibited by Gal3i cotreatment. The modulation of COL3A1 transcript expression by galectin-3 and Gal3i was also reflected in collagen type III levels in the culture medium measured with an ELISA (Figure 4K).

Genes Involved in Myocardial Fibrogenesis

The expression of the abovementioned collagens and fibrosis-associated proteins was subsequently analyzed in the LV tissue of SD and REN2 rats by using reverse transcription-quantitative polymerase chain reaction (Figure 5A–5H; primers are listed in Table II in the online-only Data Supplement). These results confirm our in vitro observations with human fibroblasts and show that Gal3i reduces the expression of pro-fibrotic genes also in vivo. We also measured the LV protein expression of galectin-3 and Hsp47 in lysates from SD and REN2 rat hearts (Figure 5I–5K). Galectin-3 and Hsp47 protein levels were increased in untreated REN2 rats. Treatment with the Gal3i resulted in decreased Hsp47 (Figure 5K) but not galectin-3 (Figure 5J) protein levels, which reflects the changes in mRNA expression (Figure 5A and 5B). Finally, we analyzed the transcript levels of matrix metalloproteinases 2 and 9, and tissue inhibitors of matrix proteases 1 and 2, in LV tissue of SD and REN2 rats. Matrix metalloproteinase-9, tissue inhibitors of matrix protease-1, and tissue inhibitors of matrix protease-2 levels were increased in untreated REN2 rats and reduced by treatment with a Gal3i (Figure VI online-only Data Supplement).

Galectin-3 Inhibition Prevents Further Progression of Established LV Remodeling

We provoked LV remodeling with TAC surgery followed by an observational period of 28 days without intervention (Figure 6A). We then treated the mice with N-Lac or saline for another 28 days. At the end of the follow-up period, no increase in LV weight (Figure 6B) was observed. Treatment with Gal3i for 28 days had no effect on LV weight, compared with the LV weights after the 28-day observational period (Figure 6B). However, a gradual progression of LV remodeling was observed in the untreated mice as evidenced by the further decline in fractional shortening (Figure 6C) and by an increase in atrial natriuretic peptide levels (Figure 6E) and fibrosis (Figure 6F and 6G). In the Gal3i-treated mice, LVEDP was lower compared with untreated mice (Figure 6D).

Discussion

The current study provides several lines of evidence that galectin-3 is an active contributor in the development of cardiac remodeling, myocardial fibrogenesis, and HF. We have demonstrated that inhibition of galectin-3 function by genetic disruption or pharmacological intervention halts the progression of cardiac remodeling, attenuates myocardial fibrogenesis, and preserves LV function. These beneficial effects can be explained, at least in part, by the lower number of...
myofibroblasts in combination with diminished collagen synthesis, processing, and cross-linking. Collectively, our results suggest that galectin-3 may be an attractive target for the prevention and treatment of HF.

Disruption of Galectin-3 Attenuates Cardiac Remodeling and Preserves Cardiac Function

To explore the hypothesis that galectin-3-targeted interventions may protect against progressive cardiac remodeling and dysfunction, 2 experimental approaches were used in well-established mouse and rat models of cardiac remodeling: (1) complete genetic disruption of galectin-3 and (2) pharmacological inhibition with an agent that specifically binds to the CRD of galectin-3. Both the genetic disruption and pharmacological inhibition of galectin-3 resulted in considerable attenuation of cardiac remodeling and, specifically, to an almost complete inhibition of cardiac fibrosis. Functionally, the inhibition of galectin-3 improved diastolic dysfunction to a large extent (less increase in end-diastolic LV pressure and improved LV relaxation) despite the presence of significant LV hypertrophy. In this respect, it is noteworthy that elevated levels of circulating galectin-3 have been shown to be strong predictors of poor outcome in patients with diastolic HF or HF with preserved LV ejection fraction.

Although the animals in our experimental models were mainly characterized by impaired diastolic function, mild (AngII, TAC-prevention) to moderate (REN2, TAC reversal) systolic dysfunction also developed over time. Inhibition of galectin-3 preserved systolic function, a finding that was above all apparent in the reversal experiment where treatment was started after 4 weeks of TAC. In the untreated mice, mild systolic dysfunction was present after 4 weeks (Figure 6C) and progressed for another 4 weeks of follow-up to overt systolic dysfunction (Figure 6C). However, when mice were treated with the galectin-3 inhibitor, progression of systolic dysfunction was attenuated. These results suggest that galectin-3 inhibition might afford functional protection against developing and progressive cardiac remodeling.

Additional evidence of the important role of galectin-3 in cardiac remodeling was obtained by treating REN2 rats with N-Lac, an established inhibitor that binds to the galectin-3 CRD. The homozygous REN2 rat model is a well-described model of rapidly progressive cardiac remodeling driven by renin overexpression with changes typical for HF, such as increased sympathetic tone, LV hypertrophy, myocardial fibrosis, and stress-related pathways. Results of our study show that the typical course of HF development, characterized by impaired LV relaxation and fast progression (within weeks) to overt HF, was attenuated by galectin-3 inhibition. It remains to be determined whether galectin-3 inhibition is equally effective in other multifactorial models of HF, such as the spontaneous hypertensive rat or postmyocardial infarction HF, as a single treatment or in addition to established HF therapy.

Mechanisms Underlying the Cardioprotective Effects of Galectin-3 Inhibition

Fibrosis is accepted as one of the main determinants of cardiac remodeling. Cessation of the fibrotic process is one of the key targets to reverse cardiac remodeling and improve prognosis. Fibroblasts, together with myofibroblasts and macrophages, have been identified as key cells in the fibrotic process. The striking observation that myocardial fibrogenesis was strongly inhibited when galectin-3 was genetically disrupted or pharmacologically inhibited leads us to investigate the effect of galectin-3 on the fibrotic process. First, we showed in REN2 rats that pharmacological inhibition of galectin-3 leads to a lower number of myofibroblasts along
with less collagen synthesis (lower PINP plasma levels) and deposition. We substantiated these changes by showing that the stiffness of the fibrotic depositions was also altered. In the collagen digestibility assay, the collagenase digested more collagens in Gal3i-treated REN2 rats (Figure 2E), indicating less cross-linked fibrotic tissue. Studies on fibrogenesis-related gene profiles in fibroblasts treated with recombinant galectin-3 revealed changes in several genes relevant to the extracellular matrix processing. In vitro incubation of human dermal fibroblasts with galectin-3 resulted in significant upregulation of genes coding for various fibrillar collagens (COL1A1, COL1A2, and COL3A1) and genes involved in the modification of (pro)collagens, including prolyl 4-hydroxylase, HSP47 (SERPIN peptidase inhibitor 1 [SERPINH1]), the procollagen C-endopeptidase enhancer, and the lysyl oxidases (LOXL2). Inhibition of P4Hs has been shown to inhibit fibrosis and preserve cardiac function in HF.27–29 Also, the antisense HSP47 was associated with less myocardial fibrosis protecting hearts from postmyocardial infarction remodeling.30 Importantly, treatment with N-Lac downregulated the expression of all these genes to baseline levels. These results suggest that galectin-3 may affect several steps involved in fibrogenesis, from enhanced synthesis of procollagen to the regulation of various enzymes involved in the processing of procollagen into mature intracellular and extracellular collagen. The activation of these pathways is attenuated by galectin-3 inhibition (Figure 7).

As the effect of galectin-3 on fibroblasts provides an explanation as to why in vivo galectin-3 inhibition results in the cessation of the fibrotic process, we validated these findings in REN2 rats. The cardiac expression of Col1a1, Col1a2, Col3a1, P4hb, Hsp47 (Serpinh1), procollagen C-endopeptidase enhancer (PCOLCE), and LOXL2 genes was upregulated, and treatment with N-Lac normalized their expression to levels similar to those of control SD rats. These observations were further substantiated by showing, both in vitro and in vivo, decreased collagen levels in culture medium (fibroblasts) and plasma (REN2 rats), increased collagen digestibility (Figure 2E), regulation of matrix metalloproteinases and tissue inhibitors of matrix proteases (Figure VI in the online-only Data Supplement), and decreased myocardial fibrosis on Gal3i treatment (Figure 2D).

Overall, our results point toward a pivotal role of galectin-3 in cardiac fibrosis. The general effect of galectin-3 on cardiac fibrosis is to reduce collagen synthesis and deposition, improve collagen digestibility, and regulate fibrogenic gene expression, thereby attenuating fibrotic process.
remodeling, attributable to myocardial fibrogenesis, seemed to be similar between mice and rats. Furthermore, the same fibrogenic effects of galectin-3 (and its inhibition by N-Lac) were observed in primary human fibroblasts and in REN2 rat hearts.

Other Observations
LV weight was increased in Gal3-KO mice and in REN2 rat treated with N-Lac. The mechanism(s) underlying this observation has not been elucidated because we were not able to detect appreciable protein expression of galectin-3 in cardiomyocytes in our preliminary experiments. It has also been reported that galectin-3 is mainly produced by macrophages homing to sites of injury. We confirmed the influx of macrophages and the colocalization of galectin-3 with macrophages in our REN2 models (Figures IV and V in the online-only Data Supplement). However, antigalectin-3 treatment did not
reduce the number of macrophages (Figure 3D). It has been suggested that the activation of macrophages is more pivotal than the number of macrophages in the development of cardiac remodeling. Usher et al.31 described mice with a deletion of the mineralocorticoid receptor in macrophages and showed they were protected against cardiac remodeling. Our findings do not exclude a role for macrophages in galectin-3–mediated HF but, from the data presented herein, we conclude that Gal3i exerts its effects primarily via binding to the CRD of galectin-3, which prevents a profibrotic effect of activated galectin-3, and not through an increase or decrease in the number of macrophages.

Clinical Perspectives
Clinical proof for a role of galectin-3 in HF comes from several studies reporting the value of galectin-3 as a biomarker in HF.6,21,32–34 In the general population, it has been recently observed that sustained elevation in galectin-3 levels may contribute to increased cardiovascular risk, all-cause mortality (Prevention of Renal and Vascular Endstage Disease [PREVEND] cohort)35 and new onset HF.36 Our data lend further support to the role of galectin-3 in cardiac remodeling as well as its potential role as a target for therapy. Future studies are being designed to establish the role of galectin-3 inhibitory compounds in HF of different pathogenesis or on top of established HF therapy. Because high galectin-3 levels may predispose for the development of HF, therapies targeted against galectin-3 may afford protection. Interestingly, although speculative, high intake of dietary cereals rich in pectins that inhibit galectin-3 has been associated with lower risk for new onset HF.37

Limitations
We studied only male animals and, given the established differences in cardiac remodeling between sexes, our results cannot be extrapolated to female animals. Furthermore, results on cardiac contractility and relaxation should be interpreted with caution because no load-independent measures of LV function were reported (end-systolic elastance [EES], end-diastolic elastance [EED], and preload recruitable stroke work [PRSW], etc). Because we only tested 1 dose of N-Lac, dose-finding studies are warranted. Finally, not all fibrotic genes responded to the same extent in our different models. This might be attributed to the differences in pathogenesis, model severity, or to differences between mice and rats. Nevertheless, and despite some gene-specific differences, the overall
response to galectin-3 interference (inhibition and knock-out) was similar.

**Conclusions**

Genetic disruption of galectin-3 and pharmacological inhibition of galectin-3 attenuated the progression of cardiac remodeling in murine and rat models of HF. Inhibition of galectin-3 largely preserved systolic and diastolic function via the inhibition of myocardial fibrosis and decreased collagen production, processing, and cross-linking. Future, more in-depth, mechanistic studies would be needed to address the precise role of galectin-3 in HF development. At later stages of remodeling, galectin-3 inhibition prevented further HF progression. Taken together, our results strongly suggest a causal role of galectin-3 in the development of cardiac remodeling and HF, and we postulate that galectin-3–targeted therapy may potentially be a useful addendum in the treatment of HF.

**Acknowledgments**

We thank Danielle Libersan, PhD, for her assistance in preparing this article. We thank Inge Vreeswijk-Baudoin and Martin Dokter for their excellent technical assistance.

**Sources of Funding**

Parts of these studies were funded by BG Medicine Inc. (Waltham, MA), provided an unrestricted research grant to the department of Cardiology of the University Medical Center Groningen, the Netherlands. Dr de Boer is supported by the Innovautional Research Incentives Scheme program of the Netherlands Organization for Scientific Research (NWO VENI, grant 916.10.117) and the Incentives Scheme program of the Netherlands Organization of Cardiology of the University Medical Center Groningen, the Netherlands Heart Foundation (Grant 2007T046).

**Disclosures**

BG Medicine Inc owns certain rights with respect to the use of galectin-3 as a biomarker. Dr de Boer and Dr van Veldhuisen received honoraria from BG Medicine. BG Medicine provided research grants to the Netherlands Heart Foundation (Grant 2007T046).

**References**


**CLINICAL PERSPECTIVE**

Cardiac remodeling is the heart’s general response to injury and is characterized by the development of myocyte hypertrophy and fibrosis formation. Progressive cardiac remodeling is the main predictor of heart failure development. Despite extensive research and advances in drug development, there is still a strong need for novel pharmacological agents that attenuate cardiac remodeling and prevent heart failure. Galectin-3 is a β-galactosidase–binding lectin and has been shown to enhance cardiac remodeling. Galectin-3 is secreted by macrophages and fibroblasts, and is important in fibrosis formation. Galectin-3 is also secreted into the circulation, where levels of galectin-3 reflect disease severity and prognosis, and as such, galectin-3 is currently being used as a biomarker. Because galectin-3 has an established causative role in tissue fibrosis, we hypothesized that disruption or inhibition of galectin-3 would inhibit myocardial fibrosis and afford cardioprotection. Using mouse and rat models of cardiac remodeling and fibrosis, we show that genetic disruption and pharmacological inhibition (with the oligosaccharide N-acetyllactosamine, N-Lac) of galectin-3 effectively attenuates myocardial fibrosis, which is accompanied with less myofibroblast activation, less collagen production, and lower collagen stiffness. This was associated with preserved cardiac function. Our results identify galectin-3 as a feasible target for therapy to prevent cardiac remodeling and heart failure.
Genetic and Pharmacological Inhibition of Galectin-3 Prevents Cardiac Remodeling by Interfering With Myocardial Fibrogenesis

Lili Yu, Willem P.T. Ruifrok, Maxi Meissner, Eelke M. Bos, Harry van Goor, Bahram Sanjabi, Pim van der Harst, Bertram Pitt, Irwin J. Goldstein, Jasper A. Koerts, Dirk J. van Veldhuisen, Ruud A. Bank, Wiek H. van Gilst, Herman H.W. Silljé and Rudolf A. de Boer

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SUPPLEMENTAL MATERIAL

Genetic and pharmacological inhibition of galectin-3 prevents cardiac remodeling by interfering with myocardial fibrogenesis

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Supplemental methods

Animal models
We studied six- to ten-week old male mice deficient for the gene encoding galectin-3 (galectin-3 knock-out mice (Gal3-KO)). The Gal3-KO homozygous mice carrying a targeted mutation in galectin-3 (a lectin, galactose binding soluble 3 or Lgals3) have been generated and are bred at Jackson Laboratory (Bar Harbor, ME, USA) and shipped to Groningen, The Netherlands. A targeting vector containing neomycin resistance and herpes simplex virus thymidine kinase genes was used to disrupt 3.7kb of sequence that includes exons 2, 3 and 4. The construct was electroporated into WW6 embryonic stem (ES) cells (derived from 129/Sv, C57BL/6 and SJL mixed background mice). Correctly targeted ES cells were injected into outbred MF-1 blastocysts. The resulting chimeric male animals were crossed with 129 female mice and the mutant strain was backcrossed with C57BL/6 for five generations. For an overview of the generation of the Gal3-KO mice, see Supplemental Figure S1. Other researchers have generated Gal3-KO mice as well.1-3 Transgene-negative male wild-type (WT) served as controls. For the reversal experiment, we used six- to eight-week old male C56Bl6/J mice (Harlan, The Netherlands).

We also studied six-week old, male, homozygous TGR(mREN2)27 rats (REN2). These rats overexpress the mouse renin-2 gene (ren-2d) and have a phenotype of severe hypertension and left ventricular (LV) hypertrophy which culminates into heart failure (HF) and low blood pressure over the course of 12-16 weeks.4-7 The rats were bred at the Max Delbrück Center for Molecular Medicine (Berlin Buch, Germany). Since Sprague Dawley (SD) rats represent the appropriate control strain for REN2 rats, age-matched male SD rats were used as controls (Harlan, The Netherlands).6

Animals were housed under standard condition. All animal studies were approved by the Animal Ethical Committee of the University of Groningen, The Netherlands, and conducted in accordance with existing guidelines for the care and use of laboratory animals.

Prevention study - mouse models
We induced cardiac remodeling by two interventions. First, cardiac remodeling was provoked by subcutaneous administration of angiotensin II (AngII) via osmotic minipumps for 14 days. Second, we induced pressure overload and cardiac remodeling by transverse aortic constriction (TAC) for 28 days. The control group received saline infusion via osmotic minipumps for 14 days. In total, six groups were studied: WT-con (control, N=10), Gal3-KO-con (N=10), WT-
AngII (N=12), Gal3-KO-AngII (N=10), WT-sham (N=7), WT-TAC (N=12), Gal3-KO-sham (N=11), and Gal3-KO-TAC (N=14).

AngII (Bachem AG, Bubendorf, Switzerland) was dissolved in 0.9% NaCl and injected into osmotic minipumps (Alzet 2004, Durect Corporation, Cupertino, CA, USA). The dose delivered by the osmotic minipumps was 2.5 µg/Kg/day. The minipumps were inserted subcutaneously on the back of the mice.

The TAC is a well established model. In brief, mice were anesthetized with oxygen and isoflurane (2%), intubated and ventilated. The thoracic cavity was opened between the second and the third intercostal cavity. Then, a blunted needle (27G) was placed on the aortic arch between both carotid arteries and, with an 8-0 nylon suture, the aorta was tied onto the needle. Immediately after, the needle was removed creating a reproducible stenosis of the aorta of about 50%. Sham-operated mice served as controls. Mice were sacrificed after 28 days.

**Reversal study - mouse model**

The TAC model was used as described above. Mice were left untreated for 28 days. After 28 days, one third of all animals were sacrificed. The remainder were given intraperitoneal injections of the galectin-3 inhibitor (Gal3i) N-acetyllactosamine (N-Lac) (Sigma-Aldrich, Zwijndrecht, The Netherlands) that targets the carbohydrate recognition domain (CRD) of galectin-3 at a final dose of 5 mg/Kg/day three times per week. N-Lac has been shown to effectively bind galectin-3. The treatment period lasted another 28 days and the total study duration was 56 days (eight weeks). Control mice were injected with saline. In total, three groups were studied: WT-TAC-control (N=9), WT-TAC-saline (N=9) and WT-TAC-Gal3i (N=9).

**Rat model**

To study the effect of galectin-3 binding protein on cardiac remodeling, we allocated SD and REN2 rats to different treatment regimens. REN2 rats were treated with N-Lac at a final dose of 5 mg/Kg/day injected intraperitoneally three times per week. SD rats were used as controls for all groups. Rats were sacrificed at week seven of the experimental protocol (when aged ~13 weeks). In total, four groups were studied: SD-con (control, N=12), REN2-con (N=18), SD-Gal3i (N=8) and REN2-Gal3i (N=10). Treatment with Gal3i did not exert any effects in control SD rats and, therefore, the results shown are limited to the REN2 groups.
**Echocardiography**

Cardiac function was assessed by echocardiography at baseline and prior to sacrifice with Vivid 7 (GE Healthcare, Chalfont St Giles, UK) equipped with a 10-MHz (rats) and a 13-MHz (mice) phase array linear transducer), as described previously. The echocardiographic measurements were performed under general anesthesia with 2% isoflurane. Both 2-dimensional (2D) images in parasternal long-axis and short-axis view and 2-D guided M-mode tracing were obtained. Parasternal long-axis (PLAX) views were obtained in order to ensure that the mitral and aortic valves and the apex were visualized. Left ventricular outflow tract (LVOT) diameter was measured in PLAX. Short-axis views were recorded at the level of mid-papillary muscles. A total of three loops was recorded and used for calculations. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from M-mode tracings. LV fractional shortening was calculated as (LVEDD – LVESD)/LVEDD × 100%. Cardiac output was calculated by echo Doppler measurements over the aortic valve (pulsed wave doppler) using the LVOT diameter. To monitor the development of cardiac hypertrophy in both mice and rats, echocardiography was performed at baseline and prior to sacrifice.

**Measurement of hemodynamic function**

Hemodynamic function was assessed invasively as described previously by introducing a 0.8-French (for mice) and a 1.4 French (for rats) microtip pressure-volume transducer (Millar Instr. Inc., Houston, TX, USA) via the right carotid artery into the aorta. A three minute period was allowed for stabilization and then systolic and diastolic blood pressure and heart rate (HR) were recorded in the aorta (average of 20 heart cycles). The catheter was then advanced into the LV. Peak systolic pressure, LV end diastolic pressure (LVEDP), maximal indices of LV contraction and relaxation (dPdmax and dPdmin) as well as the relaxation constant Tau were measured. dPdmax and dPdmin were corrected for peak systolic pressure to correct for differences in afterload, as described previously.

**Tissue procurement**

After measuring hemodynamics, blood was drawn (PINP assay) and the hearts were rapidly excised and weighed. Myocardial tissue was dissected transversally and processed for immunohistochemistry or snap frozen for molecular analysis.
**Immunohistochemistry**

Hearts were isolated and fixed with buffered 3.7% formalin for 24 hours. Thereafter, tissue sections were dehydrated and embedded in paraffin. Another slice was embedded in tissue-tec, placed on a carton disk put on a petri-dish and immediately, but slowly, frozen above liquid nitrogen. For immunohistochemistry, 3 μm-thick sections were cut on a microtome and mounted on slides. Hearts were cut at the mid-papillary level.

To measure fibrosis, Masson’s trichrome staining was performed on paraffin sections for all experimental animals. Whole stained sections were scanned (Nanozoomer 2.0-HT, Hamamatsu, Japan) and fibrosis score for the entire section was calculated at 20x magnification for mice and 10x magnification for rats (ScanScope, Aperio Technologies, Vista, CA, USA). To measure cardiomyocyte size, deparaffinized sections were stained with Gomori's silver staining. Cardiomyocyte size was measured as the cross-sectional area of transversally cut cardiomyocytes at 40x magnification for rats. Myofibroblasts were stained with a monoclonal mouse anti-human smooth muscle actin (SMA) antibody (#M0851, DAKO, Heverlee, Belgium). SMA in the entire section was calculated at 10x magnification by the SanScope software for rats. To measure the number of macrophages, macrophages were stained with a mouse anti-rat CD68 (ED1) antibody (#MCA341R, AbD Serotec, Oxford, UK) for rats. The number of macrophages was calculated for the entire section using the SanScope software at 40x magnification for rats. Proliferating cells were stained with a mouse monoclonal anti-PCNA antibody [PC10]-proliferation marker (#ab29, Abcam, Cambridge, UK). The number of proliferating cells in the entire section was calculated by the SanScope software at 40x magnification for rats.

To stain galectin-3 in cardiac tissue, an anti-galectin-3 monoclonal antibody (Thermo Fisher Scientific, Landsmeer, The Netherlands) was used. Briefly, paraffin sections were dewaxed and subjected to an antigen retrieval procedure by incubating overnight at 80°C in 0.1M Tris/HCl, pH 9.0. After three washes with TBS, endogenous peroxidase was blocked and sections were incubated with the primary antibody (anti-galectin-3 antibody, 1:100) in 1% BSA/PBS for 1 hour at room temperature. The sections were then incubated with a secondary antibody (polyclonal rabbit anti-mouse IgG/HRP, 1:100) diluted in 1% BSA/PBS buffer for two hours. The slides were first stained with 3-animo-9-ethylcarbazole and counterstained with hematoxillin. For typical examples of galectin-3 staining in cardiac tissue, see supplemental results (Supplemental Figures S3 and S4).
Enzyme-linked immunosorbent assay (ELISA)

Procollagen type I N-terminal propeptide (PINP), a marker of collagen metabolism, was analyzed in plasma using a commercial Enzyme Linked Immunosorbent Assay according to the manufacturer’s instructions (rat procollagen I N-terminal propeptide ELISA kit #E90957Ra, Uscn Life Science Inc, Wuhan, China). Human collagen type III (Human Collagen Type III (Col III) ELISA Kit, Cat No. Hu9614, TSZ ELISA, Framingham, USA) was analyzed in human dermal fibroblast supernatant (medium).

Collagen cross-linking assay

Collagen digestibility was measured following the digestion of tissue samples (for 6 hours at 37°C) with Clostridium histolyticum collagenase (Sigma) at a final concentration of 5 µg enzyme/mg tissue in a 50 mM Tris buffer containing 5 mM CaCl2, 0.15 M NaCl, 1 µM ZnCl2, 0.02% (w/v) NaN3 and 0.01% (v/v) Brij 35. After incubation, the supernatant and remaining tissue were separated and hydrolyzed in 6 M HCl at 110°C for 20-24 hours. The relative amount of collagen in the supernatant and remaining tissue was estimated by measuring the amount of hydroxyproline in the hydrolysates of both the supernatant and the remaining tissue.13

Quantitative real-time PCR

cDNA synthesis was performed with 0.5 µg total RNA using a specific cDNA synthesis kit according to the manufacturer’s protocol (Quantitect Rev. Transcriptase kit, Qiagen, Venlo, The Netherlands), as described previously.10,14,15 Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green mix according to the manufacturer’s protocol (Absolute SYBR Green ROX mix, Thermo Scientific, Breda, The Netherlands) on C1000 Thermal Cycler CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). All targets were evaluated under the same experimental conditions (95°C for 15 minutes, then 36 cycles at 95°C for 15 seconds and 60°C for 30 seconds). Samples were analyzed by quantification software (Bio-Rad CFX Manager 1.6). mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. To normalize expression data, reference genes were used. Reference genes were chosen with little sample-to-sample variability (GAPDH and 36B4). See Supplemental Tables S1, S2, and S3 for a list of primers used.
**Western blotting**

Rat hearts were homogenized in ice-cold RIPA (50 mM Tris pH 8.0, 1% nonidet P40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl) containing phosphatase inhibitor cocktail 1 (Sigma), protease inhibitor (ROCHE), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 15 mM NaVanadate for 30 min. Equal amounts of protein (20 µg) were loaded on 12% polyacrylamide gels. After electrophoresis, the gels were blotted onto nitrocellulose membranes. Membranes were then incubated with anti-galectin-3 antibody (Thermo Fisher Scientific, Landsmeer, The Netherlands), anti-Hsp47 antibody (#ab109117, Abcam, Cambridge, UK), anti-mac-2 antibody (CL8942AP, Cedarlanelabs, USA), troponin T (T6277, Sigma, Netherlands), anti-vimentin antibody (V5255, Sigma, Netherlands), anti-CD68 antibody (ab76308, Abcam, Cambridge, UK) and anti-GAPDH antibody (10R-G109a, Fitzgerald, USA) overnight at 4° C. Membranes were washed and incubated with the appropriate secondary HRP-conjugated antibody and signals were visualized with ECL and analyzed with densitometry (Syngene, Cambridge, United Kingdom).

**Human adult dermal fibroblast culture experiments**

Human adult dermal fibroblasts (primary cells) were cultured for 72 hours in culture medium (EMEM containing 1% Pen/Strep, 1% L-glutamine, 0.5% FCS and 0.173 mM ascorbic acid 2-phosphate) with or without galectin-3 (10 µg/mL; #450-38, Preprotech, Rocky Hill, NY, USA), N-Lac (10 mM) or a combination. The initial seeding density was 15,000 cells/cm². Cells were harvested and RNA was isolated. cDNA synthesis was conducted using RevertAid First Strand cDNA Synthesis Kit (#K1622, Fermentas, St. Leon-Rot, Germany) and cDNA was hybridized to a custom made low density array carrying probes for ~50 genes involved in fibrosis (collagen production, collagen cross-linking, collagen processing and collagen degradation). The complete list of gene is presented in Table S4 of the Supplemental data. Genes that were most significantly expressed (LOXL2, HSP47, PCOLCE, COL1A1, COL1A2, COL3A1, and P4HB) were verified using quantitative RT-qPCR and normalized against the reference gene GAPDH. In addition, vimentin staining, a fibroblast marker, was carried out with anti-vimentin (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein levels of collagen type 3 were measured with ELISA (human collagen 3 ELISA kit, #Hu9614, TSZ Scientific LLC, Framingham, MA, USA). Collagen type 3 protein levels were measured in singulo, therefore error bars and statistics are not provided.
Analyzes of galectin-3 expression in macrophages and in adult rat cardiac myocytes and fibroblast

Adult rat cardiomyocytes and fibroblasts were isolated using a Langendorff perfusion system with retrograde perfusion.\textsuperscript{16} The perfusion solution (10 mM HEPES (pH 7.4), 133.5 mM NaCl, 4.0 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.2 mM MgSO$_4$, 20 $\mu$M CaCl$_2$) contained 0.2mg/ml Liberase TM Research Grade (Roche) to digest the extracellular matrix. Cardiomyocytes were subsequently separated from other cells by several rounds of velocity sedimentation in a perfusion solution containing 1mg BSA/ml. Adult cardiomyocytes were directly frozen after isolation. Cardiac fibroblasts were cultured for several days in DMEM medium supplemented with 10% FCS and penicillin-streptomycin (100 IU/ml and 100ug/ml, respectively) to obtain sufficient cells for analysis. A rat alveolar macrophage cell line\textsuperscript{17} was also cultured in the same medium. Cell lysates were generated as described in the Western blot section and equal amounts of proteins were subsequently loaded on a SDS-PAGE gel.
**Supplemental Tables**

**Table S1.** List of primers used for RT-qPCR in mice

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<th>Gene symbol (name)</th>
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<th>Reverse</th>
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<td>Nppa (ANP)</td>
<td>ATGGGCTCCTTCTCCATCAC</td>
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<td>Rplp0 (36B4)</td>
<td>AAGCGCGTCTCCTGGCATTGTC</td>
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**Table S2.** List of primers used for RT-qPCR in rats

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<td>ATGGGCTCCTTCTCCATCAC</td>
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<td>Lgals3 (Galectin-3)</td>
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**Table S3.** List of primers used for RT-qPCR in human cell culture experiments

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<td>GAPDH</td>
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### Table S4. List of genes of which probes are carried by the Low Density Array

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<tr>
<th>Number</th>
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<th>Downregulated by N-Lac</th>
<th>Upregulated by Galectin-3 and downregulated by N-LAC</th>
<th>Verified by RT-qPCR</th>
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<td>Collagen modifying enzymes</td>
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<td>PLOD1</td>
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**Collagens**

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Supplemental figures and figure legends

Figure S1. Strategy for the genetic disruption of galectin-3 in mice and analysis of galectin-3 expression.

(A) Exon 2, 3 and 4 were disrupted in the Gal3-KO mice. (B) mRNA expression of galectin-3 in the left ventricles of mice from the different experimental groups (n=7-12). (C) Representative Western blot showing galectin-3 expression (anti-MAC-2) in left ventricles of mice from the different experimental groups. (D) Quantification of Western blot (n=5). Con: control (saline-infused); AngII: angiotensin II; sham: sham-operated mice; TAC: transverse aortic constriction.

* P<0.05 vs. WT-con, † P<0.05 vs. WT-AngII, § P<0.05 vs. WT-sham, ** P<0.05 vs. WT-TAC.
Figure S2. Survival of REN2 rats in the different groups.

Figure S2. shows the survival of REN2 rats in the different experimental groups. In the REN2-con group, 7 out of 18 rats (39%) died and 1 out of 10 rats (10%) died in the REN2-Gal3i group. No SD control animals died (SD-con group). Con: control; Gal3i: galectin-3 inhibitor.
**Figure S3.** Localization of galectin-3 expression in cardiac tissue.

Figure S3. shows the localization of galectin-3 in cardiac tissue from REN2 and SD rats. Immunohistochemical analysis showed that galectin-3 immunoreactivity (red-brown color) is predominantly observed at interstitial sites but not in cardiomyocytes. Con: control; Gal3i: galectin-3 inhibitor.
**Figure S4.** Localization of galectin-3.

Figure S4. Panels A-C: Lower magnification (20X) showing double labeling for galectin-3 (red), the macrophage marker ED1 (green) and the merged image with DAPI staining nuclei (blue) in the different experimental groups. Panel D: higher magnification (40X) of the merged image of galectin-3, the macrophage marker ED1 and DAPI (blue). Panels E-G: Lower magnification (20X) showing double labeling for galectin-3 (red), the fibroblast marker collagen III (green) and the merged image with DAPI staining nuclei (blue) in the different groups. Panel H: higher magnification (40X) of the merged image of galectin-3, the fibroblast marker collagen III and DAPI (blue). Con: control; Gal3i: galectin-3 inhibitor.
**Figure S5.** Protein expression of galectin-3 in cardiomyocytes, fibroblasts and macrophages.

Figure S5. shows a Western blot with lysates from different cell types. Galectin-3 was detected using anti-galectin-3, anti-troponin T (cardiomyocyte marker), anti-Vimentin (fibroblast marker) and anti-CD68 (macrophage marker) antibodies and an anti-GAPDH antibody (as loading control). CMC = adult rat cardiomyocyte; FB = fibroblast and macrophage = a rat alveolar macrophage cell line.
Figure S6. Extracellular matrix protein gene expression in rat hearts.

Figure S6. shows changes in gene expression of extracellular matrix proteins in rat hearts as determined by RT-qPCR. (A) Timp1, (B) Timp2, (c) Mmp2 and (d) Mmp9. Gal3i: galectin-3 inhibitor. †† *P*<0.05 SD-con vs. REN2-con, §§ *P*<0.05 SD-con vs. REN2-Gal3i.
Supplemental References


