Galectin-3 Marks Activated Macrophages in Failure-Prone Hypertrophied Hearts and Contributes to Cardiac Dysfunction

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Background—Inflammatory mechanisms have been proposed to be important in heart failure (HF), and cytokines have been implicated to add to the progression of HF. However, it is unclear whether such mechanisms are already activated when hypertrophied hearts still appear well-compensated and whether such early mechanisms contribute to the development of HF.

Methods and Results—In a comprehensive microarray study, galectin-3 emerged as the most robustly overexpressed gene in failing versus functionally compensated hearts from homozygous transgenic TGRmRen2-27 (Ren-2) rats. Myocardial biopsies obtained at an early stage of hypertrophy before apparent HF showed that expression of galectin-3 was increased specifically in the rats that later rapidly developed HF. Galectin-3 colocalized with activated myocardial macrophages. We found galectin-3–binding sites in rat cardiac fibroblasts and the extracellular matrix. Recombinant galectin-3 induced cardiac fibroblast proliferation, collagen production, and cyclin D1 expression. A 4-week continuous infusion of low-dose galectin-3 into the pericardial sac of healthy Sprague-Dawley rats led to left ventricular dysfunction, with a 3-fold differential increase of collagen I over collagen III. Myocardial galectin-3 expression was increased in aortic stenosis patients with depressed ejection fraction.

Conclusions—This study shows that an early increase in galectin-3 expression identifies failure-prone hypertrophied hearts. Galectin-3, a macrophage-derived mediator, induces cardiac fibroblast proliferation, collagen deposition, and ventricular dysfunction. This implies that HF therapy aimed at inflammatory responses may need to be targeted at the early stages of HF and probably needs to antagonize multiple inflammatory mediators, including galectin-3. (Circulation. 2004;110:3121-3128.)

Key Words: angiotensin ■ collagen ■ fibroblasts ■ heart failure

Despite state-of-the-art treatment, heart failure (HF) is still a progressive disorder characterized by high morbidity and mortality, suggesting that important pathogenic mechanisms remain active and unmodified by current treatment. A growing body of evidence links macrophage activation and fibrosis to the pathogenesis of HF. Accordingly, there has been increasing interest in developing therapeutic agents with anticytokine properties that might be used as adjunctive therapy in patients with HF. In particular, the growing appreciation of elevated levels of the pleiotropic cytokine tumor necrosis factor (TNF)-α in patients with HF culminated in clinical studies on TNF-α inhibition. However, these trials did not support the use of TNF-α antagonism as a treatment modality of HF. Therefore, the question arises as to whether inflammatory mechanisms merely reflect a general stress response of an organism in severe HF or whether such inflammatory response already starts early in the pathogenesis of HF and comprises a broader range of cardiotoxic mediators.

Our recent microarray study performed in a rat model of hypertensive HF allowed us to evaluate immunological mediators specific to hypertrophied hearts that have progressed to failure. Among a number of candidate genes, we have focused on galectin-3 as the most robustly overexpressed mediator in failing hearts. Galectin-3 is a member of a large family of β-galactoside–binding animal lectins. Macrophages show increased galectin-3 expression at phagocytic cups and phagosomes during the process of phagocytosis. Galectin-3 interacts with various ligands located at the extracellular matrix, including laminin, collagen, synexin, and integrins.

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The online-only Data Supplement, which contains additional information about Methods and additional figures, can be found with this article at http://www.circulationaha.org.

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The route of import for galectin-3 from extracellular milieu to cytoplasm is shown to be mediated by β₁-integrin. Extracellular galectin-3 mediates cell migration and cell-cell interactions, whereas intracellular galectin-3 regulates cell cycle and apoptosis. Galectin-3 overexpression causes changes in the expression levels of cell cycle regulators, including cyclin D₁, and the growth-promoting activity of galectin-3 is dependent predominantly on cyclin D₁ promoter activity.

Here, we report that myocardial galectin-3 expression is already increased at an early time point in rats that later rapidly progressed to HF. Furthermore, recombinant galectin-3 infused into the pericardial sac of healthy Sprague-Dawley (SD) rats induced HF and excess collagen deposition. At the molecular level, galectin-3 increased the expression of cyclin D₁ in dividing fibroblasts and failing myocardium. This study demonstrates a novel pathogenic role of macrophage activation and galectin-3 production in the deterioration of cardiac architecture and function.

Methods

Characteristics of the Ren-2 Rats

We studied 14 male homozygous Ren-2 rats and 9 age-matched SD rats (Max-Delbrück-Zentrum, Berlin, Germany). Of the 14 Ren-2 rats, 6 were euthanized at 12 to 14 weeks on clinical signs of HF, and the remaining 8 Ren-2 rats were monitored and euthanized at 17 weeks when clinical signs of failure had not appeared. At 10 weeks, none of the Ren-2 rats had developed HF, and at this stage, all the Ren-2 rats had comparable left ventricular (LV) hypertrophy, irrespective of the future consequence of either failure or prolonged compensation. We have reported separately the cDNA microarray experiment (total, 12 336 genes; Incyte Genomics; rat GEM2/3) that was performed. A small incision in the pericardial sac was made, and the catheter was fixed subcutaneously and connected to an osmotic minipump that was set to pump 0.5 μg galectin-3 per hour for a total duration of 4 weeks. The dose of galectin-3 was calculated on the basis of reported bioactivity, adjusting for the local advantage of pericardial delivery.

Measurement of Cardiac Geometry and Function

Echocardiography was performed at 0, 14, and 28 days of galectin-3 infusion in rats sedated with 2% isoflurane. Standard views were obtained in 2-D as well as M-mode with a 12-MHz transducer (Hewlett Packard) with ~220 frames per recording, and the data analysis was made by a blinded observer.

Protein Isolation and Western Blotting

A complete list for the primers and probes of the galectin-3 gene transcripts is presented as the online supplementary material (Data Addendum 1). RNA was isolated from rat LV with the RNAsafe Mini Kit following the RNaseq Mini Protocol (Qiagen) and stored at −80°C. RNA was isolated from rat and human heart biopsies with the PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer’s instructions. Optimal polymerase chain reaction (PCR) conditions were found to be 12.5 μL 2 times PCR Master Mix for Taqman assays with final concentration of 5 mMol/L MgCl₂, 300 mMol/L of each primer, 200 mMol/L of probe, and 10 ng cDNA template in a total volume of 25 μL.

Protein Isolation and Western Blotting

Protein isolation and Western blotting were performed as described previously. Primary antibodies (galectin-3, Bioreagents; collagen-I and collagen-III, Abcam; ED-1 and OX-6, a kind gift from Dr M. de Winther, Department of Molecular Genetics, University of Maastricht, the Netherlands; cyclin D₁, Cell Signaling Technologies) were diluted 1/1000 in Tris-buffered saline with Tween-20 (TBS-T). Secondary antibody (horseradish peroxidase–conjugated IgG, Cell Signaling Technology) was diluted 1/2000 in TBS-T. Protein bands were visualized by the enhanced chemiluminescence technique.

In Situ Hybridization, Immunohistochemistry, Galectin-3 Cytochemistry, and Confocal Microscopy

To localize galectin-3 mRNA, we used a nonradioactive in situ hybridization assay. A DIG-labeled oligonucleotide probe was used for the hybridization according to the manufacturer’s instructions (GeneDetect). The extent of specific binding was visualized by application of avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Laboratories). The expression of galectin-3 protein and accessible binding sites were visualized by a specific anti–galectin-3 monoclonal antibody and biotinylated galectin-3, as described previously. For confocal laser scanning microscopy experiments, galectin-3–binding sites were detected by FITC-labeled avidin. A Texas Red–labeled secondary antibody was used to visualize immunocytochemically the proliferating cell nuclear antigen (PCNA). Further details on the procedure are available elsewhere.

Cardiac Fibroblast Proliferation and Proline Incorporation Assays

Rat cardiac fibroblasts were isolated from 2-day-old neonatal SD rats, as described previously. Cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10%...
TABLE 1. Characterization of Cardiac Function in Aortic Stenosis Patients

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<tr>
<th></th>
<th>n</th>
<th>Age, y</th>
<th>Sex, % Male</th>
<th>EF, %</th>
<th>LV Mass Index, g/m²</th>
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<td>17</td>
<td>66.1±7</td>
<td>47.5</td>
<td>64±4.7</td>
<td>121±25</td>
</tr>
<tr>
<td>Decreased EF</td>
<td>5</td>
<td>67.2±4.5</td>
<td>60</td>
<td>51±5.4*</td>
<td>136±23</td>
</tr>
</tbody>
</table>

EF indicates ejection fraction; n, group size; and LV mass index, LV mass corrected for body weight.

*P<0.05.

FBS, along with 1% L-glutamate, 50 U/mL penicillin, and 0.1 g/L streptomycin and were incubated at 37°C in a humidified 5% CO₂ atmosphere. Synchronized cells were treated with murine recombinant galectin-3 (control, 10 μg/mL, and 30 μg/mL) for 24 hours. The number of dividing cells was determined by radiolabeled methyl-[³H]thymidine incorporation (0.5 μCi per well) assay. Collagen production by these cells was measured by [³H]proline uptake after a 72-hour treatment of galectin-3 (control, 10 μg/mL, and 30 μg/mL). The assays were performed in triplicate for fibroblast preparations.

Human Studies

We obtained cardiac biopsies from patients undergoing aortic valve replacement for aortic stenosis. For the study of gene expression in these human biopsies, we selected 5 aortic stenosis patients with cardiac hypertrophy and relatively depressed ejection fraction (<55%) and 17 aortic stenosis subjects with LV hypertrophy and normal or elevated ejection fraction. Patients underwent a detailed cardiovascular assessment by echocardiography before operation (Table 1). The myocardial biopsies were collected and snap-frozen in liquid nitrogen. Informed consent was obtained from the patients, and the institutional Medical Ethical Committee approved the study.

Statistical Analyses

Data are presented as mean±SEM. The paired comparisons were made by unpaired t test. For multiple comparisons, 1-way ANOVA in combination with a Dunnett’s post hoc analysis was made. Analyses were performed by use of the statistical package SPSS 10.0. Probability values of P<0.05 were considered to be statistically significant.

Results

Increased Myocardial Collagen Content in Failing Ren-2 Rats

A computer-assisted densitometric analysis of the picrosirius red–stained sections for the quantification of myocardial collagen revealed a higher degree of interstitial collagen content in the failing Ren-2 rats compared with compensated and wild-type rats (interstitial collagen volume fraction percentage: HF, 7.8±0.38; compensated, 3.8±0.54; wild-type, 2.5±0.3; P<0.05 versus compensated and wild-type).

Microarray Data Revealed an Inflammatory Gene Profile in Failing Hearts

The microarray analysis has been described separately, and the differentially expressed genes are available as supplementary material for that article. In this study, we focused on galectin-3, which emerged as the most prominently overexpressed gene with a >5-fold rise in HF rat myocardium compared with compensated hypertrophied myocardium. In addition, major histocompatibility complex antigen II (MHC-II), MHC-associated invariant chain peptide, macrophage mannose receptor, and immunoglobulin receptors genes were among the overexpressed genes.

Western Blotting Showed High Galectin-3 Expression in Failing Myocardium

Given the robust transcriptional increase in galectin-3, we measured its protein levels in the myocardium. Comparable to the results obtained in the microarray, the highest expression level of galectin-3 protein was observed in the same group of animals that had the highest degree of cardiac fibrosis and had developed HF by 12 to 14 weeks (densitometric units: HF, 94.66±9.5; compensated, 35±5.6; controls, 27.2±6.2; P<0.05 versus compensated and controls) (Figure 1a).

Localization of Galectin-3 to Activated Macrophages

We evaluated the source and distribution of galectin-3 in the rat myocardium by in situ hybridization and immunohistochemistry. Importantly, galectin-3 positivity was seen in the areas of fibrosis (Figure 2, a1). Galectin-3–positive areas colocalized with macrophage-specific staining (Figure 2, b1). These macrophages strongly expressed MHC-II antigen as well, indicating an active role of these cells in antigen presentation (Figure 2, c1). These characteristics were present only in HF rats and not in compensated rats (Figure 2, a2, a3, b2, and c2) and wild-type SD rats (Figure 2, a3, b3, and c3). The galectin-3 mRNA expression, as shown by in situ hybridization, localized to the cells infiltrated to the areas of myocardial...
dial tissue damage (Figure 2, d1). The normal SD rat myocardium, in contrast, lacked the galectin-3–producing cellular infiltrates (Figure 2, d2).

Galectin-3 Binding Sites in Extracellular Matrix and Cardiac Fibroblasts

We infused biotinylated galectin-3 into the pericardial sac to visualize galectin-3–binding sites in vivo in the myocardium. Galectin-3–binding sites localized predominantly to the myocardial matrix and fibroblasts (Figure 2e). In vitro, galectin-3–binding sites were seen as diffuse cytoplasmic staining in resting cells (Figure 3a). However, proliferating fibroblasts showed enhanced staining around the nucleus, revealing a mitosis-related alteration in staining profile (Figure 3b). We performed similar experiments in isolated cardiomyocytes to localize accessible galectin-3–binding sites. In contrast to cardiac fibroblasts, galectin-3–binding sites were absent from cardiomyocytes (Figure 3c). Confocal microscopy confirmed a compact presence of accessible galectin-3 ligands around the nucleus in proliferating (ie, PCNA-positive) cardiac fibroblasts (Figure 3, d, e, and f), reflecting that in mitotic cells, galectin-3–binding sites migrate to perinuclear areas.16

Galectin-3 Induced Fibroblast Proliferation and Collagen Production In Vitro

Exogenous recombinant galectin-3 (10 and 30 μg/mL) significantly increased cardiac fibroblast proliferation as determined by [3H]thymidine incorporation (galectin-3 at 30 μg/mL, 347±17.5 cpm; galectin-3 at 10 μg/mL, 309±4.8 cpm; control, 145±4.8 cpm; P<0.05 versus 10 μg/mL and control) (Figure 4a). We then monitored the collagen production by cardiac fibroblasts with the addition of exogenous galectin-3 using radioactive proline-incorporation assays. With 30 μg/mL of galectin-3 in the medium, proline incorporation increased by ~66% (galectin-3 at 30 μg/mL, 1066±56 cpm; control, 707±52.8 cpm; P<0.05). A lower concentration of galectin-3 failed to produce significant effects (galectin-3 at 10 μg/mL, 992±72 cpm; P=0.13) (Figure 4b).

Real-Time PCR Showed High Galectin-3 Expression in Failure-Prone Ren-2 Rat and Dysfunctional Human Hearts

To evaluate the expression of galectin-3 in the myocardium at the stage before Ren-2 rats progressed to HF (ie, 10 weeks of age), we obtained cardiac biopsies in vivo in the spontaneously beating rat heart. Measured by real-time PCR, myocardial expression of galectin-3 gene was increased only in the

Figure 2. Immunohistochemistry, in situ hybridizations, and ligand histochemistry to localize galectin-3 and its binding sites in rat myocardium. a1, b1, and c1, myocardial sections obtained from failing Ren-2 rat hearts stained for anti–galectin-3, macrophage-specific anti-CD68, and major histocompatibility complex II (MHC-II)–specific OX-6 antibody, respectively. a2, b2, and c2, galectin-3, macrophages and MHC-II probed in sections obtained from compensated Ren-2 rat hearts; a3, b3, and c3, same molecules tested in sections obtained from normal wild-type rats; d1 and d2, in situ hybridization of galectin-3 mRNA in failing Ren-2 (d1) and normal wild-type rat myocardium (d2), where intense brown staining indicates galectin-3 mRNA localized to cell infiltrates between larger nonstained myocytes in damaged myocardium of failing hearts; e, ligand histochemistry after 1 week of intrapericardial infusion of biotinylated galectin-3 to localize cell types involved in active binding of galectin-3. Magnification ×200.
rats that later progressed to HF (relative expression, 5.8±0.11), whereas it was expressed at relatively lower levels in the rats that subsequently remained compensated (3.4±0.21) and in nontransgenic control rat hearts (2.5±0.047) (Figure 5a). In human myocardial biopsies, there was significantly higher myocardial galectin-3 mRNA expression in hypertrophied hearts with relatively impaired ejection fraction compared with the compensated forms of cardiac hypertrophy (relative expression: failure, 7.08±1.17 versus hypertrophy, 4.60±0.51; P<0.05) (Figure 5b). To measure galectin-3 mRNA expression in nonhypertrophied hearts, we obtained myocardial biopsies from the patients without aortic stenosis who were undergoing cardiac surgery for coronary artery bypass grafting (CABG). Compared with the expression levels observed in aortic stenosis, real-time PCR analysis showed that galectin-3 mRNA expression was lowest in nonhypertrophied hearts from patients undergoing CABG (relative expression, normalized to cyclophilin A, 3.4±0.21, n=5).

Intrapericardial Infusion of Galectin-3 Induced LV Dysfunction and Increased Collagen I/III Ratio
To address whether chronically elevated levels of galectin-3, specifically in the heart, can induce HF and to avoid potential systemic effects of galectin-3, we designed a novel approach of intrapericardial infusion of galectin-3 in healthy rats. At baseline, there was no significant difference in cardiac function between the galectin-3– and placebo-infused rats. Rats infused with galectin-3 for 4 weeks into the pericardial space showed depressed LV ejection fraction, fractional shortening, and the amplitude of the negative slope of dP/dt max and increased lung weight–to–body weight ratio compared with rats receiving placebo infusion (Table 2). Quantification of collagen content from the LV myocardium showed increased collagen volume fraction in galectin-3–infused rats (Figure 6a). Western blotting revealed a marked increase in collagen I in galectin-3–infused myocardium (densitometric units: galectin-3–infused, 149±13; placebo, 44±6, P<0.01). However, no difference was observed in the level of collagen III (galectin-3–infused, 84±14; placebo, 70±2; P=NS) (Figure 6b). The relative abundance of collagen type I was discernible, because there was 3-fold increase in collagen type I/III ratio (Figure 6c).

Galectin-3 Induced Cyclin D1 Expression In Vivo and In Vitro
We analyzed a potential mediator of the proliferative effects of galectin-3 by measuring the inducible expression of cyclin D1, an important early cell cycle regulator. Failing Ren-2 rats had a higher level of myocardial cyclin D1 expression...
compared with compensated Ren-2 and wild-type SD rats (densitometric units: HF, 38.7±5.5; compensated, 3.7±1.7; SD, 2.3±0.6; *P<0.05 HF versus compensated) (Figure 1, b1). In proliferating cardiac fibroblasts, galectin-3 increased cyclin D1 expression (galectin-3–treated, 4.2±0.57; controls, 1.4±0.41; *P<0.05) (Figure 1, b2). In vivo, intrapericardial galectin-3 infusion also led to increased myocardial expression of cyclin D1 (galectin-3–infused, 18.6±1.33; placebo, 0.9±0.05; *P<0.05) (Figure 1, b3).

Discussion
A role for inflammatory mediators in HF has often been shown and is thought to be a rather universal response to the complex local and systemic changes in HF. In contrast to that notion, we now document that increased expression of the macrophage-derived mediator galectin-3 is already apparent in the stage of compensated hypertrophy of failure-prone hearts, before they progress to overt failure. We further demonstrate that galectin-3 induces HF in normal rats. These findings suggest that inflammatory and profibrotic mediators could still be viable therapeutic targets in HF. This supports the idea that an early recognition of failure-prone hearts and intervention with broader-spectrum antiinflammatory agents could have an additional benefit over existing treatment strategies.

Galectin-3 Structure, Functions, and Relevance for Cardiac Remodeling
Identified first as an antigen on the surface of peritoneal macrophages, galectin-3 is the only chimera-type member of the galectin family.17 It belongs to a lectin family sharing the jelly-roll–like folding pattern and calcium-independent specificity to β-galactosides as well as proteins.18,19 In addition to its antiapoptotic and growth-promoting actions, galectin-3 plays a critical role in phagocytosis by macrophages when cross-linked by the Fcγ receptor.7 In agreement with the abundant fibrosis observed in failing ren-2– and galectin-3–infused rats, increased galectin-3 expression has also been shown in a rat model of postradiation pulmonary fibrosis.20 Other studies have shown that galectin-3 expressed by liver analogues of macrophages (ie, Kupffer cells) induce the synthesis of excess fibril-forming collagens in liver.21 Earlier studies have demonstrated the relation between galectin-3 expression and the cell cycle. Nuclear galectin-3 expression is associated with cell proliferation, and this effect is mediated through enhanced cyclin D1 promoter activity.11 Cyclin D1 forms a complex with cyclin-dependent kinases and regulates progression of the early to mid G1 phase of the cell cycle.22 This suggests that galectin-3 can induce cardiac fibroblast proliferation via the activation of cyclin D1, thus allowing a macrophage-derived mediator to affect cardiac fibroblasts.

Kinetics of Galectin-3 Binding Sites in Cardiac Fibroblasts
In line with the profibrotic effects of galectin-3 in vivo, we show that galectin-3 binds to intracellular receptors and induces cardiac fibroblast proliferation and increases collagen production in vitro. Although originally discovered as a carbohydrate-binding protein, galectin-3 is known to specifically interact with intracellular targets in addition to glyco-
Echocardiographic measurements

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<tr>
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<th>Placebo†</th>
<th>Galectin-3†</th>
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<td>LV ejection fraction, %</td>
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<tr>
<td>Baseline</td>
<td>67.3±2.5</td>
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<td>4 Weeks</td>
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<td>4 Weeks</td>
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<td>4 Weeks</td>
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Hemodynamic measurements

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<tr>
<td>-dP/dt max-baseline, mm Hg/s</td>
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Tissue and body weights

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<td>Heart wt/body wt, %</td>
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<td>Lung wt/body wt, %</td>
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Significance of Interstitial Fibrosis and Collagen I Production by Galectin-3

Collagens are essential components of the myocardium, maintaining its structural and functional integrity. In the heart, fibrillar types of collagen form a delicate sheath that interconnects bundles of contractile units. Increased collagen deposition may therefore have a major impact on the diastolic and systolic function of the heart. Whereas collagen III forms an elastic network storing kinetic energy as elastic recoil, collagen I represents a stiff fibrillar protein providing tensile strength.25,26 Only the collagen I and not the collagen III promoter is studded with SP-1–binding sites. Therefore, the differential increase of collagen I over collagen III in galectin-3–infused animals could be explained by the possible differences in the molecular makeup of their promoter sites.27

Conclusions and Implications

The present study, by demonstrating macrophage activation and increased galectin-3 production preceding overt HF, expands on previous publications that have described the possible involvement of inflammatory mechanisms in the advanced stages of HF. Our finding that galectin-3 is over-expressed well before the transition to overt HF suggests the novel concept that already in the compensated phase, some hypertrophied hearts recruit macrophages and proinflammatory mechanisms. We show that exogenous galectin-3 given intrapericardially to healthy hearts over a long term, can induce cardiac dysfunction, which makes it likely that this early recruitment and activation of galectin-3–producing macrophages can drive the progression from compensated hypertrophy toward overt HF. Galectin-3 is the only member of the galectin family with an unusually broad activity including protein-carbohydrate and protein-protein interactions in nuclei, cytoplasm, plasma membrane, and extracellular matrix.23 Our in vitro data underscore that this macrophage-derived effector molecule specifically binds to cardiac fibroblasts and induces fibroblast proliferation, also reflected by its ability to upregulate cyclin D1. Relevant for fibrosis, it also induces collagen I production. Failure-prone and dysfunctional rat and human heart specimens all share an increased lectin presence. Therefore, an early recognition of failure-prone hearts and intervention with new antiinflammatory and antifibrotic agents might provide additional benefit.
over existing treatment strategies. These results shape the concept of considering galectin-3 as a new target for therapeutic intervention at an early stage of compensated hypertrophy in failure-prone hearts.

Acknowledgments
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References
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