Survivin gene therapy attenuates left ventricular systolic dysfunction in doxorubicin cardiomyopathy by reducing apoptosis and fibrosis

Paul J.H. Lee, Dmitriy Rudenko, Michael A. Kuliszewski, Christine Liao, M. Golam Kabir, Kim A. Connelly, and Howard Leong-Poi*

Division of Cardiology, Keenan Research Centre in the Li Ka Shing Knowledge Institute, 6-044 Donnelly Wing, St Michael’s Hospital, University of Toronto, 30 Bond Street, Toronto, ON, Canada M5B 1W8

Received 3 May 2013; revised 23 November 2013; accepted 27 December 2013; online publish-ahead-of-print 8 January 2014

Time for primary review: 39 days

Aims
The aim of this study was to investigate anti-apoptotic gene therapy using ultrasound-mediated plasmid delivery of survivin, an inhibitor of apoptosis protein, to prevent apoptosis and to attenuate left ventricular (LV) systolic dysfunction in a model of heart failure induced by doxorubicin.

Methods and results
Effect of survivin transduction was investigated in vitro in rat cardiomyoblasts. After survivin transduction, survivin protein was detected in cell culture supernate confirming secretion of extracellular survivin. Under doxorubicin stimulation, survivin-transduced cells had significantly reduced apoptosis; however, incubation with survivin-conditioned media also showed reduced apoptosis that was absent with null-conditioned media. Doxorubicin-induced cardiomyopathy was established in Fischer rats. Subsets of animals underwent ultrasound-mediated survivin gene delivery or empty vector gene delivery at Week 3. Control rats received doxorubicin alone. Animals were studied using PCR, immunohistochemistry, echocardiography, and invasive haemodynamic studies out to Week 6. By Week 6, LV % fractional shortening by echocardiography and systolic function by pressure–volume loops were greater in survivin treated when compared with control- and empty-treated animals. There was reduced apoptosis by TUNEL and caspase activity in survivin-treated animals compared with control and empty treated at Week 4, with reduced interstitial fibrosis at Week 6.

Conclusion
Survivin gene therapy can attenuate the progression of LV systolic dysfunction in doxorubicin cardiomyopathy. This effect can be attributed to decreased myocyte apoptosis and prevention of maladaptive LV remodelling, by both direct myocyte transfection and potentially by paracrine mechanisms.

Keywords
Survivin • Apoptosis • Gene therapy • Cardiomyopathy • Heart failure

1. Introduction
Heart failure (HF) represents the end stage of all heart disease and a major cause of morbidity and death in developed countries. Despite advances in HF therapies, long-term outcomes and rates of re-hospitalization remain suboptimal.1,2 As a consequence, the development of novel therapeutic strategies that specifically target the underlying biological processes in HF remains of vital importance.3,4 There is increasing evidence that programmed cell death, or apoptosis, contributes substantially to the pathogenesis of HF.5,6 First and foremost, it reduces the number of functioning contractile cardiomyocytes. Importantly, apoptosis of non-myectes can also have an adverse effect on the failing myocardium, contributing to adverse ventricular remodelling, and playing a key role in the transition to an end-stage decompensated stage, regardless of the underlying aetiology of HF.7 Several proven therapies to prevent and treat HF have been shown to reduce apoptosis, including beta-blockers8 and angiotensin II receptor antagonists. Thus, anti-apoptotic therapeutic strategies potentially offer an attractive approach for the treatment of HF.

A number of novel therapeutic targets to reduce cardiomyocyte apoptosis have been identified.9 While most anti-apoptotic therapies for HF are pharmaceutical in nature, a gene therapy approach to inhibiting apoptosis would be highly attractive.10 As such, molecular
targets within the signalling and effector pathways for apoptosis have been evaluated in models of HF. Previously, a human gene encoding a structurally unique apoptosis inhibitor, designated survivin, has been described. Cardiac-specific deletion of survivin results in pre-mature cardiac death, due to a reduction in total cardiomyocyte numbers, and progressive HF. In an animal model of hypertension, survivin expression was inversely correlated with apoptotic rates as well as adverse cardiac remodelling, indicating survivin’s putative role in preventing the progression to HF. Therefore, we hypothesized that gene transfer of survivin would prevent myocyte apoptosis and attenuate left ventricular (LV) systolic dysfunction in a rat model of cardiomyopathy induced by doxorubicin (DOX).

Figure 1  In vitro survivin experiments. Representative images of in vitro transduction of survivin-N-emGFP (A); and survivin concentration in the supernate by ELISA (B; *P < 0.001 vs. CTRL, **P < 0.0001 vs. CTRL, n = 10, 6, 10, 11, 8, 9, 9 for CTRL, DOX, NCM, SCM 24, 48, 72, and 96, respectively). Post-transduction, a high level of GFP expression is seen (A, bottom right), whereas control cells had no detectable survivin (scale bar = 25 μm). Supernate from SURV cells had increased survivin concentration, peaking at 72 h. CONTROL, DOX, and NCM had no detectable survivin. Survivin uptake by H9c2 from total supernate (C; *P < 0.01 vs. control, n = 6, 4, 6 for control, null transfected and survivin transfected, respectively); exosomal fraction (D; *P < 0.05 vs. CTRL, n = 12, 10, 10 for CTRL, null exo, and surv exo, respectively); non-exosomal fraction (E; †P < 0.01 vs. CTRL, ‡P < 0.01 vs. null non-exo, n = 12, 7, 8 for CTRL, null non-exo, and surv non-exo, respectively). Pre-treatment with total supernate from survivin-transduced cells led to a significant increase in survivin uptake vs. control. Pre-treatment with survivin exosomal fraction led to a modest increase in survivin uptake, but survivin non-exosomal fraction led to a significantly higher uptake of survivin protein. Pre-treatment with null supernate (both exosomal and non-exosomal) did not lead to an increase in survivin uptake. Per cent apoptosis by annexin-V/SYTOX Red staining with DOX stimulation (F; *P < 0.0001 vs. DOX, **P < 0.001 vs. DOX, n = 6 for all groups). In the SURV group, per cent apoptosis decreased significantly vs. DOX. Pre-treatment with SCM also led to a decline in the rate of apoptosis.
2. Methods
Detailed methods of the study are available in the Supplementary material online.

2.1 Cell culture
H9c2 (cardiac myoblasts; ATCC, Manassas, VA, USA) and 3T3-NIH cells (murine fibroblasts) were grown in the standard growth medium, and primary human umbilical vein endothelial cells (HUVECs) were grown in supplemented M-199 media. The media was changed every 2 days, and the cells were passaged at ~70% confluency.

2.2 In vitro adenoviral transduction
A total of $2.5 \times 10^5$ either H9c2, HUVEC or 3T3-NIH cells were grown for 24 h, inoculated with either Ad-Null (NULL) or Ad-Surv-emGFP (SURV) virus (250 MOI) in 250 µL of growth media. Supernate from cells treated with DOX was collected at 72 h. For H9c2 cells: at 24, 48, 72, and 96 h, the supernate was removed and centrifuged for the analysis of extracellular survivin by ELISA (R&D Systems, Minneapolis, MN, USA). For 3T3-NIH and HUVECs, the supernate from DOX, NULL, and SURV groups were collected at 72 h. H9c2 supernate collected from SURV and NULL at 72 h were used as survivin-conditioned media (SCM) and null-conditioned media (NCM) for subsequent experiments. Survivin concentrations were normalized to the number of cells at each time point.

2.3 DOX-induced cytotoxicity in vitro
The level of apoptosis was compared between SURV, NULL and non-transduced cells in the presence of DOX. To determine the effects of SCM, cells were cultured in either SCM or NCM for 24 h prior to DOX exposure. For each group, $2.5 \times 10^5$ cells per well were grown in 24-well dishes and cultured in 0.25 mM concentration of DOX (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 6 h. Cells were detached, pelleted, and re-suspended in 100 µL of working strength Annexin-V-Alexa-568 (Roche Diagnostics, Laval, Quebec, Canada) and 1 µL of diluted SYTOX Red (Invitrogen, Burlington, ON, Canada) for 15 min in room temperature, and the reaction was quenched with the addition of 500 µL of incubation buffer (pH 7.2, 10 mM HEPES, 140 mM NaCl, 5 mM CaCl$_2$). Stained cells were subsequently analysed using flow cytometry (BD FACS calibur II).

2.4 In vitro survivin-emGFP uptake
A total of $1.0 \times 10^5$ H9c2 cells were inoculated with either Ad-Surv-emGFP or Ad-Null. The total supernate was collected after 48 h and separated into exosomal and non-exosomal fraction by serial centrifugation, as described previously. The exosomal and non-exosomal fractions were subsequently transferred onto 1.0 × 10$^5$ H9c2 cells. After 24 h, the cells were treated with 0.25 µM DOX and the cell lysate was collected after 6 h. Survivin concentration in the cell lysate was measured using ELISA (R&D Systems, Minneapolis, MN, USA).

2.5 Animal preparation
The study protocol was approved by the Animal Care and Use Committee at St Michael’s Hospital Research Centre, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. DOX (0.5 mg/mL; Santa Cruz, Santa Cruz, CA, USA) was administered ip to male Fischer rats (n = 150; Charles Rivers, Wilmington, MA, USA) at a dosage of 2.5 mg/kg, every other day for 2 weeks, total dosage of 15 mg/kg. Inhaled isoflurane anaesthetic (0.2 L/min medical air, 3% isoflurane) was used for all in vivo studies, and the toe-pinch method was used to ensure the adequacy of anaesthetics. Animals were euthanized by means of cervical dislocation while under isoflurane-induced anaesthesia.

2.6 In vivo experimental protocol
Three weeks after the initial injection of DOX, a subset of animals (DOX + SURV, n = 50) received ultrasound-mediated survivin plasmid gene delivery. Another subset of animals underwent delivery with empty plasmid (DOX + EMP, n = 52). The DOX group (n = 48) received no treatment at Week 3, whereas CON animals (n = 21) did not receive DOX or gene therapy. Echocardiography was performed at Week 0 for baseline, Week 3 (pre-delivery), and at Week 6 to assess LV systolic function. A subset of animals were sacrificed at Day 3 (DOX n = 7; DOX + EMP n = 9; DOX + SURV n = 11) and Day 7 post-gene delivery (DOX n = 11; DOX + EMP n = 13; DOX + SURV n = 11), to assess the early effects of delivery on gene transfection

Figure 2 Ultrasound-mediated survivin transfection by immunohistochemistry. Examples of immunofluorescent staining of CON, DOX, DOX + EMP, and DOX + SURV myocardium at Week 4 (1-week post-gene delivery). (A) Robust survivin expression (white) in the DOX + SURV group was seen in cardiomyocytes (red—sarcemeric-alpha-actinin) and (B) endothelial cells (red—VWF (yellow arrows) top—arteriole; bottom—capillaries), whereas survivin protein was not detected in the CON, DOX, and DOX + EMP groups. Rabbit IgG isotype control was used to exclude any unspecific staining. DAPI (blue) was used as the nuclear stain.
Figure 3  Real-time PCR data of exogenous GFP-survivin and total survivin transcript levels. Exogenous emGFP [(A) top; n(anterior) = 10, 10, 6, 8 for CTRL, DOX, DOX + EMP, and DOX + SURV, respectively, and n(posterior) = 10, 6, 10, 7 for CTRL, DOX, DOX + EMP, and DOX + SURV, respectively. (A) bottom; n(anterior) = 10, 8, 9, 7 for CTRL, DOX, DOX + EMP, and DOX + SURV, respectively, and n(posterior) = 10, 10, 10 for CTRL, DOX, DOX + EMP, and DOX + SURV, respectively] and total survivin (rat and human) [(B) top; n = 10, 8, 8, 11 for CTRL, DOX, DOX + EMP, and DOX + SURV, respectively] expression for all groups at 4 weeks [top of (A) and (B)] and 6 weeks [bottom of (A) and (B)]. *P < 0.05 vs. CTRL anterior; **P < 0.05 vs. CTRL posterior; †P < 0.01 vs. CTRL anterior; ††P < 0.01 vs. CTRL posterior; #P < 0.01 vs. SURV.
and apoptosis. Remaining animals were sacrificed at Week 6 (3 weeks post-gene delivery).

2.7 Microbubble and DNA preparation
An expression vector driven by a CMV promoter was constructed for transfection of survivin-N-emGFP, in which the human survivin sequence was fused with emGFP at the N-terminus to create a fusion protein. The plasmid vector was acquired through SIDI.NET (Toronto, ON, USA), and was sequence-verified prior to use in vivo. For gene delivery, survivin-N-emGFP plasmid DNA (500 µg) or emGFP plasmid DNA (500 µg) was charge-coupled to cationic lipid microbubbles (1 x 10^8, volume 1 mL), as described previously.16–18

2.8 Gene delivery
For gene delivery, transthoracic ultrasound transmission to the LV was performed using an S12 transducer (Sonos 5500, Philips Ultrasound) at a frequency of 5 MHz and power 120 V during iv infusion of plasmid DNA–cationic microbubble complexes over 5 min.17,19 The ultrasound transducer was positioned transversely at the mid-papillary level, and ultrasound was transmitted during a slow sweep. A pulsing interval of 10 cardiac cycles at end-systole was used to ensure the replenishment of the bubbles in the myocardiavalusculature between each pulse of ultrasound to maximize myocyte transfection.20

2.9 Echocardiography
For LV function, M-mode and two-dimensional echocardiography in the short-axis view were performed at the mid-papillary level and in the apical four-chamber view (Sonos 5500, Philips Ultrasound, S12 transducer). Fractional shortening (FS) was derived from the measurements of the LV end-diastolic (ED) and LV end-systolic (ES) dimensions, using the following formula: \( \frac{LVEDd - LVEDd}{LVEDd} \times 100\% \). LV end-diastolic volume (LVEDV) was measured by Simpson’s monoplane technique off the apical four-chamber view. For the right ventricle (RV), two-dimensional echocardiography at the apical four-chamber view was performed to measure the right basal ventricular diastolic diameter (RVD1), and the RV fractional area change using the following formula: \( \frac{RVEDd - RVESd}{RVEDd} \times 100\% \).21

2.10 Cardiac catheterization
Cardiac catheterization was performed as previously published22 using a 2F miniaturized combined conductance catheter-micro-manometer (Scisense, London, Canada). All pressure–volume (PV) loop data were then acquired miniaturized combined conductance catheter-micro-manometer (Scisense, Canada). All pressure–volume (PV) loop data were then acquired using an S12 transducer (Sonos 5500, Philips Ultrasound, S12 transducer). Fractional shortening (FS) was derived from the measurements of the LV end-diastolic (ED) and LV end-systolic (ES) dimensions, using the following formula: \( \frac{LVEDd - LVEDd}{LVEDd} \times 100\% \). LV end-diastolic volume (LVEDV) was measured by Simpson’s monoplane technique off the apical four-chamber view. For the right ventricle (RV), two-dimensional echocardiography at the apical four-chamber view was performed to measure the right basal ventricular diastolic diameter (RVD1), and the RV fractional area change using the following formula: \( \frac{RVEDd - RVESd}{RVEDd} \times 100\% \).21

2.11 Real-time PCR
Quantitative real-time PCR for the exogenous emGFP and total survivin (endogenous rat and exogenous human) transcripts was performed using standard techniques.16,17 Beta-actin was used as an internal control gene. Data on emGFP-Survivin are reported as copy number per 1 µg of total RNA, and the data on total survivin expression are expressed as fold increase when compared with the control group. Specific primers are listed in the Supplementary material online, Methods.

2.12 Measures of apoptosis
The ApoTag Plus Fluorescein In Situ kit (Millipore, Billerica, MA, USA) was used to label apoptotic bodies according to the manufacturer’s guidelines. Post-labeling immunohistochemistry was carried out prior to mounting. Caspase-3/7 activity was measured using the Caspase-3/7-Glo assay (Promega, Madison, WI, USA), by measuring the luminescence corresponding to the amount of caspase activity from the total protein isolate. The luminescence was measured and quantified by a luminometer.

2.13 Immunohistochemistry
OCT-embedded tissue sections were fixed in 2% paraformaldehyde and permeabilized. Antibodies for mouse anti-sarcomeric-alpha-actin (1:100; Abcam, Toronto, ON, Canada), goat anti-vWF (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-survivin (1:100; Abcam, Toronto, ON, USA) were used for immunostaining. A subset of sections were also processed for apoptotic cell labelling, as described in our methods for Measures of Apoptosis. The nuclei were counterstained with DAPI (Vector Labs, Burlington, ON, USA).

2.14 Quantification of interstitial fibrosis
The extent of LV and RV fibrosis was measured by picrosirius red staining. Fibrosis was measured with a polarized light microscopy using the ImageJ software (NIH).

2.15 Statistical methods
Data are expressed as means ± standard error of the mean, unless specified otherwise. Comparisons between multiple single groups were made with one-way ANOVA with a Bonferroni correction (GraphPad Prism5, version 5.0a). When differences between means were found, a Bonferroni correction was performed. Differences were considered significant when P < 0.05.

3. Results
3.1 Survivin transfection in vitro
After transduction of H9c2 cells with the survivin-GFP gene, GFP was highly expressed in cells inoculated with the virus, indicating the presence of the survivin-GFP fusion protein both in the cytoplasm and the nucleus, whereas CONTROL cells did not exhibit any fluorescence (Figure 1A). At 48, 72, and 96 h post-transduction of H9c2 cells, survivin

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 3</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDD 6.82 ± 0.02</td>
<td>7.11 ± 0.01</td>
<td>7.07 ± 0.01</td>
</tr>
<tr>
<td>ESD 3.49 ± 0.01</td>
<td>3.66 ± 0.01</td>
<td>3.55 ± 0.01</td>
</tr>
<tr>
<td>DOX (n = 30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDD 6.87 ± 0.01</td>
<td>6.96 ± 0.01</td>
<td>6.97 ± 0.01</td>
</tr>
<tr>
<td>ESD 3.58 ± 0.01</td>
<td>3.75 ± 0.01</td>
<td>4.26 ± 0.01*</td>
</tr>
<tr>
<td>DOX + EMP (n = 30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDD 6.98 ± 0.01</td>
<td>7.05 ± 0.01</td>
<td>7.08 ± 0.05</td>
</tr>
<tr>
<td>ESD 3.52 ± 0.01</td>
<td>3.63 ± 0.01</td>
<td>4.19 ± 0.01*</td>
</tr>
<tr>
<td>DOX + SURV (n = 28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDD 6.93 ± 0.01</td>
<td>6.83 ± 0.01</td>
<td>6.82 ± 0.01</td>
</tr>
<tr>
<td>ESD 3.60 ± 0.01</td>
<td>3.64 ± 0.01</td>
<td>3.84 ± 0.01***</td>
</tr>
</tbody>
</table>

CON, non-DOX treated; DOX, doxoruubicin alone; DOX + EMP and DOX + SURV, DOX with empty and survivin-gene therapy, respectively; EDD, end-diastolic dimension (mm); ESD, end-systolic dimension (mm).

*P < 0.01 vs. CON rats.
**P < 0.05 vs. DOX rats.
***P < 0.05 vs. CON rats.
protein was detected in the SURV group supernate, peaking at 72 h (Figure 1B). Transduction of 3T3-NIH and HUVECs also led to a significant increase in survivin concentration in supernate collected at 72 h (see Supplementary material online, Figure S1A and B). Supernate collected from CONTROL cells and NULL cells had no detectable survivin in H9c2, 3T3-NIH, or HUVECs. DOX treatment did not significantly affect intracellular survivin concentration (see Supplementary material online, Figure S2).

3.2 In vitro survivin-emGFP uptake

Cells pre-treated with total supernate from either NULL or SURV groups for 24 h were analysed with ELISA for extracellular survivin protein uptake. Cells treated with NULL total supernate showed no increase in survivin, but cells treated with SURV total supernate showed a significant increase in survivin uptake (Figure 1C; 768 ± 63%). Uptake was evident with the exosomal survivin fraction of the supernate (Figure 1D; 157 ± 15%), but the uptake was even greater in cells pre-treated with the non-exosomal survivin fraction (Figure 1E; 371 ± 72%). Neither exosomal nor non-exosomal fraction of NULL supernate resulted in cellular uptake of survivin protein.

3.3 Effects of survivin on DOX-induced apoptosis in vitro

After DOX treatment, there was an increase in annexin-V positive/SYTOX Red negative cells, which was reduced by ~30% in the SURV group (Figure 1F). Pre-treatment (24 h) with SCM had a modest, but significant effect on apoptosis, with ~20% decrease in apoptosis compared with DOX. NULL and pre-treatment with NCM had no effect on the rate of apoptosis in H9c2 cells (Figure 1F).

Figure 4 Cardiac function data in all treatment groups. Representative M-mode images of CON, DOX, DOX + EMP, and DOX + SURV-treated left ventricles at Weeks 3 and 6 (A); % fractional shortening (FS) at weeks, 0, 3, and 6 (B) *p < 0.05 and **p < 0.001 vs. Week 6 CON, †p < 0.05 and ‡p < 0.001 vs. Week 6 DOX + SURV; and % change in FS (from Week 3 to 6) in all groups [(C) *p < 0.001 vs. CON, †p < 0.01 vs. DOX + SURV]. Representative pressure–volume loops at Week 6 for DOX, DOX + EMP, and DOX + SURV (D). The slope of the end-systolic PV relationship (blue line) is steepest for DOX + SURV, indicative of improved LV systolic function.
3.4 Survivin gene transfection in vivo

Ultrasound-mediated gene delivery of survivin led to myocardial transfection in cardiomyocytes (Figure 2A) as well as endothelial transfection (Figure 2B, top—arteriole; bottom—capillaries) as demonstrated by immunohistochemical staining. Analysis of the exogenous emGFP transcript expression revealed that compared with both CON and DOX groups, the DOX + EMP and DOX + SURV animals exhibited high expression of the exogenously delivered transgene at 1-week post-delivery (Figure 3A, top). At 3-week post-delivery (Week 6), the exogenous gene expression was lower, yet still demonstrated a significant amount of transgene expression when compared with DOX (Figure 3A, bottom). While the anterior wall of the LV had marginally greater transfection compared with the posterior wall in all groups at both time points, differences were not statistically significant. Analysis of the total survivin transcript level revealed that at Week 4, survivin expression was significantly increased in DOX + SURV animals, compared with DOX and DOX + EMP groups (Figure 3B, top). By Week 6, the effect of the exogenous survivin delivery diminished, with all groups showing comparable survivin expression levels (Figure 3B, bottom).

3.5 Assessment of LV function

Data on LV dimensions over time for each treatment group are shown in Table 1. Representative M-Mode echocardiographic images at the mid-papillary muscle level of the LV from each treatment group are shown in Figure 4A. Prior to gene delivery at Week 3, LV %FS was similar in CON, DOX, DOX + EMP, and DOX + SURV groups (Figure 4B). In contrast, at the 6-week time point, LV %FS was significantly greater in the DOX + SURV group when compared with the DOX group. Data on % change in LV FS are shown in Figure 4C. Whereas DOX and DOX + EMP animals exhibited a decline in LV %FS over time (−15 ± 3% and −16 ± 1%, respectively), this effect was significantly attenuated by survivin gene therapy (−5 ± 2%). LV haemodynamic and volume data at Week 6 are shown in Table 2. Survivin gene therapy was associated with improved LV systolic parameters, including significantly greater PRSW relationship index and slope of the ESPVR. LVEDV did not change in DOX + EMP-treated animals when compared with DOX animals (0.438 ± 0.007 and 0.434 ± 0.009 cm³), whereas SURV treatment led to a significant reduction in LVEDV (0.399 ± 0.007 cm³). Representative PV loops from each group are depicted in Figure 4D.

3.6 Assessment of RV function

Data on RV FAC and RVD1 at all time points are given in Supplementary material online, Table S1. DOX administration led to a mild reduction in RV systolic function as measured by RV FAC in all groups over the 6-week study period. There was no significant difference in RV FAC between the different treatment groups at Week 6.

3.7 Measures of apoptosis with survivin gene therapy

Representative images of TUNEL staining from all treatment groups are shown in Figure 5A, with apoptosis limited to cardiomyocytes. Quantitative apoptosis rates by TUNEL from all groups are shown in Figure 5B. Apoptosis was increased in the DOX and DOX + EMP groups, compared with the basal rate of apoptosis seen in the CON hearts (0.7 ± 0.1 and 0.7 ± 0.1%, respectively). Survivin gene therapy in the DOX + SURV group significantly rescued the apoptotic phenotype (0.2 ± 0.1%). Activity of caspase-3/7 was measured at Week 4 (1-week post-gene therapy). While caspase-3/7 activity was increased in the DOX and DOX + EMP groups compared with the CON, survivin gene therapy resulted in a significant reduction in caspase activity (Figure 5C).

3.8 Quantification of interstitial fibrosis

Evidence of interstitial fibrosis in the left ventricle was detected with DOX treatment, as shown by increased picrosirius red staining in the DOX and DOX + EMP-treated group (Figure 6A). When compared with the control group, the DOX and DOX + EMP groups exhibited an approximately three-fold increase in fibrosis (Figure 6B). In the DOX + SURV group, the degree of interstitial fibrosis was significantly reduced when compared with DOX alone. Similarly, DOX led to an increased interstitial collagen deposition in the RV (see Supplementary material online, Figure S3A and B). Survivin treatment, however, did not reverse the effects of DOX on the RV.

| Table 2 Haemodynamic variables by PV loops and LV end-diastolic volume by echocardiography (means ± SD) in DOX, DOX + EMP, and DOX + SURV animals at Week 6 |
|---------------------------------|-----------------|-----------------|
| Heart rate (b.p.m.)            | 321 ± 36        | 352 ± 22        | 311 ± 42        |
| ESP (mmHg)                     | 129 ± 23        | 130 ± 13        | 123 ± 14        |
| EDP (mmHg)                     | 14 ± 2          | 11 ± 2          | 9 ± 2           |
| + dp/dt (mmHg/s)               | 7274 ± 1571     | 7147 ± 1143     | 6976 ± 1091     |
| Tau Weiss                      | 14.46 ± 2.3     | 11.04 ± 1.2     | 12.4 ± 2.3      |
| ESPVR (mmHg/µL)                | 0.29 ± 0.20     | 0.35 ± 0.30     | 0.85 ± 0.50++++
| PRSW (mmHg/µL)                | 55.0 ± 13.5     | 69.8 ± 16.1     | 77.5 ± 12.7+++**
| LVEDV (cm³)                    | 0.434 ± 0.035   | 0.438 ± 0.025   | 0.399 ± 0.030**

DOX, doxorubicin alone; DOX + EMP and DOX + SURV, DOX with empty and survivin gene therapy, respectively; EDP, end-diastolic pressure; ESP, end-systolic pressure; PRSW, pre-load recruitable stroke work relationship; ESPVR, end-systolic PV relationship; LVEDV, left ventricular end-diastolic volume.

*p < 0.01 vs. DOX rats.
**p < 0.001 vs. DOX rats.
***p < 0.0001 vs. DOX rats.
****p < 0.05 vs. DOX + EMP rats.
*****p < 0.01 vs. DOX + EMP rats.
4. Discussion

Cardiomyocyte apoptosis is a prominent phenotype in the failing heart independent of the aetiology of HF. Indeed, data suggest that myocyte apoptosis may be a direct causal mechanism of HF, and as such anti-apoptotic therapy is an attractive therapeutic option. One such target is survivin, an inhibitor of apoptosis protein that acts as a caspase inhibitor to prevent programmed cell death. Survivin is highly expressed in most cancers and is associated with resistance to chemotherapy, tumour recurrence and metastasis, and reduced survival. In our study, we demonstrate that ultrasound-mediated gene delivery of survivin ameliorates myocyte apoptosis in a cardiomyopathy model induced by DOX, resulting in attenuation of LV systolic dysfunction and interstitial fibrosis. Importantly, while this anti-apoptotic effect can be attributed to direct cardiomyocyte transfection, there may also be additional paracrine effects of secreted extracellular survivin.

While survivin has been predominantly studied in the context of cancer, its role in cardiomyocyte apoptosis and HF has also been the focus of several studies. Myocardial expression of survivin is increased after acute myocardial infarction (MI), and may be indicative of more favourable LV remodelling post-MI. Similarly, survivin is elevated in end-stage HF, and is reversibly regulated after ventricular

Figure 5 Apoptosis by TUNEL and caspase activity. Representative immunofluorescent TUNEL images of CON, DOX, DOX + EMP, and DOX + SURV at Week 4 [(A) scale bar: 20 μm]; per cent apoptosis by TUNEL [(B) *P < 0.001 vs. CON, †P < 0.001 vs. DOX + SURV, n = 10, 11, 9, 10 for CONTROL, DOX, DOX + EMP, and DOX + SURV, respectively]; and caspase-3/7 activity [(C) *P < 0.001 vs. CON, †P < 0.001 vs. DOX + SURV, n = 11, 9, 13, 11 for CONTROL, DOX, DOX + EMP, and DOX + SURV, respectively] at Week 4 in the corresponding groups. DOX and DOX + EMP hearts had a higher number of apoptotic bodies indicated by FITC positivity (white arrows). Co-staining for sarcomeric-alpha-actinin (red) shows predominantly cardiomyocytes undergoing apoptosis. Caspase-3/7 activity was increased in the DOX and DOX + EMP groups compared with CON and DOX + SURV.
unloading. The seminal work in this field was performed by Levkau et al. who demonstrated that cardiac-specific deletion of survivin resulted in pre-mature cardiac death due to a dramatic reduction in total cardiomyocyte numbers leading to marked reduced LV systolic function. They also showed that adenoviral overexpression of survivin in cardiomyocytes inhibited DOX-induced apoptosis in vitro. Our present study is the first to examine survivin gene therapy in an in vivo model of HF.

We delivered targeted survivin gene therapy to the left ventricle in DOX-induced cardiomyopathy, resulting in a reduction in cardiomyocyte apoptosis and caspase activity. This modulation of apoptosis was associated with improvement in LV systolic function as assessed by echocardiography and cardiac catheterization, with improved LV FS, PRSW relationship, and ES PV relationship, coupled with a reduction in LV ED volume. The LV dp/dt showed no significant changes; however, given the load-sensitive nature of dp/dt, the load-insensitive measures of PRSW relationship, and ES PV relationship point to a significant improvement in LV contractility. DOX led to a minor reduction in RV function and increased RV fibrosis that was not attenuated by LV-targeted ultrasound-mediated survivin gene therapy. Apoptosis may lead to the recruitment of macrophages, which release fibrogenic mediators, activating myofibroblasts within the myocardium as well as release of connective tissue growth factor from apoptotic endothelial cells that induce fibrosis. Thus, the attenuation of interstitial fibrosis with survivin gene therapy may be indirectly attributed to caspase inhibition. While we have demonstrated the efficacy of survivin gene therapy in a cardiac model of disease, anti-apoptotic survivin therapy had been used in pulmonary arterial hypertension and for the ex vivo enhancement of mesenchymal stem cell therapy in MI.

Our in vitro data support the in vivo findings, with a marked inhibition of DOX-induced cell death in cardiomyoblasts. One novel aspect of our study is the finding that the protective effects of survivin gene therapy

**Figure 6** Assessment of LV fibrosis by picrosirius red staining. Representative images of the left ventricle at Week 6 in CON, DOX, DOX + EMP, and DOX + SURV ([A] scale bar: 25 μm); and the quantification of fibrosis by a polarized light microscopy ([B] †P < 0.001 vs. CON, *P < 0.001 vs. DOX + SURV; n = 10, 10, 7, 10 for CON, DOX, DOX + EMP, and DOX + SURV, respectively). Interstitial fibrosis (red staining) is increased in DOX and DOX + EMP groups, compared with CON. DOX + SURV shows less interstitial fibrosis.
may not only attributed to direct myocyte transfection, but also to the effects of secreted survivin protein. In addition, our in vitro results suggest a potential role of non-cardiomyocytes, such as fibroblasts and endothelial cells, in mediating the paracrine effect of survivin. Survivin’s dual role as an anti-apoptotic protein and a crucial member of the cell division complex has been previously described. The subcellular localization of the protein dictates the function, with anti-apoptotic function attributed primarily to the cytoplasmic fraction. Indeed, our data demonstrate that the SURV-emGFP is localized to the cytoplasm, indicating the anti-apoptotic potential of our fusion protein (Figure 1A). While these prior studies found survivin localized only to the intracellular compartment, more recent studies demonstrate survivin secretion into the extracellular space via exosomes, having biological effects on surrounding cells. In tumour cells, secreted survivin was shown to be absorbed by neighbouring cells, conferring protective anti-apoptotic effects. Results from that study also demonstrated that bone marrow stromal cells, used as normal control cells, do not uptake extracellular survivin in contrast to malignant tumour cells, indicating the possibility of cell specificity in extracellular survivin targets. Extracellular survivin interacts with neutrophils to induce the expression of adhesion molecules on the cell surface, raising the possibility that the mechanism of action for extracellular survivin’s effect on cellular function may in part be receptor mediated. Our in vitro studies demonstrate that similar to cancer cells, cardiomyoblasts also uptake survivin, leading to a reduction in apoptosis in response to doxorubicin. However, while survivin is released from cancer cells primarily via exosomes, our studies demonstrate that secretion of extracellular survivin by cardiomyoblasts occurs in both exosomal and non-exosomal fractions, with the latter being more pre-dominant. Given that DOX cardiomyopathy is mediated in part by depletion of the endogenous cardiac stem cell pool and can be rescued by exogenous progenitor cell therapy, the positive effects of survivin gene therapy may also be related to an anti-apoptotic effect on endogenous cardiac stem cells, in addition to cardiomyocytes.

There are several important limitations to our study. Survivin gene therapy was performed at a time when LV systolic function was relatively preserved, thus our study was more for prevention of progression in HF. Further studies are required to investigate the potential for reversal of LV dysfunction in various models of HF, and on the longevity of survivin gene therapy. While we did not formally assess transfection of remote organs by ultrasound-mediated gene delivery, prior studies from our lab and others have shown the target specificity of this technique. While the scope of our present study does not specifically address the mechanism by which extracellular survivin is taken up by cardiomyoblasts in vitro, this is the focus of future studies. The overall improvement in parameters of LV systolic function was relatively modest, thus repeat deliveries may be necessary to produce a more robust enhancement of LV contractility. Finally, studies are required to examine the therapeutic effect of anti-apoptotic strategies in HF of different aetiologies, such as diabetic cardiomyopathy or post-MI.

5. Conclusions

We demonstrate that survivin gene therapy using ultrasound-mediated gene delivery prevents cardiomyocyte apoptosis and interstitial fibrosis, resulting in a modest attenuation of LV systolic dysfunction in a model of cardiomyopathy induced by DOX. The anti-apoptotic effects of survivin gene therapy can be attributed to both direct myocyte transfection and potentially to the effects of extracellular-secreted survivin.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

Funding
This study was supported by an Operating Grant (FRN 62763) from the Canadian Institutes of Health Research, Ottawa, ON, Canada, and an Equipment Grant from the Canadian Foundation for Innovation, Ottawa, ON, Canada. H.L.-P. is supported by the Brazilian Ball Chair in Cardiovascular Research, St Michael’s Hospital, University of Toronto, and an Early Researcher Award, Ministry of Research and Innovation, ON, Canada.

References
21. Lang RM, Bierig M, Devereux RB, Flachskampf F, Foster E, Pellikka PA et al. Recommendations for chamber quantification: a report from the American Society of Echocardiography’s Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr 2005;18:1440–1463.


