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Original article

High-mobility group box 1-mediated heat shock protein beta 1 expression attenuates mitochondrial dysfunction and apoptosis

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ABSTRACT

Aims: Apoptosis of cardiomyocytes is thought to account for doxorubicin cardiotoxicity as it contributes to loss of myocardial tissue and contractile dysfunction. Given that high-mobility group box 1 (HMGB1) is a nuclear DNA-binding protein capable of inhibiting apoptosis, we aimed to clarify the role of HMGB1 in heat shock protein beta 1 (HSPB1) expression during doxorubicin-induced cardiomyopathy.

Methods and results: Mitochondrial damage, cardiomyocyte apoptosis, and cardiac dysfunction after doxorubicin administration were significantly attenuated in mice with cardiac-specific overexpression of HMGB1 (HMGB1-Tg) compared with wild type (WT) -mice. HSPB1 levels after doxorubicin administration were significantly higher in HMGB1-Tg mice than in WT mice. Transfection with HMGB1 increased the expression of HSPB1 at both the protein and mRNA levels, and HMGB1 inhibited mitochondrial dysfunction and apoptosis after exposure of cardiomyocytes to doxorubicin. HSPB1 silencing abrogated the inhibitory effect of HMGB1 on cardiomyocyte apoptosis. Doxorubicin increased the binding of HMGB1 to heat shock factor 2 and enhanced heat shock element promoter activity. Moreover, HMGB1 overexpression greatly enhanced heat shock element promoter activity. Silencing of heat shock factor 2 attenuated HMGB1-dependent HSPB1 expression and abrogated the ability of HMGB1 to suppress cleaved caspase-3 accumulation after doxorubicin stimulation.

Conclusions: We report the first *in vivo* and *in vitro* evidence that cardiac HMGB1 increases HSPB1 expression and attenuates cardiomyocyte apoptosis associated with doxorubicin-induced cardiomyopathy. Cardiac HMGB1 increases HSPB1 expression in cardiomyocytes in a heat shock factor 2-dependent manner.

1. Introduction

Doxorubicin, an anthracycline derivative, is an anticancer drug effective against various types of cancer [1,2]. However, the clinical utility of doxorubicin is limited by its dose-dependent cardiotoxicity, which causes dilated cardiac dysfunction and congestive heart failure associated with doxorubicin cardiomyopathy [3]. For example, approximately 4% of patients treated with doxorubicin present with dominant heart failure symptoms following administration of a cumulative doxorubicin dose of 500–550 mg/m^2, with a frequency of approximately 18% for doses of 551–600 mg/m^2, and 36% for doses in excess of 600 mg/m^2 [4]. The mechanism responsible for this cardiotoxicity is thought to be related to cardiomyocyte apoptosis [5,6], which contributes to myocardial tissue loss and severe contractile dysfunction [7].

High-mobility group box 1 (HMGB1) is a nuclear non-histone DNA-binding protein with key roles in maintaining nuclear homeostasis [8,9]. HMGB1 is highly conserved among species and organs, indicating its critical role in the modulation of cellular functions. Furthermore, the fact that HMGB1 knockout mice die shortly after birth and exhibit severe hypoglycemia shows that HMGB1 is essential for survival [10]. Previous study showed that increased HMGB1 expression inhibits apoptosis in various types of cancer cells [11,12]. Our previous studies showed that cardiac-specific HMGB1 overexpression plays a key role

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Table 1
Comparisons of histological and echocardiographic findings of HMGB1-Tg mice and their WT counterparts with and without doxorubicin administration.

	DOX (−)		DOX (+)	
	WT	HMGB1-Tg	WT	HMGB1-Tg
Histology				
Pre-BW (g)	21.9 ± 1.0	23.0 ± 1.0	22.5 ± 1.3	22.5 ± 0.6
Post-BW (g)	20.7 ± 1.5	22.4 ± 0.7	18.2 ± 1.4 **	18.0 ± 0.4 **
HW (mg)	118.0 ± 8.6	129.8 ± 5.9	94.8 ± 5.1 **	107.2 ± 4.0 *#
LW (mg)	106.0 ± 5.0	103.2 ± 9.2	146.4 ± 24.6**	113.0 ± 10.9#
HW/TL (mg/mm)	7.2 ± 1.6	7.6 ± 1.4	5.6 ± 9.2**	6.5 ± 1.2*#
LW/TL (mg/mm)	5.5 ± 3.0	5.4 ± 2.5	7.6 ± 1.3**	5.9 ± 5.9*#
Echocardiography				
HR (beats/min)	514 ± 5	516 ± 10	514 ± 8	514 ± 4
IVSd (mm)	0.74 ± 0.01	0.76 ± 0.01	0.55 ± 0.01 **	0.64 ± 0.01 *#
PWd (mm)	0.76 ± 0.01	0.76 ± 0.02	0.55 ± 0.01 **	0.65 ± 0.01 *#
LVEDD (mm)	3.18 ± 0.02	3.15 ± 0.01	3.49 ± 0.02 **	3.13 ± 0.02 *#
LVESD (mm)	1.43 ± 0.03	1.44 ± 0.02	2.04 ± 0.01**	1.61 ± 0.01*#
FS (%)	54.6 ± 0.4	55.3 ± 0.3	44.5 ± 0.1 **	50.4 ± 0.2 *#

Data are shown as the mean ± SEM; $n = 12$ each; * $p < 0.05$, ** $p < 0.01$ vs. vehicle treated WT-mice; # $p < 0.05$ vs. doxorubicin treated WT-mice.
DOX, doxorubicin; WT, wild-type littermates mice; HMGB1, high-mobility group box 1; HMGB1-Tg, cardiac-specific overexpression of HMGB1 mice; BW, body weight; HW, heart weight; LW, lung weight; TL tibial length; HR, heart rate; IVSd, intraventricular septum diameter; PWd, posterior wall diameter; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; FS = (LVEDD − LVESD) / LVEDD × 100.

in cardioprotection against myocardial infarction and pressure overload [13,14]. However, the mechanisms underlying the attenuation of cardiomyocyte dysfunction by cardiac HMGB1 remain largely unknown.

Heat shock protein beta 1 (HSPB1), also known as HSP-27 in humans, is a member of the small heat shock protein family and is involved in a wide variety of cellular processes. HSPB1 was originally described as an intracellular chaperone able to stabilize the actin cytoskeleton in response to various stresses [15]. Moreover, HMGB1 regulates HSPB1 expression and mitochondrial quality, which are associated with cell susceptibility to apoptosis in mouse embryonic fibroblasts [16]. However, to the best of our knowledge, the role of cardiac HMGB1 in regulation of HSPB1 expression and cardiac apoptosis has not been documented. Therefore, the present study clarifies the impact of cardiac HMGB1 on doxorubicin-induced cardiomyopathy.

2. Materials and methods

The methods/protocols used in the present study are detailed in the online data supplement.

2.1. Ethics statement

All experimental procedures were performed according to the animal welfare regulations of Yamagata University School of Medicine; the study protocol was approved by the Animal Subjects Committee of the university. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

2.2. Neonatal rat cardiomyocyte culture and treatment

Hearts were promptly collected from neonatal rat pups after euthanasia by decapitation, and primary cultures of neonatal rat cardiomyocytes were established [13,17,18]. HMGB1 small interfering RNA (siRNA; siHMGB1), HSPB1 siRNA (siHSPB1), pGEX-HMGB1, heat shock factor 2 short hairpin RNA (shRNA; shHSF2), and pGL4.41 (luc2P/HSE/Hygro) were transfected into cardiomyocytes by using GenomOne-Neo (Ishihara Sangyo Kaisha, Osaka, Japan) or Lipofectamine LTX plus (Life Technologies Japan, Tokyo, Japan), according to the manufacturers' instructions. Neonatal rat cardiomyocytes were stimulated with 0.5 M doxorubicin (Wako Jyunyaku Kogyo, Osaka, Japan) for 6 h.

2.3. In vivo experimental design

Eight-to-ten-week-old mice with cardiac-specific overexpression of HMGB1 (HMGB1-Tg) [14] and their wild-type (WT) littermates were injected intraperitoneally with normal saline (10 μL/mg) or a single dose of doxorubicin (17.5 mg/kg, 10 μL/mg). The mice were sacrificed 7 days after treatment with an intraperitoneal injection of ketamine (1 g/kg) and xylazine (100 mg/kg), and their hearts were immediately excised.

2.4. Western blotting

All proteins were extracted from the left ventricle and neonatal rat cardiomyocytes by homogenization in ice-cold lysis buffer [19]. Mitochondrial and cytosolic proteins were prepared using a mitochondrial isolation kit (Thermo Scientific, Rockford, IL, USA). Co-immunoprecipitation and immunoblotting were performed as previously described [13].

2.5. Real-time reverse transcription polymerase chain reaction

Real-time RT-PCR amplification was performed as previously described [20,21], and gene expression levels were normalized relative to levels of transcripts that encode glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were designed using sequences deposited in GenBank (HSPB1, NM_031970; GAPDH, NM_017008).

2.6. Transmission electron microscopy and scanning electron microscopy analysis

Cardiac tissues were fixed in 2.5% glutaraldehyde buffer. Ultrathin sections (80 nm) were cut on an RMC MTXL ultra microtome [22]. Grids were examined using a transmission electron microscope (HITACHI H-7100; Hitachi High-Technologies Corporation, Tokyo, Japan) and scanning electron microscope (HITACHI S-5000S; Hitachi High-Technologies Corporation).

2.7. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was visualized in cardiomyocytes stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Cayman Chemical

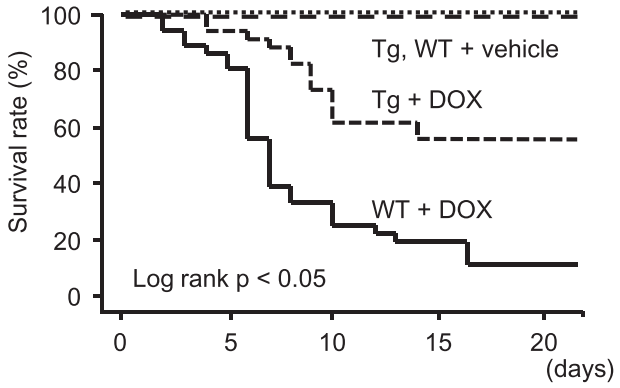


Fig. 1. Survival rate after doxorubicin administration in HMGB-1 Tg mice. Kaplan–Meier curves showing significantly higher survival rate after doxorubicin administration in HMGB1-Tg mice compared with those in WT mice ($n = 15$ each). DOX, doxorubicin; WT, wild-type littermates; HMGB1, high-mobility group box 1; HMGB1-Tg, mice with cardiac-specific HMGB1 overexpression.

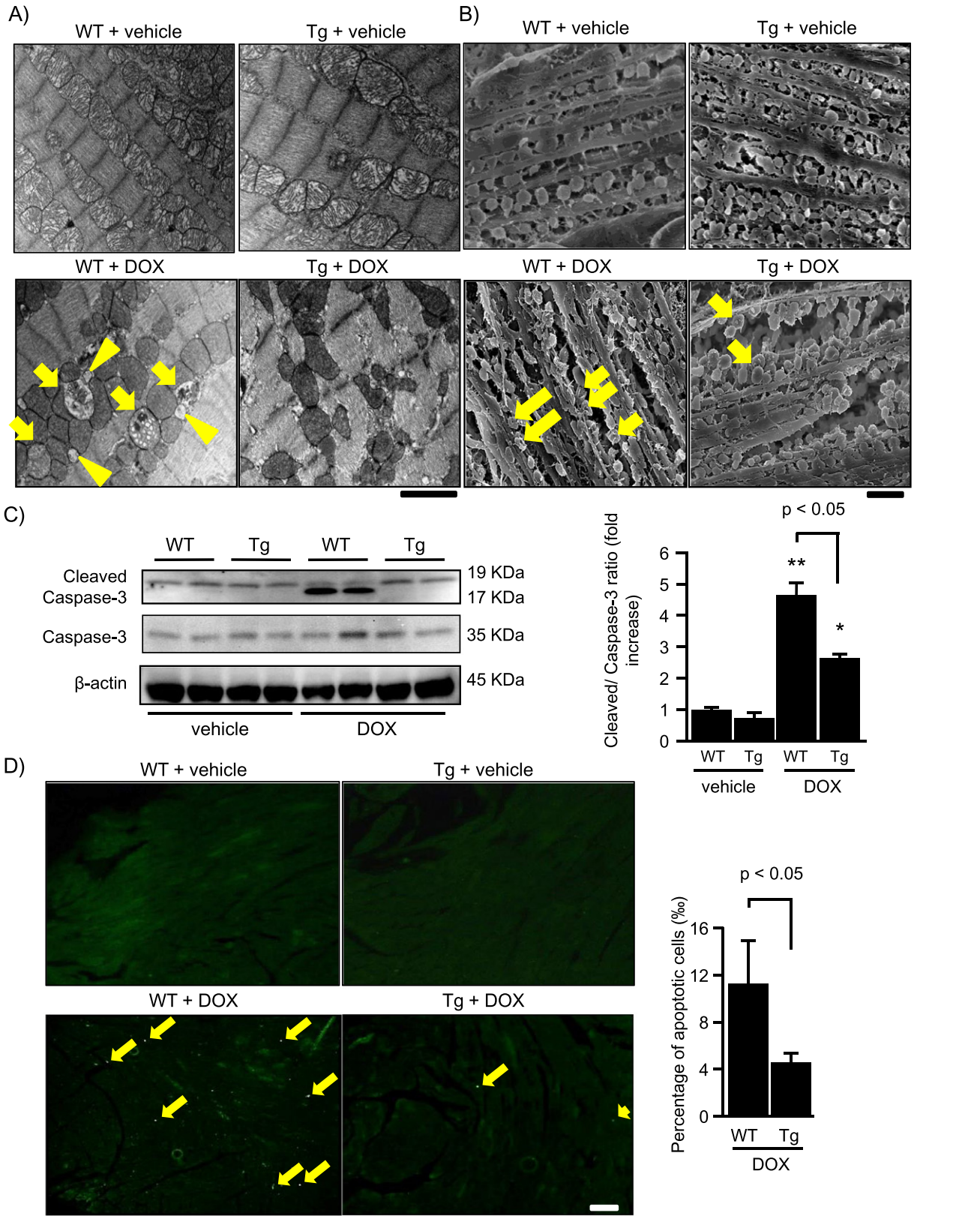


Fig. 2. Mitochondrial pathomorphological changes and cardiomyocyte apoptosis after doxorubicin administration in HMGB1-Tg mice. (A) Mitochondrial pathomorphological changes observed by transmission electron microscopy. Extensive mitochondrial collapse and vacuolization were observed in sections from doxorubicin-treated WT mice but not in doxorubicin-treated HMGB1-Tg mice. Arrows and arrowheads indicate mitochondrial vacuolization and collapse, respectively. Experiments were performed independently at least three times, and representative figures are shown. Scale bar = 1 μm. (B) Extensive vacuolization, observed using scanning electron microscopy, was suppressed in doxorubicin-treated HMGB1-Tg mice compared to that in doxorubicin-treated WT mice. Arrows indicate mitochondrial vacuolation. Scale bar = 125 μm. (C) Caspase-3 cleavage was inhibited in HMGB1-Tg mice. Bars represent means ± SEM ($n = 6$ per group); * $p < 0.05$ and ** $p < 0.01$, compared to vehicle treated WT-mice. (D) Cardiomyocyte apoptosis after doxorubicin administration was extensive in WT mice, but rare in HMGB1-Tg mice. Arrows indicate apoptotic cells. Scale bar = 10 μm. Bars represent mean ± SEM ($n = 6$ mice per group). DOX, doxorubicin; WT, wild-type littermates; HMGB1, high-mobility group box 1; HMGB1-Tg, mice with cardiac-specific HMGB1 overexpression; SEM, standard error of the mean.

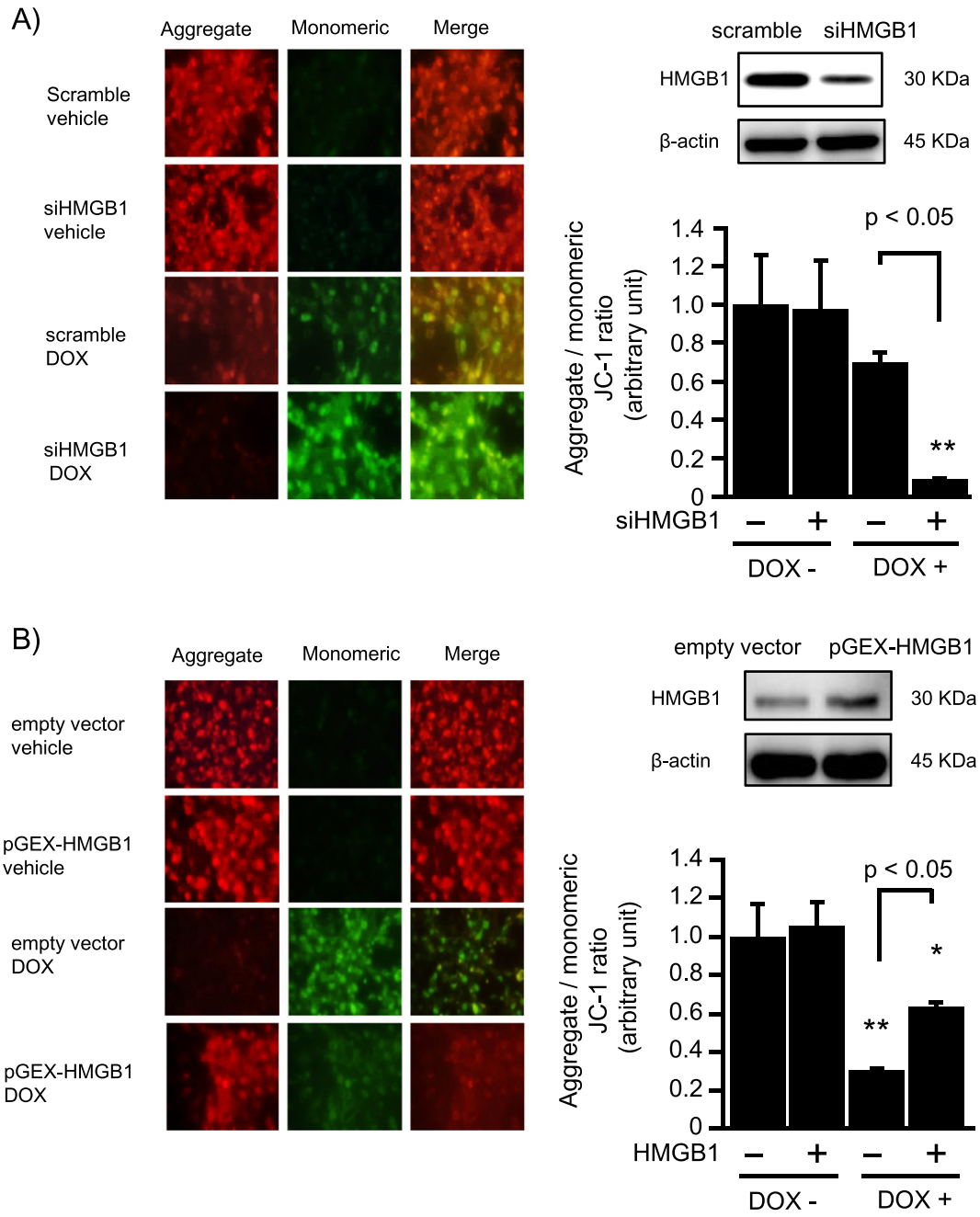


Fig. 3. Detection of mitochondrial membrane potential by JC-1 staining after doxorubicin treatment with elevated or suppressed cardiac HMGB1 levels. (A) Mitochondrial membrane potential after doxorubicin administration significantly decreased in neonatal rat cardiomyocytes following HMGB1 silencing. (B) Transfection with HMGB1 partially restored the doxorubicin-mediated decrease in mitochondrial membrane potential. Bars represent mean ± SEM ($n = 4$ each); * $p < 0.05$ and ** $p < 0.01$ compared to control. DOX, doxorubicin; HMGB1, high-mobility group box 1; si, small interfering RNA; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; SEM, standard error of the mean.

Co, Ann Arbor, MI, USA) using a laser scanning microscope (DMI3000B; Leica, Microsystems, Wetzlar, Germany) [23].

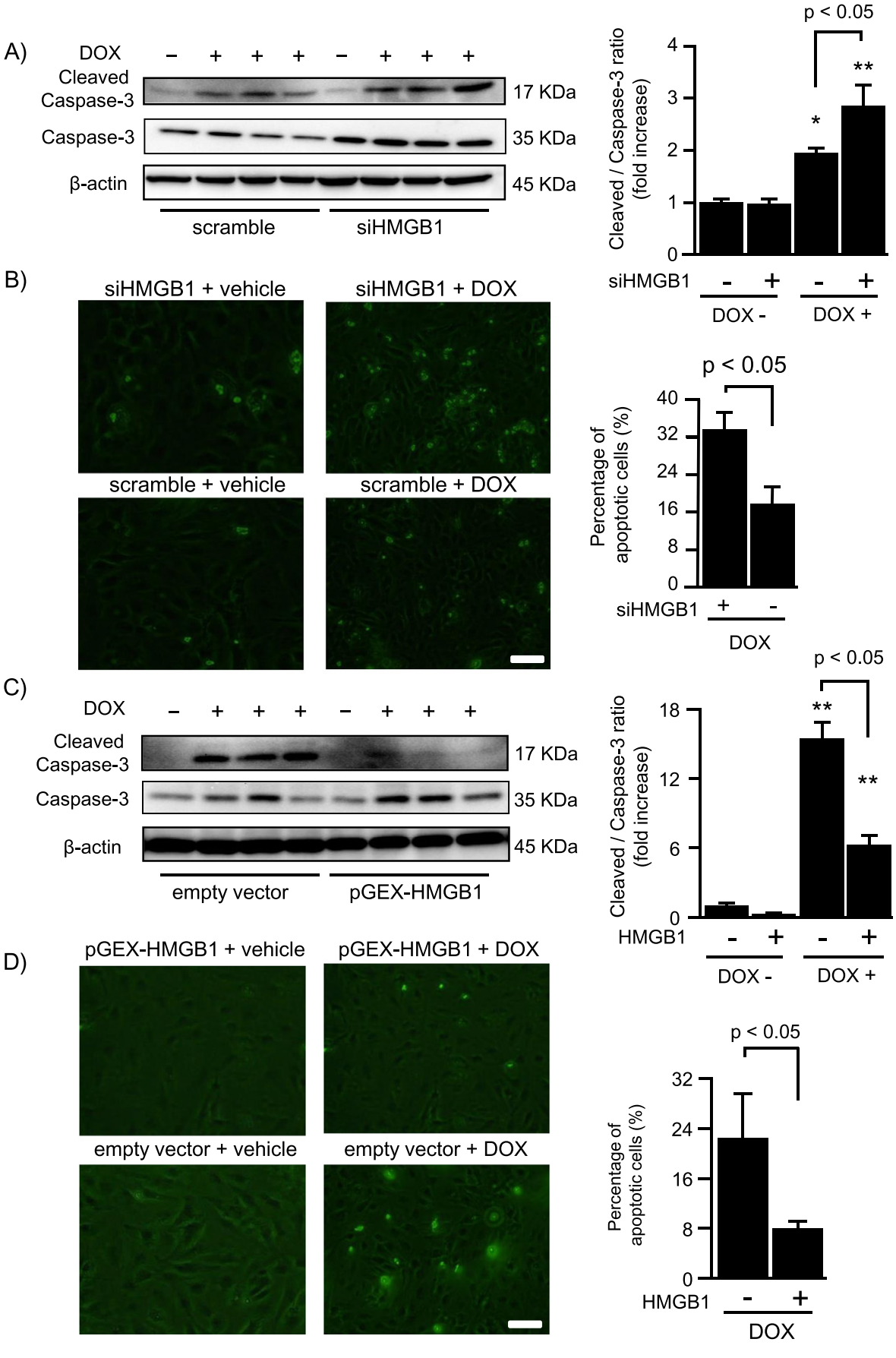
2.8. Echocardiography determination

Transthoracic echocardiography was recorded under anesthesia following intraperitoneal injection of pentobarbital sodium (35 mg/kg) with an FFsonic 8900 instrument (Fukuda Denshi Co., Tokyo, Japan) [20,22]. The adequacy of anesthesia was monitored at all times by assessing of skeletal muscle tone, respiration rate and rhythm, and response to tail pinch.

2.9. Detection of apoptotic cardiomyocytes

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using an In-Situ Cell

Fig. 4. HMGB1 modified cleaved caspase-3 abundance and cardiomyocyte apoptosis. (A) Cleaved caspase-3 levels after doxorubicin administration were significantly higher in neonatal rat cardiomyocytes after HMGB1 silencing ($n = 5$). (B) Cardiomyocyte apoptosis detected by TUNEL staining after doxorubicin administration was significantly increased in neonatal cardiomyocytes with HMGB1 silencing. Scale bar = 10 μm ($n = 5$). (C) Cleaved caspase-3 levels after doxorubicin administration were significantly suppressed in neonatal rat cardiomyocytes after HMGB1 overexpression ($n = 6$). (D) HMGB1 overexpression inhibited cardiomyocyte apoptosis after doxorubicin administration. Scale bar = 10 μm ($n = 5$). Bars represent mean ± SEM; * $p < 0.05$ and ** $p < 0.01$ compared to control. DOX, doxorubicin; HMGB1, high-mobility group box 1; SEM, standard error of the mean.


A)
DOX – + + + – + + +
Cleaved Caspase-3
Caspase-3
β-actin
17 KDa
35 KDa
45 KDa
scramble
siHMGB1
p < 0.05
Cleaved / Caspase-3 ratio (fold increase)
siHMGB1 - + - +
DOX - DOX +
B)
siHMGB1 + vehicle
siHMGB1 + DOX
scramble + vehicle
scramble + DOX
p < 0.05
Percentage of apoptotic cells (%)
siHMGB1 + -
DOX
C)
DOX – + + + – + + +
Cleaved Caspase-3
Caspase-3
β-actin
17 KDa
35 KDa
45 KDa
empty vector
pGEX-HMGB1
p < 0.05
Cleaved / Caspase-3 ratio (fold increase)
HMGB1 - + - +
DOX - DOX +
D)
pGEX-HMGB1 + vehicle
pGEX-HMGB1 + DOX
empty vector + vehicle
empty vector + DOX
p < 0.05
Percentage of apoptotic cells (%)
HMGB1 - +
DOX

Death Detection Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). For analysis, TUNEL-positive cardiomyocytes were counted in 10 randomly selected fields per section [7,17].

2.10. Chromatin immunoprecipitation assay

Hela cells were used for our chromatin immunoprecipitation assay. Hela cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM L-glutamine, and 4.5 g/L glucose. Chromatin immunoprecipitation assay with the use of HMGB1 antibody, followed by PCR analysis of the immunoprecipitated DNA fragments with primer that amplify the promoter region of *HSPB1* gene in Hela cells was performed using Shearing ChIP Kit (Nippon gene Co., LTD., Tokyo, Japan) and OneDay ChIP Kit (Nippon gene) according to the manufacturers' instructions.

2.11. Statistical analysis

Continuous data are presented as mean ± standard error of mean (SEM). Statistical differences among groups were evaluated with one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. Cumulative survival rates after administration of doxorubicin were computed using the Kaplan–Meier method and compared using the log-rank test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with a standard statistical program package (JMP version 10; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effect of cardiac HMGB1 on cardiac function in doxorubicin cardiomyopathy

We examined the role of cardiac HMGB1 in heart failure after doxorubicin administration. Heart weight, ratio of heart weight to tibial length, intraventricular septum diameter, posterior wall diameter, and fractional shortening were significantly reduced. Left ventricular end-diastolic diameter, left ventricular end-systolic diameter, lung weight, and ratio of lung weight to tibial length were significantly increased at one week after administration of doxorubicin (17.5 mg/kg), suggesting the presence of severe heart failure. However, these changes were prevented at least partially in HMGB1-Tg mice compared with WT-mice (Table 1). The survival rate after doxorubicin administration was significantly higher in HMGB1-Tg mice than in WT mice (Fig. 1).

3.2. Anti-apoptotic effect of HMGB1 on the heart after doxorubicin administration

Since mitochondrial disintegration is an early feature of doxorubicin-induced apoptosis, we evaluated mitochondrial morphology and caspase-3 cleavage, and performed TUNEL staining. Mitochondrial pathomorphological changes were observed by transmission electron microscopy (Fig. 2A) and by scanning electron microscopy (Fig. 2B) at one week after administration of doxorubicin. Extensive mitochondrial collapse and vacuolization were suppressed in doxorubicin-treated HMGB1-TG mice compared with doxorubicin-treated WT mice. Levels of cleaved caspase-3 were significantly lower in HMGB1-Tg mice than in WT mice after doxorubicin administration (Fig. 2C). Furthermore, cardiomyocyte apoptosis was reduced after doxorubicin administration in HMGB1-Tg mice as compared to that in WT mice (Figs. 2D and Supplemental Fig. I).

3.3. Effect of cardiac HMGB1 on mitochondrial membrane potential

To establish whether HMGB1 protects from doxorubicin-induced mitochondrial dysfunction, we used the JC-1 dye to assess mitochondrial membrane potential in neonatal rat cardiomyocytes before and after treatment with 0.5 M doxorubicin for 3 h. Doxorubicin decreased the ratio of red fluorescent signal (derived from aggregated dye) to green fluorescent signal (derived from the monomeric dye), implying decreased mitochondrial membrane potential after doxorubicin administration. *HMGB*1 silencing significantly decreased the JC-1 aggregate/monomer ratio after doxorubicin administration (Fig. 3A). In contrast, HMGB1 transfection prevented the decrease in the JC-1 aggregate/monomer ratio following doxorubicin administration (Fig. 3B), suggesting that HMGB1 is required for maintaining mitochondrial integrity following the exposure of cardiomyocytes to doxorubicin.

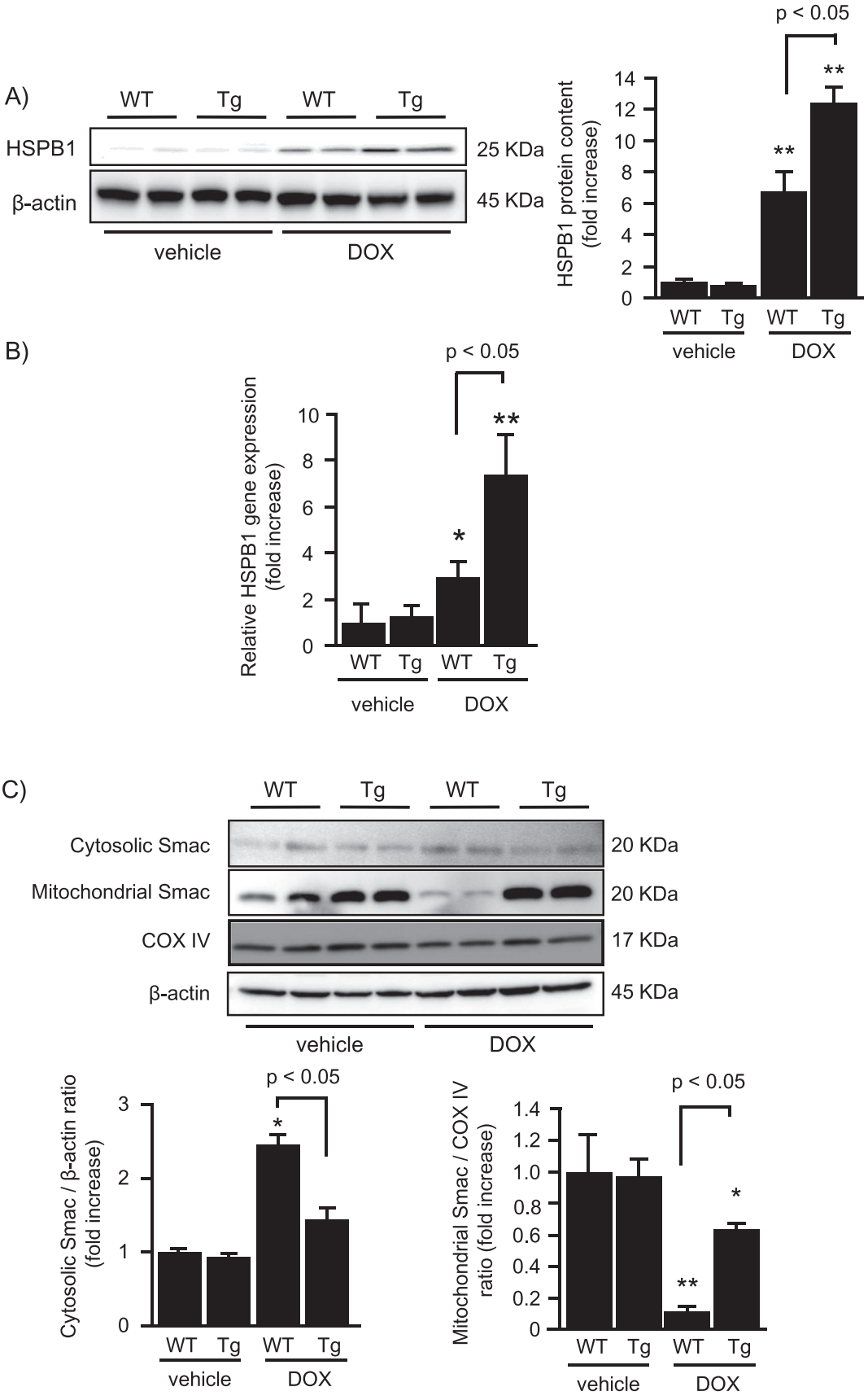
3.4. Impact of HMGB1 on cardiomyocyte apoptosis

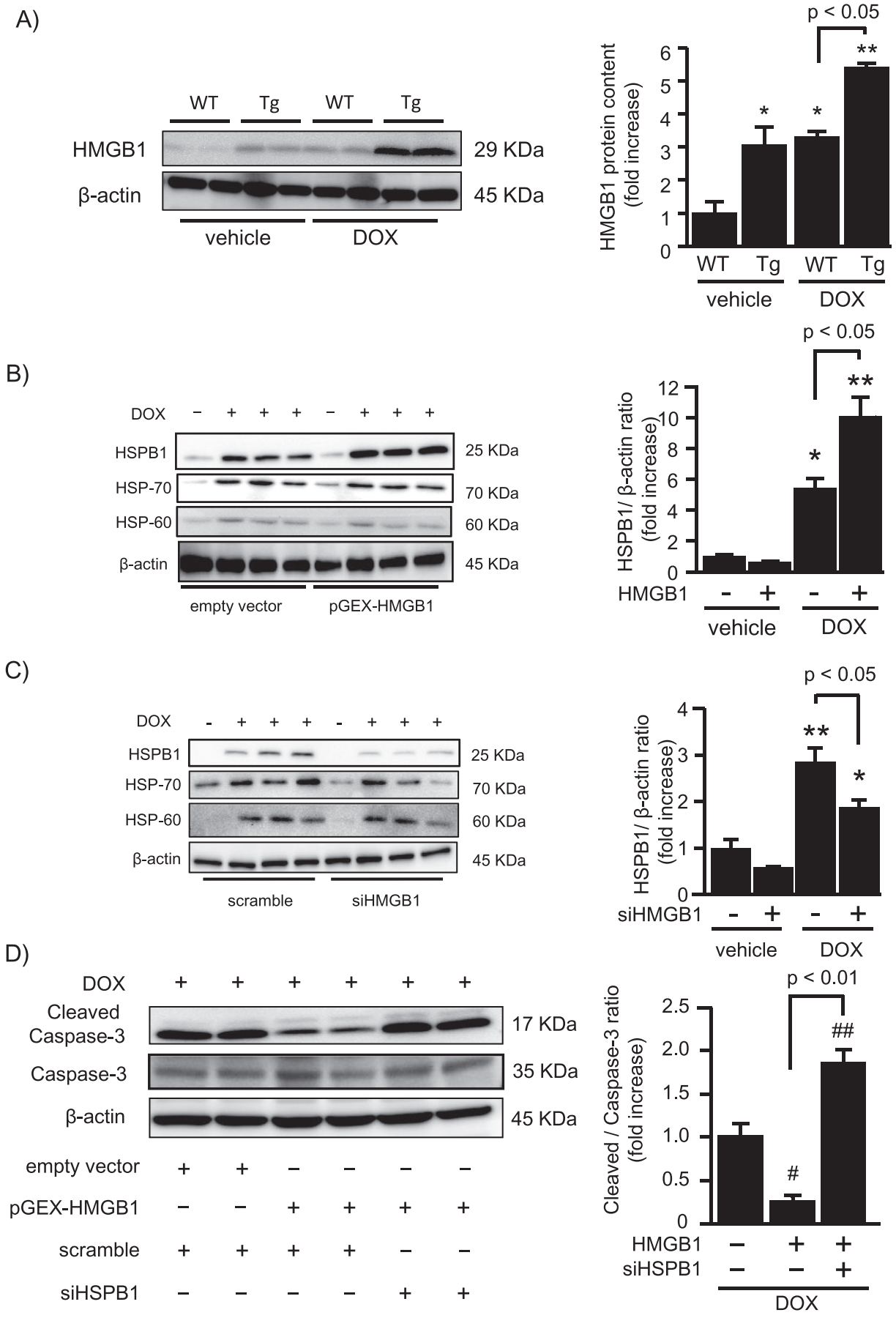
To confirm the importance of cardiac HMGB1 in controlling apoptosis in cultured cardiomyocytes, cleaved caspase-3 levels and apoptosis were evaluated in cardiomyocyte after 0.5 M doxorubicin administration for 6 h. We observed that doxorubicin significantly increased the abundance of cleaved caspase-3 and cardiomyocyte apoptosis. Moreover, siRNA-mediated *HMGB1* silencing in doxorubicin-treated cardiomyocytes significantly increased cleaved caspase-3 levels and cardiomyocyte apoptosis (Figs. 4A and B). Conversely, HMGB1 transfection significantly attenuated cleaved caspase-3 expression and apoptosis, compared with control vector transfection, after doxorubicin administration (Figs. 4C and D). These results indicate that HMGB1 attenuates mitochondrial dysfunction and cardiomyocyte apoptosis induced by doxorubicin administration.

3.5. Effect of HMGB1 on cardiac HSPB1 expression after doxorubicin administration

Recently, an association between HMGB1 and HSPB1 expression was reported [16,24], and therefore, we investigated whether HMGB1 could increase HSPB1 expression in the heart after doxorubicin administration (17.5 mg/kg). We observed that doxorubicin significantly increased both the levels of mRNA and protein of HSPB1 in the heart; however the increases were higher in HMGB1-Tg mice than in WT mice (Figs. 5A and B). The mitochondrial protein second mitochondria-derived activator of caspase (Smac) potentiates certain forms of apoptosis [25], and HSPB1 regulates the release of Smac from the mitochondria into the cytosol during apoptosis [26]. Therefore, we assessed the effect of doxorubicin on Smac localization in the heart. Less Smac was released from the mitochondria in HMGB1-Tg mice than in WT mice following doxorubicin administration (Fig. 5C). To evaluate the role of extracellular HMGB1, we measured the serum levels of HMGB1, and observed that HMGB1 concentrations were similarly increased after doxorubicin treatment in HMGB1-Tg mice and WT mice (Supplemental Fig. II). These results suggested that intracellular HMGB1 increased HSPB1 expression and attenuated cardiac apoptosis after doxorubicin administration.

Fig. 5. Intrinsic mitochondrial apoptotic pathway *in vivo*. (A) Following doxorubicin administration, HSPB1 levels were higher in HMGB1-Tg mice than in WT-mice. Bars represent means ± SEM ($n = 6$ per group); * $p < 0.05$ and ** $p < 0.01$ compared to WT mice without doxorubicin administration. (B) Doxorubicin administration significantly increased *HSPB1* mRNA abundance in WT mice. Levels of *HSPB1* mRNAs after doxorubicin administration were significantly higher in HMGB1-Tg mice than in WT mice. Bars represent means ± SEM ($n = 6$ per group); * $p < 0.05$ and ** $p < 0.01$ compared to WT mice without doxorubicin administration. (C) Doxorubicin-induced release of Smac from the mitochondria into the cytosol was prevented in HMGB1-Tg mice. Doxorubicin administration increased the abundance of cytosolic Smac in WT mice. The abundance of mitochondrial Smac was normalized to the abundance of Cox 4 prior to statistical analysis. Bars represent means ± SEM ($n = 6$ per group); * $p < 0.05$ and ** $p < 0.01$ compared to WT mice without doxorubicin administration. DOX, doxorubicin; WT, wild-type littermates; HMGB1, high-mobility group box 1; HMGB1-Tg, mice with cardiac-specific HMGB1 overexpression; Smac, Second mitochondria-derived activator of caspase; Cox 4, cytochrome C oxidase subunit 4; SEM, standard error of the mean.


A)
WT
Tg
WT
Tg
HSPB1
25 KDa
β-actin
45 KDa
vehicle
DOX
p < 0.05
HSPB1 protein content (fold increase)
B)
Relative HSPB1 gene expression (fold increase)
C)
Cytosolic Smac
20 KDa
Mitochondrial Smac
20 KDa
COX IV
17 KDa
β-actin
45 KDa
Cytosolic Smac / β-actin ratio (fold increase)
Mitochondrial Smac / COX IV ratio (fold increase)


A)
WT
Tg
WT
Tg
HMGB1
29 KDa
β-actin
45 KDa
vehicle
DOX
p < 0.05
HMGB1 protein content (fold increase)
WT
Tg
WT
Tg
vehicle
DOX
B)
DOX
HSPB1
25 KDa
HSP-70
70 KDa
HSP-60
60 KDa
β-actin
45 KDa
empty vector
pGEX-HMGB1
p < 0.05
HSPB1/ β-actin ratio (fold increase)
HMGB1
vehicle
DOX
C)
DOX
HSPB1
25 KDa
HSP-70
70 KDa
HSP-60
60 KDa
β-actin
45 KDa
scramble
siHMGB1
p < 0.05
HSPB1/ β-actin ratio (fold increase)
siHMGB1
vehicle
DOX
D)
DOX
Cleaved Caspase-3
17 KDa
Caspase-3
35 KDa
β-actin
45 KDa
empty vector
pGEX-HMGB1
scramble
siHSPB1
p < 0.01
Cleaved / Caspase-3 ratio (fold increase)
HMGB1
siHSPB1
DOX

3.6. Effect of HMGB1 on HSPB1 expression in neonatal rat cardiomyocytes

Several studies have shown that cellular levels of HMGB1 are altered by ischemia/reperfusion, infarction, and reactive oxygen species [27–29], we assessed HMGB1 expression levels upon doxorubicin administration. Doxorubicin significantly increased HMGB1 levels *in vivo*. However, the protein content was significantly higher in HMGB1-Tg mice compared to WT mice (Fig. 6A). Next, we investigated the effect of cardiac HMGB1 on HSP-expression after doxorubicin administration *in vitro*, and observed that doxorubicin significantly increased the expression levels of HSP-60, HSP-70, and HSPB1 (Figs. 6B and C). Both HSPB1 protein and mRNA levels were significantly higher in neonatal rat cardiomyocytes that overexpressed HMGB1 than in those with normal levels of HMGB1 following doxorubicin administration (Figs. 6B and Supplemental Fig. IIIA). On the contrary, HSPB1 protein levels after doxorubicin administration were lower in neonatal rat cardiomyocytes in which *HMGB1* was silenced than in doxorubicin-treated cells with normal levels of HMGB1 (Fig. 6C). However, there was no marked difference in the levels of HSP-60 or HSP-70 and *HSP-60* or *HSP-70* after doxorubicin administration in cardiomyocytes with altered levels of HMGB1 (Figs. 6B, C and Supplemental Fig. IIIB and C).

3.7. Contribution of HSPB1 to the anti-apoptotic effect of HMGB1 in cardiomyocytes

Given our demonstration that HMGB1 increased HSPB1 protein and mRNA levels, we next investigated the association between the HMGB1-mediated anti-apoptotic effect and HSPB1 expression at the protein and mRNA levels. siRNA-mediated *HSPB1* silencing increased cleaved caspase-3 levels after doxorubicin administration (Supplemental Fig. IV). Importantly, co-transfection of siHSPB1 with vector encoding HMGB1 abrogated the ability of HMGB1 to suppress cleaved caspase-3 accumulation, suggesting that the ability of HMGB1 to promote HSPB1 expression is associated with inhibition of doxorubicin-mediated apoptosis (Fig. 6D).

3.8. HMGB1-mediated heat shock factor transcriptional activity and HSPB1 promoter activity after doxorubicin administration

Since HSP gene expression is regulated by heat shock factors (HSFs) [30], we evaluated the association between HMGB1 and HSFs. Heat shock element (HSE) promoter activity increased upon 0.5 M doxorubicin stimulation for 1 h. Moreover, HMGB1 overexpression enhanced HSE promoter activity compared to empty vector transfection (Fig. 7A). To examine potential HSF-HMGB1 interaction, we performed immunoprecipitation experiments. Doxorubicin stimulation increased protein-to-protein binding of HMGB1 with HSF2 (Figs. 7B and C), but not HSF 1 or HSF 4 (Supplemental Fig. V), indicating an increase in HSF2–HMGB1 interaction in response to doxorubicin treatment in cardiomyocytes.

To confirm the impact of HSF2 on the regulation of HMGB1-mediated HSPB1 expression, HSPB1 expression after doxorubicin stimulation was assessed upon transfection with shHSF2 (Supplemental Fig. VI). Co-transfection with shHSF2 and pGEX-HMGB1 attenuated HMGB1-mediated HSPB1 expression compared to control shRNA transfection (Fig. 7D). Moreover, the ability of HMGB1 to suppress the accumulation of cleaved caspase-3 was abrogated by silencing *HSF2* (Fig. 7E). Finally, we examined HMGB1 binding to the HSPB1 promoter, using a ChIP assay with the use of HMGB1 antibody, followed by PCR analysis of the immunoprecipitated DNA fragments with primer that amplify the promoter region of *HSPB1* gene. The result of this experiment, shown in Fig. 7F, indicated that doxorubicin administration significantly increased HMGB1 binding to promoter lesion of HSPB1.

4. Discussion

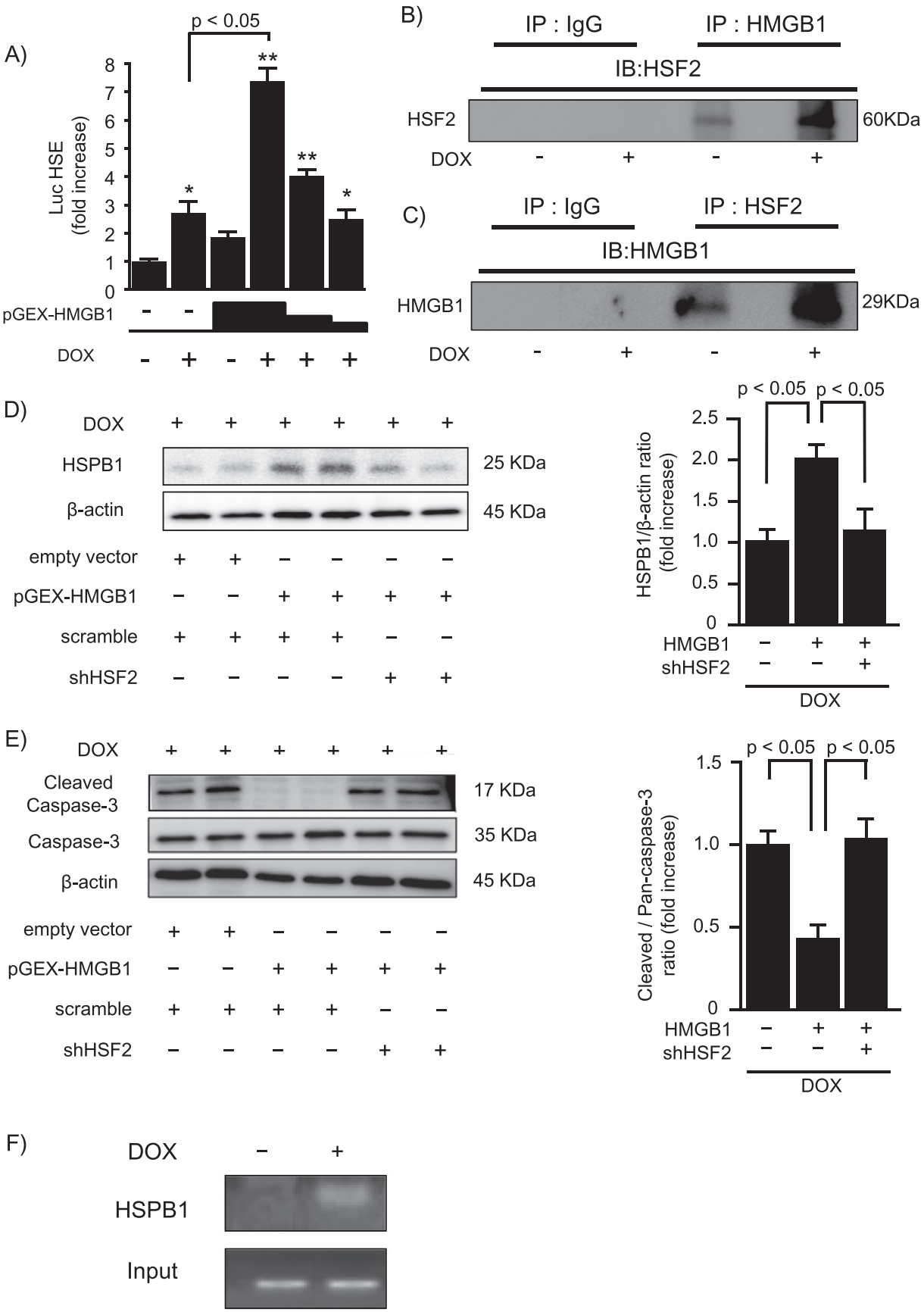
The present study demonstrated the critical role of cardiac HMGB1 in cardiomyocyte apoptosis and mitochondrial dysfunction associated with the pathogenesis of doxorubicin cardiomyopathy. The survival rate after administration of doxorubicin was significantly higher, and the mitochondrial damage was significantly lower, in HMGB1-Tg mice than in their WT counterparts. Cardiac HMGB1 increased HSPB1 expression and suppressed apoptosis after doxorubicin administration both *in vivo* and *in vitro*. HSPB1 expression was regulated by HMGB1 and HSF2 after doxorubicin stimulation.

The mechanism of pathogenesis of doxorubicin-induced cardiomyopathy is thought to relate to cardiomyocyte apoptosis. Prevention of apoptosis might be a therapeutic target for the preservation of cardiac function. The present study showed that levels of *HSPB1* mRNA and HSPB1 protein after doxorubicin administration were significantly higher in both HMGB1-transfected cardiomyocytes and HMGB1-Tg mice than in the cardiomyocytes and mice with normal levels of HMGB1, and that HMGB1 overexpression suppressed caspase-3 activation and the number of apoptotic cardiomyocytes after doxorubicin treatment. In contrast, HMGB1-mediated suppression of caspase-3 cleavage was significantly diminished by co-transfection with siHSPB1. We also showed that after doxorubicin administration, the survival rate of HMGB1-Tg mice was significantly higher than that of WT mice. This finding supported the role of cardiac HMGB1 in preventing doxorubicin-induced cardiomyopathy.

Since HSPB1 is known to prevent caspase activation [15,26], loss of HSPB1 results in mitochondrial fragmentation with decreased aerobic respiration [16,24]. Furthermore, loss of HSPB1 decreases mitochondrial membrane potential, induces Smac release from mitochondria, and activates caspase-3 [26]. In the present study, we observed extensive mitochondrial damage and vacuolization in doxorubicin-treated WT mice, but not in HMGB1-Tg mice. To the best of our knowledge, we showed, for the first time, that mitochondrial membrane potential was restored in neonatal rat cardiomyocytes that overexpress HMGB1, but not in otherwise comparable cells in which HMGB1 expression is silenced. Moreover, less Smac was released from the mitochondria of HMGB1-Tg mice than WT mice. These findings indicated that cardiac HMGB1 overexpression suppresses mitochondrial dysfunction and cardiomyocyte apoptosis by regulating the HSPB1 expression in cardiomyocytes (Fig. 8).

The 215-amino-acid protein HMGB1, which was identified as a chromosomal protein with important structural functions in chromatin organization, is ubiquitously expressed in all vertebrate nuclei [4,8,31]. HMGB1 binds to double-stranded DNA, and its interaction with other DNA-binding proteins facilitates chromatin bending [31,32]. This architectural function facilitates the binding of several transcriptional factors. The molecular mechanisms underlying the anti-apoptotic effect of HMGB1 are still unclear. However, HSF2 reportedly binds to the HSPB1 promoter in Jurkat cells [33]. Our study showed that HMGB1 overexpression enhanced HSE promoter activity, unlike transfection of an empty vector. Moreover, doxorubicin stimulation enhanced binding of HSF2 and HMGB1. Silencing HSF2 abolished the anti-apoptotic effect of HMGB1 overexpression after doxorubicin stimulation. Doxorubicin

Fig. 6. Role of HSPB1 expression in the HMGB1-dependent anti-apoptotic effect. (A) Doxorubicin administration significantly increased HMGB1 abundance. Bars represent means ± SEM ($n = 6$ per group). (B) HSPB1 expression, but not that of HSP-60 or HSP-70, was higher in neonatal rat cardiomyocytes with HMGB1 overexpression. (C) HMGB1 silencing in neonatal rat cardiomyocytes decreased HSPB1 abundance. (D) Co-transfection with siHSPB1 and pGEX-HMGB1 attenuated the ability of HMGB1 overexpression to prevent cleaved caspase-3 accumulation. Bars represent mean ± SEM ($n = 4$–6 per group) * $p < 0.05$ and ** $p < 0.01$ compared to control; # $p < 0.05$ and ## $p < 0.01$ compared to the control treated with doxorubicin. DOX, doxorubicin; HMGB1, high-mobility group box 1; HSP, heat shock protein; HSPB1, heat shock protein binding protein 1; si, small interfering RNA; SEM, standard error of the mean.


A)
p < 0.05
Luc HSE (fold increase)
pGEX-HMGB1
DOX
B)
IP : IgG
IP : HMGB1
IB:HSF2
HSF2
60KDa
DOX
C)
IP : IgG
IP : HSF2
IB:HMGB1
HMGB1
29KDa
DOX
D)
DOX
HSPB1
25 KDa
β-actin
45 KDa
empty vector
pGEX-HMGB1
scramble
shHSF2
p < 0.05 p < 0.05
HSPB1/β-actin ratio (fold increase)
HMGB1
shHSF2
DOX
E)
DOX
Cleaved Caspase-3
17 KDa
Caspase-3
35 KDa
β-actin
45 KDa
empty vector
pGEX-HMGB1
scramble
shHSF2
p < 0.05 p < 0.05
Cleaved / Pan-caspase-3 ratio (fold increase)
HMGB1
shHSF2
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F)
DOX
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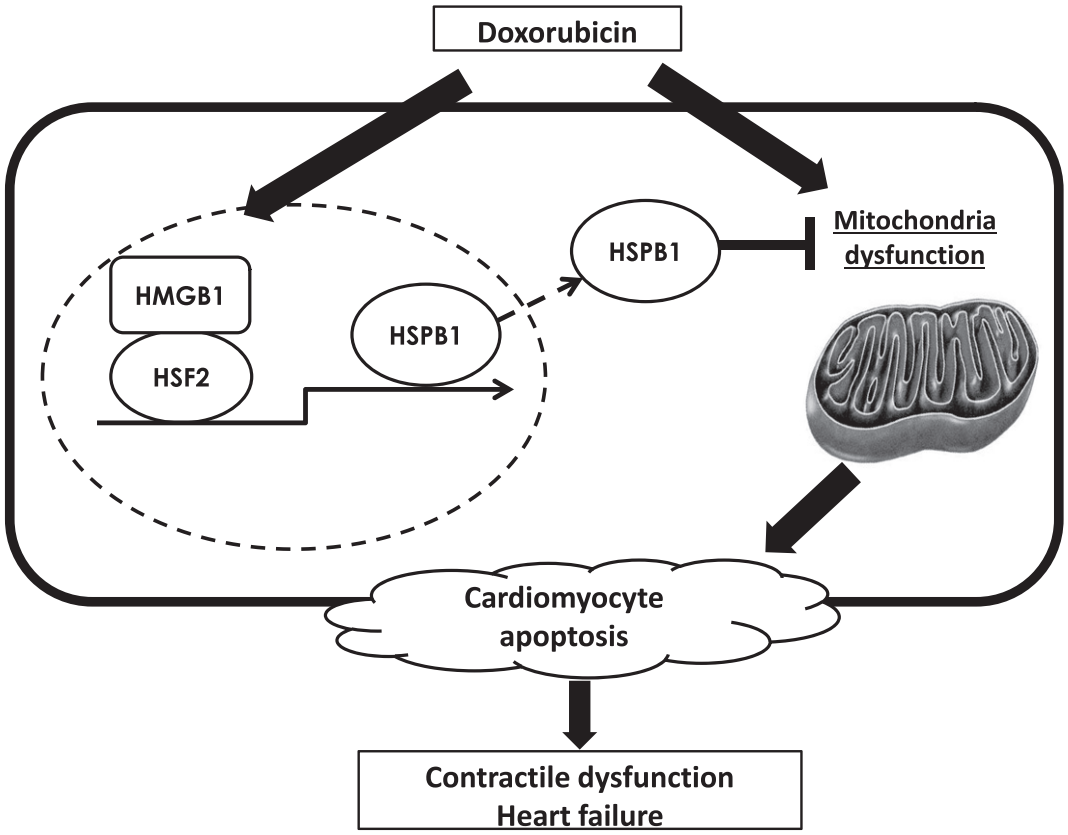

Fig. 8. Proposed mechanism for cardiac HMGB1-mediated attenuation of cardiomyocyte apoptosis and mitochondrial damage after exposure to doxorubicin. Cardiac HMGB1 increased HSPB1 expression and suppressed cardiomyocyte apoptosis after doxorubicin administration both *in vivo* and *in vitro*. HSPB1 expression was regulated by HMGB1 and HSF2 after doxorubicin stimulation. HMGB1, high-mobility group box 1; HSF2, heat shock factor 2; HSPB1, heat shock protein beta 1.

stimulation also increased in HMGB1 binding to promoter lesion of HSPB1. Taken together, cardiac HMGB1 increases HSPB1 expression in cardiomyocytes in an HSF2-dependent manner (Fig. 8). Although the mechanisms by which doxorubicin affects HMGB1 and HSF2 association have not been elucidate yet, we showed that doxorubicin increased HMGB1 expression in the heart. Therefore, it is also suspected that increased expression levels of HMGB1 enhanced the binding to HSF2 and affected HSPB1 expressions in the heart after doxorubicin expressions.

In conclusion, we report the first *in vivo* and *in vitro* evidence that cardiac HMGB1 attenuates mitochondrial dysfunction and cardiomyocyte apoptosis associated with the pathogenesis of doxorubicin-induced cardiomyopathy. These results may provide a novel therapeutic approach to combat doxorubicin-induced cardiomyopathy.

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Disclosures

The authors declare no conflicts of interest with regard to this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.02.018.

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Fig. 7. Association between HSF2 and HMGB1 in regulation of cardiomyocyte apoptosis. (A) Doxorubicin stimulation increased HSE luciferase activity. HMGB1 overexpression greatly enhanced HSE promoter activity compared to empty vector transfection. (B) Lysates from neonatal rat cardiomyocytes treated with or without doxorubicin were immunoprecipitated with an anti-HMGB1 antibody and immunoblotted with an anti-HSF2 antibody. (C) Lysates from neonatal rat cardiomyocytes treated with or without doxorubicin were immunoprecipitated with an anti-HSF2 antibody and immunoblotted with an anti-HMGB1 antibody. (D) Transfection with shHSF2 attenuated the ability of HMGB1 overexpression to increase HSPB1 expression. (E) Co-transfection with shHSF2 and pGEX-HMGB1 attenuated the ability of HMGB1 overexpression to prevent cleaved caspase-3 accumulation. Bars represent mean ± SEM (n = 4–6 per group); * $p < 0.05$ and ** $p < 0.01$ compared to control. DOX, doxorubicin; HMGB1, high-mobility group box 1; HSE, heat shock element; HSF2, heat shock factor 2; HSPB1, heat shock protein beta 1; SEM, standard error of the mean; sh, short hairpin RNA. (F) Chromatin immunoprecipitation assay with the use of HMGB1 antibody, followed by PCR analysis of the immunoprecipitated DNA fragments with primer that amplify the promoter region of *HSPB1* gene showed that HMGB1 binds *HSPB1* gene promoter 1 h after doxorubicin administration.

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