Survivin Gene Therapy Using Ultrasound-Targeted Microbubble Destruction in a Rat Model of Doxorubicin-Induced Cardiomyopathy

by

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Abstract

With the recent advent of gene therapy, anti-apoptotic therapy has been receiving spotlight as a potential modality to inhibit the deterioration of pump function in the failing heart. We hypothesized that anti-apoptotic therapy using survivin gene delivery will 1) salvage H9c2 cells exposed to doxorubicin toxicity, and 2) ameliorate the progressive decline in left ventricular function in a rat model of doxorubicin-induced cardiomyopathy. The in vitro data suggested that survivin successfully prevented cell death under doxorubicin stress by both direct and indirect/paracrine mechanisms. Doxorubicin-treated animals developed progressive left ventricular dysfunction as evident by echocardiography and invasive pressure-volume loop analysis, which was prevented by ultrasound-mediated survivin plasmid delivery, but not empty plasmid delivery. Post-mortem analysis of myocardial tissue indicated a lowered apoptotic index in survivin-treated hearts, with evidence of decrease in interstitial fibrosis. In conclusion, survivin gene therapy was shown to ameliorate doxorubicin-induced cardiomyopathy, by decreasing apoptosis and preventing adverse remodeling.

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List of Abbreviations

AAV	adeno-associated virus
ACE	angiotensin converting enzyme
AngII	Angiotensin II
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BIR	Baculoviral IAP repeat
BIRC5	baculoviral IAP repeat containing protein 5
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CABG	coronary artery bypass graft
CARD	caspase activation recruitment domain
cDNA	complementary deoxyribonucleic acid
ΔCt	change in threshold cycle
CTGF	collagen growth factor
CVD	cardiovascular diseases
DIABLO	direct IAP binding protein with low pI
DISC	death inducing sigalling complex
DMEM	Dulbecco's modified eagles media
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECG	electrocardiography
EDP	end-diastolic pressure

ELISA	enzyme-linked immunosorbent assay
emGFP	emerald green fluorescent protein
ESP	end-systolic pressure
ESPVR	end-systolic pressure volume relationship
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FasR	Fas Receptor
FMA	Fluorescent Microangiography
FS	fractional shortening
GUS	beta-glucuronidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	heart failure
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HR	heart rate
IAP	inhibitor of apoptosis protein
LV	left ventricle
LVAD	left ventricular assist device
LVEDd	left ventricular end-diastolic dimensions
LVESd	left ventricular end-systolic dimensions
MDM2	mouse double mutant
MMP	matrix metalloprotease
MOI	multiplicity of infection
NADH	reduced nicotinamide adenine dinucleotide

NCM	null-conditioned media
NES	nuclear export signal
PKC	protein kinase C
PROVED	Prospective Randomized Study of Ventricular Failure and the Efficacy of Digoxin
PRSW	preload recruitable stroke work relationship
PSR	Picrosirius red
qPCR	quantitative polymerase chain reaction
RADIANCE	The Randomized Assessment of Digoxin on Inhibitors of Angiotensin-Converting Enzyme
RCF	relative centrifugal force
RING	really interesting new gene
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RPM	rotations per minute
RV	right ventricle
SCF	stem cell factor
SCM	survivin-conditioned media
SMAC	second mitochondria-derived activator of caspases
TAH	total artificial heart
TGF-β	transforming growth factor-beta
TNFaR1	TNFa receptor type-1
TNFa	tumour necrosis factor-alpha
TRADD	tumour necrosis factor receptor type 1- associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand

TRAILR1   TRAIL receptor type-1

TUNEL   terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

UBC   ubiquitin-conjugating domain

UTMD   ultrasound-mediated microbubble destruction

VEGF   vascular endothelial growth factor

XIAP   x-linked inhibitor of apoptosis protein

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Chapter 1 - Introduction and Literature Review

1.1 Cardiovascular Diseases

Cardiovascular diseases (CVD) are of major public health and socioeconomic concern as the burden of cardiovascular disease in Canada and the United States reached approximately $20.3 and $503 billion in 2010 (Roger et al., 2011; Ross et al., 2006), respectively. According to the 2010 annual report released by the American Heart Association, the number of patients suffering from one or more cardiovascular disease-related illness reached approximately 81 million and remains the leading cause of death in both men and women (Roger et al., 2011). Some of these include, in order of increasing prevalence: high blood pressure (74,500,000), coronary heart disease (17,600,000), heart failure (HF: 5,800,000), stroke (6,400,000), and congenital cardiovascular defects (650,000-1,300,000) (Roger et al., 2011). In 2006, CVD accounted for 34.3% of all deaths in the United States. CVD related deaths were greater than any other causes of death every year since 1900, except in 1918.

Many types of CVDs can cause irreversible damage to the heart, causing neurohormonal (Bernstein, Fajardo, & Zhao, 2011; Lohse, Engelhardt, & Eschenhagen, 2003; Mendzef & Slovinski, 2004) and physical changes (Gaballa & Goldman, 2002) that may eventually develop into congestive HF, and ultimately leading to death. Paradoxically, with the advancements in medical research and improvements in surgical and pharmacological interventions, it is expected that increasing number of injured hearts survive the initial insult, and as a result, patients with these injured, aging hearts are

predisposed to developing HF later in their lifetime (*Heart and stroke foundation: Statitstics.*).

1.2 Heart Failure: Introduction and Prevalence

Heart failure is a multi-factorial clinical syndrome characterized mainly by the decline of heart's ability to pump blood, volume overload, or both. HF can be characterized by reduced systolic ejection fraction, impaired heart chamber filling and elevated filling pressures – termed systolic HF. These abnormalities can lead to pulmonary congestion, edema and dyspnea. HF affects some 500,000 people in Canada (Ross et al., 2006) and 5.8 million people in the United States, and with advances in therapies for acute myocardial infarction resulting in improved longer term survival rates, the prevalence of systolic HF is expected to increase over time. This is a syndrome with increasing prevalence in older age groups, particularly alarming in today's era of improved longevity. Prognosis for HF is poor, with 25% of patients succumbing at 1-year, and less than 50% survival rate at 5 years (Roger et al., 2011). Unlike other CVDs, HF is the end stage of all cardiac diseases. It is most often a consequence of hypertension, ischemic heart disease/myocardial infarction, valvular heart disease, diabetes, congenital heart disease, or other systemic diseases (Levy D., Larson M. G., Vasan R. S., Kannel W. B., & Ho K. K. L., 1996; McMurray & Stewart, 2000). The estimated direct and indirect cost of HF in the United States for 2010 is $39.2 billion, which is considered to be grossly underestimated, as it only account for data collected for HF as the primary diagnosis (Roger et al., 2011). Although HF has been considered to be an irreversible and progressive process characterized by physiological changes leading to the diminished contractility, recent developments have suggested that certain types of therapy may retard

the progression or even reverse the cardiomyopathic process (Levin et al., 1995; Wohlschlaeger et al., 2005).

1.3 Heart Failure: Pathophysiological Changes

The progression towards HF begins with an insult to the normal cardiac function, whose causes can include, but are not limited to: myocardial ischemia, infarction, inflammation, hypertension, and genetics. There are physiological adjustments that can be made to counteract the decline in myocardial performance, such as increase in heart rate (Mitchell, Wallace, & Skinner, 1963), contractility (Haber et al., 1993), preload (Glower et al., 1985) and contractile elements (Katz, 1994). These changes are accomplished by an increase in the activity of two neurohormonal systems, the adrenergic and renin-angiotensin-aldosterone systems (Francis, 1989). Although the two systems are quite effective in providing short-term solution to the decreased cardiac output, chronic activation will eventually outweigh the initial benefit, leading to adverse remodeling and worsening of the systolic function.

One of the mechanisms of progressive HF is the change in the ventricular structure through adverse ventricular remodeling. The failing heart takes on a characteristic shape due to a process known as eccentric hypertrophy, which was first described by Grossman and colleagues in 1975 (Grossman, Jones, & McLaurin, 1975). It was noted that the elongated, hypertrophied myocytes – sometimes increasing its size up to 100% of normal volume (Beltrami et al., 1995) – led to the increase in myocardial mass, but with minimal effect on the wall thickness. Non-myocytes, such as fibroblasts, also contribute to the remodeling process within the myocardium (Weber & Brilla, 1991). In the presence of

eccentric hypertrophy, extracellular matrix is crucial in maintaining the structural integrity of the ventricle. Fibroblasts, by controlling the production and degradation of the collagen network, maintains the wall stress, but this effect comes at a price of reduced capillary density and increased oxygen diffusion distance, which can aggravate the pre-existing ischemia and worsening of the metabolic stress of the energy-starved heart (H. N. Sabbah, Sharov, Cook J.M., Shimoyama, & Lesch, 1995). Moreover, the increased extracellular matrix and fibrogenesis adds to the structural rigidity of the myocardium, further aggravating the myocytes' ability to fully contract, reducing the ventricular contractility (Weber & Brilla, 1991).

The remodeling process can be explained by the activation of the aforementioned neurohormonal systems. The renin-angiotensin-aldosterone system is activated by the combination of altered load and the stretch of the myocytes, and results in the release of angiotensin (Ang)II (Sadoshima, Xu, Slayter, & Izumo, 1993). AngII activates protein kinase C (PKC), which in turn activates the expression of immediate/early genes that promote cell growth and hypertrophy (Komuro et al., 1991). AngII alone also induces protein synthesis, and promotes cardiomyocyte hypertrophy in vitro and in vivo (Baker & Aceto, 1990; Linz, Scholkens, & Ganten, 1989; Sadoshima et al., 1993). It is also mitogenic for cardiac fibroblasts, leading to increased collagen deposition in the myocardium (Weber & Brilla, 1991). Relating to the beta-adrenergic system, AngII acts as a facilitator for norepinephrine release (Bristow & Abraham, 1995), which is toxic to isolated cardiomyocytes (Mann, Kent, Parsons, & Cooper, 1992). Norepinephrine was shown to induce abnormal $Ca2+$ handling, decreased protein synthesis, and contractile dysfunction in cultured cardiomyocytes (Mann et al., 1992). In addition, angiotensin

induces the angiotensinogen and transforming growth factor-β (TGF-β), which are both mitogenic, and stimulates hypertrophic responses (Sadoshima & Izumo, 1993).

In addition to the structural changes, the long-term activation of the adrenergic and renin-angiotensin-aldosterone system elicits metabolic changes within the myocardium. Chronic activation of the two systems in HF settings leads to increased heart rate, wall stress and contractile mass, which are the three major factors that determine oxygen demand (Nelson et al., 1974). The metabolic need of a dilated/hypertrophied myocardium will inevitably be higher compared to that of the normal hearts. In conjunction to the higher metabolic need, the altered myocardial milieu (i.e. increased fibrosis and decreased capillary density) exacerbates the oxygen supply, leading to inadequate tissue perfusion. This results in a perpetually "energy-starved" condition in the heart (Katz, 1988), which is speculated to damage subcellular processes and myocyte function (Eichhorn & Bristow, 1996).

Given the involvement of neurohormonal system in the progressive nature of HF, it is presumed that the administration of ACE inhibitors (acting on the renin-angiotensin system) and beta-blockers (acting on adrenergic system) will have a favourable effect on mitigating the progression of HF.

1.4 Current Therapeutic Options for Heart Failure Patients

In order to determine the best course of action and therapeutic options, physicians assess the stage of HF according to different systems of classification. HF patients can be classified into 4 classes according to the functional limitations and the quality of life of patients with HF as defined by the New York Heart Association (NYHA). Other

classification systems have also been developed to evaluate the evolution, progression

and structural changes accompanying HF (Hunt et al., 2001).

New York Heart Association (NYHA)	American College of Cardiology and American Heart Association
I No symptoms, no limitation of physical activity	A No structural damage and at high risk for developing HF
II Symptoms with substantial activity – no symptoms at rest	B structural abnormality and no symptom
III Symptoms with minimal activity – no symptoms at rest	C Structural abnormality and previous or current symptoms
IV Symptoms at rest	D Refractory symptoms

Table 1 **. Different classification systems of HF symptom severity**.

1.4.1 Preventative Measures

Medical therapy, including preventative (non-pharmacological) measures, is the first-line

strategy in treating patients with HF. Strategies involved in the prevention of HF involves

understanding the pathophysiology of HF, and identifying the risk factors involved in the

development and the progression of the disease. Certain risk factors increase the chances

of developing pathological cardiac events that may eventually lead to the culmination of

HF. One of the main risk factor in the development of HF is lifestyle, such as cigarette

smoking (He et al., 2001), physical activity and obesity (Kenchaiah et al., 2002). It was

noted that the decrease in deaths from coronary heart disease (one of the main causes of

congestive HF) from 1980 to 2000, 44% of the decrease could be attributable to lifestyle

and environmental changes (Ford et al., 2007). This indicates the predominant role of

lifestyle in determining the risk of development to HF. In addition, pre-existing

cardiovascular disease, such as hypertension increases the risks of developing HF. Elevated blood pressure, especially systolic pressure, increases the risk of HF significantly (Lloyd-Jones et al., 2002). Hypertension has been identified as the cause of HF in up to 59% of women and 39% of men in the Framingham Study cohort (Levy D. et al., 1996). In addition, a cohort study of the Systolic Hypertension in the Elderly Program Cooperative Research Group noticed that HF occurrence was twice as often in patients receiving placebo, versus those receiving treatments for hypertension (Kostis et al., 1997). Accordingly, antihypertensive therapy has been shown to reduce the risk of HF. A meta-analysis study revealed a 52% reduction in HF with diuretic intervention (Moser & Hebert, 1996).

1.4.2 Pharmacological Therapy

Most patients with symptomatic HF should be routinely managed with a combination of four types of agents: 1) a diuretic, 2) digoxin, 3) beta-blocker, and 4) a blocker of the renin-angiotensin-aldosterone system, such as an angiotensin-converting enzyme (ACE) inhibitor or angiotensin-receptor blocker (M. K. Davies, Gibbs, & Lip, 2000). The utility of these drugs have allowed for the better management of HF symptoms to alleviate the effects of the failing heart on patients' quality of life, and for some drugs, offer prognostic benefits to increase survival rates. The conventional order of therapy is that patients with systolic HF are first put on a regimen of diuretics, with the goal of achieving euvolemia. Although it offers no prognostic benefits (M. K. Davies et al., 2000), it relieves the symptoms of HF, such as edema, while reducing ventricular diastolic pressure, ventricular wall stress, and maximizes sub-endocardial perfusion. Digoxin was used as a key HF drug to improve cardiac output, before the advent of the

neurohormonal hypothesis of HF. The Randomized Assessment of Digoxin on Inhibitors of Angiotensin-Converting Enzyme (RADIANCE) trial showed that withdrawal of digoxin from the cocktail of diuretics and ACE inhibitors worsened HF symptoms, and lowered exercise tolerance (Packer et al., 1993). Similarly, Prospective Randomized Study of Ventricular Failure and the Efficacy of Digoxin (PROVED) trial assessed diuretic treatments with or without digoxin, and the results were similar (Uretsky et al., 1993). Neither study reported any changes on mortality. This was expected, as digoxin, like diuretics, does not modulate the neurohormonal tone.

1.4.2.1 Angiotensin Converting Enzyme Inhibitors

Following the symptomatic treatment, ACE inhibitors (or angiotensin-receptor blockers if ACE-intolerant) and beta-blockers are administered as promptly as possibly to maximize clinical benefit and alter prognosis. ACE inhibitors have consistently shown beneficial effects on morbidity, mortality and quality of life in large-scale clinical trials (The CONSENSUS trial study group. 1987; The SOLVD investigators,1991), and are indicated in all stages of symptomatic HF. ACE inhibitors work by inhibiting the production of AngII, a vasoconstrictor and growth promoter. ACE inhibitors have also been shown to increase the concentration of bradykinin indirectly by inhibiting its degradation. Bradykinin, a vasodilator, has been shown to increase the production of nitric oxide and prostacyclin, contributing to the positive hemodynamic effects of ACE inhibition (Linz, Wiemer, Gohlke, Unger, & Scholkens, 1995; Mancini et al., 1996). Some of the side effects of the drug, however, may be also attributed to the actions of bradykinin, which are dry cough, hypotension and angioedema. ACE inhibitors also reduce the activity of the sympathetic nervous system, through the inhibition of AngII

production, which increases the release of noradrenaline and prevents its reuptake (Grassi et al., 1997). It also improves beta-receptor density (up-regulation) (Gilbert et al., 1993), variation in heart rate and baroreceptor function (Grassi et al., 1997). It has been reported that angiotensin-receptor blockers and ACE inhibitors together benefit the patients in morbidity and mortality, but the use of these two agents together are not commonly practiced due to the increased risk of adverse effects (Pitt et al., 1999).

1.4.2.2 Beta-Blockers

Asymptomatic HF patients exhibit increased cardiac adrenergic drive – the earliest detectable symptom – triggered by the decreased cardiac output and perfusion of organs, leading to the release of norepinephrine that stimulates ventricular contraction as a compensatory mechanism (Rundqvist, Elam, Bergmann-Sverrisdottir, Eisenhofer, & Friberg, 1997). However, with prolonged increase in adrenergic activity, the heart uses up more energy, leading to increased myocardial oxygen demand and oxidative stress. The vasoconstrictive effect of adrenergic activation increases both preload and afterload, increasing mechanical stress on the failing heart. Norepinephrine also down-regulates the beta-1 receptors and uncouples the beta-2 receptors, leaving myocytes less responsive to adrenergic stimuli, further decreasing the contractile function (Bristow et al., 1990). Thus the benefit in the use of beta-blockers to decrease the adrenergic drive may be explained by the energy-starved state of the failing heart.

Beta-blockers have a paradoxical effect on HF as it was once postulated to be dangerous in patients with HF due to its effects on heart rate and contractility (Nayler, Chipperfield, & Lowe, 1969). Conventional wisdom held that HF was solely due to the decline in

systolic function and was an absolute contraindication for the prescription of any medication with negative inotropic action. However, recent developments in HF mechanism recognizing the complex nature of HF involving maladaptive increase in adrenergic drive has sparked scientific endeavors revealing that it reduces morbidity and mortality and is now a common therapy supported by clinical guidelines. The initial effect of the drug decreases blood pressure and cardiac index, but long-term administration of beta-blockers is associated with increases in ejection fraction, cardiac index, decrease in LV end diastolic pressure. Beta-blockers also reverse the deleterious effects associated with LV remodeling and decrease myocardial mass and LV volume, leading to improved hemodynamics (Palazzuoli et al., 2002). Despite the clinical benefits of beta blockers, limitations of the pharmacological agent includes the requirement of careful blood pressure monitoring with the initial administration of the drug, as they can act as vasodilators (Rudolph & McMahon, 2002). HF patients often have contraindications to beta-blocker use, and are very difficult to initiate in patients, as up-titration to effective dosages requires persistence as most patients experience worsening of the symptoms at initial dosages.

Although polypharmacological therapy is essential in managing HF symptoms and prognosis, it does not offer a be-all-end-all solution to the patients suffering refractory heart failure, and the mortality rates have remained alarmingly high. Polypharmacotheraphy is of growing concern, as many patients with HF are treated with several different drugs, and patients with comorbidities, such as diabetes or arthritis, will be treated with a set of drugs for the management of those illnesses (Flesch & Erdmann, 2006). In addition, many patients are prescribed drugs to overcome the side effects of the

drugs initially prescribed to treat HF, further adding to the already sizeable list of drugs. Issues with polypharmacy encompass not only the problem of side effects associated with individual or combination therapy, but taking unnecessary therapy also may lead to a reduction in compliance with the effective drugs (Volpe, Chin, & Paneni, 2010).

1.4.3 Mechanical and Surgical Therapy

In patients with advanced HF and refractory to conventional medical therapies, certain surgical and mechanical interventions may be employed to improve the quality of life and the prognosis of the disease. Ventricular assist devices and implanted defibrillators, and biventricular pacing devices have been shown to be effective in the treatment of patients with end-stage HF. However, these intervention modalities benefit only a small population of patients, and are extremely costly, making it less appealing for the masses (Oz et al., 2003). Nonetheless, it remains the only option for patients who have no other therapeutic alternatives.

1.4.3.1 Mechanical Assist Devices and Total Artificial Heart

Mechanical assist devices such as left ventricular assist devices been developed for patients suffering from end-stage HF, who are not amenable to transplantation, either due to the lack of donor hearts, or because of contraindications for organ replacement. In these cases, LVADs are used as 'bridge to transplant' therapy until donor hearts become available, or as 'destination therapy' without the prospect of receiving a donor heart. Patients who receive LVAD as a mean to restore basic cardiac function without subsequent transplantation are known to be on a 'bridge to recovery' therapy. These devices have been explored and clinically utilized as a terminal, stand-alone therapy for

patients suffering from refractory congestive HF, as is used in the 'destination therapy' (Lietz et al., 2007; Park et al., 2005). LVAD has received more interest since its development, as it has been associated with reverse remodeling of failing hearts. Changes post-LVAD implant include decreased LV diameter and increased LV wall thickness, as well as reduction in organ size (Wohlschlaeger et al., 2005). The molecular mechanisms involved in the reversing of the remodeling processes include changes in extracellular matrix formation (via MMPs) (Y. Y. Li et al., 2001) and alteration in the neurohormonal signals that accompany normalization of volume and pressure overload with LVAD. For example, evidence of changes in beta-adrenergic signal transduction was noticed after unloading by LVAD, through a restoration of beta-adrenergic receptor density in cardiomyocytes (Ogletree-Hughes et al., 2001). In addition, up-regulation of anti-apoptotic proteins and decrease in apoptosis was demonstrated with LVAD treatment (Baba et al., 2003). Although a viable therapeutic option for transplant-ineligible HF patients, there is an increased risk for infections and hemorrhagic complications. Although patients treated with LVAD have a significant improvement in quality of life, and functional status, the risks associated with the intervention is high, with 25-40% death rate until transplantation or device removal (Birks, Yacoub, Banner, & Khaghani, 2004). Device-related complications include the production of human leukocyte antigen (HLA) antibodies, infection and device failure (Birks et al., 2004).

Similarly, total artificial heart (TAH) is currently explored as a viable bridge to transplantation option. The first implantation of TAH was done by Dr. Denton Cooley in 1969 in a patient who could not be removed from cardiopulmonary bypass following LV aneurysmectomy (Selzman, Bhati, Sheridan, Stansfield, & Mill, 2006). As of 2010,

CardioWest TAH is the only TAH that is approved for use in bridge to transplantation by the FDA, Health Canada and Consultants Europe (Nicholson & Paz, 2010). In comparison to LVAD transplantation, TAH was shown to reduce the incidence of stroke or ischemic heart attack, and had comparable rates of bleeding and infections (J. G. Copeland et al., 2003). TAH has the highest survival to transplant rate of any device (J. G. Copeland et al., 2004). The primary difference is that TAH is only used for bridge to transplant, mainly due to the large size of the console limiting the patients' mobility, while LVAD is used as either bridge or destination therapy (Nicholson & Paz, 2010). It has been shown that in a selected group of patients with end-stage HF, TAH implantation improved survival significantly: 96% of the patients survived the transplant and were discharged, and the total survival was 89% during a mean follow-up time of 20 months while control patients had a survival rate of close to 30%. However, all implanted patients suffered at least one adverse event, indicating a need for close observation of the patients post-operatively (J. G. Copeland 3rd et al., 1998). As with any surgery introducing foreign objects into the body, complications may arise with TAH implantation. Infection, severe post-operative bleeding, thromboembolism are few among a list of many (J. G. Copeland 3rd et al., 1998; J. G. Copeland et al., 2004; Nicholson & Paz, 2010). In order for TAH to be widely accepted and utilized, improvements must be made to prevent these complications.

1.4.3.2 Surgical Intervention

Surgical therapies include procedures, such as coronary artery bypass graft (CABG), valve replacements/repair, or whole organ transplant. For patients with refractory HF, the gold standard therapy is heart transplantation. Transplantation patients have fewer

rehospitalization, marked functional improvements, enhanced quality of life, and longer lives, with 50% of the patients surviving to 10 years (Boilson, Raichlin, Park, & Kushwaha, 2010). Although heart transplantation is the optimal solution for end-stage HF, its use is limited by inadequate supply to meet the demand. In Canada, 167 heart transplants were performed in 2010, with 135 patients awaiting transplant (Canadian Institute for Health Information, 2010). Typically, 10-20% patients die while on the transplant waiting list (Canadian Institute for Health Information, 2010; McManus et al., 1993). Paradoxically, advanced age, which is associated with higher incidences of HF, is often used as an exclusion criterion for heart transplant. This view is, however, being challenged with our current knowledge on mortality/morbidity relative to the younger patients (Blanche et al., 1996; Weiss, Nwakanma, Patel, & Yuh, 2008). The disparity between the supply and demand has forced the medical field to find other ways to support the severely failing hearts, including coronary artery bypass, aortic valve replacements, mitral valve repair, ventricular restoration, and the use of mechanical circulatory support devices.

1.5 Emerging Therapies for Heart Failure

Due to the side effects associated with the current treatment options, the shortage of transplant donors relative to the recipient waitlist, and the persistent high mortality rate, development of novel and improved therapeutic methods are required to alleviate this socio-economic conundrum. Recent advancements in the field of cardiac research have pointed towards the use of cellular and genetic therapies in treating heart disease.

1.5.1 Cell Therapy

HF involves the progressive loss of functional cells within the myocardium (Narula et al., 1996; Narula, Arbustini, Chandrashekhar, & Schwaiger, 2001), and naturally, the best course of therapy would involve the replenishment or replacement of damaged cells. The basic premise of cell therapy had been initially studied as a method of introducing functional myocytes or cells with potential to differentiate into myocytes into the infarcted heart in hope to repopulate the scarred myocardium. Early studies in animal models have yielded promising results. Skeletal myoblast implantation in rats showed improved heart function after ischemia, with increased contractility, attenuation in ventricular dilation and improved exercise capacity (Jain et al., 2001). Clinical studies showed that these skeletal myoblasts differentiated into functional cardiac-like myocytes, persisting in the recipient heart months after the initial transplant (Hagege et al., 2003; Hagege et al., 2006). However, doubts were cast on the hurried use of stem cells for treatment of heart disease, especially after some clinical studies reporting high rates of complications of restenosis following cell therapy, which led to the re-evaluation of the therapeutic strategies (Chien, 2004; Kang et al., 2004). There are criticisms that the clinical trials have progressed too rapidly based on the results from early exploratory studies, without first understanding the biology and the mechanisms of the benefits brought on by cellular therapy. Optimization for cell types, numbers, and cell delivery methods are currently being explored. Research is ongoing for different approaches to cell therapy: direct muscular injection, systemic injection, use of scaffolding materials, and genetically engineered and preconditioned cells. Currently the prevailing dogma is that in addition to the direct cellular effect of the engraftment of the donor cells,

paracrine effects of the cytokines secreted from the donor cells mediate the amelioration of the disease (Gnecchi, Zhang, Ni, & Dzau, 2008; Y. L. Tang et al., 2005). The field of cell therapy is still in its relative infancy, and the mechanism(s) of action is still being elucidated.

1.5.2 Gene Therapy

Gene therapy is a treatment modality involving the introduction of exogenous genes that provides previously non-existing functions to the cell, modulate and augment existing, yet non-functional ones, or substitute deleterious mutant variants with functional ones. The field of gene therapy is relatively new, and similar to cell-based therapies, is still in its infancy. However, gene therapy has been explored as one of the most promising emerging therapies with increasing knowledge of the mechanism of HF. Better understanding of the disease mechanisms, has led to the identification of novel therapeutic targets that may be difficult to modulate with existing pharmacological therapies, but may be more amenable to genetic therapies. Considering the immense changes that the heart undergoes in the process of HF, and the numerous etiologies that lead to the failing heart, it is unlikely to find a "golden bullet" gene that will single-handedly improve the heart condition. Therefore, the future of gene therapy will involve the identification of pathophysiological changes in distinct cases and strategize the therapeutic modalities in accordance to individual needs.

1.5.2.1 Gene Delivery Mechanism

Gene delivery, regardless of the target tissue, requires efficient transduction and stable expression of the gene, specifically in the tissue of interest. Ease of applicability (non-

invasive, repeatability) is also very important in clinical settings. Various delivery vehicles can be grouped into viral and non-viral categories. Currently, no delivery methods fulfill all the criteria for optimal gene delivery technique, each delivery technique having both advantages and disadvantages over the other.

1.5.2.2 Non-Viral Vector

Non-viral gene vectors include the use of plasmids or small nucleic acids for the modulation of gene expression, both upregulation and downregulation. These vectors may be injected alone, or in conjunction to some physical/chemical method to facilitate delivery into the cell. Naked DNA was first used by Isner and colleagues for treatment of hindlimb ischemia in patients using the VEGF gene (Isner et al., 1996). Myocardial injection of genes in coronary disease setting was done two years later (Schumacher, Pecher, von Specht, & Stegmann, 1998). The main advantage of direct injection of genes into the target tissue is the low immunogenicity of naked DNA/RNA (Mountain, 2000). However, due to the nature of the method of delivery (directinjection), the transfection efficiency is low (i.e. does not integrate into the genome, destroyed by host cell) (Hyvonen, Ruponen, Ronkko, Suhonen, & Urtti, 2002; Niidome & Huang, 2002).

1.5.2.3 Viral Vectors

Viral vectors are widely used in research for gene delivery due to their ability to invade the host cell, utilizing the host's expression machinery to produce the gene of interest. In viral vectors, most of the viral genome is replaced with the gene of interest rendering the virus nonpathogenic. Lentiviral, adenoviral and adeno-associated viral vectors are three viral vectors that are used widely in cardiac gene delivery.

Adenoviruses are non-integrating viruses (episomal) and have tissue tropisms for cardiac, liver, and the respiratory tract (Rapti, Chaanine, & Hajjar, 2011). A major concern for adenoviruses is its immunogenicity: safety of adenoviral therapy was raised after the death of a patient following the injection of an adenoviral vector (Yarborough & Sharp, 2009). The host immune response to the adenovirus and the cells expressing the exogenous gene may hamper the efficacy of the therapy, and deter the repeatability of the procedure, if needed.

Lentiviral vectors, unlike adenoviral vectors, can stably infect non-dividing cells (i.e. cardiomyocytes) (Davis et al., 2008). Integration into the genome increases the length of the transgene expression in the host, but also raises concerns of host cell mutagenesis or oncogenesis. Efforts to develop lentiviral vectors without the enzyme responsible for its integration may improve the safety of the vector. However, it was noted that the lack of the enzyme activity led to a reduction in gene expression duration (Bonci et al., 2003).

Adeno-associated viral (AAV) vectors have unique advantages as gene therapy vectors. These vectors are non-integrating, infect both dividing and non-dividing cells (Rapti et al., 2011), and can maintain long-term gene expression, reaching 8 years in animal models (Hadaczek et al., 2010). AAVs, depending on the serotype, have varying tropisms for various tissue types. AAV1, 6, 8, and 9 have been identified as being cardiotropic (Zincarelli, Soltys, Rengo, & Rabinowitz, 2008), 1 and 6 having been validated in human clinical trials (Rapti et al., 2011). Despite the advantages of AAV over the other viral vectors, one of the major limitations is the inability to accommodate larger gene inserts.

1.5.2.4 Gene delivery Techniques

An effective gene delivery technique must allow the gene to translocate into the nucleus to allow its transcription, but must do so by transporting the gene through various host barriers. These barriers include circulating antibodies (for viral vectors), endothelial cell barrier, and the cell/nuclear membrane.

Both viral and non-viral vectors have been used in the direct muscular injection method for gene therapy. However, the wide use of this method is impeded by the severely limited spatial distribution within the tissue, and focal expression at the initial injection site (Kawase, Ladage, & Hajjar, 2011; Kobulnik, Kuliszewski, Stewart, Lindner, & Leong-Poi, 2009). This method may be amenable to small animal studies, but in larger animals and humans, translation of these research findings may not be applicable. Locations of direct injection methods include intrapericardial (epicardial), catheter-based intramyocardial (endocardial) gene transfer, and direct intramyocardial injection during thoracotomy.

Trans-vascular gene transfer offers a diffuse expression of the gene product, but unlike direct myocardial injection, the vector must find its way through the endothelial barrier. To facilitate the transfer of vectors, capillary modulating agents are co-administered at the time of delivery to permeabilize the microvasculature. Such substances include VEGF, sildenafil, histamine, serotonin, and heparin (Rapti et al., 2011). Catheter-based intracoronary injection may occur in either antegrade or retrograde fashion (Kawase et al., 2011). Antegrade injection refers to the injection of the gene vector into the coronary artery, which allows for the homogenous expression of the gene throughout the

myocardium. Retrograde injection refers to the injection of the vector through the coronary vein. The injection site can be modified according to the disease state of the patient (i.e. patients with coronary artery blockage).

A relatively novel technique of gene delivery known as ultrasound targeted microbubble destruction utilizes ultrasound to facilitate the delivery of vectors from the vasculature to various tissues of interest, such as the myocardium (Bekeredjian, Chen, Frenkel, Grayburn, & Shohet, 2003; Bekeredjian, Chen, Grayburn, & Shohet, 2005; S. Chen et al., 2002; Vannan et al., 2002), pancreas (S. Chen et al., 2006; S. Chen et al., 2007), hindlimb (Kuliszewski, Kobulnik, Lindner, Stewart, & Leong-Poi, 2011; Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007a) (skeletal muscle), the endothelium (Lawrie et al., 2000; Taniyama et al., 2002), kidneys (Koike et al., 2005) and tumours (Hauff et al., 2005; Hayashi, Mizuno, Yoshida, & Nakao, 2009). Details of the technique will be discussed in later chapter (Section 2.1.1).

1.5.2.5 Targets of Gene Therapy

As our knowledge on the molecular and cellular mechanisms of HF increases, so do the number of potential therapeutic gene targets. Gene therapy has focused on several targets that have been shown to be dysfunctional in a HF setting. Calcium handling is known to be altered in HF (Piacentino et al., 2003), and therapies to normalize Ca^{2+} cycling include upregulation of S100A1 (Most, Remppis, Pleger, Katus, & Koch, 2007; Rohde et al., 2011) and SERCA2a. Notably, SERCA2a has undergone the phase 2 of clinical trials with favourable results, showing improvements in function and importantly, safety of the

therapy (Jessup et al., 2011). Gene therapy can also augment the angiogenic and beta-adrenergic system, by targeting VEGF (Stewart et al., 2006) and beta-adrenergic receptor type-2 (Milano et al., 1994), respectively.

Recently, it was noted that HF progression often shows an apoptotic phenotype, Induction of cell death in cardiomyocytes alone were sufficient to cause HF in animal models. Topic of apoptosis in HF will be discussed in further detail in section 1.8. Genetic targets for cell survival are being explored, and some of the potential gene targets include: Bax (downregulation), Bcl-2 (upregulation), and a host of proteins known as the inhibitor of apoptosis proteins (IAP)

1.6 Apoptosis

Apoptosis is a form of regulated cell death, which is prevalent in the development and normal physiology of multicellular organisms, and is required for normal equilibrium of cells by removing redundant or dysfunctional cells. Necrosis, an energy-independent mode of cell death, causes collateral damage due to the outpouring of cellular contents during cell lysis and the ensuing inflammatory response. Apoptosis, however, occurs in a tidy and tightly regulated manner, during which cells are rapidly phagocytosed by surrounding cells and the engulfing cells do not produce inflammatory cytokines (Elmore, 2007). Although the process of oncotic necrosis and apoptosis are recognized as separate processes, there is considerable overlap between these two processes that blurs the line between the two cell death pathways (Lemaire, Andreau, Souvannavong, & Adam, 1998; Raffray & Cohen, 1997). Two major factors that determine whether an ongoing apoptotic process converts into a necrotic process are: a decrease in the

availability of caspases, and lack of intracellular ATP (Denecker, Vercammen, Declercq, & Vandenabeele, 2001; Leist, Single, Castoldi, Kuhnle, & Nicotera, 1997). Whether a cell dies by necrosis or apoptosis also depends on a various other factors, some of which include: the tissue type, nature of cell death signals, and the physiological setting (Zeiss, 2003).

Apoptosis is characterized by specific biochemical and morphological changes including cellular and nuclear shrinkage, chromatin condensation, cell membrane blebbing, and DNA fragmentation. Cells look smaller in size, cytoplasm is dense and organelles are more tightly packed. Pyknosis as a result of chromatin condensation is the hallmark characteristic of apoptosis. Plasma blebbing is followed by nuclear fragmentation (karyorrhexis) and the cell fragments into apoptotic bodies during the budding process. These apoptotic bodies consist of cytoplasm with packed organelles, with or without a nuclear fragment. These bodies are phagocytosed, and degraded within phagolysozomes. Morphological and biochemical changes in cells undergoing apoptosis are well described in the review by Elmore (Elmore, 2007).

Apoptosis may occur via 2 main pathways: extrinsic, which involves binding of cell death ligands and therefore activating cell surface death receptors, and intrinsic pathway, which involves the mitochondrial activation and release of apoptosomes from the matrix. When encountered with an apoptotic stimuli, the cell may undergo either intrinsic, extrinsic or a combination of both forms of apoptosis, activating a unique cascade of cysteine-aspartic proteases or caspases.

1.6.1 Caspases

Caspases are proteolytic enzymes that play an essential role in apoptosis. They have proteolytic activity that allows it to cleave proteins at aspartic acid residues, with different caspases having different specificities involving recognition of neighbouring amino acids. They exist within the cells as proenzymes (inactive) and once activated, can cleave other procaspases, initiating a protease cascade. This proteolytic cascade can amplify the apoptotic signal and a rapid apoptotic process ensues. Ten major caspases have been identified, and grouped into three categories: initiator caspases (caspase -2, -8, 9-, -10), executioner caspases (caspase -3, -6, -7) and inflammatory caspases (caspase -1, -4, -5). There are other caspases (-11, -12, -13, 14) that do not fit into the categories mentioned above, but play specific roles in certain processes of apoptosis.

1.6.2 Extrinsic Apoptotic pathway

During extrinsic apoptosis initiation, death ligands, such as FasL, TNF-a, or TRAIL bind to their respective receptors, FasR, TNFaR1, or TRAILR1. Binding of the ligands to the receptor leads to the recruitment of adaptor proteins (FADD or TRADD), which then subsequently recruit procaspases-8 and -10, forming a complex known as death-inducing signaling complex (DISC). The formation of DISC allows the autoproteolytic activity of the complex to cleave the zymogenic procaspases into their active form (Kischkel et al., 1995). In some cells, the activated caspase-8 is sufficient to activate the downstream effector caspases (caspases-3/-7) (Stennicke et al., 1998), but in others, the initiator caspase-8 may go on to cleave the apoptotic protein Bid, causing the release of pro-

apoptotic mitochondrial factors, which then lead to the activation of intrinsic apoptosis pathway (D. Tang, Lahti, & Kidd, 2000).

1.6.3 Intrinsic Apoptotic Pathway

Whereas the extrinsic apoptosis initiation is dependent on specific ligand/cell surface receptor interaction, initiation of intrinsic apoptosis is much more diverse in that the initiator may both be intracellular/extracelluar, triggering intracellular signaling pathways to promote cell death. Factors that lead to the activation of the intrinsic pathway include the loss of survival factors (i.e. cytokines, growth factors, hormones), toxins, radiation, oxidative stress and hypoxia. In response to such stimuli, the mitochondrial inner trans-membrane potential is sabotaged, leading to mitochondrial outer-membrane permeabilization, releasing apoptogens stored within the matrix of the mitochondria (Saelens et al., 2004). One of these apoptogens, cytochrome c, when in the cytosol binds to Apaf-1, forming a complex known as the apoptosome (Zou, Li, Liu, & Wang, 1999). The apoptosome then acts to recruit and activate caspase-9, which is capable of activating the effector caspase-3 and 7 (P. Li et al., 1997). These effector caspases are under inhibitory regulation by the direct binding of XIAP, an inhibitor of apoptosis. Upon the release of apoptogens - such as SMAC/DIABLO and Omi/HtrA2 – XIAP binding weakens, releasing caspase-3/-7 to cleave their downstream effectors (Schimmer, 2004; van Loo et al., 2002).

Until recently, apoptosis has traditionally been considered an irreversible process with caspase activation committing a cell to death and the engulfment genes serving the purpose of dead cell removal. This view is continuously being challenged with studies

refuting the irreversible nature of the apoptotic process. For example, cells with blocked engulfment genes showed enhanced cell survival when cells were subject to weak apoptotic signal (Hoeppner, Hengartner, & Schnabel, 2001). This suggests that genes that mediate cell debris removal can also function to actively remove cells. In other words, engulfing cells may act to ensure that cells triggered to undergo apoptosis will die, rather than recover from the apoptotic stimuli.
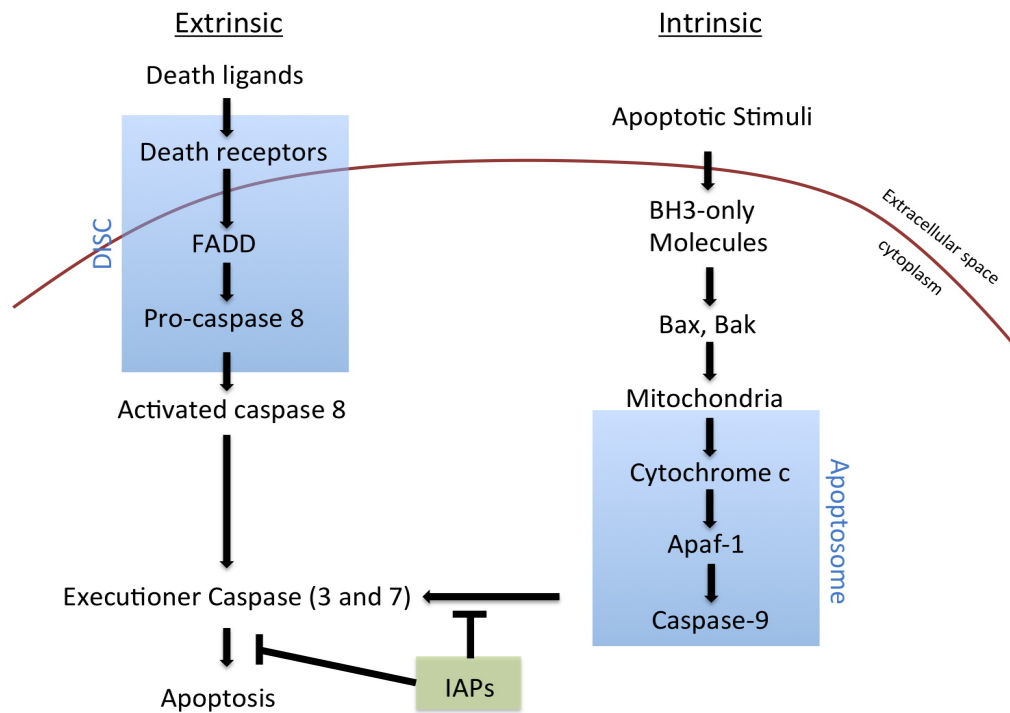

Figure 1 **Apoptotic pathways** induced by both intrinsic and extrinsic stimuli. Adapted from Xu et al. (Cell Research. 2007: 17:759-771)

1.7 Inhibitors of Apoptosis Proteins (IAP)

Inhibitor of apoptosis proteins (IAP) is a family of proteins that were first identified in insect SF-21 cells, infected with baculovirus (Crook, Clem, & Miller, 1993). IAP, encoded by the viral gene, was able to inhibit apoptosis in these cells. Subsequent studies have identified other IAPs that have between one to three baculovirus IAP repeat (BIR) domain that characterize these proteins, at the N-terminus (Wei, Fan, & Yu, 2008). The molecular structure of IAPs is well described in a review by Wei and colleagues (Wei et al., 2008). The BIR domains consist of approximately 70 amino acids that are both hydrophilic and hydrophobic in nature, which is characteristic of potential protein-protein interaction sites. Some IAPs also contain the RING finger domain at the C-terminus, which contains one zinc atom at the 3 cysteine residues, and another zinc atom at the 4 cysteine residue. This domain is responsible for ubiquitin ligase activity, which may act to degrade caspase-3 (Suzuki, Nakabayashi, & Takahashi, 2001). Other structures that are present on IAPs include, the caspase activation recruitment domain (CARD), phosphate-loop and ubiquitin-conjugating domain (UBC). BIR domain is an absolute requirement for the anti-apoptotic properties of these proteins, but not all proteins of the IAP family exhibit anti-apoptotic function. IAP acts as endogenous inhibitor of caspases, the main executioners of apoptosis. IAPs have been known to inhibit caspases by inducing their degradation, or by sequestering the active caspases away from their substrates (Tenev, Zachariou, Wilson, Ditzel, & Meier, 2005). Currently, there are 8 IAPs that have been identified in mammals (Wei et al., 2008). All IAPs are homologs with highly conserved sequences.

1.7.1 Survivin (BIRC5)

First discovered in 1997, Survivin is one of the eight proteins among the member of the IAP family (Ambrosini, Adida, & Altieri, 1997). Also known as the baculoviral IAP repeat containing protein 5 (BIRC5), it is a 429bp gene that encodes a 16.3kD protein that has a dual functionality in vivo: anti-apoptosis and cell cycle check point. Survivin has been studied extensively in the context of tumours, as its expression is highly increased in certain malignancies and offers a novel target for cancer therapies (Kanwar, Kamalapuram, & Kanwar, 2010). It is also noted that there is a positive correlation between high survivin transcript expression and poor prognosis of certain cancers, including but not limited to colorectal (Sarela, Macadam, Farmery, Markham, & Guillou, 2000), non-small cell lung cancer (Dai et al., 2010), breast cancer (Kennedy et al., 2003) and glioma (Chakravarti et al., 2002). Survivin is still scrutinized in terms of its mechanism of action in 'inhibiting' caspase activity (Banks et al., 2000; Shin et al., 2001). Although overexpression and anti-sense mediated reduction of the protein suggests that this protein is an anti-apoptotic protein in nature, the lack of caspase binding domain found in other members of IAP suggests that this may not be a direct mechanism (Ambrosini et al., 1997). For example, survivin was shown to bind to the IAP-inhibiting SMAC/DIABLO complex, which would suggest that survivin's anti-apoptotic effects may indirectly lead to inhibition of caspapses (Song, Yao, & Wu, 2003). Also, survivin is implicated in the binding to another potent inhibitor of apoptosis protein, XIAP, stabilizing the protein and further enhancing the anti-apoptotic properties of the protein (Dohi et al., 2004). However, survivin is shown to co-associate with caspase 3 in the vicinity of centrosome in mitosis (Tamm et al., 1998), and is required for

the suppression of caspase-mediated cleavage of p21waf1, which suggests that survivin may inhibit caspases in certain spatial contexts (Fukuda, Mantel, & Pelus, 2004).

Localization of survivin within the cell has been studied as one of the factors in determining the role of survivin within the cell. Survivin has been recognized to mainly occupy two subcellular pools, both the nuclear and cytoplasmic compartment, and the function-localization relationship has been studied as a potential prognostic marker in certain cancer types (Angell, 2008; Fortugno et al., 2002; F. Li, Yang, Ramnath, Javle, & Tan, 2005). It is generally believed that nuclear survivin is involved in the cell-cycle and mitotic processes, whereas cytoplasmic survivin is involved in the anti-apoptotic function (Connell, Colnaghi, & Wheatley, 2008; Stauber, Mann, & Knauer, 2007). Nuclear survivin protein is believed to be transported to the cytosol by nuclear exporter receptor, Crm1, which is regulated by a leucine-rich nuclear export signal (NES). Using Leptomycin B, a Crm1-specific inhibitor, it was possible to antagonize the proliferative activity of nuclear survivin. Furthermore, a mutation in the NES region led to the accumulation of survivin in the nucleus, unable to exit the nuclear compartment, and these mutant survivin had lower apoptotic threshold to X-irradiation than wild type survivin (Knauer, Bier, Habtemichael, & Stauber, 2006). As such, survivin localization is sometimes regarded as having considerable clinical relevance, as nuclear localization is a favourable prognostic marker in breast cancer patients, and cytoplasmic localization is unfavourable.

Recently, Survivin was also shown to be secreted into a third, extracellular pool, where it retained its physiological function as demonstrated in proliferative disease settings, such as arthritis and cancer. In 2004, a study by Bokarewa et al (Bokarewa, Lindblad,

Bokarew, & Tarkowski, 2005) reported that patients suffering from erosive rheumatoid arthritis had increased concentrations of survivin levels in the plasma as well as the synovial fluid, as opposed to patients suffering from the non-erosive type. The same study examined the survivin levels of cells in the synovial fluid; it was revealed that survivin levels in the cell lysates correlated strongly with the synovial fluid levels, denoting that the survivin was produced and secreted into the synovial space. The authors, however, were reluctant to conclude that the survivin was indeed secreted by the cells – their reasoning was that in vitro leukocyte-activating stimuli were used to induce secretion of survivin in vitro, with no avail.

A study by Khan et al in 2009 (Khan et al., 2009) demonstrated for the first time that extracellular survivin was readily taken up, and had anti-apoptotic function in cancer cells. A follow-up study in 2011 (Khan et al., 2011) by the same group showed that this secretion of survivin is mediated through exosomal bodies. Unlike the views of Bokarewa et al, the study of extracellular survivin in cancer favours the notion of active secretion, rather than passive diffusion of survivin from dead or dying cells. It was explained that the survivin concentration in the supernate was equal or higher than the concentration derived from 10% of cell lysate, but there were no dead or dying cells in the cultures from which the supernate survivin was detected (Khan et al., 2011). None of the studies examined or postulate the mode of uptake of survivin, however.

Survivin is extensively spliced to form numerous variants of varying functions, some exhibiting anti-apoptotic effects, while some aredominant negative forms of survivin (Caldas et al., 2005; Caldas, Honsey, & Altura, 2005; Mahotka, Wenzel, Springer,

Gabbert, & Gerharz, 1999). We will be using survivin to refer to the most common form of survivin (isoform 1), with potent anti-apoptotic effects.

1.7.2 Role of Survivin in Cardiac Setting and HF

Due to its clear implication in proliferative diseases, studies of survivin in the setting of cardiac research has not been extensively explored. However, it was noted that survivin protein, normally undetectable, or mildly expressed, in differentiated tissues (Iskandar & Al-Joudi, 2006, Santini et al., 2004), is further downregulated in the myocardium, induced by age and HF in a spontaneously hypertensive rat (Abbate et al., 2006). The main finding of this study dictates that the pathophysiology of hypertensive HF occurs primarily through the increase in apoptotic cells, ventricular remodeling, and a decrease in survivin expression index measured by immunohistochemistry. The correlative data between lowered apoptosis and remodeling in animals with higher survivin expression led the authors to conclude, with some reservation in making the cause-effect relationship, that the decreased survivin levels cause increased apoptosis in hypertensive rats (Abbate et al., 2006). To follow up, a landmark study by Levkau et al (Levkau et al., 2008) has sparked interest in the role that survivin plays in the normal functioning of the heart. Using survivin knock-out mice that specifically deletes the gene in cardiomyocytes, the group was able to study the effects of survivin on cardiac development and function. These animals exhibited characteristics typical of the HF syndrome, such as decreased activity, tachypnea, and hunched posture. Structural changes to the heart included massive enlargement of the cardiac cavities, pericardial effusions and atrial thrombi. Interestingly, the study found that the cardiomyocyte number was significantly lower in the survivin-deficient animals, compared to their

littermates, at birth. This difference was attributed mainly to the cell divisional functionality of survivin, rather than cell survival function. In vitro analysis of survivin effect on deficient cardiomyocytes showed that survivin was successful in preventing doxorubicin-induced cytotoxicity, and led to the normalization of cell cycle progression.

Clinically, survivin levels were noted to be increased in the remote regions of the myocardium in a post-mortem analysis of myocardial infarct patients (Santini et al., 2004). Surprisingly, peri-infarct regions had lower levels of survivin gene expression, similar to the study of the hypertensive rats (Abbate et al., 2006), myocardium expressing higher survivin levels showed favourable remodeling patterns, and decreased apoptotic index, indicating survivin's role in amelioration of adverse remodeling. However, the data suggests that survivin gene regulation post-infarct/insult is heterogeneous, at least in the setting of infarction.

A contradicting study has shown that survivin levels are upregulated in a failing heart, caused by ischemic or dilated cardiomyopathy (Levkau et al., 2008). However, this increase in survivin expression was eliminated with mechanical support by LVAD. The initial increase was assumed to be a protective measure against apoptosis, whereas the downregulation occurred after LVAD support, presumably as it was no longer required. A more recent study of the changing survivin levels with LVAD support suggested that survivin expression is related to the hypertrophy of the cells (related to survivin's role in cell division), and that the unloading by LVAD leads to the decrease in survivin expression may contribute to the decrease in cardiac hypertrophy (Wohlschlaeger et al., 2010). The contradicting findings from the two studies may be indicative of the dynamic nature of survivin expression phenotype, and as such, future studies to elucidate the

survivin expression patterns in different cardiac diseases must be completed to further progress the field of cardiac apoptosis research.

1.8 Prevalence of Apoptosis in Heart Failure

Deregulated apoptosis has been shown to be detrimental as seen in many pathologies such as cancer, autoimmune disease and recently cardiomyopathies. During the last decade, there has been accumulating evidence in both human and animal models suggesting that apoptosis may be an important mode of cell death during the development of HF (Masri & Chandrashekhar, 2008; Narula et al., 1996; Olivetti et al., 1997). The following excerpt describes well the magnitude of progressive cellular loss and the ensuing physiological effect in the presence of myocardial injury: "*The syndrome of heart failure is characterized by a group of adverse structural changes called cardiac remodeling, and an inexorable progression that continues to occur even in the absence of repeated injury. One of the hallmarks of this process is a reduction in the number of cardiomyocytes: approximately one-third of the myocytes are lost, leading to increased wall stress and in turn to a gradual further cell loss." (Masri et al. Heart Fail Rev 2008)* In myocardial ischemia/reperfusion injury and myocardial infarction, the prevalence of apoptosis is significantly elevated, leading to ventricular dysfunction and increased infarct size. The importance of apoptosis in these models is highlighted by the fact that inhibition of Fas (Miyata et al., 2010), caspases (Chandrashekhar, Sen, Anway, Shuros, & Anand, 2004), and oxidative stress (Feuerstein, Yue, Ma, & Ruffolo, 1998) significantly reduces the size of the infarct and improves the ventricular systolic function.

Although the myocardial apoptotic events at end-stage HF are not markedly increased at 0.075 to 0.119%, there is a significant increase from the level of basal apoptotic rates in control hearts (Saraste et al., 1999). It has been argued that even at these low levels of apoptosis, gradual and persistence myocyte loss over months, if not years leads to the development of cardiac dysfunction and eventually end-stage HF (Yaoita & Maruyama, 2008). Several inciting cardiac events can culminate in HF, such as hypertension, valve dysfunction that causes chronic volume or pressure overload, and infarction/ischemia, alcohol abuse, and viral myocarditis (Colucci W.S., 1997). After the initial insult, the cardiomyocytes undergo gene morphological changes to overcome the loss of contractile units, and thereby adapts to the worsened conditions. This process of hypertrophic remodeling is adaptive initially but with time, the stressed cells end up dying, and unable to replenish the destroyed cells, the heart undergoes adverse remodeling and progression to HF.

Recently, the idea that a cell with activated apoptotic mechanism will inevitably undergo apoptosis has been challenged (Masri & Chandrashekhar, 2008; Narula et al., 2001). Cells with activated caspase-3 and release of cytochrome c do not necessarily succumb to cell death, and may interrupt the process, despite the activation of the cellular death pathway. These cells display evidence of cytochrome c release and caspase-3 activation but have normal nuclei and lacked the terminal morphological features of apoptosis (Narula et al., 1999). Given the fact that HF is often characterized by apoptosis, and that apoptosis now recognized as a meta-stable state, which can be reversed to reconstitute contractile proteins and recovery of systolic function, anti-apoptotic therapy may well be a potential therapeutic intervention for HF patients.

1.9 Rationale and Novelty of the Proposed Study

Given the critical role of cell death in the progression to HF, our focus is to study the effects of anti-apoptotic therapy in HF. The study is novel compared to other anti-apoptotic studies in the heart that have been done in the past for the following reasons.

1.9.1 Ultrasound-targeted Microbubble Destruction (UTMD)

UTMD utilizes contrast agents, small gas bubbles encapsulated by a stabilizing shell, as a vehicle for gene delivery. These microbubbles have a diameter on the order of microns (Christiansen, French, Klibanov, Kaul, & Lindner, 2003), and behave similarly to red blood cells in the vasculature. Upon ultrasonic imaging, the microbubbles resonate, producing a strong signal that can be detected and displayed. The microbubble used specifically for gene delivery has a specific acoustic property due to the encapsulated gas, and can be made with cationic lipids that create positively charged microbubbles. This positive charge allows charge coupling of negatively charged plasmid DNA onto the bubble surface, with ~6700 plasmids per microbubbles (Christiansen et al., 2003). Ultrasound at high acoustic power (mechanical index of greater than 1.0) leads to the bubble destruction within the vasculature (S. Chen et al., 2002). The cavitated microbubbles propel the shell fragments/plasmid DNA into the surrounding cells through the endothelial barrier (Price, Skyba, Kaul, & Skalak, 1998), where microporation caused by the bioeffects of ultrasound and cavitating microbubbles facilitates the transfer of genes into cells (Chappell & Price, 2006). The uptake of DNA into the cells may be facilitated by several mechanisms, one of which may involve the interaction of ultrasound and microbubbles to produce hydrogen peroxide (Juffermans, Dijkmans,

Musters, Visser, & Kamp, 2006). Furthermore it has been reported that when diagnostic cardiac ultrasound was applied without the presence of contrast agents, oxygen radicals are produced in the endothelial cells (Basta et al., 2003). This can lead to an increased permeability of the cell membrane. Also, the interaction between the microbubbles and ultrasound cause a local and transient increase in temperature, increasing the fluidity and permeability of the cell membrane and facilitating gene transfer (Wu, 1998). Adverse bioeffects such as hemorrhage and vessel permeability have also been reported with the use of high powered ultrasound (i.e. mechanical index >1.0), but these effects are generally seen in less clinically applicable settings (direct ultrasound over the myocardium) and have been shown to be transient.

Previously, UTMD has been used successfully for the induction of angiogenesis in our lab. A study by Leong-Poi et al. showed that UTMD was an effective strategy of gene therapy for therapeutic angiogenesis in a model of severe chronic ischemia in rat hindlimbs (Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007b). Plasmid delivery of VEGF led to an increase in blood flow and volume, with vessel density also increased, but was diminished by week 8, indicating the need for repeated therapy. Angiogenic therapy has also been attempted in the heart using UTMD, first in 2005 where VEGF was delivered to the rat myocardium (Korpanty et al., 2005), and later in 2009 with VEGF and Stem Cell factor (SCF) were delivered after myocardial infarction (Fujii et al., 2009). Both studies demonstrated an increase in vessel density, and the study by Fujii and colleagues showed functional improvements after gene delivery. However, the result from the 2005 study saw a transient increase in capillary density at day 10 post-delivery, but the levels regressed back to the normal

levels at day 30. This is in line with the Leong-Poi study, in that expression is transient, and the therapeutic effect is not long lasting. However, recent study from our lab has demonstrated that it is possible to deliver genes in a temporally separated manner, enhancing the in vivo effects of gene therapy.

Cardiac gene delivery via UTMD has been optimized by Chen et al. in 2003 (S. Chen, Shohet, Bekeredjian, Frenkel, & Grayburn, 2003). Albumin microbubbles were used to deliver a luciferase reporter gene. The experimental protocol was carried out in order to evaluate the effects of several ultrasound parameters: ECG triggering, transducer frequency, mode of ultrasound, and acoustic power. The results found that triggered, low-frequency, high mechanical power ultrasound settings allow for maximal delivery of gene by UTMD. In another study, the same group showed that UTMD can be used repeatedly to delivery genes to the heart in a temporally separated manner (Bekeredjian et al., 2003). This allowed for the prolonged expression of the gene product within the myocardium. UTMD was also used to transfer hepatocyte growth factor (HGF) into the myocardium, for treatment of acute myocardial infarction. Kondo and colleagues described in 2004, that UTMD was successful in transfecting the myocardium with HGF, and saw structural improvements in the HGF treated group, compared with the null-vector treated group (Kondo et al., 2004).

1.9.2 Survivin Gene Therapy

Survivin has been thoroughly studied in the context of cancer biology but is yet to be explored as a candidate gene for treatment of heart failure in an in vivo setting. Numerous in vitro studies have demonstrated survivin's potential in preventing

cardiomyocyte death, and cellular apoptosis, and a recent study has revealed survivin's role in the cardiac setting (see section 1.71). Survivin is a suitable candidate for gene therapy, as it is not highly expressed in most differentiated tissues/cells (Iskandar & Al-Joudi, 2006) and its upregulation can be beneficial in offsetting the deleterious effects of many aspects of cardiac remodeling (Levkau et al., 2008). In addition, the recent findings that survivin is a secreted protein (Khan et al., 2009; Mera, Magnusson, Tarkowski, & Bokarewa, 2008) makes survivin a perfect gene therapy candidate for anti-apoptosis: the ability of survivin to exit the transfected cell and affect neighbouring cells allows UTMD with relatively low transfection efficiency to affect a larger area of the myocardium.

1.9.3 Doxorubicin Model

For our study, we used a well-described model of doxorubicin-induced cardiomyopathy in rats. This model has been employed in many studies throughout the years, as a cost-effective, reproducible model of HF (Czarnecki, 1984; Monnet & Chachques, 2005), especially in studying the effects of anti-apoptosis in cardiac tissue (X. Chen et al., 2007; De Angelis et al., 2010; Konishi et al., 2011; L. Li et al., 2006; C. Zhang et al., 2011).

Doxorubicin is a quinone-containing anthracycline antibiotic isolated in the early 1960's, used to treat a variety of cancers, but its clinical applicability is hampered by its potent dose-dependent cardiotoxic side effect (Lefrak, Pitha, Rosenheim, & Gottlieb, 1973; Singal & Iliskovic, 1998). It was reported that the occurrence of HF is 4% in patients receiving 500-550mg/m^2 and this increases to 18% in patients receiving 551-600mg/m^2 of the drug (Lefrak et al., 1973). Doxorubicin is particularly peculiar, as the cardiotoxicity of the drug persists years after cessation of the drug, with a higher

incidence of refractory congestive HF (Steinherz, Steinherz, Tan, Heller, & Murphy, 1991). There are many proposed mechanisms through which doxorubicin is believed to cause cytotoxicity but the main mechanism of this side effect is thought to be oxygen radicals generated by the redox-activation of doxorubicin (Singal, Deally, & Weinberg, 1987; Sinha, Katki, Batist, Cowan, & Myers, 1987a; Sinha, Katki, Batist, Cowan, & Myers, 1987b). The target organelle of doxorubicin toxicity is proposed to be the mitochondria, where the doxorubicin concentration is increased (Kalyanaraman et al., 2002). It has been reported that at clinically relevant plasma doxorubicin concentrations of 1 uM, intra-mitochondrial concentration is approximately 25-50 uM (Sarvazyan, 1996). In the mitochondria, nicotinamide adenine dinucleotide (NADH) dehydrogenase activates doxorubicin to a semiquinone that undergoes redox reaction to form superoxides and peroxides (K. J. Davies & Doroshow, 1986). One of the reasons why the heart is specifically a target of doxorubicin may be the presence of low levels of catalase in myocytes, which is further exacerbated by the inactivation of the enzyme by doxorubicin-induced production of peroxides (Marklund, Westman, Lundgren, & Roos, 1982; Simmons & Jamall, 1989).

Doxorubicin-induced cardiomyopathy has been studied in rats previously (Jensen, Acton, & Peters, 1984; Kaul et al., 1996; Kawasaki, Lee, Shimizu, & Ueda, 1996; Teraoka, Hirano, Yamaguchi, & Yamashina, 2000), and it has been assessed as having several features of cardiomyopathy seen in humans (Monnet & Chachques, 2005). It emulates structural as well as functional changes in patients, and it is highly reproducible, making it a valuable model to study HF.

Chapter 2 - Study Objectives

2.1 Objectives and Hypothesis

The overall objective of the study was to examine the effects of anti-apoptotic therapy by survivin gene delivery in a model of HF induced by the administration of doxorubicin. In order to achieve this, the study aims were divided into two tiers: in vitro and the in vivo parts. The main hypothesis of the study dictates that in a rat model of doxorubicin induced cardiomyopathy, survivin gene therapy will lead to the preservation of cardiac function.

2.1.1 In Vitro Experiments

H9c2 cells (rat cardiac myoblasts; ATCC) were used to study the effects of doxorubicin on apoptosis and the effects of survivin gene transfection on cell death induced by doxorubicin. The main focus of the in vitro aspect of the study was to determine the functionality of the survivin gene against doxorubicin toxicity, and its potential to abrogate cardiomyocyte apoptosis. In addition, in light of the recent development that survivin is a secreted protein (Khan et al., 2009; Khan et al., 2011; Shu et al., 2007), we endeavored to determine the presence of extracellular survivin and the effects that it may have on the level of apoptosis with doxorubicin treatment.

2.1.1.1 Hypothesis

H9c2 cells exposed to doxorubicin will undergo apoptosis, which will be abrogated by the treatment with survivin, directly by gene transfer, and indirectly in a paracrine fashion by co-incubation with the secreted survivin protein.

2.1.1.2 Approach

It is well documented that doxorubicin causes cell death in many types of cells (Damrot et al., 2006; Tan et al., 2010; C. Zhang et al., 2011), and the cardiac cell line H9c2 is one of the many that is sensitive to the toxic effects of doxorubicin. Cell death will be measured by annexin-V staining method, which detects the early changes in cellular membrane morphology (Koopman et al., 1994): With apoptosis, the composition of the outer layer of the phospholipid membrane increases the exposure of phosphatidylserine to the extracellular space. This externalization of phosphatidylserine permits the early phagocytic recognition of apoptotic cells, and allows for quick phagocytosis with minimal compromise to the surrounding tissue (Fadok et al., 1992). Fluorophore-tagged Annexin-V binds to the phosphatidylserine, and by quantifying the fluorescent signal via flow cytometry, we can indirectly measure the level of apoptosis in the cell culture. The level of Annexin-V signal was quantified using a flow cytometer. In addition, we will determine whether secreted survivin, if detectable in the supernate by enzyme-linked immunosorbent assay (ELISA), has an effect on cell survival by co-incubation with survivin-conditioned media in cell culture.

2.1.2 In Vivo Experiments

A rat model of HF was used to study the effects of survivin delivery on cardiac function. The focus of the in vivo aspect of the study was to follow the course of the functional changes that occur with doxorubicin induced cardiotoxicity, and determine the differences in LV systolic function, if any, with the appropriate treatments.

2.1.2.1 Hypothesis

With survivin gene therapy, we expect to observe a significant preservation in LV systolic function as compared to the doxorubicin-treated group or the doxorubicin-treated group with empty vector gene delivery. This maintenance of LV systolic function will be mediated by the decrease in caspase 3 and 7 activity and therefore a decrease in apoptosis in the myocardium.

2.1.2.2 Approach

LV systolic function was measured by 2D-echocardiography. Measurements of LV end systolic dimensions and end diastolic dimensions were made, and used to calculate % fractional shortening (FS), a surrogate measurement of LV systolic function. Also, using invasive conductance catheters, pressure-volume loops were generated to acquire invasive measurements of cardiac function, to further validate echocardiographic parameters. To determine the effects of survivin therapy on a cellular level, we conducted experiments to assess two measures of cardiac remodeling, cardiomyocyte apoptosis – via TUNEL staining and Caspase-3 and 7 assay – and interstitial fibrosis – via Picrosirius red (PSR) staining.

Chapter 3 - Methods and Experimental Protocol

3.1 In Vitro Protocol

3.1.1 Cell Culture

H9c2 cells (rat cardiac myoblasts; ATCC) were grown in standard growth medium of Dulbecco's Modified Eagle's Medium (DMEM, Cellgro) supplemented with penicillin and streptomycin (Gibco), and 10% (v/v) fetal bovine serum (FBS, Wisent). Cells were maintained at 37°C and 5% CO_2, and the media was replaced every 2-3 days. To prevent the loss of the myoblast population, the cells were passaged prior to reaching confluency - at approximately 70-80% confluency - at a ratio of 1:4. When necessary, cells were cryopreserved in growth media supplemented with 10% dimethylsulfoxide (DMSO, Invitrogen), and stored in -80°C. Cells between 22-25th passage were used for the subsequent experiments.

3.1.2 Adenoviral Transduction

Adenovirus for Ad-Surv-GFP and Ad-Null-GFP was constructed from Vector Biolabs. A total of 2.5×10^4 H9c2 cells were grown in 24-well plates for 24 hours, inoculated with either Ad-GFP-Null (NULL) or Ad-Surv-GFP (SURV) virus at a multiplicity of infection of 250 (250 MOI) in 250 μL of growth media. These transduced cells were used to determine the function of the exogenous survivin gene in preventing doxorubicin-induced apoptosis. At 24, 48, 72 and 96 hours, the supernate was removed from each well, centrifuged at 350 RCF for 10 minutes in room temperature to remove the cell

debris. The supernate was used immediately or kept in -80°C for analysis of extracellular survivin. Supernate collected from SURV wells at 72 hours was also used as survivin-conditioned media (SCM) for the survivin pre-treatment group. Supernate from NULL was also collected at 72 hours and used as null-conditioned media (NCM). Survivin in the supernate was measured by a commercially available Survivin ELISA kit (R&D Systems).

3.1.3 Doxorubicin-Induced Cytotoxicity In Vitro

The level of apoptosis was compared between SURV, NULL, and non-transduced cells (CONTROL) in the presence of doxorubicin. H9c2 cells were transduced with either SURV or NULL as described above, and washed the following day with PBS 3 times to remove the viral particles. The cells were allowed to grow for another 24 hours to maximize the survivin translation, prior to doxorubicin treatment. In order to determine the effects of survivin-conditioned media, non-transduced cells were cultured in either SCM or NCM for 24 hours prior to doxorubicin exposure. For each group, 2.5×10^4 cells per well were grown in 24-well dishes and cultured in 0.25 μM concentration of doxorubicin (Santa Cruz Biotechnology) for 6 hours. Cells were detached using trypsin/EDTA (Cellgro), centrifuged at 190 RCF in room temperature for 5 minutes, and resuspended in 100μl of working strength Annexin-V-Alexa-568 (Roche Diagnostics) on a horizontal tilt for 15 minutes in room temperature. The reaction was quenched with the addition of 500μl of incubation buffer (pH of 7.2, 10mM HEPES, 140mM NaCl, 5mM CaCl$_2$). Stained cells were subsequently analysed using a flow cytometer (BD FACScalibur II). It is important to note that with GFP positive cells, the signal leaked significantly into the Alexa-568 signal, increasing the readings for false positive

apoptotic signal in the SURV-GFP transduced cells. It was critical to properly compensate the machine using the appropriate controls in order to get an accurate measurement of apoptosis.
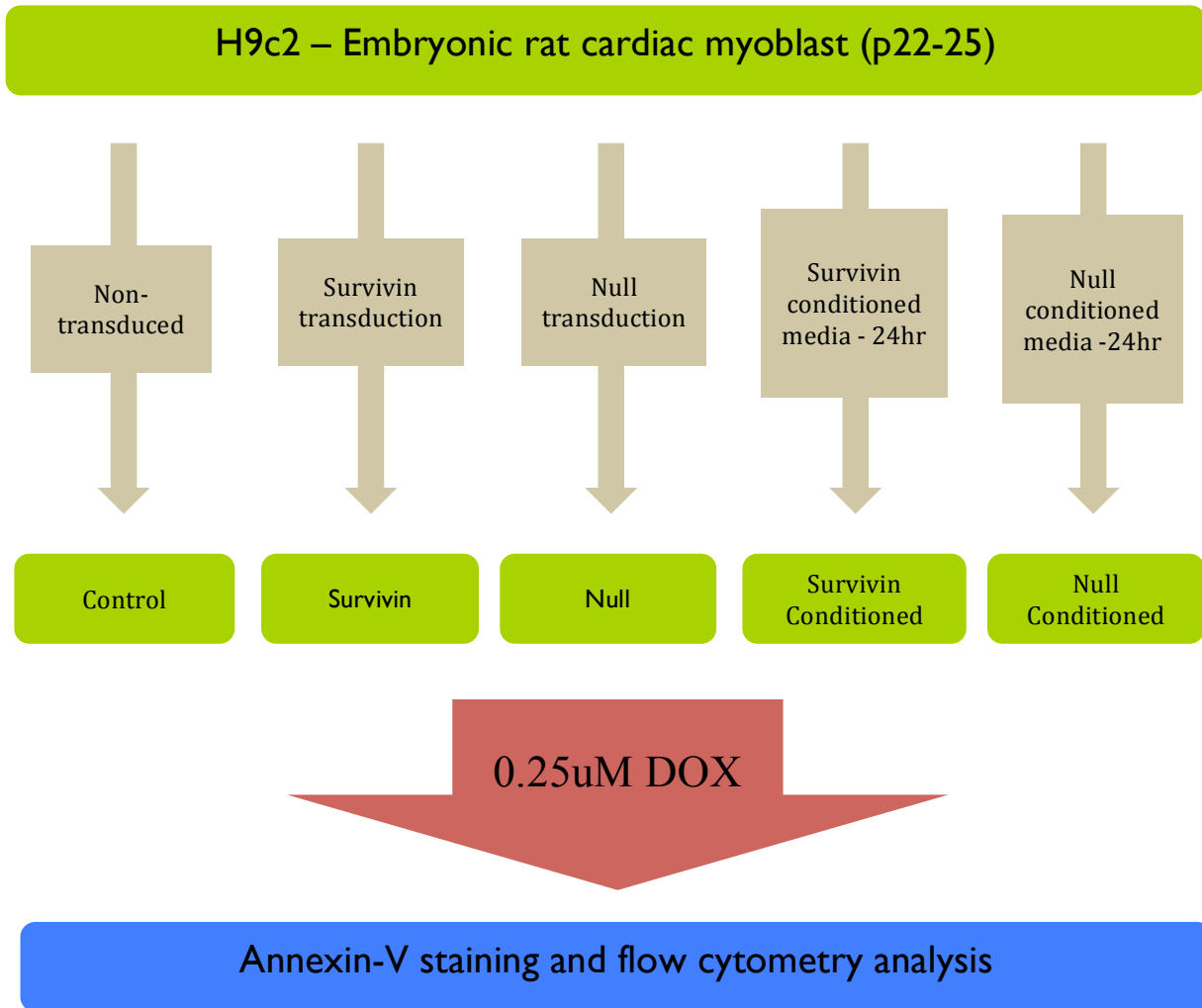

Figure 2 **In vitro experimental protocol** using H9c2 cells at passage 22-25

3.1.4 Survivin Uptake from Culture Media

In order to determine whether uptake of survivin takes place in culture, H9c2 cells were grown in 24-well plates, and incubated in either growth media, NCM or SCM for 24 hours. Subsequently, the cells were treated with doxorubicin (0.25μM) detached, washed

and analyzed by flow cytometry (BD FACScalibur II) as previously described. The percentage of survivin-positive cells was quantified by gating around the population of GFP-positive cells, and divided by the total number of cells analyzed (10,000 cells).

3.2 In Vivo Protocol

3.2.1 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. Doxorubicin (at a concentration of 0.5mg/mL, dosage of 2.5mg/kg) was administered intra-peritoneally to male Fischer rats (N=123) at a dosage of 2.5mg/kg, every other day for two weeks, totaling a dosage of 15 mg/kg of body weight.

3.2.2 Animal Experimental Protocol

Three weeks after the initial injection of doxorubicin, a subset of animals received either survivin gene therapy (DOX+SURV, N=39) or null vector delivery (DOX+NULL, N=43) by ultrasound-targeted microbubble destruction. The DOX group (N=41) received no treatment at week 3, while CON animals did not receive DOX nor gene therapy (N=21). Echocardiography was performed at week 0 for baseline function, week 3 (prior to gene therapy), and at week 6 to assess the changes in left ventricular systolic function. A subset of animals was sacrificed at week 4 and the heart tissue was harvested as follows: The heart was divided into three equal sections (the base, apex, and middle). The middle section was embedded in optimal cutting temperature (OCT) blocks, and the base and the apex tissues were divided into right ventricle (RV), LV posterior and LV

anterior. The week 4 tissue was used to assess the early effects of the therapy on exogenous gene expression, and apoptosis. The remaining animals were sacrificed at week 6, and the heart was removed as previously described, snap frozen in liquid nitrogen, and kept in -80°C until further analysis.
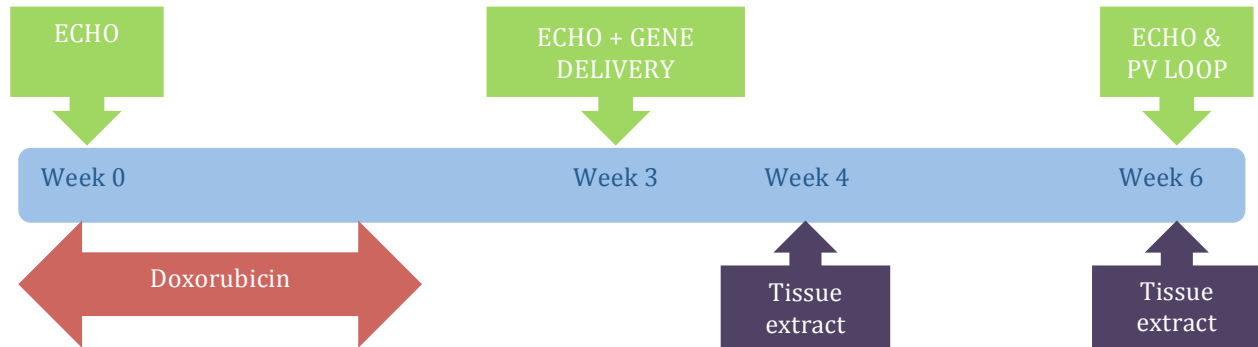
Figure 3 **In vivo experimental protocol**

3.2.3 Gene Delivery via UTMD

3.2.3.1 Cannulation of the Jugular Vein

Surgically prepared rats were anesthetized with isofluorane (0.2L/min oxygen, 3-3.5% isofluorane) and the state of its consciousness was confirmed by the toe-pinch method. A small incision (5-8mm) was made in the ventral cervical skin of the rat, slightly right of the midline, and the jugular vein was exposed and mobilized by means of blunt dissection. Two stabilizing sutures were put in place, with the thread proximal to the head (cranial) being tied off to prevent hemorrhaging. A small opening is made in the jugular vein using micro-scissors, and a sterile cannula (polyethylene tubing) attached to a stopcock and syringe was inserted into the incision. The cannula is stabilized in the

vein by the remaining suture (caudal). The patency of the cannula was tested by drawing on the syringe and visualizing a flash of blood in the catheter. After gene delivery, the cannula was removed and the caudal suture was tied off. The skin incision was sutured, and the rats received post-operative injection of anafen as analgesic. The animals were monitored for signs of infection and open sutures post-operatively.

3.2.3.2 Microbubble and DNA Preparation

An expression vector driven by a CMV promoter was constructed for transfection of Survivin-N-emGFP, or Null-N-emGFP in which the survivin or GUS sequence was fused with emGFP at the N-terminus (null stop codon) to create a fusion protein (SIDNET). For gene delivery, the plasmid-microbubble complex was prepared as previously described (Christiansen et al., 2003; Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007b). Briefly Survivin-N-emGFP or Null-N-emGFP plasmid DNA (500μg; 500ul of 1ug/uL plasmid stock) was charge-coupled to cationic lipid microbubbles (1×10^9 bubbles, volume 1 mL) by incubating the mix in room temperature for 5 minutes.

3.2.3.3 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 5MHz and at mechanical index of greater than 1.6 during intravenous infusion of plasmid DNA-cationic microbubble complexes into the jugular vein. The mechanical index of the ultrasound can be calculated by the formula: peak negative pressure (PNP) / (centre frequency)$^{1/2}$. The peak negative pressure for the delivery setting was not calculated for

our specific delivery settings. However, it can be readily measured using a needle hydrophone (Kobulnik et al., 2009). The ultrasound transducer was positioned transversely at the mid-papillary level, and ultrasound was transmitted during a slow sweep along the long axis of the heart. A pulsing interval of 10 cardiac cycles at end-systole was used to ensure the replenishment of the bubbles in the myocardial microvasculature between each pulse of ultrasound to maximize myocyte transfection. The complex was infused at a steady rate of 0.2 mL per minute using an automatic infuser. After the initial 1mL, the syringe is flushed with 1mL 0.4M saline and the infusion is resumed. This process is repeated twice to ensure the dislodgement of the residual DNA-bubble complex and thus maximal tissue transfection. The total delivery time per animal was 30 minutes.

3.2.4 Functional Assays: Echocardiography and Cardiac Catheterization

3.2.4.1 Echocardiography

Echocardiography utilizes ultrasound as a diagnostic modality to obtain images and information on ventricular function. It is a non-invasive, informative and cost effective method of measuring the LV systolic function. For transthoracic echocardiography, the rats were anesthetized with isofluorane (0.2L/min oxygen, 2% isofluorane), and the chest area was shaved and the remaining hair was removed using a hair remover cream. M-mode and 2-dimensional echocardiography were performed in the short-axis view at the mid-papillary level (Sonos 5500, Philips Ultrasound, S12 transducer). Average FS was derived from the measurements of the LV end-diastolic (EDd) and LV end-systolic (ESd)

dimensions from the M-mode images, using the following formula: %FS = [(LVEDd - LVESd) / LVEDd] x 100%.

3.2.4.2 Cardiac Catheterization

In addition to echocardiogram, which provides important visual information on heart contractility and wall motion, assessment of the hemodynamic state of the heart corroborates the echocardiographic data. Cardiac catheterization was performed at week 6, as previously published using a 2F miniaturized combined conductance catheter-micro-manometer (Scisense, London, Canada). All PV loop data were then acquired under steady state conditions and during preload reduction. Using the pressure conductance data, a range of real-time functional parameters were then calculated including: heart rate (HR), end diastolic pressure (EDP), end systolic pressure (ESP), the time constant of relaxation (Tau), the slope of the end systolic pressure volume relationship (ESPVR) and the slope of the preload recruitable stroke work relationship (PRSW).

3.2.5 Post-mortem Analysis

3.2.5.1 Gene Expression Assay

Primers for GFP were designed using Primer-BLAST available from NCBI to determine the exogenous mRNA transcription level in vivo. Survivin-specific primers were designed, but due to the similarities in human (exogenous) and rat (endogenous) survivin, we could not distinguish the differences seen in delivered and non-delivered tissues (data not shown). For RNA isolation, a small sample of tissue (2-3 mm^3), was immersed in 1

mL of TriZol (Invitrogen) and mechanically pulverized with a RNase-free micropestle. The pulverized tissue was then sonicated briefly (10-20 seconds) using an ultrasonic homogenizer and the RNA was extracted using the RNA extraction kit (BioRad). The resultant RNA was then quantified using a NanoDrop, and the quality of the RNA was confirmed by the 260/280nm absorbance ratio – for phenol contamination from TriZol – and by electrophoresis – for RNA degradation by RNase. 1ug of the RNA was reverse transcribed using a commercially available cDNA synthesis kit (BioRad). Quantitative real-time PCR (qPCR) for the various transcripts was performed using standard techniques in our laboratory. In order to determine the change in the level of survivin with doxorubicin treatment, we amplified endogenous rat survivin at weeks 0, 1, 2, 3 and 6. To determine the success of transfection using the ultrasound delivery method, we sacrificed animals and harvested the heart tissue at days 3 and week 1 post-transfection, and the tissue was snap frozen in liquid nitrogen and stored in -80°C until use. Rat beta-actin was used as an internal control for the variation in the amount of cDNA loaded for each sample. The results were expressed using the relative ΔCt method.

Primer name	Primer Sequence
Survivin Forward	GAAGCACTCCCCTGGCTGCG
Survivin Reverse	GGACGGTCCTCCGGGTCTCC
GFP Forward	AGCAAGGGCGAGGAGCTGTT
GFP Reverse	AGCTTGCCGGTGGTGCAGAT
Beta-Actin Forward	CTGCTCACCGAGGCCCCTCT
Beta-Actin Reverse	TGTGGGTGACCCCGTCTCCG

Table 2 . **Primers used for qPCR.** Targeting survivin, GFP and beta-actin transcripts.

3.2.5.2 Measures of Apoptosis

Quantification and detection of apoptosis is an integral part of this study. In order to determine the level of apoptosis in vivo, we opted for two methods, one being direct measurement of apoptotic bodies (Terminal UdT Nick-End Labelling; TUNEL), and second being an indirect measure (caspase 3 and 7 activity). For TUNEL staining, ApopTag Plus Fluorescein In Situ kit (Millipore) was used to label apoptotic bodies. Briefly, the OCT-embedded tissue was cut in 10 um thickness, and fixed in 2% paraformaldehyde. The labeling was done according to the manufacturer's recommendations. To determine the cell type undergoing apoptosis, post-labelling immunohistochemistry was carried out prior to mounting as described in the following section (see methods: immunohistochemistry). The tissue caspase 3 and 7 activity was measured using Caspase 3 and 7-Glo Assay (Promega). The kit contains an assay solution that consists of buffer-diluted substrate for activated caspase 3 and 7. Once cleaved by the caspase, the substrate becomes luminescent, which can be measured using a luminometer. The level of luminescence can therefore be used as a measure of relative activity of caspases in a given sample. Although the kit was originally designed for use in cell culture, other studies have used the kit for quantification of caspase activity in protein isolates from tissue. Briefly, 2-3 mm3 tissue was immersed in 100-150 ul of radioimmunoprecipitation assay buffer (RIPA, Sigma) buffer – a lysis buffer commonly used for the isolation of protein – supplemented with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche Diagnostics), pulverized manually by micropestles and incubated on ice for 30 minutes. The disturbed tissue was then sonicated briefly for 10-20 seconds by the ultrasonic homogenizer, and centrifuged in >14000 RCF for 10

minutes in 4°C. The supernate was collected in a separate eppendorf tube and stored in -80°C until use. To quantify the total protein, Bradford assay (Biorad) was used with known amounts of BSA (0 − 40 ug/ul) as standards. For the caspase 3 and 7 activity assay, 50ug of protein was used per sample diluted in RIPA buffer totaling 50 ul volume. 50 ul of the caspase-3 and 7 assay solution was added to the protein isolate, and mixed with a horizontal orbital mixer (at 500 RPM) and incubated in the dark for 1 hour in room. The luminescence was measured and quantified by a luminometer.

3.2.5.3 Immunohistochemistry

In order to determine the cell types undergoing apoptosis, immunohistochemical staining for specific cell markers were carried out on TUNEL stained sections. It is important to understand the pathophysiological nature of the heart disease and to determine the types of cells undergoing cell death - this information would allow future studies to specifically target cell types of interest for apoptotic therapies. Briefly, OCT-embedded tissue sections were fixed in 2% paraformaldehyde and permeabilized. Antibodies for mouse anti-sarcomeric-alpha actinin (1:100; Abcam) were used in conjunction with the TUNEL kit for double fluorophore detection. Asubset of sections that have not undergone TUNEL staining were also stained with the above cell markers, and rabbit anti-survivin (1:100; Abcam) to determine the transfected cell type. The nuclei were counterstained with 4',6-Diamidino-2-Phenylindole (DAPI, Vector). The images were taken with confocal imaging.

3.2.5.4 Quantification of Interstitial Fibrosis

Fibrosis is an important indicator of cardiac failure and remodeling. Doxorubicin treatment has been shown to lead to the accumulation of collagen fibers in the myocardium. To determine whether survivin treatment decreased the level of adverse remodeling of the ventricle, we quantified the amount of interstitial fibrosis using the PSR staining method. The PSR staining solution was made by combining 0..5g of Direct Red 80 (Sigma-Aldrich) in 500mL of 1.3% picric acid in water (Sigma-Alrich). First, OCT-embedded tissue was cut in 10um thickness, and washed twice in xylene for 5 minutes to clear the OCT compound. The tissue is then rehydrated with a sequential wash with 100% ethanol for 3 minutes (twice), 70% ethanol for 3 minutes (once) and distilled water for 5 minutes (three times). The washed sections are stained in PSR for 1 hour in room temperature. The stained tissue is then washed in 2 changes of acidified water consisting of 5mL of glacial acetic acid (Ficher) in 1L of tap water, to preserve the colour during the subsequent washes. The sections were air-dried and mounted using a resinous medium (Permount). Fibrosis can be measured by using polarized light microscopy, using the birefringent nature of collagen fibres (Whittaker, Kloner, Boughner, & Pickering, 1994).

3.2.6 Statistical Methods

Data are expressed as mean ± standard error of the mean (SEM), unless specified otherwise. Comparisons between multiple single groups were made with 1-way ANOVA with Bonferroni correction. Comparisons between multiple groups at various time points were made with a two- way analysis of variance (GraphPad Prism5, version 5.0a). When

differences between means were found, Bonferroni correction was performed. Differences were considered significant when P<0.05.

Chapter 4 - Results

4.1 In Vitro Results

4.1.1 Survivin Transfection In Vitro

After transduction with survivin-GFP gene, GFP was highly expressed in cells inoculated with the virus, indicating the presence of the survivin-GFP fusion protein, whereas CONTROL cells did not exhibit any fluorescence (Figure 4). The increase in GFP signal in the SURV-GFP inoculated cells was time-dependent, and was visually assessed for plasmid functionality but was not quantified.
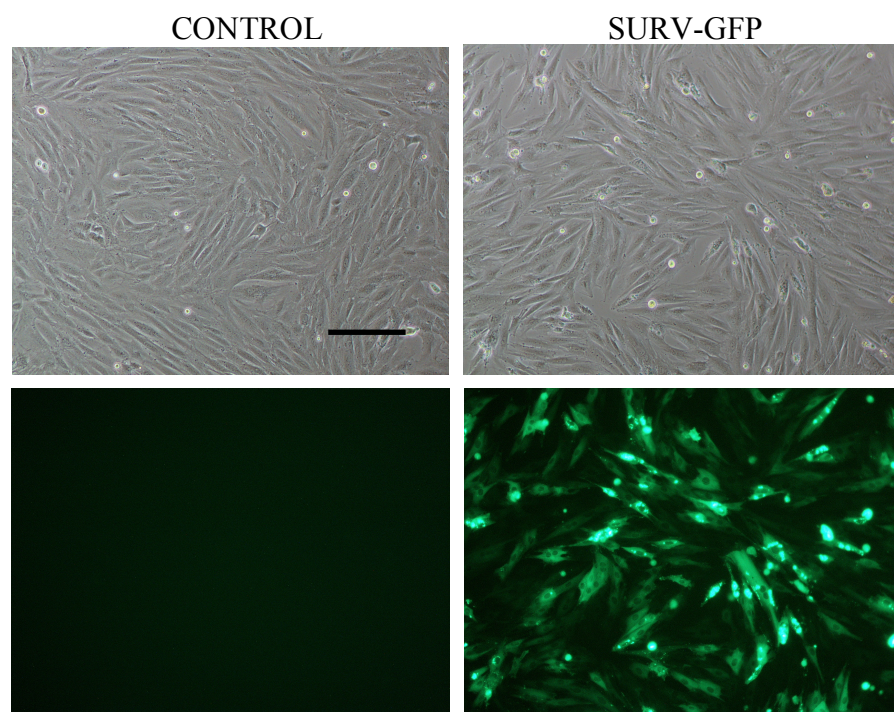

Figure 4 **Expression of exogenous survivin in vitro**. Images of cell culture dish taken with light microscopy (top row) and fluorescent microscopy (bottom row). Images on the right column show cells transduced with SURV-GFP 48 hours post-inoculation, and the images on the left column show cells that were not inoculated with any virus. Scale bar = 25um

Supernate collected from CONTROL cells had no detectable survivin as measured by survivin-specific ELISA (Figure 5). At 24, 48, 72 and 96 hours post-transduction, survivin protein was detected in the SURV group supernate (0.35 ± 0.04 ng/ml, 2.73 ± 0.001 ng/ml, 3.81 ± 0.73 ng/ml, and 2.97 ± 0.35 ng/ml, respectively), peaking at approximately 72 hours. Slight increase in concentration seen at 24 hours was not significantly different from the control samples. Similar to control, supernate collected from the NULL cells had no detectable survivin (0.002 ± 0.001 ng/ml).
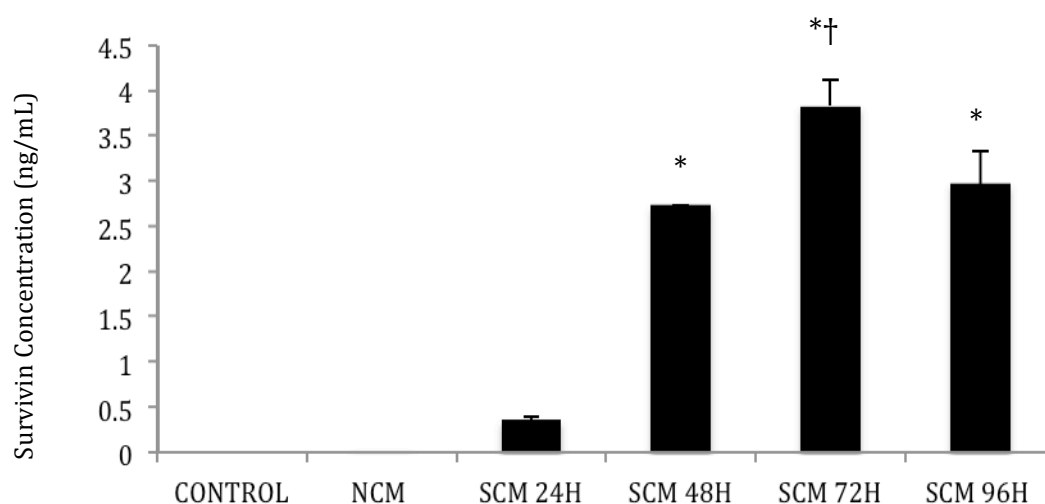
Figure 5 **Supernate survivin concentration in vitro** after transduction with either Ad-Null or Ad-Surv adenovirus. NCM was collected 72 hours post-inoculation. *P<0.001 compared with CONTROL, †P<0.05 compared with SCM 48H. N=8 for Control, 6 for all groups

4.1.2 Effects of Survivin on DOX-Induced Apoptosis In Vitro

After doxorubicin treatment, apoptosis was measured in H9c2 cells by quantification of Annexin-V positive cells by flow cytometry (Figure 6). As expected, there was a significant increase in annexin-V positive cells after the 6 hour exposure to doxorubicin (raw data not shown). All data are normalized to CONTROL values set at 100%. The

increase in apoptosis was reduced by approximately 45% by survivin transduction in the SURV-Tx group (55.87 ± 3.39%). This decrease in apoptosis was particularly important, as we used human survivin in our plasmid – this data indicates that the exogenous, human gene is translated, and has physiological function within the rat cells, and the in vivo portion of the study may ensue. Pre-treatment (24h) with SCM had a modest, but significant effect in inhibiting apoptosis (86.24 ± 2.83%), with approximately 14% decrease in apoptosis compared to the CONTROL. NULL-Tx and pre-treatment with NCM had no effect on the rate of apoptosis (101.29 ± 3.06% and 93.46 ± 2.35%, respectively).
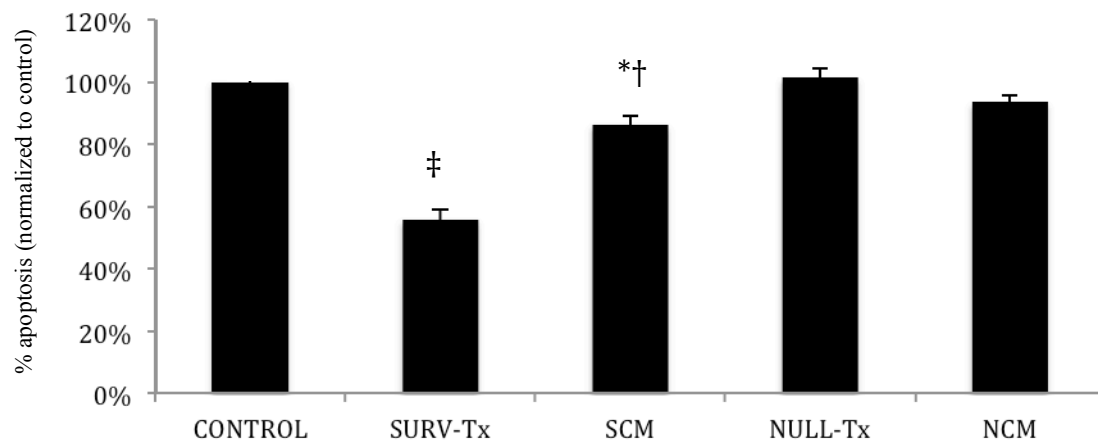
Figure 6 **Rate of apoptosis in vitro** in various groups of H9c2 cells after doxorubicin treatment. All data are normalized to control. *p<0.01 compared to CONTROL, †p<0.01 compared with SURV-Tx, ‡p<0.001 compared with CONTROL. N=9 for all groups.

4.1.3 In Vitro Survivin-emGFP Uptake

Cells were pre-treated with either regular conditioned media (CTRL), null-conditioned media (NCM) or survivin-conditioned media (SCM) for 24 hours before being analysed with flow cytometry for any GFP signal. Data plots show a distinct population of cells

that have higher GFP content in the group grown in SCM, where as cells grown in NCM did not have this population of cells (Figure 7A). Quantification of GFP+ cells from these plots revealed that 4.18 ± 0.32% of cells uptake extracellular survivin after 24-hour incubation (Figure 7B).
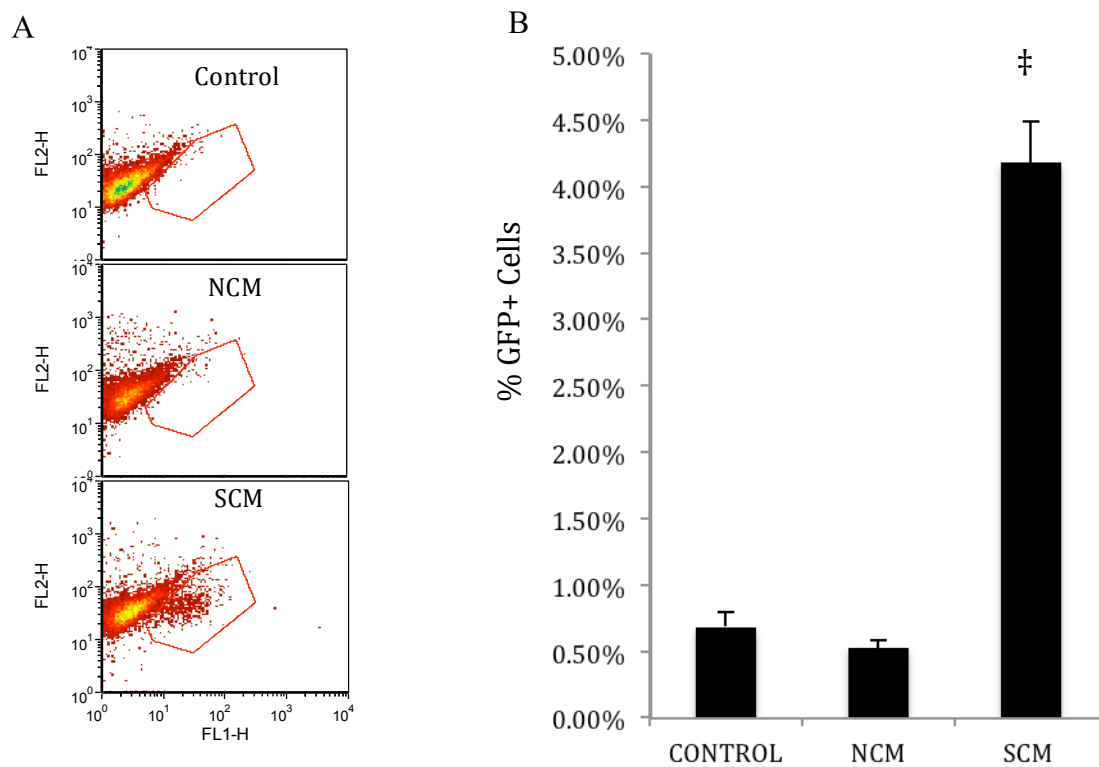
Figure 7 **Survivin uptake by H9c2** after co-incubation in either SCM or NCM for 24 hours. (A) Shows representative plots depicting cell population distribution for CTRL, NCM and SCM. FL1 = GFP, FL2 = Annexin-v-Alexa-568. The gated area shows the increase in GFP positive population in cells incubated in SCM, but not in NCM or CTRL. (B) Gated cells from the plot were quantified. % GFP+ cells were calculated by quantifying number of cells in gates divided by the total number of cells counted (10000) ‡p<0.001 compared with CONTROL. N=12 for all groups.

4.2 In Vivo Results

4.2.1 Endogenous Survivin Levels In Vivo

Prior to the delivery of the survivin gene, we sought to characterize the doxorubicin model by monitoring the level of endogenous survivin. As discussed in previous sections, the level of survivin in different models of HF differ, and it was important to establish the time course of endogenous levels of survivin to determine the optimal time point, at which delivery can yield the maximal effect. qPCR results of survivin level indicates that compared to the control, survivin levels are down-regulated at all time points after the initiation of doxorubicin administration. During the drug administration at weeks 1 and 2, the level of endogenous survivin decrease to approximately 50% and 58% of the control. At weeks 3 and 6, there was a slight trend towards an increase in survivin levels ($76.04 \pm 6.31\%$ and $70.48 \pm 10.58\%$) but the differences between post-doxorubicin weeks were not significant.
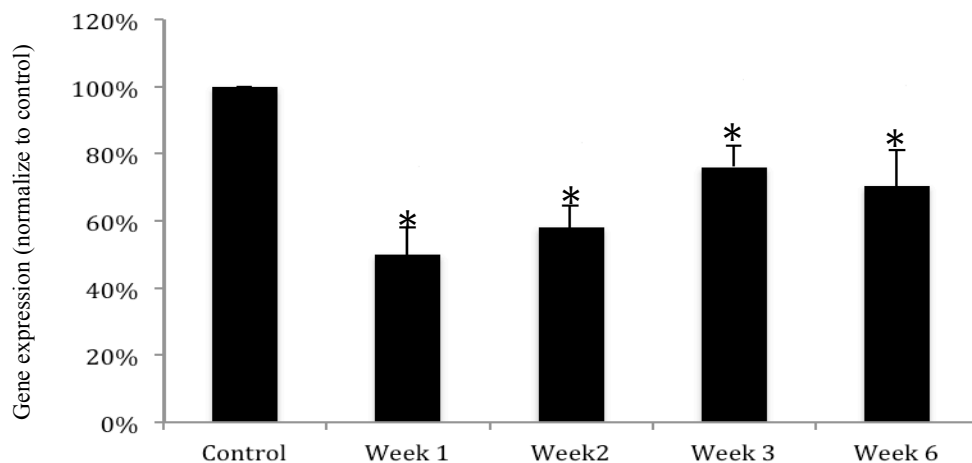
Figure 8 **Endogenous survivin transcript levels** in the myocardium after doxorubicin treatment $*p < 0.05$ compared with Control. N=6 for all groups.

4.2.2 Survivin Gene Transfection In Vivo

To determine the effectiveness of ultrasound-mediated gene delivery on transferring the exogenous gene into the heart, we detected the gene transcript levels (for transcription) and the survivin protein (for successful translation). Figure 9B shows the gene transcript levels at various time points post-delivery. As seen from previous studies in optimization of cardiac delivery using ultrasound (S. Chen et al., 2003), we saw that the maximal transfection occurred at day 3, and was diminished to half of that at day 7. In contrast, doxorubicin alone did not exhibit any increase in the exogenous gene. Figure 9A qualitatively demonstrates that ultrasound delivery of survivin gene led to the successful translation of the protein within the cardiomyocytes (white arrows)
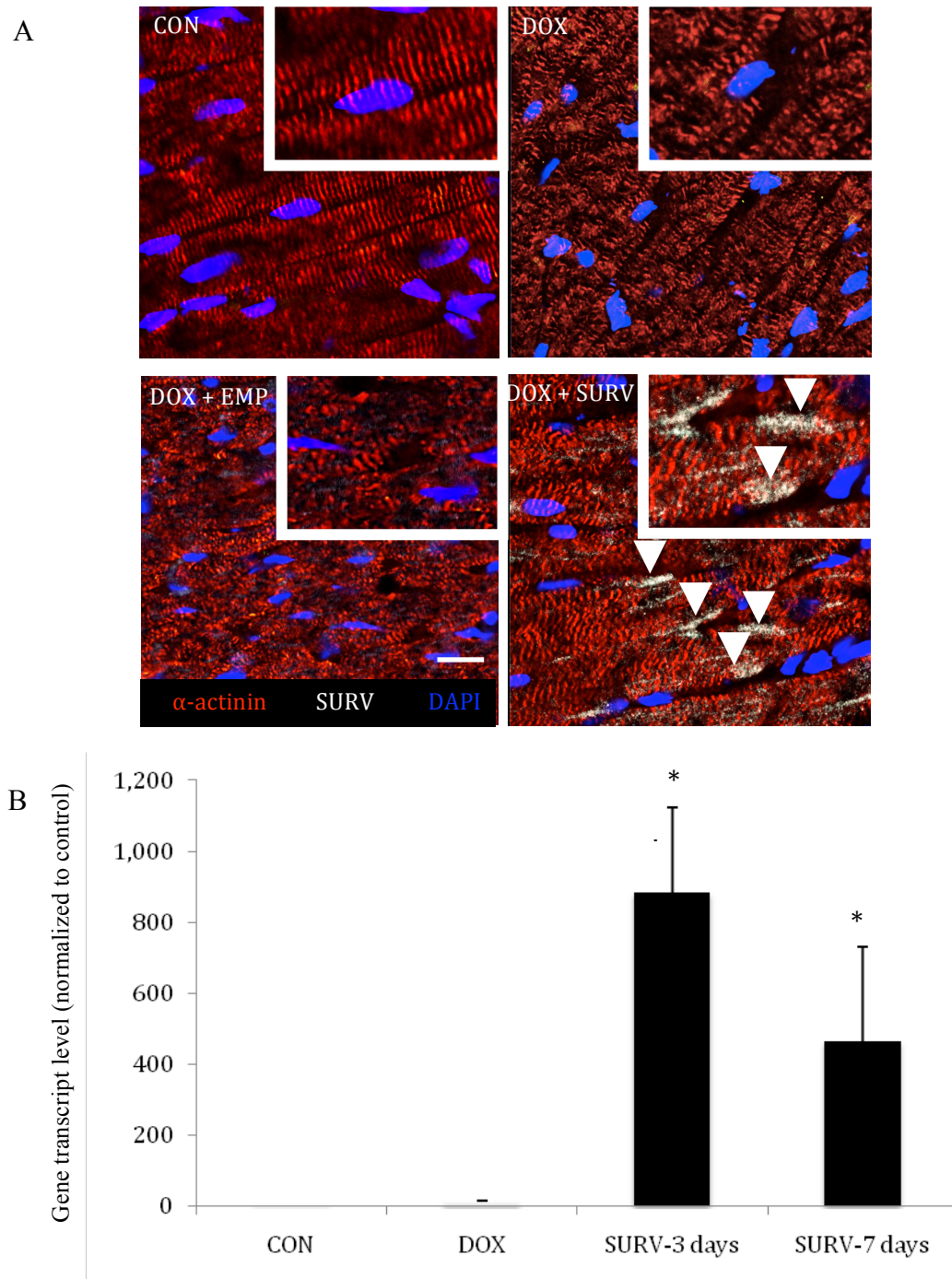
Figure 9 **Survivin gene expression levels after UTMD** (A) Immunohistochemical staining of the cardiac tissue sacrificed at week 4 (7 days post-delivery). White arrows = survivin in cardiomyocytes, yellow arrows = survivin in endothelial cells. Scale bar = 25um. Cardiomyocytes are stained with sarcomeric alpha-actinin (red) and nuclei with DAPI (blue). (B) Gene transcript levels by qPCR. Gene expression peaks at day 3, and is reduced at day 7. Values are expressed as mean ± SEM, and normalized to control (non-delivered) tissue. *p<0.001 when compared to CON, †p<0.05 when compared to SURV-7 days. N= 5 for SURV-3 days, N=10 for all other groups.

4.2.3 Assessment of LV Dimensions and Systolic Function

LV dimensions as measured from the M-mode images are summarized on Table 3. The data show a significant increase in the systolic diameter after doxorubicin treatment compared to the control at week 6 (0.4256 ± 0.01, $p<0.01$ vs CON). Survivin treatment reversed the increase in systolic dimensions (0.3841 ± 0.01, $p<0.05$ vs DOX) but empty delivery did not yield the same effect (0.4191 ± 0.005).

		Week 0	Week 3	Week 6
CON	EDD	0.6820 ± 0.02	0.7106 ± 0.01	0.7069 ± 0.007
	ESD	0.3488 ± 0.008	0.3660 ± 0.009	0.3550 ± 0.008
DOX	EDD	0.6865 ± 0.008	0.6962 ± 0.005	0.6967 ± 0.008
	ESD	0.3579 ± 0.008	0.3753 ± 0.006	$0.4256 \pm 0.01*$
DOX+EMP	EDD	0.6977 ± 0.006	0.7049 ± 0.007	0.7089 ± 0.05
	ESD	0.3515 ± 0.006	0.3628 ± 0.006	$0.4191 \pm 0.005*$
DOX+SURV	EDD	0.6928 ± 0.008	0.6830 ± 0.009	0.6816 ± 0.009
	ESD	0.3602 ± 0.006	0.3642 ± 0.006	$0.3841 \pm 0.01\dagger\#$

Table 3 . **LV dimensions for different groups at weeks 0, 3 and 6**. Measurements in cm $\pm$ SEM. *$p<0.01$ vs CON, #$p<0.05$ vs DOX, †$p<0.05$ vs CON. N=10, 30, 30, 28 for CON, DOX, DOX+EMP, DOX+SURV respectively.

Figure 10A shows the representative M-Mode echocardiographic images at the mid-papillary muscle level of the LV from each treatment group. At week 3, all groups are comparable in function to the control heart, with preserved wall motion and contractility, without systolic dysfunction. At week 6, however, DOX and DOX+EMP groups show a marked increase in the left ventricular cavity dimensions at end systole (red arrows) compared to the control, indicating an inability to contract normally. DOX+SURV shows LVESd comparable to the control. Fractional shortening was calculated from the M-mode images (Figure 10B). Prior to gene delivery at week 3, LV % fractional shortening (FS) was similar in all groups DOX, DOX+EMP and DOX+SURV animals ($46.10 \pm 0.71\%$, $48.53 \pm 0.69\%$ and $46.65 \pm 0.56\%$, respectively). In contrast, at the 6 week time point, LV %FS was significantly greater in the DOX+SURV group ($44.37 \pm 0.89\%$) as compared to the DOX ($38.99 \pm 1.23\%$, $p<0.05$ vs DOX+SURV) and DOX+EMP groups ($40.65 \pm 0.49\%$, $p<0.05$ vs DOX+SURV). Data on % change in LV %FS is shown in Figure 10C. While DOX and DOX+EMP animals exhibited a decline in LV %FS over the 6 week study period ($-14.94 \pm 2.89\%$ and $-15.74 \pm 1.30\%$), this effect was significantly attenuated by survivin gene therapy ($-4.66 \pm 2.00\%$).
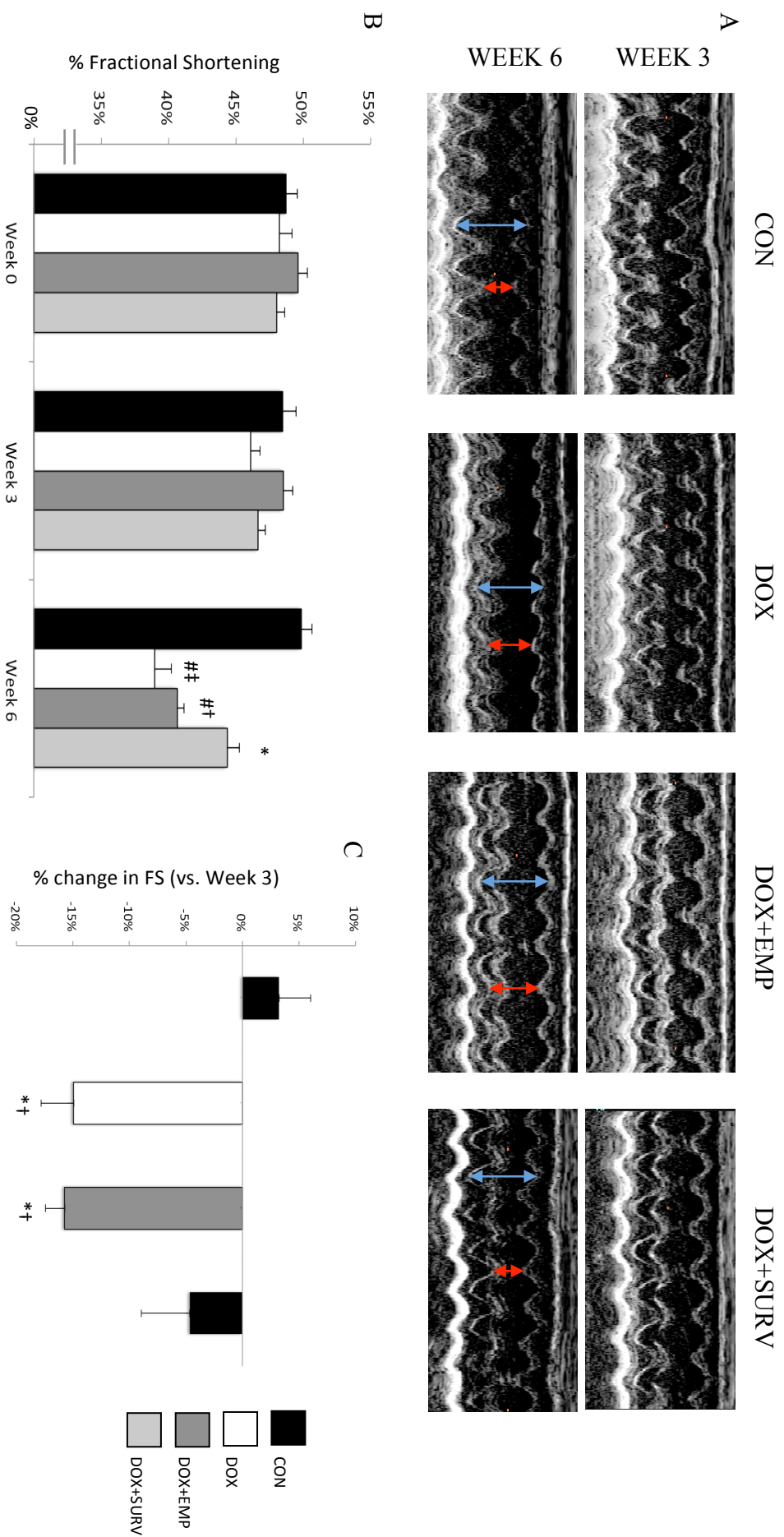
Figure 10. **Functional analysis of the heart by echocardiography.** (A) M-mode images of the left ventrical of various groups at week 3 (top panel) and week 6 (bottom panel). Red arrow = End-systolic dimension, Blue arrow = end-diastolic dimension. (B) fractional shortening of various groups at weeks 0, 3, and 6. (C) % change in fractional shortening of various groups. Change in FS is calculated by the following formula: $[1-(FS_{week6} - FS_{week3})]$ x100%. *$p<0.05$ and # $p<0.001$ compared to week-6 CON, †$p<0.05$ and ‡$p<0.001$ compared to week-6 DOX+SURV. % change in FS from week-3 to week-6 in each group. *$p<0.001$ compared to CON, †$p<0.01$ compared to DOX+SURV. N=10, 30, 30, 28 for CON, DOX, DOX+EMP, DOX+SURV, respectively.

Hemodynamic data from cardiac catheterization at week 6 is shown in Table 4. Survivin gene therapy was associated with improved systolic function, as shown by a greater preload recruitable stroke work (PRSW) relationship index and end systolic pressure volume relationship (ESPVR). Parameters for the measure of diastolic function, such as Tau Weiss and +dp/dt were not significantly altered by survivin gene therapy.

	DOX (n=15)	DOX+EMP (n=14)	DOX+SURV (n=18)
Heart Rate	321 ± 36	352 ± 22	311 ± 42
ESP (mm Hg)	129 ± 23	130 ± 13	123 ± 14
EDP (mm Hg)	14 ± 2	11 ± 2	9 ± 2
+dp/dt	7274 ± 1571	7147 ± 1143	6976 ± 1091
Tau Weiss	14.46 ± 2.3	11.04 ± 1.2	12.4 ± 2.3
ESPVR(mm Hg/uL)	0.29 ± 0.20	0.35 ± 0.30	$0.85 ± 0.50^{†, ††}$
PRSW(mm Hg/uL)	55.0 ± 13.5	69.8 ± 16.1	$77.5 ± 12.7^{*, **}$

Table 4 . **Hemodynamic Variables in DOX, DOX+EMP and DOX+SURV animals at week 6**. Data expressed as Mean ± SD. EDP, end diastolic pressure; ESP, end systolic pressure; PRSW, preload recruitable stroke work relationship; ESPVR, end systolic pressure volume relationship. For PRSW: *P<0.05 when compared with DOX + EMP rats. **P<0.0001 when compared with DOX. For ESPVR: † P<0.01 when compared with DOX + EMP rats ††P<0.001 when compared with DOX

4.2.4 Measures of Apoptosis with Survivin Gene Therapy

Representative images of TUNEL staining from all treatment groups are shown in the Figure 11A. The images demonstrate that apoptosis was predominantly limited to cardiomyocytes, indicated by the white arrows. Quantitative apoptosis rates by TUNEL

from all groups are shown in the Figure 8B. Apoptosis was increased in the DOX group

$(0.65 \pm 0.07\%)$, compared to the basal rate of apoptosis seen in the CON hearts $(0.04 \pm$

$0.02\%)$. Survivin gene therapy in the DOX+SURV group significantly rescued the

apoptotic phenotype $(0.18 \pm 0.04\%)$. DOX+EMP showed apoptotic rates similar to that

of the DOX group $(0.67 \pm 0.14\%)$. Activity of caspase-3 and caspase-7 was measured at

week 4, 1-week post-gene therapy. While caspase-3 and 7 activity was increased in the

DOX group and DOX+EMP compared to the CON $(236.5 \pm 53.1\%$ and $218.1 \pm 14.1\%)$,

in keeping with higher apoptosis rates, survivin gene therapy resulted in a significant

reduction in caspase-3/7 activity $(93.0 \pm 22.4\%)$.
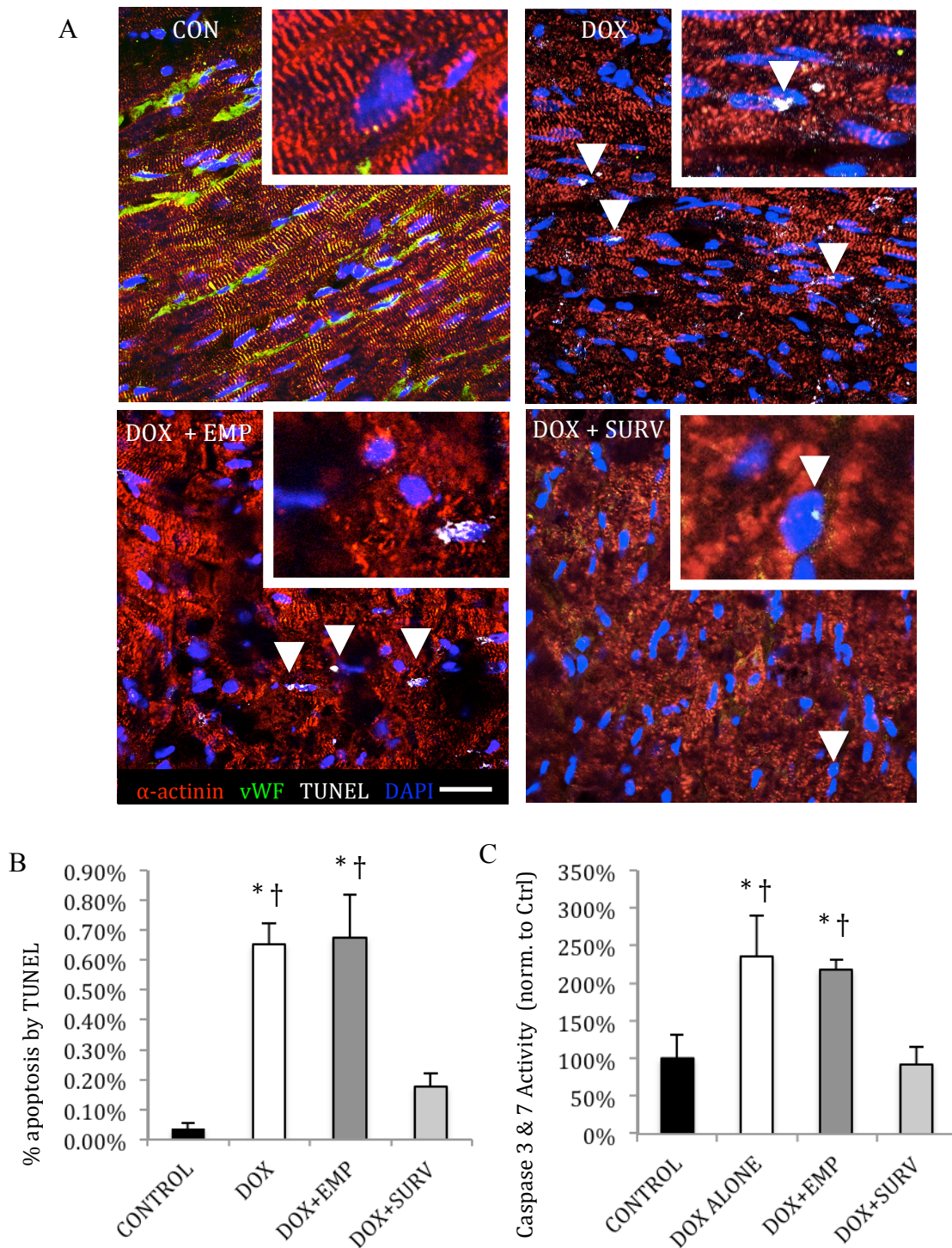
Figure 11 **Measure of Apoptosis in vivo**. (A) TUNEL staining for apoptotic bodies. Red (sarcomeric alpha-actinin) white (TUNEL+) Blue (Nuclei) Scale Bar = 25um (B) Quantification of TUNEL positive cells. (C) Caspase 3 and 7 activity in vivo. *p<0.001 compared to CON, †p<0.001 compared to DOX+SURV. N=9 to 11 for TUNEL, N=9 to 13 for caspase activity.

4.2.5 Quantification of Interstitial Fibrosis

Evidence of interstitial fibrosis in the LV was detected with doxorubicin therapy, as shown by increased PSR staining in the DOX-treated group (Figure 12A). As compared to the control group, the DOX group exhibited a 3-fold increase in fibrosis (280.44 ± 14.5%). DOX+EMP group also showed increased collagen deposition in the myocardium (296.4 ± 27.1%). Tissue sections in the DOX+SURV group showed significantly reduced degree of interstitial fibrosis as compared to DOX and DOX+EMP (151.9 ± 23.8%)

Figure 12 **Interstitial fibrosis**. (A) Representative images of PSR staining of the myocardium. Scale bar = 25um (B) Quantification of the PSR stained sections. *p<0.001 compared to CON, †p<0.001 compared to DOX+SURV. N=7 for DOX+EMP, N=10 for all other groups.

Chapter 5 - Discussion

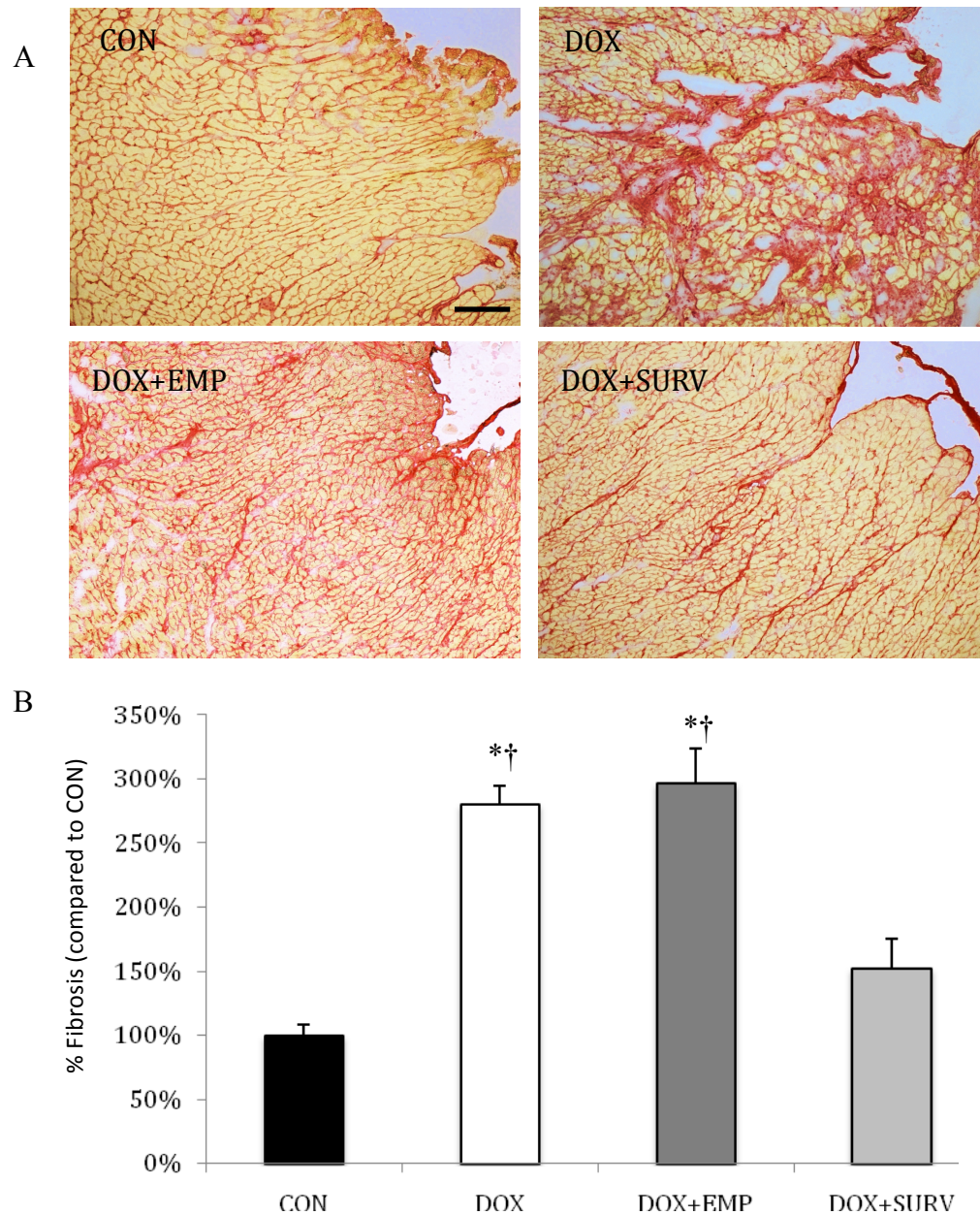## 5.1 In Vitro Experiments

The in vitro transfer of the survivin gene via viral transduction led to a high expression of the survivin-GFP protein in our H9c2 cell line. As mentioned, our plasmid encodes the human variant of survivin, which has 83% homology on the transcript level and 83% homology in the amino acid sequence compared to the rat survivin. However, the difference in the amino acid sequence may not be as critical, as 91% of the amino acid sequence being of similar properties, the function may well be preserved in rats. As an example, previous studies have delivered the human variant of the VEGF gene into rats with functional improvements in angiogenesis (Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007a). The rat-human VEGF transcript homology is 89%, but the amino acid sequence homology 80%, similar to the in rat-human survivin amino acid percent homology 85% of the amino acid sequence was of similar properties). In addition, IAP sequences are well preserved throughout species, and it was noted that a baculoviral IAP was fully functional in mammalian cells (Huang et al., 2000). Our study demonstrated that the gene was both translatable – as demonstrated by the detection of GFP detection and immunological detection of survivin by ELISA – and functional – as demonstrated by the anti-apoptotic effect under doxorubicin stress – in a rodent cell model.

When delivering exogenous genes in vivo, it is vital that the mRNA is successfully translated for protein formation (relating to the primary structure), but it is also necessary to ensure that the gene product is functional within the cells (relating to the tertiary and

quaternary structure). Our protein is a fusion protein of survivin and GFP, which may hamper the ability of survivin to form dimers (Chantalat et al., 2000; Verdecia et al., 2000) (necessary for activation) and result in the lessening in its anti-apoptotic properties. However, we have shown that our protein is successful in partly abrogating the cytotoxicity of doxorubicin in vitro, indicating its preserved function within the cell. In line with our findings, a previous study explored relationship between the localization of survivin and its function by tagging the protein to dsRed flourophore, and found no interference of the function by the fused protein (Temme et al., 2003).

In addition, the fusion protein was seen in the extracellular space as detected by ELISA, and the concentration of the "secreted" protein increased in a time-dependent manner. This increase can be either attributed to the increased production and secretion of the protein as time passes, or could be a product of the accumulation of the secreted protein over time, without changes in production or secretion by the transduced cells. The mechanism of secretion was not covered by the scope of our study. It has been noted in previous studies that survivin is secreted into the extracellular space via exosomal secretion (Khan et al., 2011). Future studies can examine the survivin secretion pathway by isolation of exosomes and detecting survivin protein specifically in the exosomal compartment. One such experiment would be to abrogate the secretion pathway via inhibitors of exosome secretion and examine the presence or absence of exosomes in the cell culture media. However, such compound that affects exosome secretion without affecting other lipid vesicle transport within the cell is yet to be isolated.

Co-incubation of extracellular survivin led to the uptake of survivin – as detected by GFP positive cells by flow cytometry – and subsequent (partial) inhibition of apoptosis under

doxorubicin stressing. The pathway of uptake was not described by previous studies of extracellular survivin: in order to strengthen the study of survivin uptake in H9c2, it may be necessary to run additional experiments to examine the effects of inhibiting well-known pathways of cellular uptake, such as clathrin- or caveolae-mediated endocytosis (Rejman, Bragonzi, & Conese, 2005). Our study is limited in that the supernate in which we believe extracellular survivin exists may also contain residual viral particles that remain infectious. The minute population of GFP positive, and therefore survivin positive cells may indeed be a result of undesirable transduction of cells by the unwashed virus. In addition, our study lacks the appropriate control for GFP-alone group. The uptake of the fusion protein may be mediated by the GFP portion of the protein, not survivin. Inclusion of a GFP containing supernate as a control may strengthen our argument for survivin uptake from the extracellular space.

Our flow cytometry analysis of doxorubicin treated cells show a potent anti-apoptotic capacity of survivin. Approximately 50% of the cells were salvaged from doxorubicin toxicity. Upon close examination of the plots, it was noticed that cell populations with higher GFP signals showed less annexin-V staining, which is indicative of dose-dependent anti-apoptotic activity. This suggests that optimization of gene transfection methods in vivo is crucial in attaining maximal therapeutic effect. To exclude the possibility of GFP-mediated anti-apoptotic effects, an appropriate GFP-alone group should be included as a control group.

Interestingly, cells incubated in survivin-conditioned media were also seen to have decreased level of apoptosis. This indicates that the protective effects of survivin may not be limited to direct transfection, but also a paracrine effect from secreted survivin

protein. We have shown that survivin was released by, and was taken up by H9c2 cells. However, there are no previous studies showing that H9c2 cell line is amenable to the uptake of secreted survivin, or if normal, non-cancerous cells are able to absorb the protein. To this note, the effects of extracellular survivin may well not be entirely by the uptake of the protein *into* the cell, but by *outside-in* communication through a yet-to-be-identified cell surface receptor. An interesting study by Mera and colleagues has shown that survivin interacts with neutrophils on the cell surface to induce expression of adhesion molecules (Mera et al., 2008), which opens the possibility that the mechanism of action through which survivin affects function may not only be through the uptake of the protein. While the scope of our study does not address the mechanism through which extracellular survivin is absorbed by cells or how it acts to inhibit apoptosis in vitro, it is necessary to elucidate the mechanism of extracellular survivin in order to ascertain the role of extracellular survivin in promoting cell survival and proliferation.

5.2 In Vivo Experiments

Prior to the delivery of the survivin gene into the failing rat hearts, we characterized the endogenous survivin levels in the myocardium after doxorubicin administration. The purpose of this was to augment the gene when the endogenous anti-apoptotic gene expression was at its lowest. Also, disease progression – or the decline in LV systolic function – was important in determining the time of gene delivery. We were interested in gene therapy when heart function was relatively maintained. Results from the gene expression analysis revealed that doxorubicin decreases survivin transcript levels at all time points after the initial administration and together with the doxorubicin induced

cardiomyopathy functional data, the optimal delivery time point was determined as the 3rd week of the study.

The decline in survivin expression following doxorubicin can be explained by the increase in DNA damage (Capranico, Dasdia, & Zunino, 1986b; Tewey, Rowe, Yang, Halligan, & Liu, 1984; Zhou et al., 2002), leading to p53 upregulation (Zhu et al., 2009). It was recently revealed that p53 represses survivin expression in acute lymphoblastic leukemia cells (Zhou et al., 2002). In this study by Zhou and colleagues, it was reported that wild type p53 was required for the downregulation of survivin after doxorubicin treatment. Another study also showed that p53 can transcriptionally inhibit survivin promoter (Mirza et al., 2002) High levels of murine double minute gene (MDM2) over-expression was able to block the survivin repression by p53, indicating the regulation of survivin by p53. Another study by Wang et al describes the survivin/MDM2/caspase-3 axis, through which survivin regulates p53 activity (Wang, Fukuda, & Pelus, 2004). The findings from this study demonstrates that the inhibition of caspase by survivin leads to the inhibition of MDM2 cleavage by caspases, and this in turn leads to the increase in MDM2, promoting the degradation and inactivation of p53. These data may suggest that survivin/p53 axis exists in a delicate balance, where perturbations of one end of the spectrum may lead to the dynamic changes on the other.

In this study, we have demonstrated the effective use of ultrasound-mediated gene delivery method to transfer the survivin gene, in turn leading to improvements in LV systolic function in a model of doxorubicin-induced cardiomyopathy in rats. DOX+SURV animals showed preservation of LV systolic function as compared to the DOX and DOX+EMP animals. The functional changes elicited by survivin gene therapy

can be attributed to the inhibition of apoptosis. Previous studies of doxorubicin cardiomyopathy models have demonstrated an increase in apoptotic cells in the myocardium, and the pathophysiology of the disease, like many other HF of varying etiologies, is recognized as being the loss of functional cells in the myocardium (X. Chen et al., 2007; De Angelis et al., 2010; Konishi et al., 2011; Nakamura et al., 2000). To measure apoptosis, two inter-related methods of apoptosis detection were used: TUNEL assay and caspase activity. The level of caspase activity in the DOX+SURV was significantly lower in than in the DOX or the DOX+EMP groups, which was expected of the IAP's ability to inhibit caspase activity. In turn, apoptotic index measured by TUNEL was lower in DOX+SURV, reflecting the fact that inhibition of caspase is adequate to inhibit apoptosis. However, the caspase activity assay used in this study only determined that the activity of the executioner caspases (-3 and -7) were increased, and does not shed light on the activity of the other caspases. Further studies are required to determine the precise pathway of apoptosis (i.e. extrinsic vs intrinsic) by assessing the activity level of various caspases.

The benefits of survivin gene therapy also included the decreased remodeling of the heart via decreased deposition of interstitial fibrosis in the myocardium. Doxorubicin-induced cardiomyopathy model involves moderate fibrotic cardiac remodeling (Bartoli et al., 2011; Feridooni, Hotchkiss, Remley-Carr, Saga, & Pasumarthi, 2011; L. Li et al., 2006). Interestingly, we found a significant decrease in fibrosis with survivin treatment. This can be explained by survivin's effect on apoptosis: Apoptosis of endothelial cells may lead to the recruitment of macrophages, which release fibrogenic mediators, activating myofibroblasts within the myocardium (Lauber et al., 2003). Also there have been

reports of endothelial release of connective tissue growth factor (CTGF) under apoptotic stress (Laplante et al., 2010). With decreased apoptosis as shown by the TUNEL data, and decreased caspase 3 and 7 activity, it is assumed that these fibrogenic factors were diminished by our survivin gene therapy.

5.3 Limitations and Future Directions

5.3.1 Efficiency of Gene Delivery Method

Although proven to be an effective method of gene delivery to the areas of tissue with high specificity and diffuse delivery area (S. Chen et al., 2003; Kobulnik et al., 2009), the gene delivery efficiency of UTMD pales in comparison to direct intramuscular injection of the gene product or viral delivery technique. Despite its lower transfection efficiency UTMD gene delivery method produces gene expression pattern that induced angiogenesis, leading to enhanced functional (increased muscle perfusion) and structural (microvascular density) endpoints (Kobulnik et al., 2009).   UMTD delivery method has been optimized in a rat cardiac delivery setting by Chen and colleagues (S. Chen et al., 2003), but the limitations of the method is that deliverable organs are limited to those that can be readily imaged by the ultrasound. Small animal studies utilizing UTMD (Bekeredjian et al., 2003; Bekeredjian, Chen, Pan, Grayburn, & Shohet, 2004; Bekeredjian et al., 2005; S. Chen et al., 2003), such as our survivin study allows for relatively efficient transfer of gene to the heart as we move the probe along the axis of the heart in a timely manner to cover the majority of the heart tissue but in large animal settings, or even clinical studies where the heart organs are much larger in size, there

needs to be a systematic protocol for the gene delivery method to maximize the coverage of the delivery in the tissue.

Delivery technique in the cardiac tissue has been optimized in a previous study using the luciferase reporter gene (S. Chen et al., 2003). Contrary to the findings in the paper, we opted to used the S12 probe for our delivery protocol, as we experienced a higher than normal mortality rates in our animals with the S3 probe. In order to compensate for the change in protocol, we modified the delivery settings in such a way that the power was over-compensated, which we believe led to comparable transfection efficiency. Also, the limitation of the ultrasound mediated gene delivery is that it is limited by perfusion. Patients suffering from HFs often suffer from co-morbidities such as coronary artery diseases and myocardial infarct (Roger et al., 2011), which may significantly hinder the effectiveness of the therapy. Surgical interventions, such as CABG to improve perfusion or gene therapy to induce vasculogenesis and increase perfusion/capillary density (Kuliszewski et al., 2011; Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007a) may prove to beneficial for subsequent anti-apoptotic gene therapy, and offer better prognosis.

5.3.2 Model of Heart Failure

The use of doxorubicin-induced cardiomyopathy as a model of heart failure may raise questions in its clinical applicability. Although the doxorubicin-induced heart failure model has been used extensively in the literature to mimic HF (Bartoli et al., 2011; X. Chen et al., 2007; Czarnecki, 1984; Jensen et al., 1984; Monnet & Chachques, 2005), the prevalence of doxorubicin-induced cardiomyopathy and the patient population suffering

from this particular etiology of heart disease is significantly smaller than other cardiomyopathies, such as ischemic cardiomyopathy (Roger et al., 2011). This study verifies that the effect of anti-apoptotic survivin therapy may have beneficial outcomes in doxorubicin-induced cardiomyopathy, but the results could be extrapolated to other forms of cardiomyopathies that involve spontaneous and progressive cardiac cell death (Abbate et al., 2006; Narula et al., 1996). As discussed previously, the myocardium undergoing heart failure undergoes progressive loss of functional cell populations by apoptosis throughout the course of the disease, even in the absence of repeated injuries (Narula et al., 1996; H. N. Sabbah & Sharov, 1998). Doxorubicin-induced cardiomyopathy is one of the many forms of cardiomyopathies that exhibit such phenotype, and it can be inferred that any form of HF, which leads to the loss of cardiomyocytes will benefit from anti-apoptotic therapy by survivin gene delivery. Nonetheless, it is vital to investigate the effects of gene therapy modality explored in this study on other models of HF, such as ischemia or ischemia/reperfusion model, in order to further validate the effectiveness of anti-apoptotic gene therapy by UTMD.

5.3.3 In Vivo Experimental Protocol

Gene therapy requires the delivery of specific genes at specific time points that are endogenously relevant. Data from our lab revealed that in a model of rat hindlimb ischemia, VEGF delivery into the ischemic skeletal muscle increased vessel density and perfusion, but at longer time points, regressed back to the diseased state (Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007b). Published data from our lab demonstrated that the delivery of VEGF and angiopoietin I in a temporally separated manner (according to the time course of the gene

expression in endogenous arteriogenesis process) led to the preservation of the arteriogenic effects (Smith et al., 2012). Accordingly, our survivin gene delivery time-point was selected to mimic the clinical situation as accurately as possible. Current knowledge on survivin in HF setting suggests that survivin is down-regulated in the peri-infarct regions of the heart (Santini et al., 2004), and in the dysfunctional myocardium of a hypertensive heart (Abbate et al., 2006). In addition, it was noted that higher expression of survivin in hypertensive myocardium was correlated to decrease in apoptosis and heart weight (Abbate et al., 2006), indicating that survivin gene expression is favorable in HF settings. In our model of doxorubicin-induced cardiomyopathy, survivin expression was down-regulated at all time-points after doxorubicin administration, suggesting that the augmentation of the gene by exogenous survivin therapy may act to reverse the adverse cardiac remodeling.

At the time of delivery (1 week after the end of doxorubicin administration) the rat heart function was relatively well-preserved. One may argue that this study examines the preventative therapy of HF, which is rarely the case in clinical settings. However, the disease course has already been initiated with the administration of doxorubicin, and we believe that this is a suitable 'treatment' model where it can be applied to patients who have suffered myocardial ischemia (with impaired systolic function) or even patients who have been diagnosed with asymptomatic HF (or with preserved ejection fraction). It has been noted that hearts undergoing failure are subject to cardiomyocyte loss at any given point in their course of the disease, and therefore it is presumed that therapy at any point after the initial cardiac event will be beneficial.

5.3.4 Length of Study

Our study examined the structural and functional changes that take place within the myocardium during a study period of 6 weeks. This is a relatively short period of time, in comparison to the chronic effects of doxorubicin, which may take years to develop in humans. In 1977, doxorubicin-induced cardiomyopathy had been established in rats, reporting that 10-20 weeks are required for the onset of cardiomyopathy (Mettler, Young, & Ward, 1977). However, we have opted for the shorter study period as doxorubicin also causes acute HF, and has been used previously by several studies (T. Li, Danelisen, Bello-Klein, & Singal, 2000; Siveski-Iliskovic, Kaul, & Singal, 1994). The purpose of the study was not limited to the treatment of doxorubicin-induced cardiomyopathy and the subsequent HF symptoms, but to establish the potential of using UTMD and survivin anti-apoptotic gene therapy in HF settings of varying etiologies.

Another concern with the relatively short study period is that we are not able to monitor the long-term effects of anti-apoptotic therapy. As mentioned earlier, doxorubicin induced cardiomyopathy persists for months/years after the drug administration, with progressive loss of cardiomyocytes. Even with the survivin treatment by UTMD, the effects of the gene therapy will be transient, and the cells will be exposed yet again to the toxicity of doxorubicin. Even after augmentative therapeutic measures (i.e. repeated gene delivery into the myocardium) concerns remain as to what the fate of the forcible inhibition of apoptosis in cells chronically exposed to cytoxicity. One of the main concerns is the apoptosis-necrosis conversion, where normally cells would undergo controlled apoptosis, forcible inhibition would tip the scale towards the necrotic pathway, causing further irreversible damage to the heart (Lemaire et al., 1998). The long-term

effect of inhibiting apoptosis has not yet been evaluated, and its usability has not been validated in humans. It is conceivable that due to the injuries already sustained, alternative forms of cell death may be activated once apoptosis is forcibly inhibited (Lemaire et al., 1998; L. J. Zhang et al., 2008), leading to the worsening of the disease prognosis. Even with inhibition of apoptosis by caspase-inhibitors, we must also note that caspase-independent apoptosis may occur (Godefroy et al., 2004; Lee et al., 2006; Shih et al., 2003), which would infer that caspase inhibition may be able to slow the progress, but may not be sufficient to reverse the disease. Promising data from Chandrashekar and colleagues have shown that systemic infusion of caspase-inhibitor for 28 days in a myocardial infarction setting led to improvements the left ventricular systolic function, as well as structural changes (Chandrashekhar et al., 2004). As the scope of this study does not address this issue, further studies are required to better understand the pathophysiology of HF, and the effect of apoptosis in HF of different etiologies, in order to better design the therapeutic modalities for clinical use.

5.3.5 Changes in the Endothelial Cells and the Vasculature

Doxorubicin toxicity has been demonstrated in vitro by numerous independent studies, and it is plausible that the decline in heart function may be partly due to the diminished endothelial function (Mailloux et al., 2001; Wolf & Baynes, 2006). Doxorubicin is also implicated in the expression of VEGF, a potent angiogenic protein, whereby it decreases its expression in vitro, and this may lead to impedance of vascular growth (Duyndam et al., 2007). In support of the role of the endothelium in the setting of doxorubicin-induced cardiomyopathy, Hamed and colleagues demonstrated an improvement in LV function after treatment with erythropoietin, which led to a significant increase in capillary density

compared to the non-treated group (Hamed et al., 2006). Although this study relies on a simple immunohistochemical stain for endothelial cells and manual quantification, a more suitable and systematically quantitative technique would be the use of fluorescent microangiography (FMA). FMA, in conjunction with confocal microscopy allows for the visualization of the vasculature in a 3D framework, providing a better understanding of the overall structure of the vascular network (Leong-Poi, Kuliszewski, Lekas, Sibbald, Teichert-Kuliszewska, Klibanov, Stewart, & Lindner, 2007a). Using this technique, it is possible to determine the vascular density of the cardiac tissue, which would shed light on the effect of survivin therapy on endothelial cell function. However, it is important to note that an increase in vascular density alone does not equate to an improvement in function. Our lab employs contrast enhanced ultrasound to quantify perfusion of the myocardial tissue, which may offer more insight into not only the structure but the functional aspect of the myocardial vascular system, pre- and post-doxorubicin treatment.

5.3.6 Other Supplementary Assays and Experiments

Assays and potential experiments to further solidify the work – gene expression analysis of genes related to doxorubicin-induced cardiomyopathy may further strengthen the study. For example, doxorubicin, a genotoxic drug, induces DNA breaks (Capranico, Dasdia, & Zunino, 1986a; L'Ecuyer et al., 2006) and the activity of p53 increases appropriately (Liu, Mao, Ding, & Liang, 2008; Zhu et al., 2009). Survivin-p53 pathway is well-studied in the literature and there is evidence to support that survivin and p53 regulate each other (Wang et al., 2004; Zhou et al., 2002). p53 also has implications in apoptosis (Gottlieb & Oren, 1998; Lowe, Ruley, Jacks, & Housman, 1993) and can further reinforce the apoptotic data we demonstrate in this study. Analysis of p53

expression may allow for the better understanding of doxorubicin-induced cardiomyopathy and explain the effect of survivin on apoptosis, in addition to the caspase inhibition demonstrated in this study. Gene expression analysis of other relevant factors, such as CTGF (Laplante et al., 2010), Bax/Bcl-2 (Yin, Oltvai, & Korsmeyer, 1994), or MDM-2 (Zhou et al., 2002), that have implications to fibrogenic activity, apoptosis, or survivin pathway can also be investigated as an exploratory experiment to elucidate further the mechanism of the beneficial effects of survivin gene therapy.

There is a convincing, emerging school of thought that challenges the existing dogma that the heart is a terminally differentiated organ. Rather, the heart contains a pool of cells with stem cell like properties, and can replace cardiac cells in vivo. Recent study by De Angelis and colleagues has reported that doxorubicin cardiomyopathy is mediated by the decrease in cardiac stem cell pool (De Angelis et al., 2010). The results from the study indicates that cardiac progenitor cells are exquisitely sensitive to doxororubicin cytotoxicity, and that by the end of the 6 week study period, doxorubicin eradicates almost all of the CPCs in the myocardium. Therefore, salvaging the CPCs from death may lead to the preservation of endogenous restorative mechanism and ultimately to the rescue of the failing heart. Since our gene delivery technique is diffuse, it is entirely possible that a subset of the CPC population may have been transfected with the exogenous survivin gene. An interesting adjunct to the study involves the immunohistochemical staining for the CPC, with a concomitant analysis for TUNEL positive cells. If it is found that doxorubicin affects CPC viability in vivo, it may be worthwhile to explore cell therapy using CPC that has been transfected with a pro-survival gene to enhance their survival and engraftment in the new environment.

Chapter 6 - Conclusions

We have demonstrated that anti-apoptotic therapy of the survivin gene by ultrasound mediated gene delivery in the myocardium ameliorates the deterioration of LV systolic function caused by doxorubicin-induced cardiotoxicity in a rodent in vivo model. In vitro data suggests that survivin gene transfer in h9c2 cells leads to a significant decrease in cell death after exposure to doxorubicin, indicative of survivin's role in salvaging cardiac function by preventing cardiomyocyte loss. This anti-apoptotic effect can be mostly attributed to the direct gene transfer into the cell acting on the cells expressing the exogenous gene, or via a second, less potent, paracrine mechanism through which secreted extracellular survivin acts on the neighbouring cell population in an as yet to be defined manner. In vivo results of survivin gene delivery mirror the in vitro results, with decreased apoptosis in DOX+SURV group, compared with DOX treated and DOX+EMP plasmid delivered groups. This decrease in apoptosis, in conjunction with decreased interstitial fibrosis, led to the preservation of LV systolic function at week 6 as demonstrated by both a non-invasive echocardiogram and invasive hemodynamic measures by pressure-volume loop experiments.

The results from this study provides further evidence that ultrasound mediated gene delivery method is a viable, safe and effective method for in vivo gene transfection, and anti-apoptotic gene therapy using UTMD in HF settings may lead to the amelioration of left ventricular systolic dysfunction where cardiac cell loss is prominent.

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