

Therapeutic inhibition of galectin-3 improves cardiomyocyte apoptosis and survival during heart failure

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Abstract. Galectin-3 is an important mediator of cardiac fibrosis and heart failure. Cell viability of cardiomyocytes was measured using a CCK-8 assay; flow cytometry was employed for the detection of the cell cycle and cardiomyocytes apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blotting was performed to examine the expression of associated genes and proteins. The present study demonstrated that overexpression of galectin-3 significantly decreased the viability of cardiomyocytes in a time-dependent manner, with simultaneous arrest of the cell cycle and induction of apoptosis. The expression levels of proliferating cell nuclear antigen (PCNA) and B-cell lymphoma 2 (Bcl-2) were decreased and Bcl-2-associated X protein (Bax) was increased in cardiomyocytes with galectin-3 overexpression. However, inhibition of galectin-3 by intravenous tail vein injection of a galectin-3-targeting short hairpin RNA-expressing vector during hypertension-induced heart failure in Dahl hypertensive rats increased rat survival and body weight. Inhibition of galectin-3 also increased the expression of PCNA and Bcl-2 and reduced the expression of Bax in the cardiac tissue of hypertensive rats. These results demonstrate the therapeutic potential of targeting galectin-3 for the treatment of cardiac disease.

Introduction

Chronic and acute stress to the heart results in a pathological remodeling response accompanied by cardiomyocyte hypertrophy, fibrosis, myocyte degeneration and apoptosis, finally contributing to heart failure (HF) (1). HF is a progressive disorder characterized by poor quality of life and poor

prognosis. In recent years HF has reached endemic proportions in the industrialized world, with 5.7 million patients affected by this disease in the United States (2). Similarly striking data are also observed in Europe and Asia, and the incidence of critical risk factors for HF have also markedly increased (3,4). The prevalence of HF is expected to rise due to the aging population and improved treatment of cardiovascular diseases that precede HF (5). Therefore, it is imperative that novel methods to improve the treatment of HF are identified.

Cardiac remodeling is an important determinant in the clinical outcome of HF; it is linked to disease progression and poor prognosis (6). Cardiac fibrosis is an important contributor to the pathophysiology of cardiac remodeling caused by hypertension. Galectin-3 is a β -galactoside-binding lectin that appears to be a mediator of cardiac fibrosis in several experimental studies (7,8). The potential HF-promoting actions of galectin-3 have been described (9), although the precise mechanisms still warrant further investigation. Galectin-3 is produced by macrophages and fibroblasts, but not by cardiomyocytes, in rat and mouse models of progressive cardiac remodeling (10,11). The pro-fibrotic characteristics of galectin-3 have also been demonstrated in models of pathological fibrosis in kidney and liver, suggesting that fibrosis may be a hallmark of cardiac remodeling and HF (12). Although several prognostic studies of patients with HF have indicated the independent predictive value of galectin-3 for cardiovascular outcomes, others have questioned whether galectin-3 is truly independently associated with adverse cardiac events (13,14). The observations provide evidence for the hypothesis that longstanding elevated galectin-3 may not signify disease, but likely represents a unique phenotype at high risk for the development and progression of heart failure.

The mechanism of galectin-3 as a promoter of the development of HF has not been investigated. The present study aimed to explore the mechanism by which galectin-3 may be associated with cardiomyocyte apoptosis and survival during HF.

Materials and methods

Isolation of cardiomyocytes and cell culture. Isolation of cardiomyocytes and cell culture was performed as previously described (15). In brief, animals were anesthetized by an intraperitoneal injection of chloral hydrate (300 mg/kg).

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Perfusion with fixatives (4% paraformaldehyde, 2.5% glutaraldehyde) was used as the primary method of euthanasia. The heart was excised and perfused using the retrograde Langendorff technique. Subsequently, the perfusion buffer was supplemented with 0.6% collagenase II and 0.6% bovine serum albumin (both from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the buffer was recirculated for 12–15 min at 25°C. The dissociated cells, including the majority of the cardiomyocytes, were filtered through a 200 μ m mesh. The cardiomyocytes were preplated for 1 h [2 mM L-glutamine and 10% FBS in Dulbecco's modified Eagle's medium (DMEM); Invitrogen; Thermo Fisher Scientific, Inc.] at 37°C in a humidified atmosphere of 5% CO₂ and 95% air to enrich the culture, and were subsequently cultured in serum-free DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Overexpression and construction of stable cell lines. The galectin-3 coding sequence was purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and the sequence was cloned into the pcDNA3.1(+) vector (Sangon Biotech Co., Ltd.). The primers used were as follows: Galectin-3 forward, 5'-CGGGATCCTTAGATCATGGCGTGGTTAGC-3' and reverse, 5'-CGGGATCCTTAGATCATGGCGTGGTTAGC-3'. The sequences were annealed and digested using *Eco*RI and *Bam*HI, and were ligated into the pcDNA3.1(+) vector. An empty pcDNA3.1(+) vector served as the negative control (NC). The pcDNA3.1(+) vector encoding galectin-3 (1 μ g) was subsequently transfected into the cardiomyocytes using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. All assays were performed 48 h following transfection.

Cell viability analysis. Following transfection, cardiomyocytes were plated into 96-well plates at 1x10³ cells/well in a final volume of 100 μ l, and cultured overnight. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay, according to the manufacturer's instructions. In brief, at 24, 48 and 72 h following transfection, 10 μ l CCK-8 solution was added to each well and incubated for 1 h at 37°C and 5% CO₂. Following incubation, absorbance was measured at 450 nm using a microplate reader (SM600 Labsystem; Shanghai Utrao Medical Instrument Co., Ltd., Shanghai, China).

Cell cycle and apoptosis analysis. For the cell cycle assay, 3x10⁴ transfected cells were collected and fixed in 70% ethanol at -20°C overnight. The cells were subsequently washed in PBS and resuspended in staining solution containing 20 μ g/ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 200 μ g/ml RNase A. Cell cycle was assessed by flow cytometry, using BD Accuri C6 software version 1.0.264.21 (BD Biosciences, Franklin Lakes, NJ, USA). For the cell apoptosis assay, 3x10⁴ transfected cells were collected by trypsinization (Jrdun Biotechnology Co., Ltd., Shanghai, China) and incubated with 195 μ l Annexin V-fluorescein isothiocyanate for 15 min at 4°C and 5 μ l PI for 5 min at 4°C, prior to analysis using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Following this, the results were subsequently analyzed using CellQuest software (version 5.1; BD Biosciences).

Animals and treatment. A total of 18 male Dahl salt-sensitive rats (weight, 200–250 g) purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were fed a high-salt (HS) diet (8.0% NaCl) starting at 8 weeks of age, which progresses to a model of congestive HF (16). Rats (6/cage) were housed in an animal facility at 25°C, with a relative humidity of 60–70% and received food and water *ad libitum*. After 1 week on the HS diet, 25 μ g/kg lentivirus containing gal-3 short hairpin (sh)RNA sequence (pLKO-shGAL3 cat. TRC0000029305; OpenBiosystems; Thermo Fisher Scientific, Inc.) was intravenously injected via the tail vein (6/group). The shRNA scramble sequence was used as a transduction control for 3 weeks. After 4 weeks on the HS diet, the rats were sacrificed and weighed. Cardiac tissue samples were collected at the indicated time-points for molecular or histological examination, which were frozen at -80°C and fixed with 4% paraformaldehyde for 48 h at 25°C. The survival time of rats with galectin-3 knockdown treatment was measured during the 4 weeks. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (17). The study was approved by the ethics committee of Zhongnan Hospital of Wuhan University (Wuhan, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. For *in vitro* and *in vivo* RT-qPCR analyses, RNA was extracted from cardiomyocytes or cardiac tissue with TRIzol (Life Technologies; Thermo Fisher Scientific, Inc.). A total of 1 μ g RNA was used to generate cDNA using a M-MLV RT Reagent kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). To detect the level of galectin-3, proliferating cell nuclear antigen (PCNA), B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax), qPCR was performed with a SYBR Green qPCR Master mix (Takara Biotechnology Co., Ltd., Dalian, China) and data collection was conducted using an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. GAPDH was used as an endogenous control for normalization. The gene expression was calculated using the 2^{- $\Delta\Delta$ C_q} method (18). The primers (Sangon Biotech Co., Ltd.) used were as follows: Galectin-3 forward, 5'-CATTGTGTGTAACACGAAGCAGGAC-3' and reverse, 5'-CTGCAGTAGGTGAGCATCGTTGA-3'; GAPDH forward, 5'-GGAATCCACTGGCGTCTTCA-3' and reverse, 5'-GGTTCACGCCCATCACAAAC-3'; Bcl-2 forward, 5'-CCACCTGTGGTCCATCTGAC-3' and reverse, 5'-CAATCCTCCCCCAGTTCACC-3'; Bax forward, 5'-GTCATCTCGTCTGGTACGG-3' and reverse, 5'-CACACACAAAGCTGCTCC-3'; PCNA forward, 5'-CCCTGGAGCCCTTGAAGAAG-3' and reverse, 5'-AGATGCACAACTTCTCGGCA-3'.

Western blot analysis. Mouse monoclonal antibodies against galectin-3 (1:1,000; cat. no. sc-374253) and the rabbit polyclonal antibodies against Bax (1:500; cat. no. sc-493) and

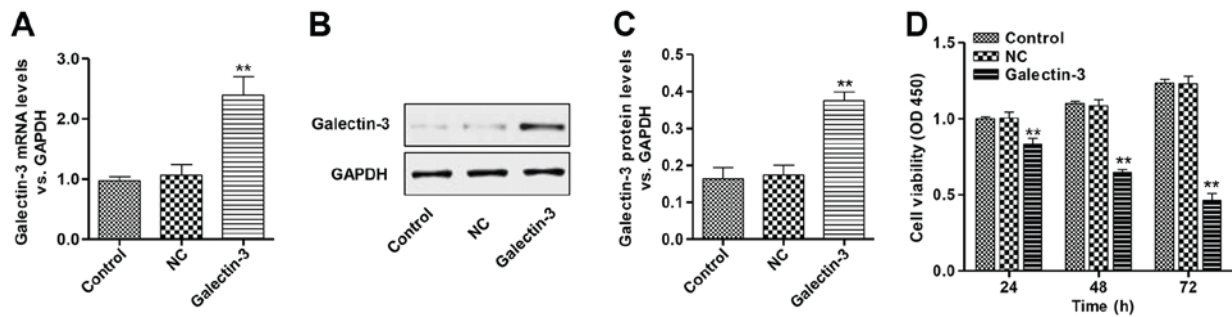


Figure 1. Effects of galectin-3 overexpression on the cell viability of cardiomyocytes. (A) Reverse transcription-quantitative polymerase chain reaction analysis of galectin-3 expression profiles in three treatment groups. (B) Western blot analysis and (C) semi-quantification of galectin-3 expression profiles in three treatment groups. (D) Cell viability of cardiomyocytes with galectin-3 overexpression was measured by Cell Counting Kit-8 assay. Data are presented as the mean \pm standard deviation (n=3). **P<0.01 vs. NC group. NC, negative control; OD, optical density.

Bcl-2 (1:1,000; cat. no. sc-492) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit monoclonal antibodies against PCNA (1:1,000; cat. no. 13110) and GAPDH (1:1,000; cat. no. 5174) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit (1:1,000; cat. no. A0208) and anti-mouse (1:1,000; cat. no. A0216) secondary antibodies were purchased from Beyotime Institute of Biotechnology (Haimen, China). Proteins were detected by western blot analysis as previously described (19). The total cell lysates were extracted using radioimmunoprecipitation buffer (Amyjet Scientific, Inc., Wuhan, China) according to the manufacturer's instructions. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (cat. no. PICPI23223; Thermo Fisher Scientific, Inc.). A total of 15 μ l of proteins were subjected to 10 or 15% SDS-PAGE, transferred to polyvinylidene fluoride membranes (Sigma-Aldrich; Merck KGaA) and blocked in fat-free milk overnight at 4°C. The membranes were subsequently incubated with primary antibodies for 2 h at 25°C and then with secondary antibodies for 1 h at 37°C, and were visualized using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA) and signals were semi-quantified by densitometry (Quantity One software, version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data were presented as the mean \pm standard deviation with three repeats and were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were assessed using a one-way analysis of variance, followed by Tukey's post hoc test. Survival analysis was performed by the Kaplan-Meier method, and subjected to the log rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Galectin-3 overexpression inhibits the viability of cardiomyocytes. To investigate the role of galectin-3 in cardiomyocytes, a galectin-3 overexpression vector was constructed; the galectin-3 coding sequence was cloned into a pCDNA3.1(+) vector, and the empty vector was used as NC. At 48 h after transfection, the expression of galectin-3 was determined using RT-qPCR (Fig. 1A) and western blotting

(Fig. 1B and C). The overexpression vector significantly increased the expression of galectin-3 in cardiomyocytes at the mRNA and protein levels.

To determine the impact of galectin-3 overexpression on the viability of cardiomyocytes, the cell viability of galectin-3-overexpressing cardiomyocytes was analyzed by CCK-8 assay. Cell viability was significantly reduced in the galectin-3-overexpressing cardiomyocytes compared with the control and NC groups (Fig. 1D). There was no significant difference between the control and NC groups. These data suggest galectin-3 may have a role in the cell viability of cardiomyocytes.

Galectin-3 overexpression induces cell cycle arrest and apoptosis of cardiomyocytes. The potential inhibitory effect of galectin-3 overexpression on cell cycle progression was investigated. As presented in Fig. 2A, overexpression of galectin-3 resulted in a lower number of cells in the G₀-G₁ phase (54.54 \pm 4.18%) compared with the NC group (69.11 \pm 3.17%). Furthermore, there was a higher number of cells in the S (26.94 \pm 1.39%) and G₂-M phases (16.64 \pm 1.52%), compared with the corresponding NC groups (S phase, 19.59 \pm 2.24%; G₂-M phase, 9.86 \pm 1.46%). These data suggest that galectin-3 overexpression may induce cell cycle arrest at S and G₂-M phases, which may be associated with reduced cell viability in galectin-3-overexpressing cardiomyocytes.

To assess the effects of galectin-3 overexpression on cell apoptosis, Annexin V/PI staining was performed (Fig. 2B). The ratio of cells undergoing apoptosis was significantly increased to 27.17 \pm 2.32% in galectin-3-overexpressing cardiomyocytes, compared with the NC group (4.33 \pm 1.31%). These results indicate that galectin-3 may promote apoptosis in cardiomyocytes.

Effects of galectin-3 overexpression on PCNA, Bcl-2 and Bax expression in cardiomyocytes. PCNA is a protein that is synthesized in early G₁ and S phases of the cell cycle and functions in cell cycle progression, DNA replication and DNA repair (20). The proteins of the Bcl-2 family perform critical roles in the regulation of apoptosis by functioning as promoters (i.e., Bax) or as inhibitors (i.e., Bcl-2) of cell death progression (21). RT-qPCR and western blot analysis were performed to detect the mRNA and protein expression levels of PCNA, Bcl-2 and Bax. Galectin-3 overexpression resulted in a marked reduction in the levels of PCNA and Bcl-2, with a concomitant increase in the level of Bax compared with the corresponding NC groups (Fig. 3). These

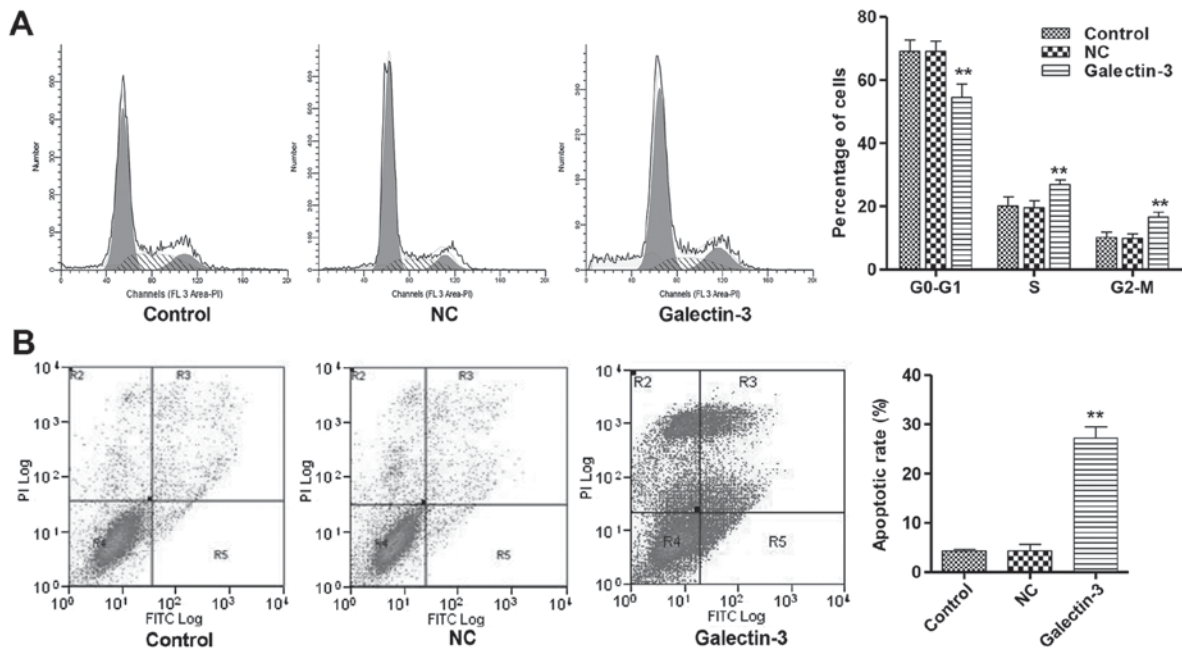


Figure 2. Effects of galectin-3 overexpression on cell cycle progression and apoptosis of cardiomyocytes. (A) Cell cycle analysis of cardiomyocytes with galectin-3 overexpression was performed by PI staining and flow cytometry. (B) Apoptosis of cardiomyocytes with galectin-3 overexpression was measured by Annexin V-FITC/PI staining and flow cytometry. Data are presented as the mean \pm standard deviation ($n=3$). ** $P<0.01$ vs. NC group. PI, propidium iodide; FITC, fluorescein isothiocyanate; NC, negative control.

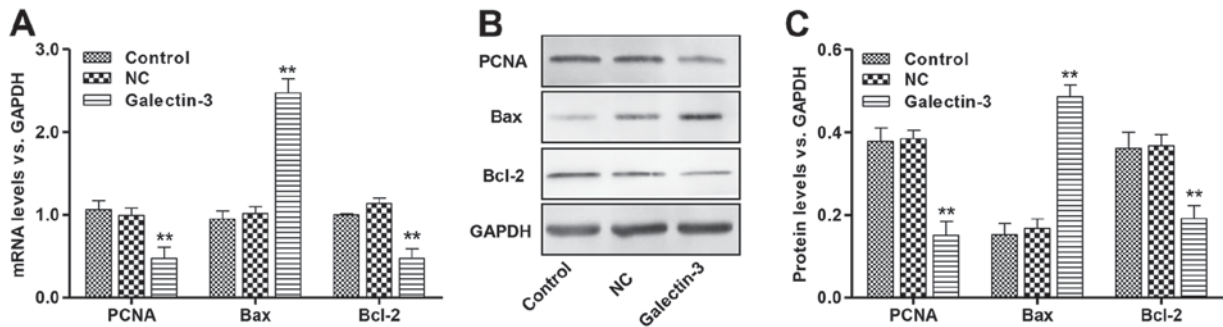


Figure 3. Effects of galectin-3 overexpression on PCNA, Bcl-2 and Bax expression in cardiomyocytes. (A) Reverse transcription-quantitative polymerase chain reaction of PCNA, Bcl-2 and Bax expression profiles in the three treatment groups. (B) Western blot analysis and (C) semi-quantification of PCNA, Bcl-2 and Bax expression profiles in the three treatment groups. Data are presented as the mean \pm standard deviation ($n=3$). ** $P<0.01$ vs. NC group. PCNA, proliferating cell nuclear antigen; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; NC, negative control.

results demonstrate that galectin-3 overexpression may decrease PCNA expression and decrease the ratio of Bcl-2/Bax, which may contribute to the observed increase in cell apoptosis.

Therapeutic silencing of galectin-3 improves cardiomyocyte survival during heart failure. The potential therapeutic impact of galectin-3 inhibition was investigated *in vivo*. Dahl salt-sensitive rats were fed a HS diet (8.0% NaCl), which causes the rats to progress to a model of heart failure. After 1 week on the HS diet, rats were intravenously injected with galectin-3 shRNA or shNC, stably expressed in the pLKO.1 lentiviral vector. Knockdown of galectin-3 significantly decreased the expression levels of galectin-3 in the cardiac tissue of rats with heart failure (Fig. 4A-C). After 4 weeks on the HS diet, the control and shNC rats started to exhibit signs of discomfort and were sacrificed; however, intravenous injection of galectin-3 shRNA significantly increased the

survival rate (Fig. 4D). As a surrogate indicator of overall health, body weight was regularly monitored for the duration of the study (1). All the rats gained weight together until day 14 of the HS diet, whereas rats in the control and shNC groups after day 14 of HS diet exhibited significantly reduced weight gain, compared with the rats injected with galectin-3 shRNA (Fig. 4E).

Effect of galectin-3 silencing on PCNA, Bcl-2 and Bax expression in Dahl hypertensive rats. To investigate the regulation of proliferation- and apoptosis-associated genes, PCNA, Bcl-2 and Bax mRNA and protein levels were assessed in the cardiac tissue of Dahl hypertensive rats. PCNA and Bcl-2 mRNA levels were significantly increased in HS-induced rats injected with galectin-3 shRNA (Fig. 5A). Furthermore, treatment with galectin-3 shRNA significantly decreased the Bax mRNA expression level in HS-induced rats. The altered regulation

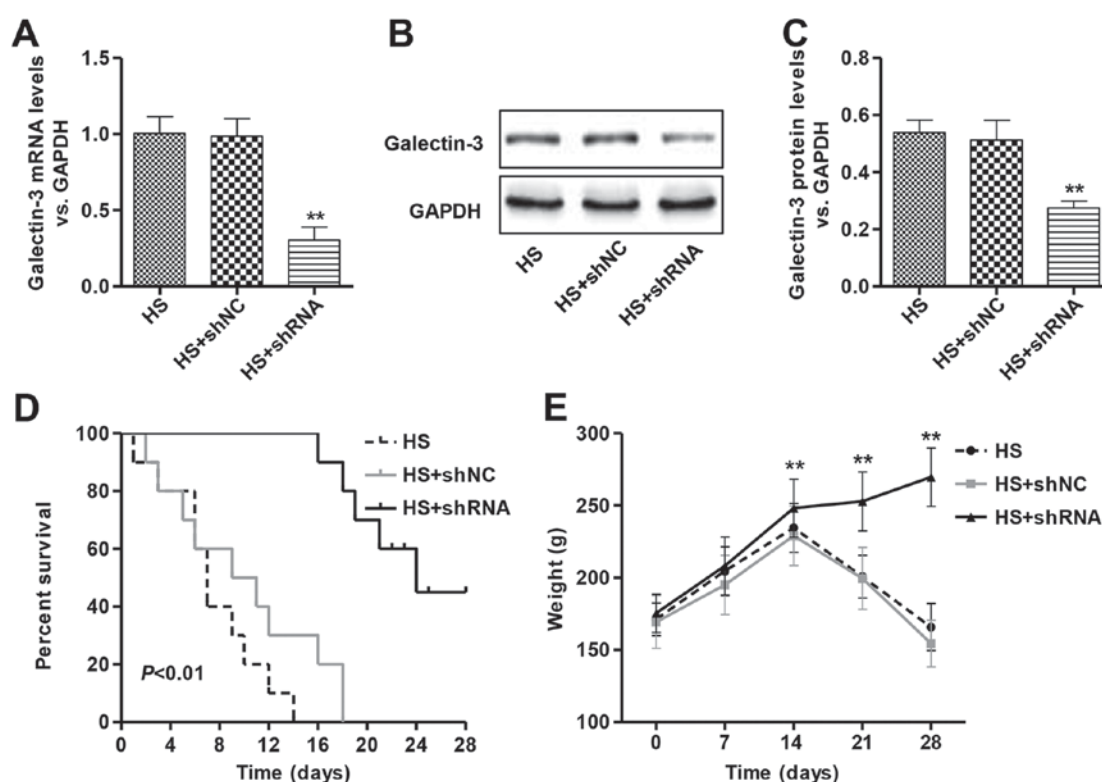


Figure 4. Therapeutic effect of galectin-3 knockdown on Dahl hypertensive rats. (A) Reverse transcription-quantitative polymerase chain reaction of galectin-3 expression profiles in the cardiac tissue of hypertensive rats. (B) Western blot analysis and (C) semi-quantification of galectin-3 expression profiles in the cardiac tissue of hypertensive rats. (D) Kaplan-Meier survival curves of the Dahl hypertensive rat model, demonstrating a pronounced decrease in survival in response to an 8% HS diet. Survival is significantly improved with galectin-3 inhibition treatment. (E) Body weight analysis of Dahl hypertensive rats on the 8% HS diet reveals significant reductions in weight after 14 days. This was not observed in rats that received galectin-3 inhibition treatment. Data are presented as the mean \pm standard deviation (n=6/group). **P<0.01 vs. HS + shNC group. HS, high-salt; shRNA, short hairpin RNA; shNC, negative control shRNA.

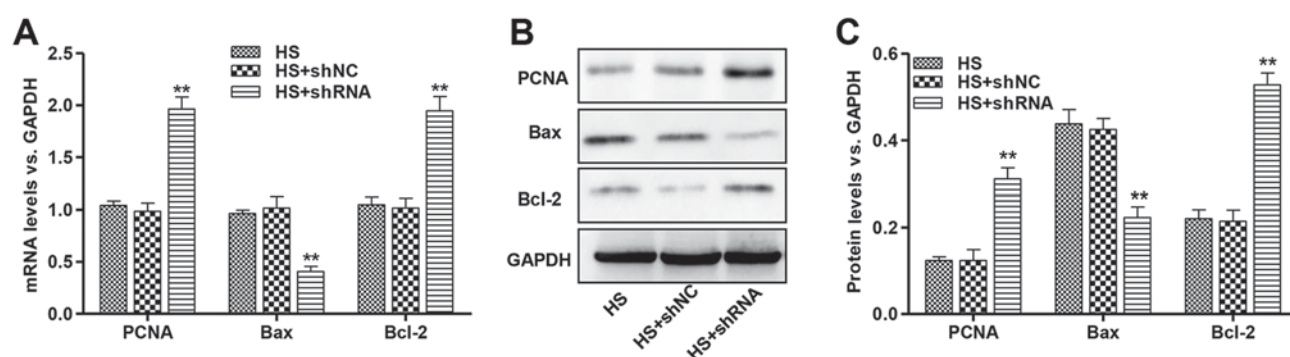


Figure 5. Effect of galectin-3 knockdown on PCNA, Bcl-2 and Bax expression in Dahl hypertensive rats. (A) Reverse transcription-quantitative polymerase chain reaction analysis of PCNA, Bcl-2 and Bax expression profiles in the cardiac tissue of hypertensive rats after 4 weeks on the HS diet. (B) Western blot analysis and (C) semi-quantification of PCNA, Bcl-2 and Bax expression profiles in the cardiac tissue of hypertensive rats after 4 weeks on the HS diet. Data are presented as the mean \pm standard deviation (n=6). **P<0.01 vs. HS+shNC groups. PCNA, proliferating cell nuclear antigen; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; HS, high-salt; shRNA, short hairpin RNA; shNC, negative control shRNA.

of these proteins was confirmed by western blot analysis (Fig. 5B and C). These results demonstrate that galectin-3 knockdown may increase PCNA and the Bcl-2/Bax ratio, which may lead to an increase in cardiomyocyte survival.

Discussion

Galectin-3 is expressed in various tissues and cell types and is involved in several biological processes, including cell proliferation, cell cycle, apoptosis and angiogenesis (22).

An increased concentration of galectin-3 has been identified in patients with HF and this protein may be promising for high-risk patient identification (23). The present study demonstrated that higher levels of galectin-3, a marker of cardiac fibrosis (24), are associated with the development and progression of HF. To the best of our knowledge, this is the first study to report the association of galectin-3 with cardiomyocyte proliferation and apoptosis *in vitro*, and with survival *in vivo*. The present study has also indicated that the HF-promoting galectin-3 mechanisms may be associated

with decreased expression of PCNA and Bcl-2, and increased expression of Bax.

Experimental evidence has suggested that galectin-3 is the most predominantly overexpressed gene in transgenic Ren-2 rats that progress to HF (7). The present study revealed that overexpression of galectin-3 in cardiomyocytes resulted in suppression of cell proliferation in a time-dependent manner, accompanied by apoptosis and cell cycle arrest at S and G₂-M phases. These data suggest that galectin-3 overexpression may contribute to cardiac dysfunction by inducing cardiomyocyte cell cycle arrest and apoptosis. In agreement with these findings, infusion of galectin-3 into the pericardial space leads to cardiac dysfunction in rats, a process that appears to be mediated via the transforming growth factor- β /SMAD family member 3 signaling pathway (8). However, several studies have indicated that galectin-3 overexpression regulates the cell cycle and apoptosis via alterations in the expression levels of cell cycle regulators, including cyclin D1. Notably, the growth-promoting activity of galectin-3 is predominantly dependent on cyclin D1 promoter activity (25,26). The expression of PCNA and Bcl-2 were significantly decreased in galectin-3 overexpressing cardiomyocytes, whereas the Bax expression was elevated, suggesting that galectin-3 may arrest the cell cycle and induce apoptosis via regulation of the PCNA and Bcl-2/Bax pathways.

Experimental evidence for the involvement of galectin-3 in HF development came from studies that galectin-3 inhibition in mice resulted in a marked reduction of cardiac fibrosis, and thus, may be beneficial in the prevention of HF (8,27). Similarly, in the present study, the therapeutic effects of galectin-3 inhibition in the Dahl hypertensive rats provided evidence that intravenous tail vein injections of galectin-3-shRNA is sufficient to deliver galectin-3-shRNA effectively to the heart in vivo, and that galectin-3 inhibition results in the increased survival and weight gain of Dahl hypertensive rats, compared with rats without galectin-3 inhibition. Cell cycle and apoptosis-associated proteins, including PCNA, Bcl-2 and Bax, were also detected in Dahl hypertensive rats. The results indicated that galectin-3 inhibition significantly increased the expression of PCNA and Bcl-2, and decreased the expression of Bax, compared with Dahl hypertensive rats without galectin-3 inhibition. These results suggested that PCNA and the Bcl-2/Bax pathway were also involved in the galectin-3 silencing-dependent promotion of the survival and weight gain of Dahl hypertensive rats.

Subsequent pharmacokinetic and efficacy studies in larger mammals are required to establish whether inhibition of galectin-3 is able to establish a comparable therapeutic effect in larger animals. In addition, therapeutic galectin-3 inhibition would likely involve combination with the current standard treatments in used for patients with HF; thus, it will be important to assess whether galectin-3 inhibition, in conjunction with current treatments, adds to the beneficial effects of these drugs. Taken together, this study demonstrates that overexpression of galectin-3 in cardiomyocytes inhibits cell proliferation, arrests the cell cycle and induces apoptosis. Galectin-3 inhibition in Dahl hypertensive rats increased survival and further validates galectin-3 as a potential target for cardiac disease therapy.

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